THE IDENTIFICATION OF NOVEL AUTOANTIGENS BY MEANS OF SEROLOGICAL SCREENING OF A cDNA EXPRESSION LIBRARY CONSTRUCTED FROM MULTIPLE SCLEROSIS BRAIN TISSUES

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The Identification of Novel Autoantigens by means of Serological Screening of a cDNA Expression Library Constructed from Multiple Sclerosis Brain Tissues.

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Abstract

Multiple sclerosis is the most common demyelinating disease of the central nervous system. It typically affects young adults, and as with many autoimmune diseases, MS affects more women than men.

A large number of studies have concentrated on the identification of the antigen(s) responsible for inciting MS, but the "causative agent(s)" has yet to be found. Some of these studies demonstrated that T and B lymphocytes isolated from MS patients are reactive with autoantigens, such as myelin basic protein, myelin oligodendrocyte protein, proteolipid protein, and myelin associated protein, and with viruses, such as Epstein-Barr virus, measles, and varicella-zoster. However, a role for any of these antigens in the initiation of multiple sclerosis has not yet been established.

This study was undertaken as an attempt to identify potential autoantigens in multiple sclerosis by using a modification of the SEREX (serological identification of antigens by recombinant expression cloning) technique developed by Sahin et al. (1995). This technique, unlike many of the techniques previously used by investigators to identify autoantigens in MS, makes no *a priori* assumptions as to the identity of the autoantigen. Messenger RNA was isolated from multiple sclerosis brain tissues and used to construct a cDNA library in a lambda phage vector. This vector was transfected into *Escherichia coli*, protein expression was induced with isopropyl β-D-thiogalactopyranoside, and the proteins were transferred to nitrocellulose membranes. The membranes were screened with patients' sera and positive clones were detected with a color reaction which recognizes IgG in patients' sera bound to the recombinant protein.

Positive clones were subcloned to clonality and sequenced, and the sequences compared with DNA and RNA sequences in various databases. Three positive clones were used as probes in Northern blotting experiments to determine their relative expression levels in various tissues. One of these clones was identified as testican, the other two appear to be related gene products of the clone F4 transmembrane protein and KIAA0530.

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Abbreviations

ACTH adrenocorticotropic hormone

BBB blood-brain barrier

bp base pairs

CNS central nervous system

cDNA complementary DNA

Cop-1 Copolymer-1

CSF cerebrospinal fluid

CNP 2', 3'-cyclic nucleotide 3'-phosphodiesterase

CR3 complement receptor type 3

DEPC diethyl pyrocarbonate

DTH delayed type hypersensitivity

EAE experimental allergic (autoimmune) encephalomyelitis

EDTA ethylenediaminetetraacetic acid

ELISA enzyme linked immunosorbent assay

EST expressed sequence tags

hnRNA heterogeneous nuclear RNA

HTLV-I human T cell lymphotrophic virus type I

ICAM intercellular adhesion molecules

IgG immunoglobulin G

IgM immunoglobulin M

IL interleukin

IFN-γ interferon gamma

IPTG isopropyl β-D-thiogalactopyranoside

LFA lymphocyte function-associated antigen

MAG myelin-associated protein

MBP myelin basic protein

MHV mouse hepatitis virus

MMLV-RT Moloney murine leukemia virus reverse transcriptase

MOG myelin oligodendrocyte protein

MRI magnetic resonance imaging

mRNA messenger RNA

MS multiple sclerosis

O.D. optical density

PCR polymerase chain reaction

PLP proteolipid protein

PNS peripheral nervous system

rIL-2 recombinant IL-2

rMOG recombinant myelin oligodendrocyte protein

RT-PCT reverse transcriptase polymerase chain reaction

SDS sodium dodecyl sulfate

SEREX serological identification of antigens by recombinant expression cloning

TAL-H human transaldolase gene

TCR T cell receptor

TEMED N, N, N', N' –tetramethylethylenediamine

TGF transforming growth factor

TMEV Theiler's murine encephalomyelitis virus

TNF-α tumor necrosis factor alpha

Tween 20 polyoxyethylene-sorbitan mono-laurate

VCAM vascular cell adhesion molecules

VLA very late antigen

X-gal 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Chapter One Introduction

1.0 Multiple Sclerosis

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS) (Antel and Arnason, 1991). Some authors estimate the risk of developing this disease as 1in 800 for individuals of Northern European descent (Bell and Lathrop, 1996). MS typically occurs in young adults with the mean age of onset about 30 years of age (Coyle, 1996) and affects twice the number of women as men (Steinman, 1996). Multiple sclerosis is generally believed to be an autoimmune disease with genetic and environmental components.

Although experimenters have yet to identify a "susceptibility gene(s)" for this disease, there are a number of pieces of evidence which do indicate a genetic predisposition for MS. First, the incidence of MS among immediate relatives of MS patients is 15 to 20 times higher than expected in the absence of a genetic component (Compston, 1991a). Second, the concordance rate of multiple sclerosis in monozygotic twins is 25 to 30 percent (Oksenberg and Hauser, 1997), whereas the concordance is only about 4 percent for dizygotic twins (Ebers et al., 1986). Third, there is an association of the HLA-DRB1*1501-DQA1*0102-DQB1*0602 haplotype, also known as the DR2 haplotype, in caucasian MS patients who are of Northern European descent (Oksenberg and Hauser, 1997). Wucherpfennig et al. (1991) estimated that between 50 and 70 percent of patients with multiple sclerosis carry this DR2 haplotype which is found in only 20 to 30 percent of the normal population in North America. Fourth, multiple genetic regions have been linked to multiple sclerosis in gene mapping studies (Ebers et

al., 1996; Kuokkanen et al., 1996; The Multiple Sclerosis Genetics Group, 1996; Sawcer et al, 1996).

From the genetic work completed to date, it appears that multiple sclerosis is not simply a genetic disease. Instead multiple sclerosis seems to be due to multiple genes which confer susceptibility in combination with environmental factors (Wucherpfennig et al., 1991; Compston et al., 1994; The Multiple Sclerosis Genetics Group, 1996; Sawcer et al., 1997). The importance of environmental factors can be deduced from the fact that there is an incomplete penetrance of multiple sclerosis in monozygotic twins (Stinissen et al., 1997). Compston et al. (1994), suggested that genetic factors are neither necessary nor sufficient for the development of MS. It is possible that the HLA associations observed in MS patients do not reflect a genetic predisposition to MS, but rather that these loci are linked markers to one or more of the other 200 genes known to map to this region (Oksenberg and Hauser, 1997). Perhaps the best summary of the genetics of multiple sclerosis is as follows: "Autoimmune diseases result from the action of environmental factors on a predisposed genotype. Autoimmune diseases are not inherited as entities but as constitutions which confer an increased probability of developing disease." (Baxter, 1997).

1.1 Clinical Features and Diagnosis of Multiple Sclerosis

Multiple sclerosis may include any of a large number of clinical features. Some of the most commonly occurring symptoms and clinical features include: optic neuritis, vertigo, vomiting, scanning speech, weakness, spasticity, bladder and bowel dysfunction,

electric shock-like sensation on flexion of the neck, tingling or tightness of the extremities, band-like sensations about the trunk, depression, weight loss, fatigue, and intention tremor (Tourtellotte et al., 1983; Antel and Arnason, 1991; Raine, 1997a). The manifestation of these symptoms is extremely variable, with the number of symptoms experienced by each patient and the order in which they occur varying from patient to patient. The fact that there is no "pattern" of symptoms can be explained by the observation that myelinated axons anywhere in the central nervous system can be involved (McFarland and McFarlin, 1995).

Since a laboratory test specific for multiple sclerosis has yet to be elucidated, the diagnosis of the disease relies mainly on clinical history and neurologic examination. Perhaps the most widely used set of criteria for the clinical diagnosis of MS are those proposed by Poser et al. in 1983. These authors divided multiple sclerosis diagnosis into clinically definite, laboratory-supported definite, clinically probable, and laboratory-supported probable multiple sclerosis. In general, the age of onset must be between 10 to 59 years inclusive, and no other condition(s) must be able to account for the symptoms.

A. Clinically Definite Multiple Sclerosis

1. Two attacks and clinical evidence of two separate lesions
2. Two attacks; clinical evidence of one lesion and paraclinical evidence of another separate lesion.

B. Laboratory-supported Definite Multiple Sclerosis

1. Two attacks; either clinical or paraclinical evidence of one lesion; and cerebrospinal fluid (CSF) oligoclonal bands or increased intrathecal synthesis of immunoglobulin G (IgG).
2. One attack; clinical evidence of two separate lesions; and CSF oligoclonal bands or increased intrathecal synthesis of IgG.
3. One attack; clinical evidence of one lesion and paraclinical evidence of another, separate lesion; and CSF oligoclonal bands or increased intrathecal synthesis of IgG.

C. Clinically Probable Multiple Sclerosis

1. Two attacks and clinical evidence of one lesion.
2. One attack and clinical evidence of two separate lesions.
3. One attack; clinical evidence of one lesion and paraclinical evidence of another separate lesion.

D. Laboratory-supported Probable Multiple Sclerosis

1. Two attacks and CSF oligoclonal bands or increased intrathecal synthesis of IgG.

In each of these categories the attacks must involve different parts of the CNS, must occur at least one month apart, and must each last at least 24 hours. In the laboratory-supported criteria oligoclonal bands must not be present in patient's serum and the level of IgG in the serum must be normal.

From reading this list of criteria it is easy to see how the development of a specific laboratory test for MS would be useful. There are a number of laboratory findings, which can be used as supporting information when a diagnosis of multiple sclerosis is suspected based on clinical and neurological observations (including oligoclonal bands and increased synthesis of IgG as mentioned above):

1. An increased synthesis rate of CSF IgG, observed in 80 to 90 percent of MS patients (Link and Tibbling, 1977; Tourtellotte, 1985).
2. The presence of oligoclonal bands of IgG in the CSF of more than 90 percent of patients with multiple sclerosis (Mehta et al., 1982; Gonsette et al., 1984; Tourtellotte, 1985). These bands are believed to be synthesized within the CNS by a limited number of B lymphocytes (Compston et al., 1994; Garren et al., 1998). It is important to note that the detection of such bands is not exclusive to MS, as oligoclonal bands are also found in diseases such as syphilis, herpes simplex encephalitis, and subacute sclerosing panencephalitis (Chu et al., 1983; Antel and Arnason, 1991; McFarland and McFarlin, 1995). Nevertheless the detection of these bands is helpful in the diagnosis of multiple sclerosis.
3. Evoked potentials response tests show slowing of conduction indicative of loss of myelin from axons in approximately 80 percent of MS patients (Antel and Arnason, 1991; Waxman, 1998).

4. "Multifocal cerebral white matter lesions" are detected with magnetic resonance imaging (MRI) in more than 90 percent of patients with MS (Antel and Arnason, 1991; McFarland and McFarlin, 1995; Miller, 1996).

1.2 Clinical Course of Multiple Sclerosis

Like the symptoms of multiple sclerosis, the course and severity of this disease are variable and unpredictable. The majority of patients, approximately 85 percent, endure a relapsing/remitting type of disease (Coyle, 1996) which can continue for more than 30 years. Relapses may present with new symptoms, the worsening of old symptoms, or both (Raine, 1997a). For between 40 and 65 percent of patients who present with a relapsing/remitting disease, a secondary progressive disease develops (Coyle, 1996). That is to say that although the patient experiences relapses followed by remissions, the extent of recovery from each relapse is not complete and so with time a gradual worsening of the patient is observed.

In contrast to this relative majority of multiple sclerosis patients, there is a minority of patients for whom the disease is primary progressive. These patients do not undergo the relapsing/remitting form of disease, but rather become progressively worse from the onset of disease without any remissions (McDonald, 1994).

There is also a very small group of multiple sclerosis patients for whom the disease has a progressive-relapsing course (Lublin and Reingold, 1996). Similar to the primary progressive patients, the progressive-relapsing patients experience a progressive disease from onset. However, in contrast to the primary progressive patients, they also

experience clear acute relapses. Recovery from these relapses may or may not be complete. As well, there is continuing progression of the disease between these relapses.

1.3 The Immunopathology of the Multiple Sclerosis Lesion

The characteristic pathology of multiple sclerosis includes inflammation of the CNS, demyelination with relative sparing of axons, and astroglial scarring (Sobel, 1995; Lassmann et al., 1998). Although this is the characteristic pathology of MS, the incidence of inflammation, demyelination, and remyelination differs not only with the form and stages of the disease, but also from patient to patient (Bruck et al., 1994; Ozawa et al., 1994). Based on their observations of the variability of MS pathology, Lucchinetti et al. (1996) suggested the following classifications: (1) demyelination with relative preservation of oligodendrocytes, (2) myelin destruction with concomitant and complete destruction of oligodendrocytes, (3) primary destruction or disturbance of myelinating cells with secondary demyelination. This lack of a characteristic pathology may reflect the actions of different immune responses.

Demyelination leads to the formation of multifocal lesions known as plaques, which are typical of MS. These plaques are well demarcated from the surrounding tissue, can be of various sizes, and can be formed in almost any region of the CNS (Antel and Arnason, 1991; Richardson, 1994; Martin, 1997). As previously mentioned, this variable distribution pattern is thought to be partly responsible for the fact that there is no common course of symptoms between patients. However, there are a few areas where plaques are know to predominate; the cerebellum, the optic nerves and tract, the brain

stem, the spinal cord, and the periventricular white matter (Lucchinetti and Rodriguez, 1997; Martin, 1997; Stinissen et al., 1997). Plaques are generally limited to white matter (Boccaccio and Steinman, 1996) and to the CNS (Antel and Arnason, 1991).

Typically the acute MS lesion is inflammatory and contains cytokine secreting T lymphocytes (mostly $CD4^+$ T lymphocytes), activated macrophages and microglia, and a few B lymphocytes and plasma cells (Prineas, 1985; Hauser et al., 1986; Raine 1991a,1994; Lassmann et al., 1998). The chronic MS lesion differs from the acute lesion in several ways: it contains more $CD8^+$ T lymphocytes than $CD4^+$ T lymphocytes (Raine, 1994), the number of B lymphocytes and plasma cells is greater (Prineas and Wright, 1978; Ozawa et al., 1994; Bruck et al., 1995), there is less inflammation, and glial scar formation occurs due to incomplete remyelination (Martin, 1997). The topic of remyelination is discussed later.

In general, the inflammation and demyelination observed in multiple sclerosis are believed to occur with "relative sparing of axons". However, this does not mean that axons are entirely protected. Some investigators suggest that axonal loss occurs in all MS plaques to some degree (Rodriguez and Scheithauer, 1994; Storch and Lassmann, 1997). Evidence of axonal injury has been demonstrated in the brains of patients with multiple sclerosis, and is believed to be mediated by inflammation (Ferguson et al., 1997; Trapp et al., 1998). McDonald (1994) even cited inflammation as the "earliest detectable event in the development of a new lesion". Perhaps the demyelination of the axons makes them susceptible to damage by macrophages, lymphocytes, or antibodies (Rodriguez and Scheithauer, 1994).

Whatever the mechanism of injury to the axons, it seems that axonal loss may be directly related to clinical deficits (McDonald, 1994; Ferguson et al., 1997; Trapp et al., 1998). Losseff et al. (1996) had already proposed that axonal loss was a more reliable correlate of permanent neurological deficit than the total extent of demyelination.

1.4 The Target of Demyelination in Multiple Sclerosis

Before addressing the specifics of the immunology of multiple sclerosis it is important to consider the possible targets of this immune response. The most likely candidates are the myelin sheath, or components of the myelin sheath, and the oligodendrocyte. To date there is no verdict as to which of these, if either, is actually the target in MS.

1.4.1 Myelin Composition and Function

In the central nervous system, myelin sheaths are actually multiply wrapped oligodendrocyte cell membranes (Richardson, 1994). In the peripheral nervous system (PNS), myelin is produced by Schwann cells. Myelin is composed mostly of lipids, particularly cholesterol and cerebrosides (Williams and Deber, 1993). The protein content of myelin is low. Proteolipid protein (PLP), the most abundant myelin protein, accounts for approximately 50 percent of the total myelin (Williams and Deber, 1993). Myelin basic protein (MBP) accounts for 25 to 30 percent of myelin (Stinissen et al., 1997). Myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) are minor constituents (Stinissen et al., 1997). MOG and PLP are found only in CNS myelin (McFarland and

McFarlin, 1995; Bernard et al., 1997; Stinissen et al., 1997). Since MS is known to preferentially affect the CNS, this makes MOG and PLP good potential targets. In addition to this, MOG is located on the extracellular surface of oligodendrocytes and the myelin sheath, making it easily accessible for an immune attack (Bernard et al., 1997; Stinissen et al., 1997). PLP is an integral hydrophobic membrane protein that may also be accessible to the immune system through its extramembranous domains (Sobel et al., 1994).

Myelin acts as an insulator for axons and as such increases the speed of conduction of an impulse along the axon from the nerve cell body to the synaptic junction (Deber and Reynolds, 1991; Lemke, 1996; Waxman, 1998).

The majority of evidence pointing to myelin or myelin components as possible targets in multiple sclerosis consists of the isolation of T lymphocytes, plasma cells, and antibodies specific for CNP, MOG, MBP, PLP, or MAG. These observations are fully discussed in the section " The Roles Played by Specific Immune Cells in Multiple Sclerosis".

1.4.2 Oligodendrocytes

The preservation of oligodendrocytes not only varies with the type of plaque observed, but also with the individual MS patient investigated (Bruck et al., 1994; Lucchinetti et al., 1996). Typically chronic inactive lesions show a loss of oligodendrocytes when compared with the normal surrounding tissue (Perier and Gregoire, 1965; Ozawa et al., 1994; Richardson, 1994; Rodriguez and Miller, 1994). The state of oligodendrocytes in early, active lesions is more complex. Oligodendrocytes

have been reported to be present in such lesions, sometimes even in "high density" (Raine et al., 1981; Prineas, 1985; Raine, 1997b; Raine and Wu, 1993; Bruck et al., 1994; Ozawa et al., 1994). It has also been reported that there is a "striking loss" of oligodendrocytes in the early stages of demyelination followed by proliferation of these cells at the edges of plaques (Prineas et al., 1989,1993a). The best summary of these observations was given by Bruck et al. (1994) who stated that in actively demyelinating plaques oligodendrocytes are intact, but that the number of oligodendrocytes present ranges from normal to absent. The presence of oligodendrocytes in these early plaques is considered by many to be proof that this cell is not a primary target in multiple sclerosis (Raine et al., 1981; Ozawa et al, 1994).

Rodriguez et al. (1993) and Rodriguez and Scheithauer (1994) proposed that demyelination is the result of "dying-back oligodendrogliopathy". That is, it is not the oligodendrocyte itself which is damaged in MS, but rather its "luxury" function, namely myelination. Such damage would result in the production of abnormal myelin, which may elicit an immune response. Support for this theory comes from the observation of degeneration in the inner myelin sheaths and in the inner glial loops of oligodendrocytes and the accumulation of organelles within the inner glial loops of the myelin sheaths (Kirk, 1979; Ludwin and Johnson, 1981; Rodriguez, 1985; Rodriguez et al., 1993). Rodriguez et al. (1993) argue that if demyelination is solely the result of a lymphocytic reaction in the CNS, then the outer myelin sheath should be destroyed first as this is the structure to which the lymphocytes have access. They therefore suggest that the

oligodendrocyte or some other cell(s) necessary for the production and maintenance of myelin is the target in multiple sclerosis.

Lending support to this hypothesis, is a study by Banki et al. (1994). They found antibodies to a recombinant human transaldolase in the serum and CSF of a subset of MS patients, but not in normal individuals or patients with other neurological disorders. They also demonstrated that this transaldolase was able to induce proliferation of peripheral blood lymphocytes from patients with multiple sclerosis. The human transaldolase gene (TAL-H) is expressed selectively in oligodendrocytes at high levels and may have a role in the initiation of multiple sclerosis.

In direct contrast, Poser (1993) argues against the oligodendrocyte as a multiple sclerosis target based on the following observations:

1. The areas of demyelination in MS are sharply demarcated. A single oligodendrocyte typically myelinates several axons, it is unlikely that the area over which a particular oligodendrocyte extends would have sharply defined edges.
2. The lesions in MS are scattered throughout the white matter, while diseases in which the oligodendrocyte is known to be the primary target show large confluent areas of demyelination.

1.5 The Immunology of Multiple Sclerosis

The immune system is thought to be responsible for the damage incurred in multiple sclerosis. At the present time, it is not known if the destructive immune response is primarily directed against the CNS or if the damage seen in MS is the result of an immune response to some other antigen or infectious agent.

In a normal individual the central nervous system is a sequestered site, due to the presence of an intact blood brain barrier (BBB). The BBB is composed mostly of non-fenestrated endothelial cells connected via tight junctions (Steinman, 1996). As its name implies, the blood brain barrier functions to separate the brain from blood, blood derived leukocytes, and serum proteins (Williams et al., 1994a). Therefore, in order for T and B lymphocytes and blood monocytes to enter the CNS and for inflammation and demyelination to occur, the BBB must somehow be breached. Blood brain barrier breakdown has been observed in MS patients by using MRI and is considered to be an early feature in lesion formation (Kermode et al., 1990; McDonald et al., 1992; McDonald, 1994). In fact Compston (1991b) referred to penetration of the BBB as the "primary disease process without which none of the events directly responsible for myelin injury would occur".

Activated T lymphocytes are able to penetrate the BBB in an antigen non-specific manner, that is the antigen for which a T lymphocyte is specific need not be present in the CNS (Wekerle et al., 1986; Hickey et al., 1991; Williams et al., 1994a; McFarland, 1995). This movement across the BBB is dependent upon the expression of certain adhesion molecules. In the normal CNS, adhesion molecule expression on endothelial

cells is low or absent (Wong and Korovini-Zis, 1992; Washington et al., 1994; Cannella and Raine, 1995; Brosnan and Raine, 1996). As a result, the endothelial cell layer is relatively resistant to leukocyte adhesion and allows only a few cells, such as activated lymphocytes, to enter the CNS (Hafler and Weiner, 1987).

Endothelial cells in MS lesions are activated (Sobel, 1995) and have increased expression of vascular cell adhesion molecules (VCAMs) and intercellular adhesion molecules (ICAMs) particularly VCAM-1 and ICAM-1 (Sobel et al., 1990; Brocke et al., 1994; Washington et al., 1994; Cannella and Raine, 1995; Bo et al., 1996). The levels of soluble VCAM-1 and ICAM-1 are significantly increased in the CSF of relapsing MS patients, as compared with patients with other neurologic diseases (Droogan et al., 1996). The increased expression of adhesion molecules may be due to the action of cytokines, such as interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), which are secreted by activated T lymphocytes and macrophages (Dustin et al., 1986; Sharief et al., 1993a; Williams et al., 1994a). In fact, levels of free circulating soluble ICAM-1 in serum and CSF of patients with active MS correlate with TNF-α levels and BBB disruption (Sharief et al., 1993b). The levels of VCAM-1 and L-selectin have been shown to correlate with the concentration of the soluble TNF receptor (Hartung et al., 1995a).

Activated T lymphocytes which express very late antigen-4 (VLA-4) are able to cross the BBB by binding to the VCAM-1 molecule on endothelial cells (Brocke et al., 1994). The binding of ICAM-1 with lymphocyte function- associated- antigen-1 (LFA-1), expressed on activated T lymphocytes, may also be involved in T lymphocyte

penetration of the blood brain barrier (Sobel et al., 1990). T lymphocytes in the CSF and plaques of multiple sclerosis patients have increased expression of LFA-1 and VLA-4 (Svenningsson et al., 1993; Cannella and Raine, 1995). Almost all invading leukocytes in MS lesions express LFA-1 (Bo et al., 1996).

Several studies have shown that increased expression of adhesion molecules correlate with disease activity. For example, Dore-Duffy et al. (1995) showed that serum levels of ICAM-1 and endothelial leukocyte adhesion molecule (E-selectin) were increased in patients with chronic progressive MS as compared with controls. These same patients also had increased CSF levels of VCAM-1 and E-selectin when compared with controls. Another study found VCAM-1 and L-selectin to be increased in the serum of both relapsing/remitting and chronic progressive patients when compared with controls (Hartung et al., 1995a). Increased serum levels of ICAM-1 were also found during relapses of MS (Hartung et al., 1993; Sharief et al., 1993b; Tsukada et al., 1993).

The activated T lymphocytes that have broken through one "layer" of the blood brain barrier must cross the extracellular matrix composed of type IV collagen. In order to accomplish this task, matrix metalloproteases specific for type IV collagen are required. These are known as gelatinase A and B (Steinman, 1996). It is possible that these proteases are secreted by the T lymphocytes themselves (Coyle, 1996). The importance of these gelatinases is indicated by the detection of both gelatinase A and B in the tissue surrounding inflamed brain endothelium (Steinman, 1996), and in the CSF of MS patients (Gijbels et al., 1992). Gelatinase B is also present in MS lesions, endothelial cells, pericytes, macrophages and astrocytes (Steinman, 1996). A study by Rosenberg et

al. (1996) found gelatinase B to be increased in patients with MRI evidence of BBB breakdown.

Once inside the CNS the activated T lymphocytes specific for CNS antigens remain, T lymphocytes which do not find their specific antigens return to the peripheral circulation (Hickey et al., 1991; Williams et al., 1994a). The remaining T lymphocytes are able to incite a number of responses through the secretion of cytokines. These include the induction of inflammation (Williams et al., 1994a), the activation of endothelial cells, the recruitment of lymphocytes and macrophages (McFarland, 1995), and alterations in the permeability of the BBB thereby allowing the entry of B lymphocytes, macrophages, immunoglobulins, and complement (Williams et al., 1994a; Gay and Esiri, 1991). It is also possible that the cytokines themselves may directly damage the tissue of the CNS (Wisniewski and Bloom, 1975; Probert et al., 1995).

An interesting study by Barnes et al. (1991) suggested that the repair of the BBB may not be complete in multiple sclerosis and that it may take quite some time for the BBB to be restored. This finding has important implications for the disease. In theory, the opening of the BBB for an extended period of time would allow exchanges in both directions between the CNS and the peripheral blood system which could aid in the perpetuation of MS.

1.6 The Roles Played by Specific Immune Cells in Multiple Sclerosis

Once T and B lymphocytes as well as macrophages have gained entry into the CNS it is important to consider the roles played by each type of cell in the induction and perpetuation of multiple sclerosis.

1.6.1 The Role of the T Lymphocyte

Since the discovery that the animal model of multiple sclerosis, experimental allergic (autoimmune) encephalomyelitis (EAE), is mediated by $CD4^+$ T lymphocytes (Pettinelli and McFarlin, 1981; Swanborg, 1983), many investigators have concluded that MS must also be a T lymphocyte mediated disease. There are a number of observations that indicate a role for T lymphocytes in multiple sclerosis. To date, a decision regarding the exact role of the T lymphocyte cannot be made. These observations are listed below:

1. T lymphocytes are nearly always found in and around MS lesions (Adams, 1983; Poser, 1993), and have even been identified beyond the plaque/normal tissue border (Prineas and Connell, 1978). In addition, increased numbers of activated lymphocytes have been demonstrated in the CSF of MS patients (Noronha et al., 1980; Bellamy et al., 1985)
2. The fact that there is an association between certain patients with multiple sclerosis and the class II MHC molecule, HLA-DR2, is evidence of T lymphocyte involvement in MS as $CD4^+$ T lymphocytes are known to recognize antigen in the context of class II MHC molecules. In addition, the T lymphocyte response to myelin basic protein has been found to be particularly restricted by HLA-DR (Pette et al., 1990).

3. T lymphocytes specific for MBP, PLP, and MOG, or peptides of these proteins, have been isolated from the blood and CSF of multiple sclerosis patients, patients with other neurologic diseases, and healthy individuals. However, in each of these studies the frequency of MBP-, PLP-, or MOG-specific T lymphocytes is highest in MS patients (Ota et al., 1990; Olsson et al., 1990a,1992; Sun et al., 1991a,b; Chou et al., 1992). Another study found T lymphocytes specific for MAG could be isolated from the peripheral blood of MS patients, but not from controls (Johnson et al., 1986). Wallstrom et al. (1998) recently found that peripheral blood lymphocytes isolated from MS patients with the HLA haplotype DR2 showed greater numbers of cells secreting IFN-γ in response to several MOG peptides than lymphocytes from HLA matched normal controls. Studies by Kerlero de Rosbo et al. (1993,1997) found T lymphocytes isolated from multiple sclerosis patients predominantly responded to MOG and MOG peptides over MBP, PLP, and MAG.
4. The fact that autoreactive T lymphocytes could be isolated from MS patients and healthy individuals implies that the presence of these cells is not enough to produce multiple sclerosis (Ewing and Bernard, 1998). This led some investigators to search for differences between the cells of the two groups. Allegretta et al. (1990) found increased rates of somatic mutations in MBP-specific T lymphocytes isolated from the blood of patients with MS. Somatic mutations are a marker of antigen-driven T lymphocyte proliferation or

activation. As these mutations were not observed in MBP-specific T lymphocytes of healthy individuals, it can be concluded that these T lymphocytes are not activated. Zhang et al. (1994) were also able to show that MBP-reactive T lymphocytes of MS patients are in a different state of activation than those of controls. They demonstrated a higher frequency of MBP- and PLP-specific T lymphocytes from the blood and CSF of MS patients over controls after primary stimulation with recombinant IL-2 (rIL-2). Bieganowska et al. (1997) also demonstrated a high frequency of activated autoreactive T lymphocytes in MS patients. Hermans et al. (1997) found that a higher percentage of primary T lymphocyte cultures produce IFN-γ in response to MBP in MS patients than in controls. The MBP-specific T lymphocytes isolated from MS patients also produced higher levels of TNF-α than those of controls. These differences in cytokine secretion may be the result of *in vivo* activation of T lymphocytes specific for MBP in patients with multiple sclerosis. These findings are extremely important to the understanding of multiple sclerosis as it is known that only activated T lymphocytes are able to traverse the blood brain barrier.

The events leading to the activation and proliferation of autoreactive T lymphocytes in MS are still under investigation. Such cells may be present in MS in high frequencies as part of the immune response to the target antigen(s). In contrast, the autoreactive T lymphocytes may be a part of the normal T cell repertoire and are activated by the release of myelin breakdown products into the peripheral circulation

(Martin et al., 1992a; Poser, 1993; Hohlfeld et al., 1995). This implies that the myelin reactive T lymphocytes themselves are not sufficient to induce demyelination.

1.6.1.1 T Cell Receptor Usage in Multiple Sclerosis

Much work has concentrated upon elucidating the specific T cell receptor (TCR) genes used by the MBP-specific T lymphocytes in multiple sclerosis. Unfortunately it seems that with each study differing results were obtained. For example, Kotzin et al. (1991) found Vβ5.2 and 6.1 to be over-represented, while other studies demonstrated that the TCR usage was more heterogeneous (Martin et al, 1992b; Meinl et al., 1993). Wucherpfennig et al. (1990) showed that the Vβ chain used by the TCR depended upon the epitope recognized by the T lymphocyte line. For example, T lymphocyte lines specific for MBP84-102 predominantly expressed Vβ17 or 12. In contrast, Vβ17 was rarely detected in T lymphocyte lines specific for MBP143-168. Oksenberg et al. (1993) found rearranged Vβ5.2 genes in all MS patients who were HLA-DRB1*1501, DQA1*0102, DQB1*0602, DPB1*0401. However, these same patients frequently had Vβ6, 7, 8, and 12 as well. It is now thought that the TCR-V gene usage has limited combinations in any given individual, but that between individuals there is much heterogeneity (Wucherpfennig et al., 1990; Ben-Nun et al., 1991; Meinl et al., 1993; Vandevyer et al., 1995; Hafler et al., 1996). There is also evidence that TCR gene usage is not static during the course of multiple sclerosis. Whereas it has been reported that the TCR Vβ gene transcripts are diverse in acute lesions, but more restricted in chronic lesions (Wucherpfennig et al., 1992), and that TCR usage becomes more diverse with the duration of the disease (Utz et al., 1994).

1.6.1.2 How Specific is the T Cell Receptor?

The T cell receptor was considered for many decades to be extremely antigen specific, however recent studies question whether the TCR is as stringent as previously believed. In 1995 Wucherpfennig and Strominger were investigating molecular mimicry in multiple sclerosis using various viral and bacterial peptides. They were successful in identifying several peptides capable of stimulating MBP-specific T lymphocytes, but more importantly they noticed that only one of these peptides would have been identified as a possible mimic by sequence homology with the sequence of MBP. The other peptides shown to mimic MBP, but having less sequence homology, had amino acid substitutions which the T cell receptor "tolerated". Thus the TCR was not as restricted as others believed.

A more recent report by Hemmer et al. (1998a) pointed out that the majority of the interaction between a T lymphocyte and an antigen-presenting cell involves contact between the T cell receptor and the MHC molecule and not with the antigenic peptide. Garboczi et al. (1996) found they could change key T cell receptor contact positions of peptides without affecting the stability of the TCR-MHC complex. Experiments using sets of peptides with single or multiple amino acid substitutions have shown that the T cell receptor is able to recognize a number of modified peptides (Wucherpfennig et al., 1994; Evavold et al., 1995; Chen et al., 1996; Vergelli et al., 1997). It was also shown that T lymphocyte clones could be stimulated with peptides which were not at all homologous with the antigen used to establish the T lymphocyte clone (Hemmer et al., 1998b). This led to the conclusion that although some of the amino acids in a peptide

sequence are more important than others, none of them is strictly required for T lymphocyte recognition. Taken together, these observations indicate that the antigen recognition by T lymphocytes is highly degenerate, that the TCR-MHC interaction contributes more to the stimulation of a T lymphocyte than the TCR-MHC-peptide interaction, and that T cell recognition of antigenic peptides is less restricted than originally thought.

1.6.1.3 Cytokine Production by T Lymphocytes in the CNS of Multiple Sclerosis Patients

It has been suggested that the number of T lymphocytes present in the CNS of MS patients is not large enough to inflict direct injury on myelin (Richardson, 1994). Perhaps the T lymphocytes are able to indirectly injure myelin sheaths through the release of various cytokines. Activated T lymphocytes are capable of releasing numerous cytokines known to be involved in immune responses and in inflammation and possibly in demyelination (Ewing and Bernard, 1998).

The cytokines produced by $CD4^+$ T lymphocytes are typically divided into the pro-inflammatory and anti- inflammatory categories. The pro-inflammatory cytokines include tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-2, and interferon-γ. These cytokines are produced by a subset of $CD4^+$ T lymphocytes known as T_h1 lymphocytes. The anti-inflammatory cytokines include transforming growth factor-β (TGF-β), interferon-α (IFN-α), interferon- β (IFN-β), interleukin-4 (IL-4), and interleukin-10 (IL-10). These cytokines are produced by the T_h2 subset of $CD4^+$ T lymphocytes. It should be noted that the classification of T_h1 and T_h2 derives from

murine models. It has not been proven that this simplistic sub-typing is applicable to humans. In fact, Navikas and Link (1996) argue that this system does not reflect the complexity of the interaction of cytokines seen in humans and should not be used when discussing human immunology. In the following discussion of cytokines in multiple sclerosis, cytokines will be referred to as pro-inflammatory or anti-inflammatory.

It is believed that the pro-inflammatory cytokines promote the immune responses leading to the damage observed in multiple sclerosis, while the anti-inflammatory cytokines act to down-regulate this effect (Navikas and Link, 1996). These two groups of cytokines may even act simultaneously. Probably the most important, and certainly the most studied, cytokines involved in the disease process are IFN-γ, TNF-α, TNF-β, and TGF-β. The evidence pointing to a role for each of these cytokines is discussed below.

Although these cytokines may play important roles in the initiation and perpetuation of multiple sclerosis, it must be remembered that the cytokine profile observed in MS patients is not exclusive to this disease. Similar cytokine patterns are seen in other inflammatory, non-demyelinating, CNS diseases (Brosnan et al., 1995; Navikas and Link, 1996).

A. Interferon-γ

IFN-γ is present in active MS lesions (Traugott and Lebon, 1988a,b) but not in the surrounding normal tissue (Voskuhl et al., 1993; Vartanian et al., 1995). As well, the number of mononuclear cells isolated from blood and CSF which secrete IFN-γ in response to *in vitro* stimulation with myelin proteins or viral antigens is greater in MS patients than in control subjects (Olsson et al., 1990a; Sun et al., 1991a,b; Link et al.,

1992). Levels of mononuclear cells expressing IFN-γ messenger RNA (mRNA) are increased in the blood and CSF of MS patients, as compared with controls (Link et al., 1994a,b). Link et al. (1994a) also found that multiple sclerosis patients with moderate or severe disability had high levels of IFN-γ expressing cells. The production of IFN-γ has been shown to increase before exacerbations of multiple sclerosis (Beck et al., 1988).

Perhaps the most convincing piece of evidence suggesting a role for IFN-γ in the pathogenesis of multiple sclerosis is the demonstration that administration of IFN-γ to MS patients results in exacerbations of the disease (Panitch et al., 1987). On the other hand, administration of IFN-α or IFN-β reduces the rate and severity of exacerbations (The Interferon-β Multiple Sclerosis Group, 1993; Durelli et al., 1994). This is an important finding since IFN-β reduces IFN-γ production and interferes with the induction of MHC class II expression by IFN-γ (Noronha et al., 1993; Durelli et al., 1994).

The effects of IFN-γ include up-regulation of MHC class II molecules on microglia, and therefore increased antigen presentation (Vass and Lassmann, 1990; Ewing and Bernard, 1998), activation of macrophages, and up-regulation of adhesion molecules which promotes T cell homing to the CNS (Dustin et al., 1986; Pober et al., 1986; Wong and Korovini-Zis, 1992; Navikas and Link, 1996). This cytokine has also been shown to induce apoptosis of oligodendrocytes *in vitro* (Vartanian et al., 1995).

B. Tumor Necrosis Factor

Tumor necrosis factor-α and TNF-β (lymphotoxin) are present in the plaques and CSF of multiple sclerosis patients (Hofman et al., 1989; Selmaj et al., 1991a; Cannella

and Raine, 1995). The level of TNF-α is typically low in normal CNS tissue, but is increased in MS lesions (Cannella and Raine, 1995). Patients with multiple sclerosis also have higher serum TNF-α receptor levels than controls (Hartung et al., 1995a). As well, there is an increase in the number of both mononuclear cells and MBP specific cells expressing TNF-α mRNA in the peripheral blood and CSF of MS patients (Navikas et al., 1996).

Relationships have been detected between TNF and different stages of MS. CSF levels of TNF-α are elevated in active MS as compared with stable MS or controls (Sharief and Hentges, 1991; Trotter et al., 1991; Sharief and Thompson, 1992). In the chronic progressive patients these high levels were found to correlate with the degree of disability and the rate of neurologic deficit. Increased levels of TNF-α and TNF-β mRNA have been identified prior to clinical relapses (Rieckmann et al., 1995). In active multiple sclerosis TNF-α levels have been shown to correlate with BBB damage (Sharief and Thompson, 1992).

The effects of TNF-α are similar to those of IFN-γ, such as the up-regulation of the expression of adhesion molecules (Pober et al., 1986,1987; Wong and Korovini-Zis, 1992; Barten and Ruddle, 1994), and the induction of MHC class II expression (Cannella and Raine, 1989). In addition, TNF-α may induce the production of other cytokines (Zoja et al., 1991) and may act synergistically with IFN-γ (Navikas and Link, 1996). TNF-α and TNF-β have also been shown to damage myelin and oligodendrocytes *in vitro* (Brosnan et al., 1988; Selmaj and Raine, 1988; Selmaj et al., 1991b; Zajicek et al., 1992a).

C. Transforming Growth Factor-β

Increased levels of mononuclear cells expressing TGF-β have been detected in the CSF and peripheral blood of patients with MS (Link et al., 1994a). Further studies have directly and indirectly demonstrated that TGF-β is associated with remissions (Correale et al., 1995a). For example, TGF-β mRNA levels have been shown to decline prior to relapse (Rieckmann et al., 1995), while TGF-β like activity is detected more frequently in MS patients recovering from exacerbations than from stable patients (Beck et al., 1991). As well, MS patients with no or slight disability had high levels of TGF-β mRNA expressing cells (Link et al., 1994a).

1.6.2 The Role of the B Lymphocyte

There is no doubt that T lymphocytes play an integral role in the development and progression of multiple sclerosis. However, there now seems to be consensus that multiple sclerosis is not solely the result of the actions of autoreactive T lymphocytes. At a recent conference it was stated that, "... the pathogenesis of multiple sclerosis lesions is more complex, and cannot be explained on the basis of a T lymphocyte mediated response alone. The mechanisms of demyelination may be diverse in different patients" (Lassmann et al., 1998). There are a number of immune anomalies in multiple sclerosis patients which suggest that B lymphocytes and their immunoglobulins are important mediators in the pathogenesis of MS. Some of these abnormalities have already been mentioned: the presence of oligoclonal IgG in CSF (Link and Tibbling, 1977; Tourtellotte, 1985), the elevated synthesis rate of IgG in CSF (Mehta et al., 1982; Gonsette et al., 1984; Tourtellotte, 1985), and the presence of high numbers of B

lymphocytes in chronic MS lesions (Prineas and Wright, 1978; Bruck et al, 1995). Others include: high concentrations of oligoclonal IgG in the brain of MS patients (Mattson et al., 1980; Owens et al., 1998), oligoclonal bands in the serum of at least 50 percent of MS patients (Zeman et al., 1996a), a large increase in the proportion of IgG producing cells in the CSF of MS patients compared with controls (Link et al., 1989) and high levels of IgG heavy chain mRNA in MS plaques as compared with normal white matter (Owens et al., 1998). The B lymphocyte response in acute multiple sclerosis is limited and restricted, suggesting a specific local immune response, that is intrathecal synthesis of immunoglobulins (Owens et al., 1998).

In general, MS patients who are negative for the presence of oligoclonal bands tend to have less disability than those patients who do possess these bands (Stendahl-Bodin and Link, 1980; Zeman et al., 1996b). It was also noted in a small study that serum antibodies reactive with brain tissue were not detected in most of the patients with relapsing/remitting multiple sclerosis. Of the patients which did test positive for such antibodies, the majority had a chronic progressive form of MS (Henneberg et al, 1991).

The identification of autoantigens and/or antigens for which the majority of the antibodies are specific has been the topic of research for many years. To date the mystery has not been resolved, but a number of antigens have been found to react with some of the antibodies.

Several studies have identified antibodies in the CSF of MS patients which are shown to react with a variety of viral antigens. These include measles, mumps, rubella, herpes simplex type 1, varicella-zoster, vaccinia, Epstein-Barr virus, cytomegalovirus,

and adenovirus (Adams and Imagawa, 1962; Adams et al., 1970; Vartdal et al., 1980; Bray et al., 1983; Baig et al., 1989; Reder and Arnason, 1985). These anti-viral antibodies have not been found to account for the oligoclonal bands characteristic of multiple sclerosis (Vartdal et al., 1980).

Other studies have discovered antibodies and/or antibody secreting cells specific for myelin components in the CSF of MS patients. These myelin components include MBP (Panitch et al., 1980; Gorny et al., 1983; Wajgt and Gorny, 1983; Cruz et al., 1987; Link et al., 1989,1990; Baig et al., 1991; Gerritse et al., 1994), MAG (Link et al., 1989; Moller et al., 1989; Wajgt and Gorny, 1983; Baig et al., 1991), PLP (Sun et al., 1991a), and MOG (Sun et al., 1991b). These antibody responses were not found to be specific for MS as they were also detected in control subjects and patients with other neurologic diseases. In general, the number of antibody secreting cells was higher in MS patients than in controls or patients with other neurologic diseases. The antibody producing responses appeared to be sequestered in the CNS (Olsson, 1990b) as cells secreting antibodies against MAG and MBP were rarely detected in peripheral blood (Link et al., 1989; Baig et al., 1991). A study by Gerritse et al. (1994) found antibody secreting cells specific for MBP to be present in both plaques and normal CNS tissue of MS patients, but absent from the CNS tissues of patients with other neurologic diseases. As with the anti-viral antibodies, the myelin reactive antibodies do not represent a significant amount of the oligoclonal bands in the CSF of MS patients (Kaiser et al., 1997). This is reinforced by the finding that the absorption of CSF from multiple sclerosis patients with

myelin does not remove oligoclonal bands from the CSF samples (Nobile-Orazio et al., 1987).

Similarly, Walsh and Murray (1998) demonstrated the presence of antibodies to 2', 3'-cyclic nucleotide 3'-phosphodiesterase in the serum of 74 percent of MS patients tested. These antibodies were immunoglobulin M (IgM) and were also present in the CSF of patients. They also found CNP to be a part of immune complexes in MS brain. CNP is a membrane associated protein of the oligodendrocyte (Sprinkle, 1989), but is also expressed by lymphocytes (Bernier et al., 1987) and in retina (Giulian and Moore, 1980). The immune response to CNP may explain the retinal manifestations that often are present in patients with multiple sclerosis

Circulating immune complexes in the CSF and serum of MS patients have been shown to contain MBP (Dasgupta et al., 1983; Coyle, 1985). Dissociation of these complexes resulted in an increase in anti-MBP specific antibodies (Warren and Catz, 1987). The level of bound versus free anti-MBP antibodies differs depending upon the phase of MS investigated. In the chronic progressive form the majority of antibodies appear to be bound, whereas during and after relapses most of the antibodies seem to be free (Warren and Catz, 1987,1989).

There are a number of ways in which the antibody secreting cells or the antibodies they produce could contribute to the pathogenesis of multiple sclerosis:

1. The antibody-secreting cells express MHC class II and therefore may act as antigen-presenting cells to activated T lymphocytes (Gerritse et al., 1994; Sobel, 1995).

2. The antibodies may participate in antibody-dependent cell mediated cytotoxicity (Baig et al., 1991; Gerritse et al., 1994). Satoh et al. (1991) showed that *in vitro* cytolysis of bovine oligodendrocytes is due to the action of human killer cells in the presence of anti-oligodendrocyte antiserum.
3. The antibodies may be important in complement-mediated lysis. IgG and complement can be observed attached to oligodendrocytes in the CNS of MS patients (Prineas and Graham, 1981; Yamada et al., 1990a), and are deposited in areas of active demyelination (Storch et al., 1998). Activated complement and terminal complement complexes have been identified in the CNS tissue and CSF of patients with MS (Lumsden, 1971; Morgan et al., 1984; Sanders et al., 1986; Compston et al., 1987,1989). As well, *in vitro* studies have shown that human oligodendrocytes are not susceptible to complement attack in the absence of antibody (Zajicek et al., 1995), and that the degradation of MBP and PLP mRNA in cultured oligodendrocytes can be enhanced by the addition of activated complement complexes (Shirazi et al., 1993). C9neo antigen, a marker of activated lytic complement complexes, is present only in active areas of demyelination (Storch et al., 1998) and the concentration of C9 is decreased in the CSF of multiple sclerosis patients compared with controls (Morgan et al., 1984). Macrophages and vessel walls in MS plaques frequently possess particles coated with C3d, C9, IgG and IgM, but these particles are not observable in normal CNS tissue (Gay and Esiri, 1991). In

addition, macrophages in MS lesions contain degradation products with myelin antigens, immunoglobulin, and C9neo antigen (Storch et al, 1998). Gay and Esiri (1991) state that, "When complement and immunoglobulin are detected at the same location in tissues....this provides evidence that antibody has reacted with antigen and that complement has been fixed by the classical pathway.".

4. The binding of antibodies to the myelin membrane may activate myelin proteases (Kerlero de Rosbo and Bernard, 1989). A study by these two experimenters showed MBP degradation to be greater if myelin was incubated with antibodies from MS patients than if myelin was incubated without such antibodies or with control antibodies. They believed the degradation was due to the action of a neutral protease. It has been shown that MAG and MBP are susceptible to neutral protease degradation (Sato et al., 1982). A similar, but more specific study by Menon et al. (1997) identified anti-MOG monoclonal antibodies as the antibodies responsible for MBP degradation. This degradation was not observed when anti-MAG or anti-MBP antibodies were added.
5. The production of TNF-α by B lymphocytes may aid in damaging myelin and/or the oligodendrocytes (Hartung et al., 1995b).

Regardless of the probable roles for these antibodies, it remains a possibility that the antibodies are merely the products of an immune response elicited by the breakdown of myelin (Baig et al., 1991). In which case, the antibodies are not important to the

pathogenesis of multiple sclerosis. It is also possible that the antibodies important in the pathogenesis of MS are bound to their antigen(s). If this is so, then the targets of these antibodies will not be identified with conventional strategies (Cash et al., 1992; Olsson, 1994). Or perhaps the antibodies are specific for an infectious agent or CNS component which has not yet been identified (Martin et al., 1992a).

1.6.3 The Role of Macrophages

According to Richardson (1994), "Macrophages are far more likely candidates as cellular agents of demyelination (than T lymphocytes)". Although macrophages are present in all MS lesions, they are most numerous in active lesions where they are generally found to contain myelin degradation products giving them a "foamy" appearance (Adams, 1983; Esiri and Reading, 1987; Esiri and Morris, 1991; Richardson, 1994; Bruck et al., 1994,1995,1996). Macrophages have been found to outnumber T lymphocytes several fold in acute and chronic lesions (Bruck et al, 1994; Lassmann et al., 1994).

Macrophages may contribute to demyelination in a number of ways:

1. Macrophages have been observed actively phagocytosing myelin (Raine et al., 1981) and, as mentioned above, are frequently found to contain byproducts of myelin breakdown. Whether this phagocytosis of myelin is active removal of myelin or scavenging of the debris resulting from myelin degradation is still under debate (Richardson, 1994). However, Prineas et al. (1984) did report macrophages in active MS plaques seemingly stripping and phagocytosing MAG-positive fragments from intact myelin sheaths.

2. Macrophages may release cytokines, such as TNF-α, which are capable of damaging myelin and oligodendrocytes (Selmaj and Raine, 1 988) and up-regulate the expression of adhesion molecules (Pober et al., 1987; Barten and Ruddle, 1994).
3. These cells may participate in complement-mediated lysis. Activated macrophages produce complement (Levi-Strauss and Mallat, 1987) and receptors for components of complement, such as C3b (Gay and Esiri, 1991). Macrophages in active lesions not only contain myelin degradation products, but also C9neo antigen and immunoglobulin (Storch et al., 1998). The addition of antibodies against complement receptor type 3 (CR3) or cobra venom factor to cultures was shown to inhibit phagocytosis of myelin by macrophages (Bruck and Friede, 1990). An interesting experiment by De Jong and Smith (1997) investigated the role of complement in mediating demyelination. They found that complement was required for maximal myelin phagocytosis by cultured macrophages. However, if the myelin was previously sonicated, complement was no longer needed. This suggests that complement is necessary to fragment myelin, making it easier for the macrophages to phagocytose.
4. Macrophages in actively demyelinating lesions express MHC class II molecules (Esiri and Reading, 1987; Bo et al., 1994a) which suggests that these cells may act as antigen-presenting cells to $CD4^+$ T lymphocytes. In addition, macrophages in MS lesions have also been shown to express co-

stimulatory molecules, such as B7 (Williams et al., 1994b; De Simone et al., 1995; Windhagen et al., 1995a), also implying an antigen-presenting role for these cells. It is interesting to note that B7 molecules are not normally expressed in CNS tissue (Williams et al., 1994a).

5. These cells may participate in antibody-dependent cell mediated cytotoxicity. Scolding and Compston (1991) found that macrophages were able to lyse oligodendrocytes *in vitro* in the presence of anti-myelin antibodies.
6. Macrophages have been shown to phagocytose myelin via receptor mediated phagocytosis. Briefly, this refers to the phagocytosis of myelin being mediated by clathrin-coated pits present on the surface of "foamy" macrophages with IgG acting as the ligand between the myelin debris and the pits (Prineas and Connell, 1978; Prineas, 1985; Raine and Scheinberg, 1988).
7. Macrophages may damage myelin sheaths through the release of various mediators, including proteolytic enzymes, nitric oxide, and oxygen radicals (MacMicking et al., 1992; Merrill et al., 1993; Brosnan et al., 1994; Cross et al., 1994). Oxygen radicals and nitric oxide have been detected in the center and edge of acute MS lesions (Bo et al., 1994b).

1.6.4 The Role of Microglia

Although microglia are a part of the central nervous system, they are essentially the indigenous macrophages of the CNS. Typically, these cells possess a dendritic morphology, but upon activation they take on the morphology of a phagocytic

macrophage (Bo et al., 1994a; Ulvestad et al., 1994a). The potential roles for microglia in demyelination resemble those of macrophages:

1. Microglia may act as antigen-presenting cells for $CD4^+$ T lymphocytes. The low levels of MHC class II molecules constitutively expressed by these cells are up-regulated in active MS lesions due to the release of IFN-γ (Vass and Lassmann, 1990; Williams et al., 1993,1994b; Bo et al., 1994a; Ulvestad et al., 1994a). These cells also express B7 co-stimulatory molecules (Williams et al., 1993, 1994b; De Simone et al., 1995), molecules which are not typical of CNS tissue. Expression of B7 molecules can be up-regulated by stimulation with IFN-γ (Williams et al., 1994a,b; De Simone et al., 1995). This finding is especially important when it is remembered that activated T lymphocytes produce IFN-γ. The administration of monoclonal antibody specific for this co-stimulatory molecule inhibits antigen presentation by microglia *in vitro* (Williams et al., 1994b).
2. Activated microglia produce cytokines, such as TNF- α (Hartung et al., 1995b), which are capable of damaging myelin and oligodendrocytes.
3. Microglia express receptors for the immunoglobulin constant region suggesting a role in antibody-dependent cell mediated cytotoxicity. These receptors are more frequent in MS plaques than in the surrounding "normal" tissue (Ulvestad et al., 1994a,b,c).
4. Microglia also express complement receptors and some components of complement (Levi-Strauss and Mallat, 1987; Ulvestad et al., 1994a),

indicative of a role in complement-mediated lysis. It has been demonstrated that activated rat microglia are able to phagocytose MBP in the presence of complement (Zajicek et al., 1992a).

5. These cells express the adhesion molecules, ICAM-1, LFA-1, and LFA-3, (Williams et al., 1993; De Simone et al., 1995).
6. Microglia also produce nitric oxide and oxygen radicals (Griot et al., 1989; Merrill et al., 1993; Brosnan et al., 1994).

1.7 Hypotheses of Demyelination in Multiple Sclerosis

The cause of multiple sclerosis is still unknown. Many investigators believe MS to be an autoimmune disease, while others point to a viral etiology. Several hypotheses have been put forward to explain the inability to identify a specific antigen or infectious agent responsible for the initiation of MS. It is possible that multiple sclerosis is not actually the result of a single infectious agent or autoantigen, but instead represents a common immunopathologic path resulting from a multitude of antigens (Kennedy and Steiner, 1994; Sobel, 1995). This may explain the heterogeneity with respect to clinical symptoms, pathology, and response to immunomodulatory treatments observed between patients with multiple sclerosis (Lassmann and Vass, 1995; Bieganowska et al., 1997; Lassmann et al., 1998). In contrast, it could be a common endpoint for a variety of immunological mechanisms (Lucchinetti et al., 1996). It is also possible that the target

antigen(s) in multiple sclerosis is an as yet unknown protein, or infectious agent (Bansil et al., 1995).

1.7.1 Autoimmune Hypothesis

The majority of investigators believe that multiple sclerosis is an autoimmune disease. Perhaps the strongest "evidence" supporting such a conclusion is first, that EAE can be induced in susceptible animals through immunization with components of nervous tissue or even nervous tissue homogenates and second, that MS is associated with over-representation of HLA-DR2. Some investigators suggest that the failure to identify an infectious agent responsible for multiple sclerosis is also evidence of an autoimmune pathology.

It has already been established that only activated T lymphocytes are able to gain access to the CNS. Of the activated T lymphocytes which enter the CNS only those specific for CNS antigens remain. As myelin components are sequestered in the CNS, these T lymphocytes must be activated in the periphery. Putting the requirements for activation and CNS specificity together with the autoimmunity theory leads to a number of probable mechanisms by which activation of T lymphocytes can lead to the inflammation and demyelination characteristic of multiple sclerosis. These include molecular mimicry, activation by superantigens, and T cell/T cell activation.

1.7.1.1 Molecular Mimicry

Molecular mimicry is best explained as the sharing of an epitope(s) between a virus or bacterium which is capable of inducing an immune response against the host's self proteins (Wucherpfennig et al., 1991). Therefore, an immune response may

eliminate the immunogen which initiated the response but may also lead to cross-reaction with one or more self-antigens which share an epitope(s) with the immunogen (von Herrath and Oldstone, 1996). In this way, an immune response directed at a viral or bacterial antigen can inadvertently activate autoreactive cells. This may be the case in MS. Thus, the T lymphocyte specific for a CNS constituent would be "accidentally" activated by a viral or bacterial antigen with homology for the autoantigen, would cross the BBB, become reactivated by its autoantigen, and the inflammatory process would ensue.

There is evidence of molecular mimicry in multiple sclerosis. Wucherpfennig and Strominger (1995) found that MBP-specific T lymphocyte clones isolated from MS patients could also be stimulated with seven viral peptides and a bacterial peptide, including peptides from Epstein-Barr virus, influenza, adenovirus, and *Pseudomonas aeruginosa*. Atkins et al. (1990) found sequence homologies between rubella and measles and PLP. These results led both groups to conclude that it is unlikely that MS is the result of autoimmunity initiated by a single virus. Banki et al. (1994) demonstrated cross-reactive epitopes between recombinant human transaldolase and human T cell lymphotropic virus type I (HTLV-I). Alvord (1985) and Jahnke et al. (1985) identified sequence homologies between a number of viruses, Epstein-Barr virus, influenza, adenovirus, and measles, and MBP. Talbot et al. (1996) demonstrated that T lymphocyte lines isolated from MS patients were cross-reactive for MBP and coronavirus.

Lodge et al. (1996) suggested that molecular mimicry would explain the induction of MS if the immune response were directed against a viral or bacterial antigen which mimics an epitope of myelin which is normally hidden, that is a cryptic epitope.

However, with the finding that the T cell receptor is able to recognize peptides which have limited if any sequence homology (Hemmer et al., 1998b), the number of potential molecular mimics increases dramatically.

1.7.1.2 Superantigens

It has also been suggested that autoimmunity may result from the activation of autoreactive T lymphocytes by superantigens (Brocke et al., 1994,1996). Superantigens are proteins produced by microorganisms which are capable of "linking" the class II MHC molecule on the antigen presenting cell with the Vβ portion of the T cell receptor. This "linkage" is independent of the specificity of the T lymphocyte or the MHC class II molecule as the superantigen does not bind to the peptide-binding groove of the MHC molecule (Scherer et al., 1993). The binding of a superantigen is restricted only by the Vβ expressed by the T cell receptor. For example, staphylococcal enterotoxin A can activate T lymphocytes bearing T cell receptors with the Vβ3, 12, 14, 15, 17, or 20 chains. If we assume that an individual expresses Vβ1-60, and that each of these Vβ gene segments is equally expressed, then 1 out of every 60 T lymphocytes expresses a given Vβ chain in its T cell receptor. Therefore, superantigens are capable of activating a large number of αβ T lymphocytes in an individual (Scherer et al., 1993). In this situation, the superantigen-activated T lymphocyte would cross the BBB, be reactivated by its corresponding autoantigen and induce inflammation.

Hermans et al. (1997) found that MBP-specific T lymphocyte clones isolated from MS patients produced IFN-γ after stimulation with staphylococcal enterotoxin B (a superantigen) and that the amount of IFN-γ produced was comparable with the amount produced after stimulation with MBP. A study by Zhang et al. (1995) found MBP-reactive T lymphocyte clones capable of responding to staphylococcal enterotoxins A and B and toxic shock syndrome toxin-1.

1.7.1.3 Epitope Spreading

Epitope spreading is an interesting phenomenon that may explain why MS patients have T and B lymphocytes specific for so many CNS antigens and why patients have relapses. Simply defined, epitope spreading is the "...development of immune responses to endogenous epitopes secondary to the release of self-antigens during a chronic autoimmune or inflammatory response" (Vanderlugt and Miller, 1996). Epitope spreading in MS could be the result of myelin degradation. With the subsequent release of myelin components, cryptic epitopes are "uncovered". Therefore, autoreactive T lymphocytes specific for cryptic myelin epitopes are now able to recognize their specific antigens (Lehmann et al., 1992; Vanderlugt and Miller, 1996). Epitope spreading has been demonstrated in MS by the finding that T lymphocytes isolated from MS patients are specific for multiple MBP or PLP epitopes (Olsson et al., 1992; Correale et al., 1995b).

It must be noted that epitope spreading is not cross-reactivity. The autoreactive T lymphocytes are activated by the self-antigens released during the disease process, regardless of the antigen which gave rise to the initial immune response.

1.7.1.4 T Cell/T Cell Activation

There is some evidence for T cell/T cell activation. Activated T lymphocytes can activate nearby, resting T lymphocytes via adhesion molecules (Brod et al., 1990). In this case, the activated T lymphocyte could activate a resting autoreactive T lymphocyte. This theory does not place any restrictions on the viral or bacterial antigens activating the first T lymphocyte. There are no requirements that these antigens share any sequence homology with the autoantigens for which the autoreactive T lymphocyte is specific (Wucherpfennig et al., 1991)

1.7.2 Viral Hypothesis

There are researchers who believe that multiple sclerosis is a viral disease and that the inflammatory response observed in the disease is purely an anti-viral response (Wucherpfennig et al., 1991). To date there is no conclusive evidence which links a specific virus or viruses to multiple sclerosis (Lucchinetti and Rodriguez, 1997), but there is indirect evidence which implicates environmental factors in the acquisition of the disease (Cook et al., 1996).

1. Intrathecal synthesis of antibodies against viral antigens is detectable is approximately 80 percent of patients with multiple sclerosis (Schadlich et al., 1990). As well, a number of studies have shown that titers of measles antibodies in both the serum and CSF are higher in MS patients than in normal controls (Adams and Imagawa, 1962; Schadlich et al., 1987). However, these increased antibody titers do not represent the major antibody response in multiple sclerosis (Owens et al., 1997).

2. The presence of oligoclonal bands in CSF is not exclusive to MS patients and similar bands have been observed in CNS infections, such as syphilis and subacute sclerosing panencephalitis (Antel and Arnason, 1991; McFarland and McFarlin, 1995). In most of these diseases the oligoclonal bands are specific for the virus responsible for disease induction (Vandvik et al., 1976). For example, in subacute sclerosing panencephalitis the majority of the oligoclonal bands are antibodies against measles virus. Gilden et al. (1996) stated that the presence of oligoclonal bands in CSF is found almost exclusively in infectious disorders of the CNS.
3. The majority of multiple sclerosis patients are positive for Epstein-Barr virus antibodies (Dalgleish, 1997).
4. There are DNA and RNA viruses capable of causing spontaneous demyelination (Cook et al., 1996).
5. There appears to be a world-wide pattern of MS prevalence, with a north to south gradient in North America and Europe, a low prevalence in Asia, Africa, and South America, and a south to north gradient in Australia (Kurtzke, 1993).

Many viruses have and are being considered as candidates for multiple sclerosis induction based on their ability to explain the worldwide distribution of MS, their ability to induce demyelination in humans or animals, or the fact that antibodies against the virus are elevated in the serum and CSF of MS patients, their viral genome has been identified in MS brain, or they have been isolated from MS tissue (Burks et al., 1980; Murray et al.,

1992; Cook et al 1995,1996). These viruses include measles, human coronavirus, Epstein-Barr virus, several retroviruses, and human herpes virus type 6. The evidence implicating these viruses in multiple sclerosis is "sketchy" to say the least.

There are a variety of ways in which viral infections could give rise to the damage to myelin sheaths observed in multiple sclerosis.

1. The bystander effect. The damage incurred by the CNS may result from an immune system attack against a virus residing in the CNS, such as measles or rabies (Johnson et al., 1984; Steinman and Conlon, 1997).
2. During an anti-viral response, immune cells may gain access to the CNS, and encounter CNS antigens. As these antigens are normally sequestered, the immune cells would recognize them as foreign and attack them (Steinman and Conlon, 1997).
3. Viral infections may activate transcription in a hypermutable area of the genome, such as an Alu repeat. This may result in the expression of neoantigens which the immune system would treat as foreign and attack (Panning and Smiley, 1995; Archelos et al., 1998).
4. Systemic viral infections often cause cytokine release by immune cells. These cytokines may induce changes in the endothelial cells of the CNS, such as the up-regulation of adhesion molecules, which could in turn effect inflammation and demyelination of the CNS (Allen and Brankin, 1993; Cristallo et al., 1997).

5. Viruses capable of causing latent or persistent infections may lead to chronic stimulation of autoreactive T lymphocytes via epitope spreading (Kennedy and Steiner, 1994; Wucherpfennig and Strominger, 1995; Miller et al., 1997).

1.8 Remyelination of Multiple Sclerosis Plaques

For many years myelin repair or remyelination was not thought to occur in multiple sclerosis or was believed to be so negligible that it was unimportant. This opinion has changed. Numerous studies have reported the presence of shadow plaques, thinly myelinated nerve fibers with short internodes at the edge of inactive plaques (Perier and Gregoire, 1965; Suzuki et al., 1969; Gledhill and McDonald, 1977; Lassmann, 1983; Prineas et al., 1993a; Rodriguez et al., 1993; Bruck et al., 1994; Rodriguez and Scheithauer, 1994). These observations are now known to represent remyelination.

Remyelination is now believed to be a common occurrence in multiple sclerosis lesions (Storch and Lassmann, 1997). Evidence of myelin repair is found in both chronic and acute MS lesions (Raine, 1994,1997b), but is typically observed at the edge of chronic (inactive) plaques (Raine 1997b; Lassmann et al., 1998). Remyelination can occur simultaneously with demyelination (Lassmann, 1983; Ghatak et al., 1989; Prineas et al., 1993a; Rodriguez and Scheithauer, 1994), and in the presence of inflammation (Rodriguez and Scheithauer, 1994; Hunter and Rodriguez, 1995). Usually remyelination repairs only a small area of a plaque, but areas of extensive myelin repair have been seen (Lassmann, 1983; Prineas et al., 1993a; Rodriguez and Scheithauer, 1994; Raine, 1997b).

It has been suggested that entire plaques may be repaired (Lassmann et al., 1998). The myelin sheath produced by remyelination is chemically and structurally normal, but is always shorter and thinner than that found in normal CNS tissue (Ludwin, 1996,1997).

The extent of remyelination varies between multiple sclerosis patients. In general, spontaneous remyelination occurs in MS plaques which have a high density of oligodendrocytes (Bruck et al., 1994; Ozawa et al., 1994), and is more effective and complete in MS lesions in the early stages of disease (Lassmann, 1983; Prineas, 1985; Raine and Wu, 1993). Remyelinated plaques are not protected against further demyelination (Prineas et al., 1993a; Raine 1997b) and are just as susceptible to demyelination as any normal myelinated areas of the CNS.

Very little is known about what triggers remyelination to begin or to terminate, why some lesions remyelinate, while others do not, or why remyelination in MS lesions is typically incomplete. It is known that myelin repair requires proliferation of an adequate number of oligodendrocytes which have access to the axon(s) (Ludwin, 1996,1997; Raine, 1997b). It has been suggested that demyelinated lesions remyelinate unless they are interrupted by recurrent demyelination (Prineas et al., 1993a) or are somehow prevented from doing so by the presence of certain immune factors in the demyelinated lesion (Ludwin, 1980; Rodriguez and Lindsley, 1992). Prineas et al. (1993b) suggested that permanent myelin loss may be partly due to recurrent demyelination in a previously remyelinated area. Another possibility is that there are "remyelination promoting factors" present in some MS lesions. Therefore, lesions devoid of such factors would not undergo remyelination (Rodriguez and Lindsley, 1992).

For example, insulin-like growth factor has been shown to enhance oligodendrocyte regeneration in culture and increase remyelination in an animal model of MS (Yao et al., 1995). A simpler explanation is that there are not enough oligodendrocytes present in the plaque to carry out the amount of myelin repair needed (Ludwin, 1996,1997) or that the remyelination process is eventually overwhelmed and demyelination again ensues (Raine, 1994).

There is still much debate as to whether the myelin repair observed in multiple sclerosis is the work of mature oligodendrocytes or progenitor cells (Ludwin, 1996,1997; Lassmann et al., 1997). Coyle (1996) suggested that both mature oligodendrocytes and progenitor cells may be responsible for remyelination.

Remyelination in young patients or patients with acute or early MS may be rapid and extensive (perhaps even repairing the entire lesion) and may explain the quick recovery from relapses often observed in such patients (Lassmann, 1983; Lassmann et al., 1998). Remyelination may also play a role in remissions observed in relapsing/remitting patients regardless of age or length of illness.

1.9 Treatment of Multiple Sclerosis

To date there is no cure for multiple sclerosis or even a treatment that can completely stop progression of the disease. There are therapies that can treat symptoms, reduce exacerbation rates, and/or halt the formation of new lesions. The most common treatments are the administration of steroids, interferons, immunosuppressives, or immunomodulating agents. There are also a few treatments which may be effective but

are not yet widely used, these include tolerance induction, the administration of chimeric monoclonal antibodies, and T lymphocyte vaccination. In general, the agents used in the treatment of multiple sclerosis were derived from the EAE models. A major problem in the clinical testing of therapeutic agents in multiple sclerosis is the unpredictable nature of the disease. It is often difficult to conclude if improvements are due to the action of the agent being tested or if they are part of the natural course of the disease.

1.9.1 Steroids

Panitch (1996) refers to steroids, such as methylprednisolone, and agents which induce steroid production, such as adrenocorticotropic hormone (ACTH), as the mainstay of MS therapy for the past 20 years. These drugs do not have an immunomodulating effect but rather act by improving the conduction through a demyelinated nerve, and/or reducing the edema and inflammation in the MS lesion (Antel and Arnason, 1991). Steroids and ACTH are able to reduce the duration of exacerbations, but seem to have no effect on the degree of recovery (Miller et al. 1961; Rose et al., 1970; Durelli et al., 1986; Milligan et al., 1987).

1.9.2 Global Immunosuppression

Drugs which cause non-specific immunosuppression, including azathioprine and methotrexate, have been used in the treatment of multiple sclerosis for a number of years. The effectiveness of these drugs is still controversial. The general immune suppression which results from taking these drugs may have serious consequences. Studies of azathioprine have shown conflicting results with regard to the benefits of taking the drug. A review of the most controlled clinical trials concluded that patients given azathioprine

have a greater chance of being relapse free, than patients who are not receiving the drug (Yudkin et al., 1991). Methotrexate reduces the exacerbation rates in some relapsing/remitting MS patients (Currier et al., 1993) and is able to slow the rate of disease progression in some chronic progressive patients (Goodkin et al., 1995).

1.9.3 Interferons

Several recombinant interferons have been used in clinical trials. IFN alpha-2a was shown to reduce the clinical exacerbation rate and the development of new lesions. It was also able to prevent the existing lesions from becoming larger (Durelli et al., 1994). Even though IFN alpha-2a is effective in the treatment of multiple sclerosis, the majority of research has focused on the IFN beta-1a and -1b as therapeutic agents. IFN beta-1a reduces the exacerbation rate and the number and volume of MS lesions in relapsing/remitting multiple sclerosis (Jacobs, et al., 1995,1996; Pozzilli et al., 1996). A more recent study, indicates that treatment with IFN beta-1a not only reduces exacerbations, but can also delay the progression of the disease (PRISMS Study Group, 1998). Similarly, IFN beta-1b reduces the rate and severity of relapses and the number of active lesions in relapsing/remitting MS (Paty and Li, 1993). In addition, IFN beta-1b has recently been shown to be effective in delaying the sustained neurological deterioration observed in secondary progressive multiple sclerosis patients (European Study Group on Interferon β-1b in Secondary Progressive MS, 1998).

These clinical trials are evidence that IFN beta can alter the course of multiple sclerosis possibly through the down-regulation of pro-inflammatory cytokines and/or the up-regulation of anti-inflammatory cytokines (Arnason et al., 1997).

1.9.4 Copolymer-1

Copolymer-1 (Cop-1) is a synthetic polypeptide randomly composed of tyrosine, glutamate, alanine, and lysine. It was produced as a mimic of the antigenic properties of MBP, but was later found to be able to down-regulate EAE and to inhibit MBP- and MOG-specific T lymphocyte lines isolated from mice and humans (McFarland and McFarlin, 1995; Polman and Hartung, 1995; Ben-Nun et al., 1996). Administration of Cop-1 to relapsing/remitting multiple sclerosis patients caused a reduction in relapses and improved disability (Bornstein et al., 1987; Johnson et al., 1995). Copolymer 1 is not as effective in the treatment of chronic progressive MS patients (Bornstein et al., 1991).

Copolymer 1 is believed to compete with MBP and MOG for binding to MHC class II molecules and can even displace MBP that is already bound to MHC class II molecules (Teitelbaum et al., 1988; Racke et al., 1992a; Fridkis-Hareli et al., 1994; Ben-Nun et al., 1996). It has also been demonstrated that monoclonal antibodies have cross reactivity between myelin basic protein and Cop-1 (Teitelbaum et al., 1991).

1.9.5 Tolerance Induction

A few studies have been undertaken to analyze the effects of the oral administration of myelin. Weiner et al. (1993) found that bovine myelin given orally to relapsing/remitting MS patients reduced relapses in almost 50 percent of the subjects and reduced the frequency of MBP-specific T lymphocytes. Men, who were HLA-DR2 negative received the most benefit from this treatment. More recent studies have found that the oral administration of bovine myelin to relapsing/remitting MS patients induced a change in the cytokine pattern of the T lymphocytes. T lymphocytes isolated from these

subjects showed a higher frequency of TGF-β1 secreting cells after stimulation with MBP or PLP, than T lymphocytes from MS patients who were not given oral myelin (Fukaura et al., 1996; Hafler et al., 1997).

Tolerance can be induced via other routes as well. Warren et al. (1997) were able to induce tolerance to MBP by single or repeated intravenous injections of a soluble MBP peptide as indicated by the disappearance of anti-MBP antibodies

1.9.6 Chimeric Anti-CD4 Monoclonal Antibody

The administration of a chimeric anti-CD4 monoclonal antibody to chronic progressive MS patients was shown to decrease the number of circulating $CD4^+$ T lymphocytes and to prevent the development of new lesions (Lindsey et al., 1994; Moreau et al., 1994). These antibodies consist of a human constant region coupled with a mouse variable region. Two of these chimeric antibodies have actually been tested, CAMPATH-1H (anti-CDw52) and CM-T412. The CM-T412 is thought to be the most promising as its side effects are less devastating (Lindsey et al., 1994).

1.9.7 T Lymphocyte Vaccination

The immunization of multiple sclerosis patients with irradiated, autologous, MBP-specific T lymphocytes leads to the depletion of circulating MBP-reactive T lymphocytes in the subjects (Zhang et al., 1993; Medaer et al., 1995). In relapsing/remitting MS patients this immunization decreased exacerbations and stabilized the lesions (Medaer et al., 1995). No effects were noticed with chronic progressive patients.

1.10 Animal Models of Multiple Sclerosis

There are several animal models of multiple sclerosis. The three most investigated and best representative of multiple sclerosis are the experimental allergic (autoimmune) encephalomyelitis model, the coronavirus model, and the Theiler's murine encephalomyelitis virus (TMEV) model.

1.10.1 Experimental Allergic (Autoimmune) Encephalomyelitis

Experimental allergic (or autoimmune) encephalomyelitis is an animal model of multiple sclerosis which is typically induced in rodents, mainly rats and mice. This disease can also be induced in marmosets but differs in many ways from the disease produced in rodents, therefore the marmoset model will be discussed separately.

1.10.1.1 Induction of Experimental Allergic Encephalomyelitis

Experimental allergic encephalomyelitis can be induced in rodents by the injection of CNS homogenates (Brown and McFarlin, 1981; Brown et al., 1982; Munoz et al., 1984), the injection of CNS myelin proteins, such as PLP (Yamamura et al., 1986; Satoh et al., 1987), MBP (Panitch and Ciccone, 1981; Pender, 1988), or MOG (Johns et al., 1995), or by the injection of peptides of MBP (Fritz et al., 1983; Sakai et al., 1988a), PLP (Tuohy et al., 1989; Greer et al., 1992), or MOG (Johns et al., 1995; Kerlero de Rosbo et al., 1995; Mendel et al., 1996). EAE has also been induced in (PL/J X SJL/L)F_1 mice by using a viral peptide with limited homology (only 5 amino acids) to MBP (Gautam et al., 1998). In each case the antigens are emulsified in complete Freund's adjuvant before injection. In the murine models it is often necessary to administer *Bordetella pertussis* as an ancillary adjuvant. This microorganism causes an increase in

BBB permeability through the action of a histamine sensitizing factor (Linthicum, 1982). The ability to produce EAE by immunizing animals with CNS tissue homogenates or proteins strongly suggests that MS is an autoimmune disease.

EAE can also be adoptively transferred to naive, syngeneic animals by $CD4^+$ T lymphocytes activated *in vitro* by MBP, PLP, and MOG (Paterson, 1960; Pettinelli and McFarlin, 1981; Mokhtarian et al., 1984; Vandenbark et al., 1985; Zamvil et al., 1985; Satoh et al., 1987; Mendel et al., 1995,1996) or by a synthetic peptide of MBP (Sun et al. 1992).

1.10.1.2 The Course of Experimental Allergic Encephalomyelitis

The amount of demyelination, the clinical course and the symptoms of EAE vary with the species and strain of animal used, the inducing antigen, the route of inoculation, the dose of antigen, the age of the animal at the time of sensitization, and the sex of the animal (Stone et al., 1969; Lassmann and Wisniewski, 1979; Lublin, 1996).

The symptoms of EAE generally include weight loss, hind limb paralysis, loss of tail tonicity, and hind and forelimb paralysis (Swanborg 1988; Goverman and Brabb, 1996). For some animals the induction of EAE is fatal, others recover and are resistant to further induction, while others develop a chronic relapsing disease.

In general, mice develop a chronic relapsing disease with considerable inflammation and demyelination (Mokhtarian et al., 1984; Zamvil et al., 1985). By contrast, rats develop an acute monophasic disease with extensive inflammation but little demyelination (Lassmann and Linington , 1987; Schluesener et al., 1987; Martin et al.,

1992a). However, there are many variations of EAE producible in rodents including a chronic relapsing disease with considerable demyelination in rats.

1.10.1.3 The Immunology of Experimental Allergic Encephalomyelitis

The immunology of experimental allergic encephalomyelitis is in many ways identical or very similar to that of multiple sclerosis. In fact, much of what is known of the immunology of multiple sclerosis comes from investigations in this animal model.

1.10.1.3.1 The T Lymphocyte in Experimental Allergic Encephalomyelitis

EAE is mediated by $CD4^+$ T lymphocytes of the T_h1 subset which are specific for myelin antigens (Pettinelli and McFarlin, 1981; Swanborg, 1983; Ando et al., 1989; Baron et al., 1993; Wekerle et al., 1994; Bradl and Linington, 1996; Goverman and Brabb, 1996). It is interesting then that encephalitogenic T lymphocytes specific for myelin are found in normal Lewis rats and probably in other rodents as well (Wekerle et al., 1994). There are a number of observations which show that the development of EAE is due to the actions of T_h1 lymphocytes:

1. MBP-reactive T lymphocytes are found in fresh relapsing lesions but not in silent chronic lesions (Cross et al., 1993a).
2. Labelled MBP-sensitized T lymphocytes can be seen to form part of the inflammatory infiltration of the CNS during acute and early chronic EAE (Cross et al., 1990)
3. The majority of cells in the inflammatory infiltrate are $CD4^+$ T lymphocytes (Stinissen et al., 1997).

4. Adoptive transfer of activated $CD4^+$ T lymphocyte lines induces EAE (Pettinelli and McFarlin, 1981).
5. Antibodies against CD4 inhibit EAE (Waldor et al., 1985).
6. Encephalitogenic T lymphocyte lines express and secrete IFN-γ, TNF-α and -β, and IL-2, cytokines known to be secreted by T_h1 lymphocytes (Ando et al., 1989; Powell et al., 1990; Mustafa et al., 1991,1993).
7. EAE cannot be passively transferred by the injection of serum (Paterson, 1971).
8. The induction of T lymphocyte tolerance to MBP prevents the development of EAE (Miller et al., 1991).

However, these T lymphocytes cannot give rise to inflammation and demyelination without crossing the BBB and entering the CNS. The series of events concerning the entry of T lymphocytes into the CNS and subsequent induction of inflammation in EAE are very similar to those of multiple sclerosis. In fact, the majority of information regarding this process in MS has been elucidated and extrapolated from the animal models.

When an animal is inoculated with a CNS antigen, antigen presenting cells in the draining lymph nodes process the antigen and present it to $CD4^+$ T lymphocytes in the context of MHC class II (Lublin, 1996). This leads to the activation of $CD4^+$ T lymphocytes specific for CNS antigen. Alternatively, CNS antigen-specific T lymphocytes may be activated *in vitro* and then injected into the animal. In either case, the activated T lymphocytes home to the CNS (Cross et al., 1990). In fact after adoptive

transfer, MBP-responsive T lymphocytes are among the first cells to infiltrate the CNS (Raine, 1994). Activated T lymphocytes are able to enter the CNS regardless of their antigen specificity, or their ability to distinguish their particular antigen from the other CNS components (Wekerle et al., 1986; Sedgwick et al., 1990; Hickey et al., 1991).

T lymphocytes are able to breach the BBB in large part due to the up-regulation of adhesion molecules, such as VCAM-1 and ICAM-1, on the endothelial cells of the CNS and the up-regulation of their respective ligands, VLA-4 and LFA-1, on the activated T lymphocytes (Baron et al., 1993; Dopp et al., 1994). The importance of adhesion molecules in EAE is shown by the changes in adhesion molecule expression during the disease. For example, up-regulation of ICAM-1 on endothelial cells and LFA-1 on $CD4^+$ T lymphocytes in the CNS occurs during acute disease. These adhesion molecules are subsequently down-regulated during remissions and are up-regulated again during any further relapses (Cannella et al., 1990,1991). As well, several studies using monoclonal antibodies against various adhesion molecules have also shown these molecules to be necessary for the production of EAE. Archelos et al. (1993) showed that the administration of anti-ICAM-1 antibodies reduced CNS inflammatory infiltrates and suppressed EAE. Yednock et al. (1992) prevented the accumulation of leukocytes in the CNS, and therefore the development of EAE, through the injection of anti-VLA-4 antibodies. Baron et al. (1993) demonstrated that a loss of VLA-4 molecules on T lymphocytes, reduced their ability to enter the CNS. Studies using anti-LFA-1 monoclonal antibodies have found conflicting results. Cannella et al. (1993) found that the administration of anti-LFA-1 antibodies had adverse effects in one group of EAE

mice tested, but overall there was no significant effect on EAE. In contrast, Welsh et al. (1993) found that anti-LFA-1 antibodies increased the severity of EAE and even resulted in early mortality in some animals. Cannella et al. (1993) suggested that the effects of anit-LFA-1 antibodies were dependent upon the stage of disease at which they were injected. In addition, the onset of signs of EAE has been shown to correlate with the attachment and infiltration of lymphocytes, mostly LFA-1 positive T lymphocytes, and the appearance of ICAM-1 on vessels of the CNS (Raine et al., 1990). These authors also observed that pseudopodia from lymphocytes attach and penetrate endothelial cells of the CNS.

Once the activated T lymphocytes have crossed the BBB and entered the CNS, those which are specific for CNS antigens are reactivated by resident antigen-presenting cells in the CNS, such as microglia (Hickey and Kimura, 1988; Hohlfeld et al., 1995) and remain in the CNS. T lymphocytes which are unable to detect their specific antigens within the CNS traffic out. The re-stimulated T lymphocytes initiate an inflammatory response through the release of an array of cytokines, including TNF-α and IFN-γ. These cytokines lead to the increased permeability of the BBB to serum proteins and immunoglobulin, the recruitment of B lymphocytes, macrophages, and other T lymphocytes, the up-regulation of adhesion molecules on endothelial cells, and the up-regulation of MHC class II molecules on local antigen-presenting cells (Powell et al., 1990; Wekerle et al., 1994; Hohlfeld et al., 1995; Lublin, 1996). In EAE this inflammatory pathology is generally associated with neurologic deficit (Wekerle et al., 1994).

A. Cytokine Production by T Lymphocytes in Experimental Allergic Encephalomyelitis

It is suggested that the cytokine patterns of lesions in EAE vary depending upon the stage of disease. During early disease pro-inflammatory cytokines, such as IL-1, IL-2, TNF-α, and IFN-γ are dominant while anti-inflammatory cytokines, such as TGF- β and IL-10 are almost absent. During the recovery phase the levels of these cytokines switch with TGF-β and IL-10 dominating and the inflammatory cytokines being down-regulated (Khoury et al., 1992; Kennedy et al., 1992). Kennedy et al. (1992) also demonstrated that the peak of mRNA expression for IL-2, Il-4, IL-6 and IFN-γ occurs before the peak of clinical severity. Declines in the mRNA levels of these cytokines occurred concomitantly with an increase in the IL-10 mRNA level. These changes were associated with stabilization of clinical symptoms. The importance of cytokines in EAE, particularly TNF-α and IFN-γ, has been demonstrated in many experiments. TNF is believed to be the most important cytokine in the pathogenic process in EAE (Lublin, 1996). Transgenic mice over-expressing TNF-α spontaneously develop a chronic inflammatory demyelinating disease and eventually die due to worsening neurological symptoms (Probert et al., 1995). TNF-α causes myelin damage and is cytotoxic to oligodendrocytes (Selmaj and Raine, 1988; Zajicek et al., 1992a). Lymphotoxin also injures oligodendrocytes and is more potent than TNF-α (Selmaj et al., 1991b). TNF also promotes up-regulation of adhesion molecules. Anti-TNF antibodies have been shown to reduce or prevent up-regulation of VCAM-1 (Barten and Ruddle, 1994). Antibodies against TNF or the TNF receptor can inhibit the development of EAE (Ruddle et al.,

1990; Selmaj et al., 1991c; Baker et al., 1994; Barten and Ruddle, 1994; Selmaj and Raine, 1995). Levels of TNF have even been found to correlate with disease severity (Owens and Sriram, 1995).

A role for IFN-γ in EAE is indicated by numerous observations. An increase in cells producing IFN-γ and in the level of IFN-γ is seen during the early phase of EAE (Mustafa et al., 1991; Kennedy et al., 1992; Merrill et al., 1992). Administration of IFN-γ ameliorates EAE (Voorthuis et al., 1990; Kalman et al., 1992), while administration of anti- IFN-γ antibodies enhances EAE (Billiau et al., 1988; Lublin et al., 1993). IFN-γ is believed to up-regulate expression of class II MHC molecules and adhesion molecules of endothelial cells in the CNS (McCarron et al., 1986; Cannella et al., 1991; Baron et al., 1993) and is therefore important in the homing of lymphocytes into the CNS.

TGF-β is known to be important in the recovery phase of EAE. This cytokine inhibits IFN-γ induced up-regulation of class II MHC molecules and inhibits T lymphocyte activation (Lublin, 1996). By inhibiting the activation of T lymphocytes, TGF-β is able to block the production of IFN-γ and lymphotoxin by encephalitogenic T lymphocytes (McDonald and Swanborg, 1988; Karpus and Swanborg, 1989; Stevens et al., 1994). TGF-β also interferes with the active and adoptive transfer of EAE (Kuruvilla et al., 1991; Johns et al., 1991; Stevens et al., 1994). For example, the induction of EAE by MBP or PLP can be prevented by TGF-β secreting T lymphocyte clones isolated from animals tolerized with MBP (Chen et al., 1994). Administration of TGF-β has been shown to reduce the severity of EAE, to prevent the development of EAE, and to reduce

the occurrence of relapses (Johns et al., 1991; Kuruvilla et al., 1991; Racke et al., 1991). Animals treated with anti- TGF-β antibodies showed worsening of clinical and pathological features of EAE (Racke et al., 1992b; Johns and Sriram, 1993).

B. T Cell Receptor Usage in Experimental Allergic Encephalomyelitis

As in multiple sclerosis, the issue of TCR restriction in EAE models is unresolved and complex. The TCR response varies with the species and strain of animal used, as well as with the antigen and even the epitope used. Many authors found evidence of preferential usage of the Vβ8 chain of the T cell receptor in PL/J mice (Acha-Orbea et al., 1988; Urban et al., 1988; Zamvil et al., 1988). Later it was observed that the response to MBP1-9 in the PL/J mouse was restricted to Vβ8.2 (Zamvil and Steinman, 1990). However, deletion of the Vβ8 TCR from PL/J mice was not able to prevent the development of EAE in all the mice tested, as a small percentage of the $V\beta8^-$ mice did present with EAE (Zamvil et al., 1988; Kalman et al., 1993). Another study found that the use of anti-Vβ8.2 monoclonal antibodies could also suppress EAE in PL/J mice (Swanborg, 1995). Similar studies with SJL/J mice demonstrated that about 50 percent of the T cell clones specific for MBP89-101 from these mice express Vβ17a. However, depletion of T lymphocytes expressing this Vβ chain does not suppress EAE in this strain of mouse (Sakai et al., 1988b). It was concluded that these mice use a broad repertoire of Vβ elements (Sakai et al., 1988b; Su and Sriram, 1992).

Other investigators have not found TCR Vβ usage to be as restricted. For example, Mendel et al. (1995,1996) found that the TCRs of T lymphocyte lines from H-

2b mice (C57BL/6J and C3H.SW) immunized with MOG35-55 TCR used Vβ1, 2, 6, 8, 10, 14, 15, but Vβ 8.2 usage was preferential. T lymphocyte clones isolated from SJL/J mice immunized with PLP139-151 were found to use Vβ2, 6, 10, and 17a, with no preferential usage of Vβ chains (Kuchroo et al., 1992).

The same conflicting results are observed with studies of TCR usage in rats. The T lymphocyte response to MBP in Lewis rats has been shown to use Vβ8.2 exclusively (Chluba et al., 1989; Zhang and Heber-Katz, 1992). In contrast, Sun et al. (1992) found that T lymphocyte lines specific for synthetic peptide MBP87-99 did not use Vβ8. A study by Gold et al. (1992) found that lymph node T lymphocytes from Lewis rats responsive to MBP72-89 preferentially use Vβ8.2. They also found that the T cell receptor usage differs depending upon the location from which the cells are taken. T lymphocyte clones isolated from the spinal cord specific for synthetic peptides of MBP used Vβ6 predominantly, while T lymphocyte clones with the same specificity isolated from lymph nodes used an array of Vβ chains including Vβ6. Furthermore, Sun et al. (1994) reported that T lymphocyte lines from Lewis rats reactive with MBP68-88 demonstrated diverse TCR usage. They also found that the majority of Vβ8 positive cells were not encephalitogenic and that T lymphocytes expressing other T cell receptors were encephalitogenic.

It should be noted that the T cell receptor usage is not static during the course of the disease. Karin et al. (1993) used a T lymphocyte line specific for MBP68-86 which expressed Vβ8, 10, 5, 16, and 19 to induce EAE. They found that less than 24 hours

after injection of T lymphocytes the majority of Vβ transcripts in the brains of these animals were the same as those of the transferred T lymphocyte line. Two days after the transfer Vβ8 was predominant, but with the onset of paralysis the TCR usage became diverse with respect to Vβ. During the recovery phase the TCR usage was more restricted with Vβ8, 9, 10, and 19 predominating. Offner et al. (1993) also found that during the T lymphocyte response to MBP in Lewis rats Vβ usage changes over the course of the disease, especially as the rats recover.

In general, the TCR V gene usage is restricted within an individual animal, but there is much diversity of the TCR V gene usage between animals (Hafler et al., 1996). T cell receptor V gene usage also appears to differ with the location from which the T lymphocytes are isolated and the epitope to which the cells are exposed.

1.10.1.3.2 Other Immune Cells Implicated in Experimental Allergic Encephalomyelitis

T lymphocytes are not the only cells important in the production and perpetuation of EAE. Macrophages are also found in EAE lesions (Lassmann et al., 1993), in fact macrophages are the cell type most often associated with demyelinating lesions (Genain et al., 1995a). The inhibition of macrophage enzymes or the depletion of macrophages ameliorates the clinical signs of EAE and inhibits demyelination (Brosnan et al., 1981; Huitinga et al., 1990; Tran et al., 1998). Tran et al. (1998) concluded that the depletion of macrophages inhibited leukocytes from migrating across the BBB and/or inhibited the accumulation of these cells in the CNS. Macrophages may damage oligodendrocytes indirectly via the release of various cytokines, such as TNF-α, (Genain et al., 1995a) or

the release of nitric oxide or reactive oxygen species (Okuda et al., 1995; Ruuls et al., 1995). These cells have even been observed phagocytosing myelin in lesions of EAE (Lampert, 1978; Raine et al., 1980; Prineas and Graham, 1981). Just as important in EAE are B lymphocytes, or more specifically their antibodies. Lassmann et al (1983) found a correlation between the appearance of a humoral immune response in sera, CSF, and brain extracts with the development of large demyelinated plaques in chronic relapsing EAE. Although antibodies to MBP, MOG, and PLP have been identified in the CSF and serum of animals with chronic EAE (Lassmann and Linington, 1987; Sobel, 1995), the majority of studies have concentrated on the role of anti-MOG antibodies in EAE.

1.10.1.3.3 The Importance of MOG and Anti-MOG Antibodies in Experimental Allergic Encephalomyelitis

Before discussing the role of anti-MOG antibodies in EAE, the importance of the MOG protein in the induction of EAE will be addressed. A single injection of purified MOG or MOG peptide can produce relapsing/remitting EAE in rats with extensive demyelination (Johns et al., 1995). Antibodies isolated from the sera of these animals were reactive with MOG, but not MBP, MAG, or PLP and were able to degrade MBP *in vitro*. Chronic relapsing demyelinating EAE can also be induced by the injection of a synthetic MOG peptide (MOG35-55) (Ichikawa et al., 1996a). These experimenters were unable to induce disease with other synthetic MOG peptides. Injection of the peptide also induced a strong humoral response which was restricted to MOG35-55.

Prior to these studies it had been demonstrated that the transfer of T lymphocytes specific for MOG35-55 into naive Lewis rats led to CNS inflammation, and severe BBB

dysfunction, without demyelination or neurological deficit. However, co-transfer of these T lymphocytes with anti-MOG monoclonal antibodies induced a rapidly progressive form of EAE with extensive demyelination (Linington et al., 1993).

This was only one of an array of experiments which demonstrated the necessity of anti-MOG antibodies for demyelination and more severe EAE. Anti-MOG antibodies were identified in rats with chronic relapsing EAE induced by intrathecal injection of sera from guinea pigs with EAE (Linington and Lassmann, 1987). More importantly, the serum demyelinating activity of the rats *in vivo* was found to be proportional to the anti-MOG antibody titer. In 1992, Linington et al. used repeated co-transfer of MBP specific T lymphocytes and monoclonal anti-MOG antibody to induce chronic relapsing EAE in Lewis rats. Their observations showed that the formation of the large, focal demyelinated lesions seen in these animals was dependent upon an autoantibody response to an appropriate myelin antigen. They also injected anti-MOG antibodies into Lewis rats made increasingly resistant to further induction of EAE by repeated injections of MBP specific T lymphocytes. The administration of the monoclonal antibodies 4 days after each injection of T lymphocytes resulted in severe clinical relapses. Similarly, the injection of anti-MOG antibodies to Lewis rats after the onset of EAE induced by MBP specific T lymphocytes or T lymphocyte lines enhanced the clinical impairment and primary demyelination seen in these animals (Lassmann and Linington, 1987; Lassmann et al., 1988; Linington et al., 1988). EAE induced by MBP-specific T lymphocytes and anti-MOG antibodies appeared more quickly, progressed very rapidly, and was more severe than when induced by a similar number of T lymphocytes alone (Namer et al.,

1994). Anti-MOG antibodies can also induce demyelination when given intravenously to rats immunized with MBP (Schluesener et al., 1987; Piddlesden et al., 1993). The latter studies reported increased disease severity after the injection of the antibodies. Schluesener et al. (1987) also found that the injection of anti-MOG antibodies into SJL mice during remission induced fatal relapses. Levels of anti-MOG antibodies have been shown to be higher during relapses than in the initial disease (Morris et al., 1997).

The demyelination observed with anti-MOG antibodies cannot be repeated by the administration of anti-MBP, anti-PLP, or anti-MAG antibodies (Seil and Agrawal, 1980; Seil et al., 1981; Schluesener et al., 1987; Kerlero de Rosbo et al. 1990)

Many experimenters have concluded from studies such as these that myelin-reactive T lymphocytes and anti-MOG antibodies act synergistically. There is also direct evidence for this theory. A study of EAE induced in Lewis rats by MBP-specific T lymphocytes and anti-MOG antibody showed that the structure and size of the lesions was dependent upon the number of T lymphocytes which were injected into the animal (Lassmann et al., 1988). High numbers off T lymphocytes with anti-MOG antibody resulted in ubiquitous perivenous demyelination, whereas low number of T lymphocytes with anti-MOG antibody resulted in focal confluent demyelinated lesions. Severe demyelination in Lewis rats immunized with MOG35-55 was observed only in the presence of high levels of anti-MOG antibodies (Ichikawa et al., 1996b). This study also showed that the anti-MOG antibodies alone were not sufficient to produce clinical disease. It is believed that in the T lymphocyte-mediated model of EAE in the Lewis rat encephalitogenic T lymphocytes are responsible for inflammation in the CNS, but are not

capable of inducing demyelination. Demyelination depends not only upon the action of these T lymphocytes, but also upon the action of autoantibodies (Linington et al., 1992; Wekerle et al., 1994).

It is important to note that although anti-MOG antibodies appear to be important mediators in demyelination, the majority of the oligoclonal bands found in the sera and CSF of animals with EAE are specific for *Mycobacterium tuberculosis*, a component of the adjuvant used in the induction of the disease (Whitacre et al., 1981,1982; Mehta et al., 1985).

The question now becomes what is the mechanism responsible for the demyelination elicited by these autoantibodies? A number of mechanisms have been proposed including the activation of complement, enhanced phagocytosis of myelin by macrophages, and activation of myelin proteases with subsequent MBP degradation.

Anti-MOG antibodies can lyse cultured oligodendrocytes, but only in the presence of complement. If complement is removed or heat inactivated, lysis is no longer possible (Linington et al., 1989). This lysis was also found to be dependent upon the presence of the Fc portion of the antibody molecule as incubation of the oligodendrocytes with the $F(ab)'_2$ fragment of the antibody did not lyse the cells. These results are evidence of the importance of complement activation by anti-MOG antibodies. Other studies have used cobra venom factor, a decomplementation agent, to demonstrate the involvement of complement in demyelination. For example, Linington et al. (1989) were able to completely suppress the clinical signs of EAE by administering cobra venom factor to Lewis rats which had been immunized with MBP and given anti-MOG

monoclonal antibody. The injection of the decomplementation factor did not completely prevent demyelination. The authors suggested that the demyelination seen in these animals may be indicative of a role for macrophages in demyelination, or it may be the result of complement synthesized within the CNS by activated macrophages, or the activation of other complement components which were not susceptible to cobra venom factor. Piddlesden et al. (1991) also found that the injection of cobra venom factor into rats immunized with MBP suppressed the clinical signs of EAE and reduced inflammation in the CNS.

In 1990, Kerlero de Rosbo et al. concluded that the induction of demyelination by anti-MOG monoclonal antibodies does not occur in the absence of complement. Piddlesden et al. (1993) expanded upon this by demonstrating that the ability of anti-MOG antibodies to induce demyelination is related to their ability to fix complement.

Antibody-dependent cell mediated cytotoxicity involving macrophages and microglia is a major mechanism for antibody-mediated demyelination *in vivo* (Brosnan et al., 1981; Vass et al., 1992). Anti-MOG antibodies may enhance phagocytosis of myelin by macrophages via antibody-dependent complement activation on the myelin surface. This would lead to the release of complement derived macrophage activators and opsonization of the membrane with C3b. As macrophages express the receptor for this complement component, phagocytosis could result (Linington et al., 1989). There is also evidence of Fc receptor mediated phagocytosis in EAE as the injection of the $F(ab)'_2$ fragment of the anti-MOG monoclonal antibody did not affect the clinical course of EAE (Linington et al., 1989). However, this observation may instead support a role for

complement. Complement can also bind Fc, this may lead to Fc-receptor mediated macrophage attack. Alternatively, it has also been shown that anti-MOG antibodies can activate myelin proteases that are able to degrade myelin (Johns et al., 1995).

The role of anti-MOG antibodies in demyelination is complicated by studies showing that the lysis of rat oligodendrocytes *in vitro* is mediated by complement and can occur in the absence of anti-myelin antibodies (Scolding et al., 1989a,b; Zajicek et al., 1992b). Rat oligodendrocytes lack CD59, a complement regulatory protein, the absence of this protein is responsible for the antibody-independent complement mediated lysis observed in these experiments (Wing et al., 1992; Piddlesden and Morgan, 1993). In contrast, human oligodendrocytes are not sensitive to complement-mediated lysis in the absence of antibody and do express CD59 (Zajicek et al., 1995).

1.10.1.4 Is EAE a Good Model of Multiple Sclerosis?

There are, of course, arguments for and against the use of EAE as a model for multiple sclerosis. First of all, it must be remembered that EAE, as originally described, is a disease induced in inbred strains of rodents, while MS is a spontaneous disease in an outbred population (Raine, 1994; Goverman and Brabb, 1996). According to Dinter et al. (1997), using inbred animals restricts the immunological response and limits the validity of the model. It has also been suggested that the phylogenetic difference between rodents and humans may limit the usefulness of EAE as a model for a human disease (Massacesi et al., 1995). Another concern is that the etiology of EAE is in no way similar to that of MS (Wekerle et al., 1994).

When one compares the clinical aspects of EAE with those of MS, a number of differences are discovered. As yet there is no EAE model which presents with all the clinical and pathological aspects of multiple sclerosis (Wekerle et al., 1994; Genain and Hauser, 1997). Linington et al. (1989) stated that observations of animals with EAE often give conflicting results due to the comparison of models which have different modes of induction, clinical course and pathological responses.

In addition, conflicting results have been observed between the EAE models and MS. For example, rat oligodendrocytes do not express CD59 and are therefore susceptible to antibody-independent complement mediated lysis, whereas human oligodendrocytes do express CD59 and are not susceptible to such lysis. As well, IFN-γ ameliorates EAE (Voorthuis et al., 1990; Kalman et al., 1992), but when given to patients with MS it exacerbates the disease (Panitch et al., 1987). This brings into question the usefulness of the animal models for the prediction of treatment for multiple sclerosis patients.

Some experimenters believe that the EAE models of multiple sclerosis are not representative of the human disease. "Current studies of patients with MS indicate that the immunological mechanisms in MS are considerably more complicated than in EAE" (Utz and McFarland, 1994). "... a far reaching conclusion based, for example on an EAE model in a single species or strain (or on a single molecule such as MBP) will likely prove to be an oversimplification, and perhaps of uncertain relevance to human disease" (Sobel et al., 1994).

However, there are reasons to continue work with animal models. Although the models are not perfect representatives of multiple sclerosis, they can be used to study the basic immunologic mechanisms that are responsible for the autoimmune inflammatory disease of the CNS and the formation of lesions (Richardson, 1994; Genain and Hauser, 1996). In addition, the use of inbred rodents enables the investigator to study the genetics of the disease and adoptive transfer of EAE (Swanborg, 1995). More specifically, the use of murine models is advantageous since there are a barrage of mouse specific reagents available, as well, congenic strains, mutants and transgenic animals are easily developed (Wekerle et al., 1994; Swanborg, 1995). Of course, rodent models are useful in the designing and preliminary testing of potential therapies.

1.10.1.5 Epitope Spreading in Experimental Allergic Encephalomyelitis

There is circumstantial evidence that epitope spreading is responsible for the relapses seen in EAE (Tuohy, 1994). In fact, both inter- and intramolecular determinant spreading has been shown to occur in EAE. Intramolecular determinant spreading has been demonstrated by the following experiments:

1. One of the first reports of epitope spreading, was made be Perry and Barzaga in 1987. They demonstrated that in B10.PL X SJL/J mice with EAE induced by MBP the primary response was directed against an MBP peptide presented by I-A^u. After relapse the immune response included I-E^u restricted epitopes of MBP. A later study found the immune response to MBP to spread from I-A^u restricted peptides to I-A^s restricted peptides (McCarron et al., 1990).

2. Chronic MBP-induced EAE is distinguished by spreading of the autoreactive response to determinants of MBP not involved in the original immunization (McCarron et al., 1990; Lehmann et al., 1992).
3. Determinants of MBP that are cryptic after primary immunization of (SJL x B10.PL)F_1 mice can become immunogenic during the course of EAE (Lehmann et al., 1992).
4. T lymphocytes isolated from mice with EAE induced by T lymphocytes reactive with the immunodominant PLP139-151 peptide, are specific for PLP178-191 (McRae et al., 1995). Furthermore, these PLP178-191 reactive T lymphocytes were able to transfer relapsing EAE to naive syngeneic animals. Another study using the PLP139-151 peptide to induce EAE in (SWR X SJL)F_1 mice found the T lymphocyte response to include PLP249-273 and PLP173-198 (Yu et al., 1996).
5. Relapsing EAE was induced in SJL/J mice by the adoptive transfer of T lymphocytes specific for PLP139-151. After the animals recovered from the attack, T lymphocytes were found to proliferate in response to PLP178-191. This study also found that T lymphocytes isolated from mice with relapsing EAE induced by immunization with PLP139-151 or by adoptive transfer of T lymphocytes specific for PLP139-151 could be used to transfer EAE to naive recipients after being stimulated with either PLP139-151 or PLP178-191 (Miller et al., 1995).

Intermolecular determinant spreading has been observed in the following experiments:

1. Spleen cells isolated from SJL/J mice with EAE induced by passive transfer of MBP87-99 specific cells were able to transfer EAE after stimulation with PLP139-151 (Cross et al., 1993b).
2. (SJL/J X PL/J)F_1 mice immunized with MBP developed relapsing EAE, and their T lymphocytes were shown to develop reactivity to PLP during the course of the disease (Perry et al., 1991).
3. T lymphocytes from mice with EAE induced by MBP84-104 specific T lymphocytes developed a response to PLP139-151 (McRae et al., 1995).
4. (SWR X SJL)F_1 mice with PLP139-151 induced EAE had T lymphocytes reactive with other myelin antigens, including MBP87-99 (Yu et al., 1996).
5. Relapsing EAE was induced in SJL/J mice with MBP84-104. These animals developed T lymphocyte responses to PLP139-151 during and after clinical relapse (Miller et al., 1995).

Miller et al. (1995) explained the role of epitope spreading as follows, "As a result of myelin damage and opening of the blood-brain barrier during acute disease, T lymphocytes specific for endogenous epitopes on the same and/or different myelin proteins are primed and expand either in the periphery or locally in the CNS. These secondary T lymphocytes initiate an additional round of myelin destruction, leading to a clinical relapse by production of additional pro-inflammatory cytokines, similar to bystander demyelination operative during acute disease." McRae et al. (1995) and Yu et

al. (1996) concluded that relapsing paralysis or relapsing clinical episodes were the result of epitope spreading.

The focus now became how to prevent epitope spreading. Miller et al. (1995) were able to abrogate epitope spreading by inducing tolerance to the initiating epitope prior to the primary clinical episode. They also found that inducing tolerance to PLP139-151 after the transfer of PLP139-151 specific T lymphocytes, but before the onset of clinical signs of EAE, not only prevented intramolecular determinant spreading to PLP178-191 but also prevented the development of relapsing EAE. Not only did this show that it was possible to prevent epitope spreading, but also confirmed that damage to the CNS was necessary for the development of this phenomenon. McRae et al. (1995) were able to prevent relapsing EAE by inducing tolerance to intact PLP after the primary disease episode. Yu et al. (1996) concluded that the induction of tolerance to the spreading epitopes after the onset of EAE prevents progression of the disease.

Therefore, it seems that myelin breakdown due to the action of T lymphocytes specific for a myelin component leads to the release of autoantigens which in turn may activate other T lymphocytes and perpetuate the disease (Cross et al., 1993b; Goverman and Brabb, 1996).

1.10.2 The Marmoset Model of Experimental Allergic Encephalomyelitis

The marmoset model of EAE warrants a separate discussion as it differs in many respects from the rodent models of EAE. Marmosets are non-human primates and are therefore phylogenetically closer to humans than rodents. The species of marmoset used

as a model for multiple sclerosis is *Callithrix jacchus*. Although the individual animals in multiple births are derived from separate ova that are fertilized independently, the placentae of the embryos fuse. This leads to cross circulation of bone marrow derived elements between the fetuses. Therefore each animal is genetically distinct, but each is tolerant of the other's bone marrow derived cell populations (Picus et al., 1985). This natural bone marrow chimerism is extremely important in the EAE model as it allows adoptive transfer of T lymphocytes between outbred animals (Massacesi et al., 1995).

Chronic relapsing/remitting EAE can be induced in *Callithrix jacchus* by immunization with human CNS white matter (Massacesi et al., 1995). This EAE is very similar both clinically and pathologically to multiple sclerosis, with early and prominent demyelination leading to the formation of plaques. The demyelination appeared to be mediated by the macrophage infiltration observed within the lesions.

Genain et al. (1994) found MBP-reactive T lymphocytes to be abundant in unimmunized, healthy marmosets and to recognize a number of MBP epitopes. T lymphocytes from these animals were stimulated with MBP and used to adoptively transfer inflammatory CNS disease to other normal marmosets. The adoptive transfer produced a mild disease, with no macrophage infiltration and no demyelination, T lymphocytes isolated from the immunized animals were responsive to MBP but not PLP. Two important conclusions can be made from this experiment, first of all, MBP reactive T lymphocytes are capable of inducing inflammation and clinical signs but are not sufficient for plaque formation. Secondly, "naturally occurring populations of circulating

T lymphocytes in a healthy immunologically normal outbred individual can mediate an autoimmune disease."

A similar study by Massacesi et al. (1995) used MBP-reactive T lymphocyte lines from animals immunized with human white matter to adoptively transfer EAE. This resulted in the induction of the clinical signs of EAE in these animals, but did not produce demyelination.

An interesting study by Genain et al. (1995a) showed that the clinical signs of EAE could be induced in *C. jacchus* by immunization with human white matter, MBP, PLP, a recombinant fusion protein of MOG (rMOG), and combinations of either PLP + MBP, or PLP + MBP + rMOG. However, the characteristic pathology of EAE induced by immunization with white matter (as described by Massacesi et al., 1995) was only reproducible by immunization with either rMOG or rMOG + PLP + MBP. Analysis of the antibody repertoire of the animals showed that antibodies against the immunizing proteins were detected in animals immunized with MBP, PLP, and rMOG. Marmosets immunized with white matter or with PLP possessed antibodies to all 3 proteins (PLP, MBP, and rMOG). It is thought that the presence of anti-MBP and anti-rMOG antibodies in the PLP immunized animals is the result of epitope spreading. In any case, from these results it was concluded that in order to produce the MS-like lesion in *C. jacchus* a T lymphocyte response to MBP or MOG and an antibody response to MOG are required.

Further experimentation by Genain et al. (1995a) supported this conclusion. Adoptive transfer of anti-white matter, anti-rMOG, or anti-MOG monoclonal antibodies to MBP immunized marmosets led to demyelination and clinical deterioration, while

immunization with complete Freund's adjuvant and *Bordetella pertussis* followed by anti-MOG monoclonal antibodies did not induce demyelination. Genain and Hauser (1996) suggested that the T lymphocytes were responsible for opening the BBB which then enabled the pathogenic antibodies to enter the CNS.

A more recent study by McFarland et al. (1999) showed that immunization of marmosets with MP4, a chimeric molecule containing the known human epitopes of MBP and PLP, but none of the epitopes of MOG or other myelin antigens, induced EAE in these animals. Some animals developed an inflammatory disease, while others developed a demyelinating disease. Investigation of the demyelinating and nondemyelinating lesions demonstrated immunoglobulin deposits and anti-MOG antibodies to be present only in the demyelinating lesions. The authors suggest that the intermolecular determinant spreading to the MOG determinants resulted in the B lymphocyte response which was necessary for the demyelinating disease.

A role for macrophages in this demyelination was elucidated by the administration of Rolipram to marmosets with EAE induced with white matter (Genain et al., 1995b). Rolipram is a type IV phosphodiesterse inhibitor, which is known to suppress the release of TNF-α from macrophages (Schade and Schudt, 1993; Semmler et al., 1993). The administration of rolipram to these animals completely prevented EAE and demyelination.

EAE in *C. jacchus* is partly mediated by $CD4^+$ T lymphocytes that produce IL-2, IFN-γ, and TNF-α in response to MBP (Genain and Hauser, 1996). There does not appear to be any preferential usage of TCR Vβ genes (Uccelli et al., 1997). The

mechanisms mediating demyelination in the marmosets are thought to be the same as those occurring in the rodent EAE model so they will not be described here.

There are many advantages of using the marmoset model of EAE over the rodent models for the study of multiple sclerosis. These advantages include: the clinical and pathological similarity between marmoset EAE and human multiple sclerosis; the ability to perform sequential studies of CSF, T lymphocyte, and antibody responses in these animals; the similarity of immune and nervous system genes and proteins between humans and non-human primates; and the ability to perform adoptive transfers between siblings (Genain and Hauser, 1996). As well, the MS-like lesion observed in *C. jacchus* appears to be due to a diverse immune response to multiple myelin antigens. This is also believed to be the case in MS.

1.10.3 Viral Models of Multiple Sclerosis

The most extensively studied viral models of multiple sclerosis and the two that most closely resemble MS are the Theiler's murine encephalomyelitis virus model and the coronavirus model. In analyzing viral models of MS it is important to consider three questions: (1) Is the damage to oligodendrocytes due to the virus itself or to the immune response directed against the virus? (2) Is the activation of autoantigen-specific T lymphocytes the result of tissue destruction brought about by the viral infection? (Bradl and Linington, 1996). For example, the virus may damage certain tissues thereby releasing sequestered antigens which then become the target of an autoimmune response (Horowitz et al., 1998).

1.10.3.1 Theiler's Murine Encephalomyelitis Virus

Theiler's murine encephalomyelitis virus is a natural enteric picornavirus in mice (Rodriguez et al., 1988; Bradl and Linington, 1996) and is also indigenous to colony-bred mice (Lipton, 1975; McFarland and McFarlin, 1995). This RNA virus preferentially infects the white mater in the CNS of susceptible mice (Steinman and Conlon, 1997). Infection with TMEV first presents as poliomyelitis. After this initial infection, the virus is quickly cleared from the peripheral circulation but takes up residence in the glial cells of the CNS (Clatch et al., 1990; Tsunoda and Fujinami, 1996; Steinman and Conlon, 1997). Within a month TMEV induces a chronic progressive $CD4^+$ T lymphocyte (T_h1 lymphocyte) mediated inflammatory, demyelinating disease (Lipton, 1975; Lipton and Dal Canto, 1976; Clatch et al., 1986; Bradl and Linington, 1996; Steinman and Conlon, 1997). The resulting disease has a relapsing/remitting course and demyelination reminiscent of chronic progressive multiple sclerosis (Bradl and Linington, 1996; Steinman and Conlon, 1997).

The proposed mechanism of demyelination in TMEV infection is similar to that proposed for EAE. TMEV-specific $CD4^+$ T lymphocytes respond to the virus residing in the CNS and produce pro-inflammatory cytokines which recruit and activate macrophages and other lymphocytes (Rodriguez et al., 1986a,1997; Miller et al., 1990; Peterson, et al., 1993).

Evidence of an immune mediated mechanism for demyelination was gained from several observations:

1. During the chronic phase, the CNS infiltrate is composed mostly of macrophages and T lymphocytes, about half of which are $CD4^+$ (Lipton and Dal Canto, 1976; Clatch et al., 1990). During active demyelination the infiltrate consists of plasma cells, lymphocytes, and macrophages (Dal Canto and Lipton, 1975).
2. Demyelination late in the disease can be prevented by cyclophosphamide (Lipton and Dal Canto, 1976).
3. The administration of anti I-A monoclonal antibodies to animals with persistent TMEV infection reversed chronic paralysis and reduced inflammation and demyelination (Rodriguez et al., 1986b; Friedmann et al., 1987). Fatalities were noted in animals given the antibodies before a persistent infection had occurred.
4. Additionally, studies have shown that mice depleted of $CD4^+$ T lymphocytes experience diminished demyelination (Welsh et al., 1987; Gerety et al., 1994). This is contradicted by experiments showing that depletion of the CD4 or MHC class II genes results in severe demyelination and clinical illness (Roder and Hickey, 1996), and that the administration of monoclonal antibodies to deplete MHC class II molecules early in the disease can lead to increased demyelination and even death (Rodriguez and Sriram, 1988). These conflicting results may be due to the different strains of mice, viral isolates or antibodies used. It is also possible that the time of the depletion of the T lymphocytes may be an important factor. Welsh et al. (1987) suggested that

depletion of T lymphocytes before injection of TMEV may render the mice unable to clear the virus.

In later stages of the disease, there is evidence of epitope spreading as T lymphocytes reactive with several PLP peptides and with MOG92-106 have been identified (Miller et al, 1995,1997). The authors suggested that as there is no cross-reactivity between these peptides and TMEV, these T lymphocyte responses are due to the breakdown of myelin and the subsequent release of autoantigens. No such T lymphocyte reactivity to myelin antigens was demonstrated early in the disease (Miller et al., 1987). Further evidence of epitope spreading comes from the finding that the initial infection with TMEV is not dependent upon an immune response to myelin antigens. The induction of tolerance with TMEV epitopes inhibits disease induction and decreases the frequency and severity of virus induced demyelination (Karpus et al., 1995), while the induction of tolerance with myelin antigens does not (Miller et al., 1990). Furthermore, TMEV demyelination cannot be transferred by adoptive transfer of MBP-stimulated T lymphocytes from TMEV-infected animals (Barbano and Dal Canto, 1984), but the adoptive transfer of $CD4^+$ T lymphocytes specific for a viral protein increases the incidence of clinical disease in TMEV-infected mice (Gerety et al., 1994). Epitope spreading may explain the finding that the distribution of lesions moves from grey matter to white matter during TMEV infection (Tsunoda and Fujinami, 1996).

$CD8^+$ T lymphocytes may also be important in the clinical signs of TMEV infection and/or the demyelination induced by the virus. Transgenic mice with no $CD8^+$ T lymphocytes show demyelination and inflammation and high viral titers, but only a

mild illness (Roder and Hickey, 1996). The deletion of class I MHC molecules by monoclonal antibodies resulted in less inflammation and fewer lesions and was independent of the stage of disease at which the antibodies were given (Rodriguez and Sriram, 1988). However, Rodriguez (1997) reported that the deletion of class I MHC genes results in severe demyelination. In addition, class I molecule expression is up-regulated in the CNS of mice infected with TMEV (Tsunoda and Fujinami, 1996) and $CD8^+$ T lymphocytes specific for capsid proteins of TMEV have been found in animals infected with TMEV (Lin et al., 1995).

There is also circumstantial evidence to support the importance of a humoral response in TMEV-induced demyelination. For example, Yamada et al. (1990b) identified a monoclonal antibody that reacts with both TMEV and myelin. As well, TMEV can produce demyelination in nude mice (Johnson, 1985). Mice persistently infected with TMEV have elevated levels of IgG and oligoclonal bands in CSF, the majority of IgG present in the serum and CSF of these mice is specific for virus antigens (Roos et al., 1987; Rodriguez et al., 1988).

It has been suggested that macrophages may be a final effector in demyelination, as TMEV antigens and RNA have been found in these cells in demyelinating lesions in chronic TMEV infection (Tsunoda and Fujinami, 1996). As well, TMEV-infected macrophages possess MBP degrading proteolytic activity (Rodriguez and Quddus, 1986). Macrophages are the most highly infected cells during both the acute and chronic phases of TMEV induced disease (Dal Canto and Lipton, 1982; Clatch et al, 1990)

Susceptibility to the late disease depends upon the genetic background of the mouse, or more specifically the ability of the mouse to generate an MHC class II restricted, T lymphocyte mediated delayed type hypersensitivity (DTH) response to the virus (Clatch et al., 1985,1986; Miller et al., 1987). The DTH response to the virus residing in the CNS leads to secondary demyelination due to the virus induced inflammatory response (McFarland and McFarlin, 1995).

1.10.3.2 Coronavirus

Infection with mouse hepatitis virus (MHV), particularly the JHM strain, produces encephalomyelitis with a demyelinating pathology similar to that of multiple sclerosis (Weiner, 1973; Bradl and Linington, 1996). Although JHM is a mouse coronavirus, it can produce demyelinating disease in mice, rats, and primates (Gerber et al., 1985). The rat adapted JHM virus produces a relapsing disease in the Lewis rat (Watanabe et al., 1983). In mice the JHM virus leads to persistent CNS infection and demyelination due to the direct infection of oligodendrocytes (Herndon et al., 1975; Johnson, 1985; Dal Canto, 1996).

The lesions in MHV-JHM infection have been shown to contain macrophages, T lymphocytes, B lymphocytes, plasma cells, IgG, and complement C9, suggesting an immune response is responsible for the demyelination (Zimprich et al., 1991). This demyelination has been found to be partly the result of an antibody response to a viral spike protein (Yamada et al., 1990b), displayed on the surface of infected oligodendrocytes in the lesions (Zimprich et al., 1991; Bradl and Linington, 1996).

There is also a role for T lymphocytes in this process. Irradiation of mice infected with the JHM virus prevented demyelination, while the transfer of T lymphocytes from infected mice to these irradiated mice restored demyelination (Wang et al., 1990).

Epitope spreading is also known to occur in JHM infected animals. Splenic T lymphocytes from Lewis rats infected with JHM were found to respond to MBP and were able to transfer relapsing EAE when activated with MBP (Watanabe et al., 1983; ter Meulen, 1988). There is no cross reactivity between JHM and MBP (McFarland and McFarlin, 1995). Therefore coronavirus is able to stimulate T cell mediated autoimmune reactions (Watanabe et al., 1983; Kyuwa et al., 1991).

2.0 Hypothesis

To date, the antigens against which the serum and CSF IgG antibodies of MS patients are directed have not been fully identified. MS lesions are typically restricted to the white matter of the CNS, the optic nerve, and the retina (Antel and Arnason, 1991; Boccaccio and Steinman, 1996; Kerrison, et al., 1994; Lucchinetti and Rodriguez, 1997; Martin, 1997; Stinisson et al., 1997), one would expect that the expression of autoantigen(s) important to MS would also be limited to these areas. My experimental hypothesis was that potential autoantigens in multiple sclerosis could be identified by using a modification of the SEREX, (serological identification of antigens by recombinant expression cloning) method of Sahin et al. (1995). In this thesis, SEREX involves construction of a cDNA library: (a) by preparation of cDNA from mRNA isolated from MS brain tissues; (b) by packaging the cDNA into a lambda phage vector

(λZapII); (c) by transfecting the vector into *Escherichia coli*; (d) and by colour detection of expressed proteins induced by isopropyl β-D-thiogalactopyranoside. The recombinant proteins are transferred to nitrocellulose membranes and screened with patients' sera. The antigens are detected by a colour reaction which recognizes IgG in patients' sera bound to the recombinant protein. Positive clones are subcloned to clonality, sequenced, and the sequences compared with DNA and RNA sequences in various databases.

The rationale of using a cDNA expression library prepared from MS lesions is that serological screening should lead to the identification of both previously identified and novel MS autoantigens. This technique is advantageous compared with many other approaches to identify autoantigens since it makes no *a priori* assumptions as to the MS autoantigens.

Molecular targets potentially relevant to MS are then used as probes in Northern blotting experiments to determine their relative expression levels in various tissues.

2.1 Background

SEREX was developed for use in the identification of antigens in tumours. Sahin et al. (1995) screened cDNA expression libraries constructed from various malignant tumors with autologous sera. They identified some antigens with restricted expression in each tumor, showing that tumors express multiple antigens which are able to elicit immune responses. A similar study by Scanlan et al. (1998) used the SEREX method to identify distinct antigens in colon cancer.

The identification of novel autoantigenic targets in MS could lead, in the long

term, to novel therapies. For example, once an autoantigen is identified as a major immune target in MS, immunodominant epitopes could be defined. Altered peptide ligands could then, in theory, be prepared for treatment. Altered peptide ligands are peptides which are modified so that they bind to the T cell receptor in a sub-optimal way and subsequently do not result in the same T lymphocyte response as that initiated by the original peptide (Steinman, 1996). It is also possible that altered peptide ligands bind with the same affinity as the native peptide, but result in different signaling mechanisms (Hafler and Weiner, 1995). Several investigators have already demonstrated the usefulness of altered peptide ligands in causing a switch in the cytokine patterns to decrease the production of IFN-γ and increase the production of TGF-β (Windhagen et al., 1995b; Ausubel et al., 1997)

Of course, there are certain difficulties in screening such a library. Antibodies recognize a particular conformational epitope. The recombinant proteins produced in such a library may not undergo the same post-translational modifications as the proteins produced in the MS patients. In contrast, they may be subjected to certain modifications in the bacterial system which do not occur in the human system. Also, although IgG is known to be increased in MS CSF and serum, there is no way of knowing how much of this increase is directed towards an MS specific antigen (Owens et al., 1996). There is also no way of knowing if the antigen responsible for inducing MS is actually eliciting an antibody response when the serum is taken (Tureci et al., 1997). It is possible that individual libraries would have to be screened with autologous serum as different

antigens may be responsible for disease induction in different patients (Owens et al., 1996).

2.2 Studies Relevant to the Master's Project Discussed in this Thesis

A number of investigators have used various molecular techniques, including cDNA libraries, and peptide libraries, in an attempt to identify peptides, infectious agents and/or autoantigens specific to multiple sclerosis patients. Some of these experiments are discussed in the following paragraphs.

Owens et al. (1996) used mRNA isolated from chronic and acute multiple sclerosis plaques and from periplaque white matter to construct a directional cDNA expression library. The resulting libraries were screened with CSF from MS patients, or with IgG extracted from MS sera or CSF. These authors also used subtracted cDNA libraries in order to enrich mRNA sequences which were unique or over-represented in the MS plaque as compared with normal white matter.

Screening of these libraries did not detect any sequences unique to MS, nor did it detect MBP or any other myelin specific proteins. They did suggest several reasons to account for the absence of detection of myelin specific antigens: "(1) the in-frame expression products are unstable or toxic, (2) the antibodies may recognize post-translational modifications or secondary systems not formed by bacterial systems (3) the antigen(s) recognized by MS antibodies may be rare or present only in acute plaques", and (4) the anti-myelin antibodies are at low concentration in the samples used.

Cortese et al. (1996) screened random peptide libraries displayed on phage with sera and CSF of MS patients and with sera of controls. They coined the term phagotope to refer to phage displayed epitopes, that is the phage displayed peptides which reacted with antibody.

Screening with two CSF samples, CSF1 and CSF2, showed several hundred phagotopes are recognized by IgG in CSF1 while substantially fewer phagotopes were recognized by CSF2. Of these positive clones, 32 were chosen for further study based on their strong reactivity. Sequencing of these clones revealed three classes of amino acid homology. Class I phagotopes showed related sequences with a highly conserved KPPNP motif, class II phagotopes showed distinct but highly homologous sequences. Both class I and II phagotopes were recognized by CSF1. Phagotopes recognized by CSF2 were grouped as class III, and showed distinct but homologous sequences. The phagotopes recognized by CSF2 are totally unrelated to those recognized by CSF1, and do not react with CSF1. In addition, the phagotopes identified with CSF1 and 2 rarely react with CSF from other MS patients.

It was found that the CSF1 and CSF2 selected phagotopes not only reacted with sera obtained from the same patient, but also with sera from a number of MS patients and healthy controls. Further studies demonstrated that for the majority of phagotopes the specific activity of the antibodies reacting with the phagotopes is greater in the CSF than in the serum.

These results and similar results obtained by this group in 1998 led to the following conclusions: (1) "antibodies displaying the same binding specificity are present

in the CSF and serum of the same patient, (2) these antibodies may be directed against ubiquitous antigens, (3) some of these antibodies are specifically enriched in the CSF", (4) it seems that each MS patient has a different set of intrathecally produced antibodies in the CSF, and (5) anti-phagotope antibodies are found in the sera of healthy individuals as well as that of MS patients .

Becker et al. (1997) constructed a normalized cDNA library from plaques of a chronic progressive MS patient. This involved hybridizing single stranded plasmids with RNA transcripts and removing the resulting DNA:RNA hybrids by chromatography. By removing these hybrids they were able to increase the frequency of rare cDNA clones and decrease the frequency of abundant cDNAs (Soares, et al., 1994). Expressed sequence tags (ESTs) were produced through the sequencing of cDNA clones isolated from the library. Analysis of the cDNA library showed the majority of cDNAs represented unknown or uncharacterized genes. However, 54 cDNAs corresponding to genes known to be involved in immune activation were identified. Most of these encoded products which are important in antigen processing and presentation, such as MHC molecules, and cell adhesion molecules. Others encoded inflammatory mediators, such as cytokines and cytokine receptors, and genes typically associated with T and B lymphocyte recognition, activation, and regulation. Comparison with normalized adult brain libraries showed only 16 of the 54 cDNAs identified in the MS library to be present in normal brain. In addition, 19 cDNAs encoding known or suspected autoantigens were identified in the MS library. These included MBP and PLP, as well as 16 autoantigens known to be associated with other autoimmune diseases, for example systemic lupus erythematosus,

insulin dependent diabetes mellitus, and Graves disease. Only 3 of these 16 genes were found in the normal adult brain libraries. This study did not identify any cDNA sequences identical to those of any infectious agents.

Archelos et al. (1998) used an oligodendrocyte-precursor cell line to construct two expression libraries, which were subsequently screened with pooled CSF from MS patients and patients with other neurological diseases. Examination of the pooled CSF prior to its use in the screening did not detect the presence of anti-MBP, -MOG, or -MAG antibodies in the MS patients nor in the patients with other neurologic diseases. This screening identified only 6 positive clones, none of which corresponded to a known protein. However, 5 of the 6 clones contained a common 7 amino acid sequence (EKPCLEK) which is highly homologous to the translated consensus *Alu* repeat epitope. After concluding that the positive responses to each of these 5 clones was due to the presence of the EKPCLEK motif, the authors created a synthetic peptide, the *Alu-6* peptide, which included this motif. This peptide was composed of 12 amino acids, EKPCLEKPKKKK, and was used in Enzyme Linked Immunosorbent Assays (ELISAs) so that a number of serum samples could be tested for reactivity with this peptide. Approximately 44% of relapsing/ remitting MS patients tested positive for antibodies recognizing the *Alu-6* peptide. A lower percentage of primary and secondary progressive MS patients, healthy controls, and patients with other neurologic diseases were also found to possess such antibodies. In addition, the antibody titers of the relapsing/remitting MS patients were higher than those of the other MS patients, the

controls, and the patients with other neurologic diseases. Comparable results were obtained when CSF was used in the ELISAs.

Alu repeats are short, repetitive elements found in the introns of almost every known protein-encoding gene of humans and other primates (Makalowski et al., 1994). They are approximately 300 bp in length, are scattered throughout the human genome (Bloom et al., 1996), and may account for as much as 5% of the entire human genome (Garren et al., 1998). These elements are so named due to the presence of a single recognition site for the Alu I restriction endonuclease, AG↓ CT, located near the middle of the Alu element (Bloom et al., 1996). There are a couple of mechanisms by which these repeats may lead to disease. *Alu* elements contain numerous stop codons and are therefore considered to cause premature termination if they are present within transcripts. For this reason, the *de* novo insertion of *Alu* repeats may result in genetic disease. The splicing of intragenic Alu elements into mRNA may have similar consequences (Makalowski et al., 1994). It is also known that the oligo(A) sequences of *Alu* repeats are often associated with genomic instability (Chen et al., 1995), this may result in pathogenic genetic rearrangement. Alu repeats have also been shown to be able to "..insert themselves into or in the vicinity of *cis*-acting sequences or into open reading frames or genes" , where they can function and may even direct transcription (Szmulewicz et al., 1998). Alu insertions have been directly and indirectly linked to a number of human illnesses including hypertension, breast cancer, and colon cancer.

The *Alu* peptides discovered by Archelos et al. (1998) may represent translated *Alu* repeat epitopes, epitopes of an unknown oligodendrocyte protein, or regulatory molecules.

Chapter Two Materials and Methods

2.0 Isolation of Total RNA

Brain tissue from an MS patient was generously provided by Dr. Barry Rewcastle, a neuropathologist at the University of Calgary, Calgary, Alberta. Sufficient white matter plaques were removed from this frozen sample to make up 1.89 g of tissue. TRIzol™ reagent (Gibco BRL, Gaithersburg, MD) was used to isolate total RNA from these plaques. The tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle and added to 19.0 ml of TRIzol™ reagent (1 ml of TRIzol™ reagent for every 50-100 mg of tissue), in a 30 ml Nalgene Oak Ridge polypropylene centrifuge tube (Nalge Nunc International, Rochester, N.Y.). This mixture was incubated at room temperature for 5 minutes and then centrifuged at 12 000 g for 10 minutes at 4°C, to sediment any unsolubilized material. The supernatant was transferred to a fresh tube and 3.8 ml of chloroform were added (0.2 ml of chloroform per ml of TRIzol™ reagent used). The tube was vigorously shaken for 15 seconds, incubated at room temperature for 3 minutes, and centrifuged at 12 000 g for 15 minutes at 4°C. The supernatant was transferred to a fresh tube and the RNA precipitated with 9.5 ml of isopropanol (0.5 ml per ml of TRIzol™ reagent) followed by incubation at –20°C overnight.

The next day, the precipitate was centrifuged at 12 000 g for 10 minutes at 4°C and the pellet washed with 19 ml of 75% (v/v) ethanol [diluted in diethyl pyrocarbonate treated water (DEPC-H_2O)] (1 ml of 75% ethanol per ml of TRIzol™ reagent). This was vortexed briefly and centrifuged at 7500 g for 5 minutes at 4°C. The pellet was dried in a

bell vacuum and resuspended in 500 µl of DEPC-H_2O, then incubated at 60°C for 10 minutes.

Spectrophotometric readings were taken using the Warburg program of the Soft-Pac™ Module of the Beckman D4®-64 Spectrophotometer (Beckman Instruments Inc., Fullerton, CA) to check the purity and the amount of RNA isolated.

2.1 Isolation of Messenger RNA (mRNA)

The Poly (A) Quik® mRNA isolation kit (Stratagene, La Jolla, CA) was used to isolate mRNA from the total RNA. The RNA sample was incubated at 65°C for 5 minutes and immediately placed on ice. Fifty-five µl of 10X sample buffer [100 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 M NaCl] was added to the denatured RNA sample. The Poly (A) column was attached to a 3cc. Luer lock syringe and the storage buffer pushed out. Next, two 200 µl aliquots of high salt buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 M NaCl) were pushed through the column. The RNA sample was then pushed through the column, collected in a 1.5 ml microcentrifuge tube, and re-applied to the column. This was followed by another wash with 200 µl of high salt buffer and three washes with low salt buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M NaCl). The mRNA was eluted from the column with four 200 µl aliquots of preheated (65°) elution buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and the eluate collected in four 1.5 ml microcentrifuge tubes. The mRNA was precipitated with 500 µl of cold 100% ethanol, per tube, and incubated overnight at –20°C.

The following day the precipitate was centrifuged at 12 000 g for 30 minutes at 4°C, washed with 100 µl of 70% ethanol (DEPC-H_2O), and dried in a bell vacuum. The pellet was resuspended in 39 µl of DEPC-H_2O and spectrophotometric readings were taken as before.

2.2 Construction of a cDNA Library

The cDNA library was constructed using the ZAP Express™ cDNA Synthesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

2.2.1 First Strand Synthesis

A hybrid poly (dT) linker-primer and Moloney murine leukemia virus reverse transcriptase (MMLV-RT) were used to synthesize first strand cDNA from the RNA template. The linker-primer contains an Xho I restriction enzyme recognition site which ensures that the cDNA is inserted in the vector in the sense orientation. The reaction was carried out in a 0.5 ml microcentrifuge tube with 5.0 µl of 10X first strand buffer, 3.0 µl of first strand methyl nucleotide mixture, 2.0 µl of linker-primer (1.4 µg/µl), 8.0 µl of DEPC-H_2O, 1.0 µl of RNase Block Ribonuclease Inhibitor (40 U/µl), and 29.5 µl of mRNA (5 µg). The first strand methyl nucleotide mixture contains normal dATP, dGTP, and dTTP, but the dCTP is a 5'-methyl analog of dCTP. The addition of the 5'-methyl dCTP to the first strand cDNA protects against digestion of the cDNA by Xho I in a later step. The reaction mixture was briefly vortexed and incubated for 10 minutes at room temperature to allow the template and the primer to anneal. Following this incubation, 1.5 µl of MMLV-RT (50 U/µl) was added. The reaction tube was vortexed, centrifuged briefly, and incubated at 37°C for 1 hour. Then immediately placed on ice.

2.2.2 Second Strand Synthesis

The first strand cDNA synthesis was now complete. Next, 20.0 µl of 10X second strand buffer, 6.0 µl of second strand nucleotide mixture, 115.9 µl of sterile H_2O, 2.0 µl of RNase H (1.5 U/µl), and 11.1 µl of DNA Polymerase I (9.0 U/µl) were added. The reaction tube was vortexed, centrifuged briefly, and incubated at 16°C for 2.5 hours. Then placed on ice.

During the second strand synthesis, the RNase H breaks the mRNA template into fragments which are subsequently used as primers for the DNA Polymerase I. The DNA Polymerase I translates the RNA fragments into second strand cDNA. The second strand nucleotide mixture contains normal dCTP to reduce the chances of 5'-methyl dCTP becoming incorporated into the second strand cDNA.

2.2.3 Blunting of cDNA Termini

At this point the ends of the newly synthesized cDNA strands were uneven. To produce blunt ends, 23 µl of blunting dNTP mix, and 2.0 µl of cloned *Pfu* DNA Polymerase (2.5 U/µl) were added. The tube was vortexed, centrifuged briefly, and incubated at 72°C for exactly 30 minutes. This incubation was followed by a phenol-chloroform extraction to isolate the cDNA. Two hundred µl of phenol-chloroform [1:1(v/v)] were added to the reaction mixture. The tube was vortexed and then centrifuged for 2 minutes to separate the organic and aqueous layers. The aqueous layer, containing the cDNA, was transferred to a fresh 1.5 ml microcentrifuge tube and an equal volume of chloroform was added. The tube was vortexed and then centrifuged for 2 minutes. The aqueous layer was transferred to a fresh 1.5 ml tube and the cDNA was

precipitated with 20.0 µl of 3 M sodium acetate and 400 µl of 100% ethanol. The tube was inverted several times to mix and incubated at –20°C overnight.

The next day the precipitate was centrifuged at 16 000 g for 60 minutes at 4°C. The pellet was washed with 75% (v/v) ethanol, centrifuged at 16 000 g for 2 minutes at 4°C, dried under a bell vacuum, and resuspended in 9.0 µl of EcoR I adapters. The reaction mixture was incubated at 4°C for 30 minutes to resuspend the cDNA.

2.2.4 Ligating the EcoR I Adapters

Next, the EcoR I adapters were ligated to the blunt ended cDNA strands by the addition of 1.0 µl of 10X ligase buffer (500 mM Tris-HCl pH 7.5, 70 mM $MgCl_2$, 10 mM dithiothreitol), 1.0 µl of 10 mM rATP, and 1.0 µl of T4 DNA ligase (4 U/µl). The tube was incubated at 8°C overnight. The following day the reaction mixture was incubated at 70°C for 30 minutes to inactivate the ligase, centrifuged briefly, and cooled to room temperature.

2.2.5 Kinasing the EcoR I Adapters

The EcoR I adapters were kinased so they were able to ligate into the dephosphorylated vector arms. When the reaction tube had reached room temperature 1.0 µl of 10X ligase buffer, 2.0 µl of 10 mM rATP, 6.0 µl of sterile H_2O, and 1.0 µl of T4 polynucleotide kinase (10 U/µl) were added. The tube was incubated at 37°C for 30 minutes, and then incubated at 70°C for 30 minutes, to inactivate the kinase. The tube was centrifuged and cooled to room temperature.

2.2.6 Xho I Digestion

As there is an Xho I recognition site in the linker-primer, digestion of the newly synthesized cDNA with Xho I removes the residual primer and the EcoR I adapter from the 3' end of the cDNA. This digestion allows the cDNA to be oriented in the vector in the sense direction due to over-hanging Xho I and EcoR I sites. The digestion required the addition of 28.0 µl of Xho I buffer supplement, and 3.0 µl of Xho I (40 U/µl). The reaction tube was incubated at 37°C for 1.5 hours. The tube was cooled to room temperature and 5.0 µl of 10X STE buffer (1 M NaCl, 200 mM Tris-HCl pH 7.5, 100 mM EDTA) was added. The cDNA was then size fractionated using a Sephacryl S-500 Spin Column.

2.2.7 Size Fractionation of cDNA

A 1 ml syringe was plugged with a small amount of cotton and filled with Sephacryl S-500. The syringe was placed in a 15 ml Falcon tube and centrifuged at 385 g for 2 minutes. The syringe was again filled with Sephacryl S-500 and centrifuged at 385 g for 2 minutes. The Sephacryl S-500 column was washed with two 300 µl aliquots of 1X STE. The column was then transferred to a fresh Falcon tube with a 1.5 ml microcentrifuge tube in the bottom to collect fractions. Note that each fraction was collected in a fresh 1.5 ml microcentrifuge tube. The cDNA was pipetted into the spin column and centrifuged at 385 g for 2 minutes. This first fraction was removed and stored in a 1.5 ml tube. Sixty µl of 1X STE was loaded onto the column and centrifuged at 385 g for 2 minutes. The second fraction was transferred to a fresh tube. Sixty µl of

1X STE was loaded on the column and centrifuged at 385 g for 2 minutes. The third fraction was collected.

An equal volume of phenol-chloroform [1:1(v/v)] was added to each fraction. The tubes were vortexed and centrifuged at 16 000 g for 2 minutes to separate the aqueous and organic layers. The aqueous layers were transferred to fresh 1.5 ml tubes and equal volumes of chloroform were added. The tubes were vortexed and centrifuged for 2 minutes. The aqueous layers were transferred to fresh tubes. The cDNA fractions were precipitated by adding twice the volume of 100% ethanol and incubating overnight at –20°C.

The following day the precipitates were centrifuged at 16 000 g for 60 minutes at 4°C, washed with 200 µl of 80% (v/v) ethanol, and dried under a bell vacuum. Each fraction was resuspended in 3.0 µl of H_2O.

2.2.8 Quantitation of cDNA Fractions by Ethidium Bromide Assay

The relative concentrations of the cDNA fractions were determined by spotting 0.5 µl of each sample and 0.5 µl of known DNA standards on a 0.8% (w/v) agarose / 0.5X TBE (0.045 M Tris-borate, 0.001 M EDTA) gel. The gel also contained 4 µl of a 10 mg/ml solution of ethidium bromide. The relative concentrations of the fractions were determined by comparing the intensities of the fractions with those of the known samples under ultraviolet light.

2.2.9 Ligation of the cDNA into the Zap Express Vector

The ligation of cDNA into the Zap Express vector gives the best results if 100 ng of cDNA is ligated into 1 µg of vector. The first ligation reaction was set up as follows:

1.25 µl of fraction 2 (80 ng/µl), 0.5 µl of 10X ligase buffer, 0.5 µl of 10 mM rATP pH 7.5, 1.0 µl of ZAP Express vector (1 µg/µl), 1.25 µl of sterile H_2O, 0.5 µl of T4 DNA ligase (4 U/µl). The reaction tube was incubated at 4°C for 48 hours. The ligation reaction was repeated with the remaining 1.25 µl of fraction 2 (80 ng/µl), and with 2.5 µl of fraction 3 (40 ng/µl).

2.2.10 Packaging of the cDNA Library

One µl of the ligated cDNA was added to the packaging extract just as the extract began to melt. The reaction was immediately placed on ice, briefly centrifuged and incubated at room temperature for 1 hour and 38 minutes. Next, 500 µl of SM buffer (5.8 g of NaCl, 2.0 g of $MgSO_4 \cdot 7H_2O$, 50.0 ml of 1 M Tris-HCl pH 7.5, 5.0 ml of 2% (w/v) gelatin) and 20 µl of chloroform were added to the reaction tube and centrifuged briefly to sediment the debris. The supernatant containing the phages with cDNA inserts was transferred to a fresh tube.

2.2.11 Plating and Titering of the cDNA Library

The size of the library was determined by transfecting the phages into the host cells, XLI-Blue MRF' bacteria, and counting the number of resulting plaques. The XLI-Blue MRF' bacteria were cultured in LB broth (per liter: 10 g of bacto-tryptone, 5 g of bacto yeast extract, and 10 g of NaCl) supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$, to an optical density (O.D.) of 0.5 to 1.0 at 600nm. The culture was centrifuged at 500 g for 10 minutes. The media were discarded and the bacteria resuspended in half the original volume with 10 mM $MgSO_4$. One µl of the packaged reaction, was added to 200 µl of XLI-Blue MRF' cells. Fifteen µl of 0.5 M isopropyl β-D-thiogalactopyranoside

(IPTG) and 50 µl of 250 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) were added to 2 ml of melted NZY top agar (per liter: 5 g of NaCl, 2 g of $MgSO_4 \cdot 7H_2O$, 5 g of bacto yeast extract, 10 g of casein hydrolysate, 0.7% w/v agarose). The NZY top agar was heated until boiling and then 2 ml aliquots were placed in Falcon tubes and incubated at 58°C. The phage and bacteria were incubated for 15 minutes at 37°C and then added to the NZY top agar. This was quickly poured over a 150 mm x 15 mm NZY agar plate (per liter: 5 g of NaCl, 2 g of $MgSO_4 \cdot H_2O$, 5 g of bacto yeast extract, 10 g of casein hydrolysate, 15 g of bacto agar) and allowed to solidify before being inverted and incubated overnight at 37°C. The next day, the number of recombinant and non-recombinant clones were counted.

2.2.12 Amplification of the cDNA Library

The library was amplified to increase the number of each clone and to stabilize the library. The phages were plated so that there were approximately 50 000 plaques per plate. This time 600 µl XLI-Blue MRF' cells were used. As before, the phage/bacteria mixture was incubated for 15 minutes at 37°C. This was then added to the melted NZY top agar and poured over NZY agar plates. When the top agar had solidified, the plates were incubated at 37°C until the plaques were 1-2 mm in diameter, approximately 9 hours. As soon as the plaques reached this size, 10 ml of SM buffer was added to each plate. The plates were incubated at 4°C overnight with gentle shaking to allow the phages to diffuse into the buffer. The next day the SM buffer was recovered from each plate and pooled. The plates were rinsed with an additional 2 ml of buffer, which was added to the pooled buffer. Chloroform was added to a final concentration of 5%, the

tube was mixed well and incubated for 15 minutes at room temperature. The cell debris was sedimented by centrifugation at 500 g for 10 minutes. The supernatant was recovered and stored at 4°C. Chloroform was then added to a final concentration of 0.3%. The resulting library was designated as the MS2 cDNA library.

2.3 Primary Screening of the MS2 cDNA Library

After amplifying the library, several dilutions were plated to find the most suitable dilution for screening. The best dilution was found to be 1/50. One µl of the 1/50 dilution of the library was added to 600 µl of XLI-Blue MRF' cells and incubated for 15 minutes at 37°C. Fifteen µl of 2 M IPTG was added and the mixture was incubated again for 15 minutes at 37°C. This was added to 9 ml of melted NZY top agar and immediately poured onto an NZY agar plate. After the agar had solidified, the plates were incubated overnight at 37°C. The following day the plates were overlaid with NitroPure nitrocellulose transfer membranes, 0.45 µm 137 mm, (Micron Separations Inc. Westboro, MA) and incubated at 37°C for 3 hours. A needle was used to punch holes through the membrane and the agar simultaneously. These holes would be used later in the screening to match up the positive clones on the membrane with the clones on the corresponding agar plate. The membranes were removed from the plates and washed three times, 10 minutes per wash, with a solution of 1X TBS (140 mM NaCl, 3.0 mM KCl, 25 mM Tris pH7.4) and 0.05% polyoxyethylene-sorbitan mono-laurate (Tween 20), with shaking at room temperature. Next, the membranes were blocked for 1 hour with 5% skim milk powder in 1X TBS and rinsed 5 times with 1X TBS.

Before the patients' sera were used for screening the library, *Escherichia coli* antibodies were removed by chromatography on glutaraldehyde activated silica to which an *Eschericia coli* lysate was coupled. (This chromatography was carried out by Dr. David Haegert.) Five of the sera were pooled together in a 1/200 dilution in 1X TBS / 0.5% milk / 0.1% sodium azide. Each membrane was incubated overnight with shaking at room temperature in 20 ml of the pooled serum. [The sera used in this study were obtained from a number of different patient populations in different countries. As the criteria for determining the different subtypes of MS differ somewhat between countries, it was decided that the 18 sera used in this project would be chosen at random from the samples available. The 5 sera which were pooled were also chosen at random from these 18 sera.]

The following day the serum was collected and the membranes rinsed five times with 1X TBS. The secondary antibody, alkaline phosphatase-conjugated AffiniPure anti-human IgG, Fcγ fragment specific antibody (Jackson Immuno Research Laboratories Inc., West Grove, PA) was added to the membranes at a concentration of 1/5000 in 1X TBS / 0.5% milk. The membranes were incubated at room temperature for 1 hour with shaking and then rinsed five times with 1X TBS. The bound antibody was detected with the BioRad Alkaline Phosphatase Conjugate Substrate Kit (BioRad, Hercules, CA). Two hundred and fifty µl of colour reagent A (nitroblue tetrazolium in dimethyl formamide with $MgCl_2$) and colour reagent B (5-bromo-4-chloro-3-indolyl phosphate in dimethyl formamide) were used per 100 ml of alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

Positive clones are detected as dark purple rings on a lighter purple background. The holes in the membranes are aligned with the corresponding plates and the positive clones are removed from the agar with a 1 ml pipette tip. These agar "plugs" containing the positive clones were incubated overnight at 4°C in 500 µl of SM buffer to allow the phage to diffuse into the buffer.

2.4 Secondary Screening of the MS2 cDNA Library

The following day 1/10 dilutions were made of these plugs and 1 µl of the diluted phage was incubated with 200 µl of bacteria at 37°C for 15 minutes. Fifteen µl of 2 M IPTG was added to the phage/bacteria mixture and incubated at 37°C for 15 minutes. This was added to 2 ml of melted NZY top agar, and poured onto one quarter of an NZY agar plate. The plaque densities were checked the next day and the volumes and dilutions were adjusted to give the appropriate number of plaques per quarter, 15-30, for secondary screening.

At this stage each clone was screened with the secondary antibody alone and with the pooled sera. Screening with the secondary antibody alone allowed the identification of clones which expressed immunoglobulin, such clones were discarded. Screening with the pooled sera, for a second time, allowed the identification of false positives. Any clones which were negative on this secondary screening were discarded. Clones which were positive with secondary screening, and which were not identified as immunoglobulin, were subjected to further "subcloning" until clonality was achieved. This was followed by screening with individual sera to determine the number of sera with which each clone was reactive.

2.5 Amplification of Positive Clones by Polymerase Chain Reaction

Before the clones could be sequenced, each clone had to be amplified. This was accomplished using the T3 (5' AATTAACCCTCACTAAAGGG 3') and T7 (5' GTAATACGACTCACTATAGGGC 3') primers which are derived from the sequence of the Zap Express Vector. One hundred µl of each clone was heated to 95°C for 5 minutes and then placed on ice, to denature the DNA. Ten µl of this denatured sample was added to a 0.5 ml microcentrifuge tube containing 90 µl of PCR cocktail. Each cocktail contained 10 µl of 10X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 4 µl of 50 mM $MgCl_2$, 5 µl of 4 mM dNTP, 5 µl of 10 µM T3 primer, 5 µl of 10 µM T7 primer, 0.5 µl Taq DNA Polymerase (5 U/µl) (Gibco BRL, Gaithersburg, MD), and sterile H_2O to a final volume of 90 µl. The final concentrations were: 1X PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM for each primer, 2.5 U Taq DNA Polymerase. The parameters used for the polymerase chain reaction were as follows: 95°C for 5 minutes, 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, for 34 cycles, and then 72°C for 7 minutes. The reaction tubes were stored at 4°C. The amplified clones were electrophoresed on a 1% agarose / 0.5X TBE gel to check for clonality and to check the relative sizes of each clone. The "cleanest" samples of each clone were chosen to be used for cycle sequencing.

2.6 Purification of PCR Products

The PCR products were purified using the Wizard® PCR Preps DNA Purification System (Promega, Madison, WI) according to the manufacturer's directions. The PCR product was transferred to a 1.5 ml microcentrifuge tube and 100 µl of Wizard™ PCR

Preps Direct Purification Buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton® X-100) was added. This mixture was vortexed briefly to mix. Next 1 ml of PCR Preps DNA Purification Resin was added and the slurry vortexed 3 times over 60 seconds. The Wizard™ PCR Preps Minicolumns (one for each sample to be purified) were attached to 3cc. Luer lock syringes and the DNA / resin mixture pushed through the columns. The Minicolumns were washed with 2 ml of 80% isopropanol, placed in 1.5 ml microcentrifuge tubes, and centrifuged at 10 000 g for 2 minutes to dry the resin. The DNA fragments were eluted with 50 µl of sterile H_2O, which was applied directly to the Minicolumns. After 1 minute, the columns were centrifuged at 10 000 g for 20 seconds in a 1.5 ml tube. The DNA was transferred to a new 1.5 ml microcentrifuge tube.

2.7 Cycle Sequencing of Purified PCR Products

The positive clones were sequenced using the fmol™ DNA Sequencing System (Promega, Madison, WI) and the T3 primer. The T3 primer was first labelled with [γ-^{33}P] dATP. This reaction was carried out as follows: 2.0 µl of (10 µM) T3 primer, 6.0 µl of [γ-^{33}P] dATP (2000 Ci/mmol), 2.0 µl of T4 polynucleotide kinase 10X buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM dithiothreitol, 1.0 mM spermidine), 2.0 µl of T4 Polynucleotide Kinase (10 U/µl), and sterile H_2O added to a final volume of 10 µl. The reaction tube was incubated at 37°C for 30 minutes and then at 90°C for 2 minutes, before being stored at 4°C.

For each sample, four 0.5 µl microcentrifuge tubes were labelled, one for each d/ddNTP. Each tube contained 2.0 µl of the d/dd A,C,G, or T Nucleotide Mix. A cocktail for each sample was prepared by adding 5.0 µl of 5X fmol™ Sequencing buffer

(250 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$), 1.0 µl of template DNA, 1.5 µl of [γ–^{33}P] dATP labelled T3 primer, 8.5 µl of sterile H_2O, 1.0 µl of Sequencing Grade Taq DNA Polymerase (5 U/µl). The cocktails were centrifuged briefly and 4 µl was added to each of the four tubes containing one of the d/ddNTP. The parameters used for cycle sequencing were as follows: 95°C for 3 minutes, 95°C for 30 seconds, 42°C for 30 seconds, 70°C for 1 minute, 70°C for 1 minute, for 29 cycles, then stored at 4°C. After the cycle sequencing had finished 3 µl of fmol™ Stop Solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each sample.

The products of the cycle sequencing were electrophoresed on a 38 cm x 50 cm 8% acrylamide / 7M urea gel (40.0 ml of 30% acrylamide / bis solution 19:1, 15.0 ml 10X TBE, 63.0 g urea, 20.0 ml deionized H_2O). These ingredients were mixed until all the urea was in solution, the volume was adjusted to 150 ml with deionized H_2O and the mixture filtered and degassed for 5 minutes. The gel was poured in the same manner as other acrylamide gels. First, the plug was poured and allowed to polymerize and then the remainder of the gel was poured. The plug was made of 35 ml of the gel mixture with 180 µl of 10% (w/v) ammonium persulfate and the same volume of N, N, N', N' – tetramethylethylenediamine (TEMED). The remainder of the gel was poured after the addition of 288 µl of 10% (w/v) ammonium persulfate and 115 µl of TEMED.

The sequencing gel was preheated to 50°C by running at 110 Volts for approximately 1 hour. The samples were incubated at 70°C for 10 minutes and then immediately placed on ice. Three µl of each sample were loaded on the sequencing gel and electrophoresed for approximately 4 hours at 110 Volts.

The gel was removed from the apparatus, fixed for 15 minutes in 10% methanol / 10% acetic acid, and dried for 2 hours at 80°C. The gel was exposed to film overnight at –70°C and developed the following day. The sequences of the clones were read from this gel and sent to FASTA and BLAST (Pearson and Lipman, 1988; Altschul et al., 1990,1997) for comparison with RNA and DNA databases. These nucleotide sequences were also analyzed for translation in 6 frames using the Baylor College of Medicine Human Genome Sequencing Center website.

2.8 Northern Blot Analysis of Three Positive Clones

2.8.1 Isolation of RNA from Multiple Normal Tissues

From the results of the sequencing experiments it was decided that the tissue distribution of three of the positive clones would be analyzed by Northern blot. Freshly frozen specimens of adrenal, brain, cervix, colon, kidney, liver, spleen, testes, and thyroid were generously obtained by Dr. Desmond Robb, and TRIzol™ reagent was used, as previously described, to isolate total RNA from each sample. Spectrophotometric readings were taken to check the purity and the amount of RNA isolated.

2.8.2 Dot Blotting of the Isolated RNA

A dot blot was carried out using the isolated RNA from each of the tissues, to ensure that the RNA samples were not degraded and to normalize the amount of RNA. Spectrophotometric readings were used to calculate the volumes of resuspended RNA needed to give 1 μg of RNA in a final volume of 10 μl. The samples were denatured at 65°C for 15 minutes, and then placed on ice. Hybond-NX (Amersham Life Science,

Buckinghamshire, England) nylon membrane was labelled with each of the samples approximately 2 centimeters apart. The denatured samples were slowly pipetted onto the membrane. The membrane was air dried for 5 minutes, then the RNA was cross-linked with ultraviolet light (254 nm) for 3 minutes. Next, 5 ml of pre-heated (65°C) ExpressHyb™ Hybridization Solution (Clontech, Palo Alto, CA) was added to the blot in a hybridization bag. The blot was pre-hybridized at 65°C for 15 minutes. The [α–^{32}P] dCTP (3000 Ci/mmol) labelled human beta-actin cDNA control probe (Clontech, Palo Alta, CA) was added to the hybridization solution and was allowed to hybridize at 65°C for 1 hour. The beta-actin probe was labelled using the rediprime™ II random prime labelling system (Amersham Pharmacia Biotech, Buckinghamshire, England) described in the section "Preparation of Probes for Northern Blotting".

Following the hybridization, the blot was washed in 2X SSC (3.0 M NaCl, 0.3 M Na_3-citrate pH 7.0) / 0.1% SDS (sodium dodecyl sulfate) at room temperature for 15 minutes. This wash was repeated, and the blot was then washed in 0.1X SSC / 0.1% SDS at 65°C for 1 hour.

The blot was exposed to Biomax™ film (NEN™ Life Science Products, Boston, MA) for 3 hours at –70°C. The ChemiImager ™ 4000 Low Light Imaging System (Canberra Packard Canada, Montreal, Canada) was used to measure the intensity of the samples on the blot. This information was used to normalize the amount of RNA loaded onto the agarose / formaldehyde gel.

2.8.3 Agarose / Formaldehyde Gel Electrophoresis of RNA Samples

Twenty μg of each RNA sample was electrophoresed on a 3% formaldehyde / 1% agarose gel (1.755 g of agarose, 127.5 ml of DEPC-H_2O, 15.938 ml of 10X MOPS, and 15.938 ml of 37% formaldehyde). MOPS is composed of 200 mM MOPS (3-[N-morpholino] propanesulfonic acid) pH 7.0, 50 mM Na_3-acetate, and 5 mM Na_2-EDTA. In addition, a 0.24 -09.5 Kb RNA Ladder (Gibco BRL) was electrophoresed on the same gel in order to give standards with which to compare the sizes of the transcripts obtained by probing with each of the 3 clones.

A solution of 8% formaldehyde, 65% formamide, and 1.0X MOPS was made, and added to the RNA samples to be loaded on the gel, 3.16 μl per μl of RNA and DEPC-H_2O. The samples were incubated at 70°C for 12 minutes, centrifuged briefly, and 2 μl of 0.1 mg/ml ethidium bromide in DEPC-H_2O and 2.0 μl of RNA loading buffer (60% glycerol, 50 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol) were added. The samples were loaded on the formaldehyde gel and electrophoresed at 5 volts / cm until the xylene cyanol band was approximately half way down the gel. The gel was then photographed under ultraviolet light using the ChemiImager ™ 4000 Low Light Imaging System to check the quality of the RNA samples.

2.8.4 Capillary Transfer of RNA

The RNA was transferred from the formaldehyde gel to a nylon membrane for probing. Hybond-NX nylon membrane was cut to the size of the gel minus the wells. The membrane was briefly soaked in DEPC-H_2O and then soaked for 15 minutes in 10X SSC made with DEPC-H_2O. The gel tray was inverted in a glass dish containing about

300 ml of 10X SSC. It is important that the gel tray is not submerged. A piece of Whatman #3 filter paper was draped over the gel tray so that the ends hung down into the 10X SSC to act as wicks. A sterile Pasteur pipette, soaked in 10X SSC, was used to roll air bubbles out of the filter paper. The wells were cut off the gel and a nick made in the upper right hand corner. The gel was then laid upside down on the gel tray and the bubbles rolled out in the same manner. Next the nylon membrane was placed over the gel and the bubbles rolled out. A nick was made in the membrane to correspond with the nick in the gel. Two pieces of filter paper slightly larger than the gel were laid on top of this arrangement. Saran Wrap™ was placed around the gel and the glass dish to prevent the evapouration of SSC during the transfer. Blotting paper (approximately 12cm high) was placed on top of the filter paper and this whole assembly was covered with Saran Wrap™. Finally, a 500 g weight was placed on top. The transfer was carried out overnight at room temperature.

The following day, the transfer was disassembled and everything was discarded, except the nylon membrane. The membrane was allowed to air dry for approximately 30 minutes and was then baked at 80°C for 2 hours, to immobilize the RNA on the membrane. The bands corresponding to the 0.24 – 9.5 Kb RNA marker were visualized under UV light and a pencil used to mark the positions of these bands on the membrane. A standard plot was produced by graphing the size of the standard against the distance migrated. This plot was used to estimate the sizes of the transcripts detected by the Northern blotting experiments.

2.8.5 Preparation of Probes for Northern Blotting

The probes used in the Northern blotting experiments were produced by amplifying the three positive clones with custom designed primers (Gibco BRL, Gaithersburg, MD). The sequences of these primers are given in Table 2-1. The primers were designed using the sequences of the clones and the Primer3 Test Pre-Release Program (Whitehead Institute for Biomedical Research). The use of custom designed primers ensured that the Zap Express vector was not a part of the probe.

The cDNA inserts were amplified with the polymerase chain reaction using their specific primers. The PCR cocktail used for this set of experiments was the same as previously described with the exception that the final concentration of $MgCl_2$ was changed from 2.0 mM to 1.5 mM, making the PCR reaction more stringent. The reaction parameters were also changed to make the reaction more stringent: 95°C for 5 minutes, 94°C for 30 seconds, 56°C (57°C) for 30 seconds, 72°C for 45 seconds, for 35 cycles, then 72°C for 7 minutes, and stored at 4°C. One probe, MS2 58.4, had an annealing temperature of 57°C, while the other two probes, MS2G383 and MS2 7.0, had an annealing temperature of 56°C. The templates in each PCR were 0.5 µl of the purified T3 / T7 PCR products.

The amplified probes were purified using the Wizard® PCR Preps DNA Purification System (Promega, Madison, WI) and then diluted to 5 ng/µl. Each of these probes was labelled using the rediprime™ II random prime labelling system (Amersham Pharmacia Biotech). Twenty- five ng of the probe was added to 40 µl of 1X TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). This mixture was boiled for 5 minutes and

Table 2-1. Sequences of the custom primers used for amplification of the probes used in Northern blotting experiments, and the clones for which they are specific.

Clone	Primer Identification Number	Sequence (5' to 3')
Ya 10.3	MS2G383P	CCT GCT TGA CCC TTC AGA GA
Ya 10.3	MS2G383M	CCA GCT CCC GTT CAT ATT CT
7.0	MS2 7.0P	TGA TTG TGA AGG CTG TGA CC
7.0	MS2 7.0M	TTT CTT GGT TTG CCT TGC TT
58.4	MS2 58.4P	TTC CCA AGA ACG AGA AGG AA
58.4	MS258.4M	TGA GAG TTT GAA TCC ACA GCA

immediately placed on ice. The denatured probe was added to a rediprime™ II random prime labelling system reaction tube (containing dATP, dGTP, dTTP, exonuclease free Klenow enzyme, and random primers). Five µl of [α–^{32}P] dCTP (3000 Ci/mmol) was also added to the tube and the ingredients mixed by pipetting up and down several times. The reaction tube was incubated at 37°C for 45 minutes.

Unincorporated nucleotides were removed from the labelled probe by Sephadex G-50 chromatography. A 1cc. syringe was plugged with sterile glass wool and filled with Sephadex G-50 suspended in 1X TE, pH8.0. The syringe was placed in a Falcon tube and centrifuged at 200 g for 3 minutes. The syringe was again filled with Sephadex G-50 and centrifuged at 200 g for 3 minutes. The syringe was then placed in a Falcon tube with a 1.5 ml microcentrifuge tube in the bottom and the labelled probe was added to the center of the resin. This was centrifuged for 5 minutes at 200 g. Fifty µl of 1X TE buffer was added to the center of the resin and the column centrifuged at 200 g for 5 minutes. The probe was transferred to a new microcentrifuge tube and 1µl was removed for counting in the scintillation counter. The volume of probe to be added to the Northern blot was calculated using the following formula: 1.5×10^6/CPM = the number of µl of probe required per ml of hybridization solution.

2.8.6 Hybridization of Probes

The blot was briefly soaked in 2X SSC and sealed in a hybridization bag. Ten ml of prewarmed (65°C) ExpressHyb™ Hybridization Solution was added and the blot was prehybridized at 65°C for 30 minutes with gentle rocking. The appropriate amount of

probe was boiled for 5 minutes, cooled on ice, and briefly centrifuged before being added to the prehybridized blot. At this stage it is important that the labelled probe be added to the hybridization solution in the hybridization bag and not directly onto the blot. The blot was hybridized at 65°C for 60 minutes with gentle rocking.

Next, the blot was washed with 1X SSC / 1% SDS at room temperature for 15 minutes, then with 0.5X SSC / 0.5% SDS at room temperature for 15 minutes, and finally with 0.1X SSC / 0.1% SDS at room temperature for 15 minutes. If the background was still high when checked with a Geiger counter, the blot was washed in 0.1X SSC / 0.1% SDS at 42°C for 15 minutes. The blot was exposed to Biomax™ film (NEN™ Life Science Products, Boston, MA) overnight at –70°C and developed the next day. If the film was weak the blot was exposed for a longer period of time before being developed.

The same blot was used for two of the probes, ya 10.3 and 58.4. In addition, the blot was also probed with beta-actin as a control probe. The remaining probe, 7.0, was tested on a multi-tissue blot purchased from Clontech, which was generously provided by Mr. Mike Witcher (Dr. Allan Pater Laboratory, Memorial University of Newfoundland, St. John's, Newfoundland, Canada). This blot was also hybridized with beta-actin as a control. After each hybridization was complete the blots were "stripped" by incubating in a boiling solution of 0.1% SDS until counts were no longer detected with the Geiger counter. The blots were exposed overnight at –70°C and developed the next day to ensure that there was no radioactivity present. Before the blots were used again they were rinsed in 2X SSC.

Chapter Three Results

3.0 Construction and Screening of the MS2 cDNA Library

Messenger RNA isolated from several multiple sclerosis plaques was used to construct a directionally cloned cDNA expression library in the λ Zap Express vector. This library, denoted MS2, contained approximately 800 000 clones, with less than 4% non-recombinants. Primary serological screening of the library with pooled patients' sera identified 62 positive clones. Of these, secondary screening showed 30 to be false positives, while 26 were IgG. Photographs of a representative NZY plate used for primary screening, and of a nitrocellulose membrane used in the secondary screening process are in Figures 3-1 and 3-2, respectively.

The remaining 6 positive clones were partially sequenced and the nucleotide sequences sent to FASTA and BLAST for comparison with DNA and RNA databases. As the time allotted for the completion of the Master's project did not permit the study of all 6 clones, it was decided that only 3 of the clones would be investigated. These 3 clones were selected based on the number of sera with which each reacted, and their percent homology with known proteins. Two of the "rejected" clones encoded unknown proteins. The product of one of these clones reacted with 4 out of 18 sera, while the other reacted with 3 out of 18. The third clone encoded a ribosomal protein and the product of this clone reacted with 2 out of 18 sera. Due to the amount of time required to characterize a previously unknown protein, by others in the research laboratory of D.G. Haegert, it was decided that the remaining clones would be left for analysis in a future project. The clone numbers, the FASTA identification, the percent homology, the

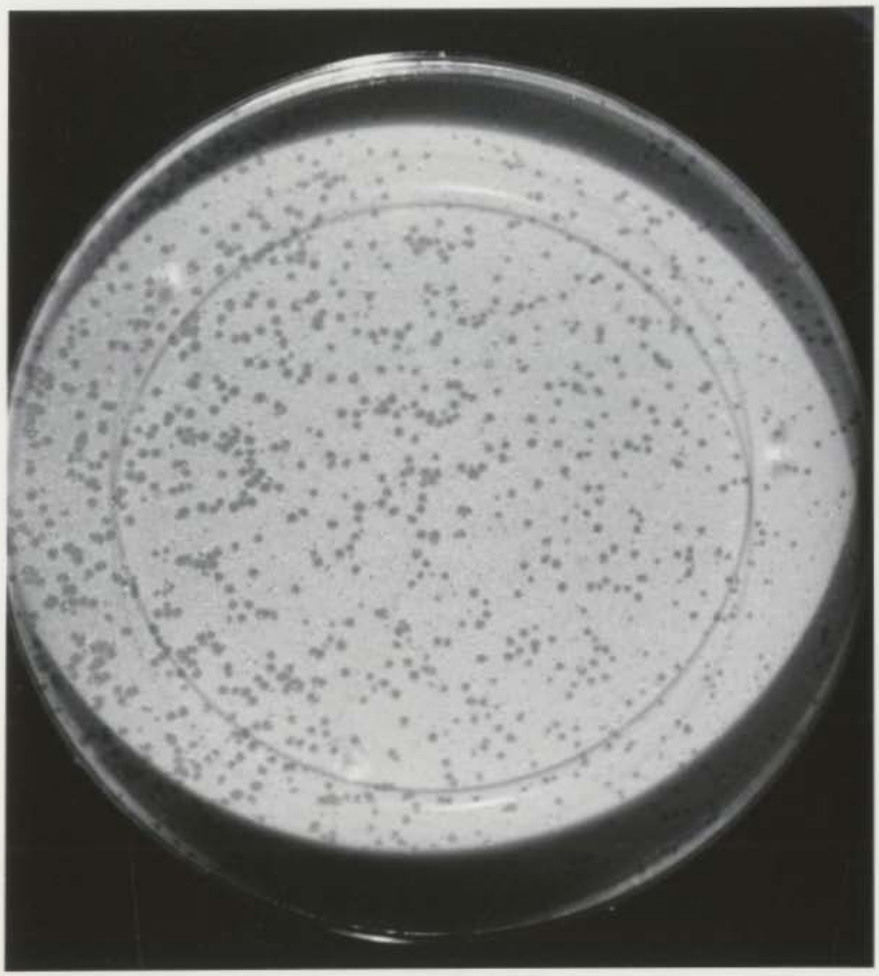

Figure 3-1. A photograph of an NZY agar plate used for primary screening. The plate was incubated overnight at 37°C with 600 µl of XLI Blue MRF' bacteria and a suitable volume of MS2 cDNA phage to give an appropriate number of plaques for screening. The plaques are identified as "holes" in the lawn of bacteria.

Figure 3-2. Photograph of a nitrocellulose membrane used for secondary screening. A quarter of the NZY agar plate was incubated overnight at 37°C with 200 µl of XLI Blue MRF' bacteria and an appropriate volume of a dilution of the "plug". The quarter was overlaid with the nitrocellulose membrane and incubated for 3 hours at 37°C. The membrane was subsequently removed, washed with 0.05% Tween 20 / 1X TBS, blocked with 5% milk / 1X TBS, then incubated with 1/2500 patients' serum. Positive clones were detected with alkaline phosphatase-conjugated AffiniPure anti-human IgG, Fcγ fragment specific antibody. Positive clones are identified as dark purple circles against a light purple background, or in this case a dark circle on a lighter background.

number of sera with which each clone was reactive, the RETRIEVE accession code, and the approximate size of the "selected" clones are given in Table 3-1. The results obtained from the BLAST searches are not given in Table 3-1 as they were virtually identical to those of the FASTA searches.

Four hundred eighty-six base pairs (bp) of clone ya 10.3 were sequenced from the 5' end. (The preliminary sequencing of ya 10.3 was performed by myself, however, the extension of the sequence and the selection of internal primers was done by Mr. Dante Galutira.) A FASTA search indicated that this sequence is 98% homologous with that of testican. The ya 10.3 clone was approximately 1165 bp and the product of this clone reacted with 2 of the 18 sera tested. A search of the BLAST dbEST database indicated that this molecule is expressed in human fetal brain and retinal pigment epithelium. (In each of the dbEST searches described in this thesis a score of 100 was used as the cutoff point of significance as described by Blond et al. (1999)). The ya 10.3 sequence was also submitted to the Baylor College of Medicine Human Genome Sequencing Center website and analyzed for translation in 6 reading frames. One hundred sixty-one amino acids were identified in frame +3 with no stop codons. Therefore, the ya 10.3 sequence lies within an open reading frame of at least 161 amino acids. This derived amino acid sequence was compared with the 439 amino acid sequence of testican. Amino acids 1 to 157 of the ya 10.3 sequence were 97% homologous with amino acids 255 to 411 of the testican protein. Therefore, ya 10.3 corresponds to a part of the open reading frame of the testican gene. The remaining 4 amino acids of the derived ya 10.3 sequence were not homologous with the testican amino acid sequence. This is unexpected as the nucleotide

Table 3-1. The FASTA identification, percent homology, RETRIEVE accession code, estimated size, and number of sera which gave positives, for each of the three positive clones found to be suitable for further study.

Clone	FASTA Identification	Percent Homology	RETRIEVE Accession Code	Number of Sera which gave Positives /18	Approximate Size (bp)
Ya 10.3	Testican	98	X73608	2	1165
7.0	*H. sapiens* mRNA for KIAA0530	97	Ab011102	2	2015
58.4	*H. sapiens* clone F4 transmembrane protein	94	L09749	3	1165

sequences of clone ya 10.3 and testican were 98% homologous. However, closer examination of the FASTA results revealed that this homology was obtained through the insertion of a "gap" in the testican sequence at position 1663, thereby causing a frame shift. If the sequencing results are correct then clone ya 10.3 and testican are not identical sequences, but rather are related sequences. Of course, it is possible that sequencing errors in either clone may be responsible for the differences in the amino acid sequences.

Two hundred thirty-seven base pairs of clone 7.0 were sequenced from the 5' end and this sequence corresponded most closely to *Homo sapiens* mRNA for KIAA0530. The sequence had 97% homology. The 7.0 clone was approximately 2015 bp and the product of this clone reacted with 2 out of 18 sera. A search of the BLAST dbEST database demonstrated that this molecule is expressed in germinal center B lymphocytes, an ovarian cancer, and normal kidney. The 7.0 sequence was also analyzed for translation in 6 reading frames. Seventy-nine amino acids were identified in frame +1 with no stop codons; i.e. the 7.0 sequence lies within an open reading frame of at least 79 amino acids. A comparison of the clone 7.0 derived amino acid sequence with the known amino acid sequence of KIAA0530 demonstrated 100% homology between the putative amino acids 1 to 67 of clone 7.0 and 1083 to 1149 of KIAA0530. The remaining 12 amino acids of the putative 7.0 sequence were not homologous with the KIAA0530 sequence. This at first seems surprising, as the nucleotide sequences of the two were 97% homologous. However, closer examination of the FASTA results revealed that this homology was obtained by adding "gaps" to the clone 7.0 sequence at positions 200 and

221. This explains the different reading frames between the two sequences. Assuming that the sequencing of clone 7.0 is correct, the results suggest that clone 7.0 and KIAA0530 may be related sequences but not identical sequences. Alternatively, sequencing errors in either clone would lead to the observed differences in amino acid sequence.

Two hundred fifty-seven base pairs of clone 58.4 were sequenced from the 5' end. This sequence corresponded most closely to *Homo sapiens* clone F4 transmembrane protein in the FASTA database. The homology was 94%. This clone was the same size as ya 10.3 and the product of this clone reacted with 3 out of 18 sera. A search of the BLAST dbEST database did not find any matches with a score greater than 100. The 58.4 sequence was also analyzed for translation in 6 frames. Eighty-five amino acids were identified in frame +1 with no stop codons; i.e. the 58.4 sequence lies within an open reading frame of at least 85 amino acids. Although the amino acid sequence corresponding to the clone F4 transmembrane protein has not been reported, I obtained the nucleotide sequence from BLAST using the accession code L09749 and submitted the sequence to the Baylor College of Medicine Human Genome Sequencing Center website for translation in 6 reading frames. Only 64 amino acids were translated before a stop codon was identified. As well, all 6 reading frames contained multiple stop codons. This leads me to believe that the sequence submitted by Andrews (1993, unpublished) to the database does not correspond to the "clone F4 transmembrane protein". The submitted nucleotide sequence does not contain an open reading frame. Thus the 58.4 sequence

could be the 3' end of some other sequence that shares homology to the putative clone F4 transmembrane protein.

Although three clones were not investigated in this study, each was partially sequenced from the 5' end and the resulting sequences were compared with the BLAST dbEST database and were analyzed for translation in 6 frames. There were no matches with scores greater than 100 for the two sequences which encoded unknown proteins. The third sequence was found to encode a molecule known to be expressed in a large number of tissues including thymus, small intestine, T lymphocytes, retina, and fetal brain and kidney. For simplicity these clones will be referred to as A, B, and C. Clones A and B encode unknown proteins, clone C encodes a ribosomal protein. Analysis of the 118 base pair sequence of clone A for translation in 6 reading frames identified 37 amino acids in frame +1 before a stop codon was identified. This means that at most the clone A sequence contains an open reading frame corresponding to 37 amino acids and the remainder of the 3' sequence is non-coding. The same analysis of the 144 base pair sequence of clone B identified 47 amino acids in frame +3 with no stop codons. Analysis of the translation in 6 reading frames of the 244 base pair sequence of clone C identified 80 amino acids in frame +3 with no stop codons. Therefore, clones B and C likely contain open reading frames corresponding to at least 47 and 80 amino acids, respectively.

3.1 Northern Blotting Experiments

Northern blotting was done with PCR-amplified fragments corresponding to 428 base pairs of clone ya 10.3, 155 base pairs of clone 7.0, and 190 base pairs of clone 58.4. The results of these Northern blotting experiments are shown in Figures 3-3 and 3-4. As described in the section "Dot Blotting of the Isolated RNA", the RNA samples used for the Northern blotting experiments were normalized before being electrophoresed on an agarose / formaldehyde gel by performing a dot blot with a beta-actin probe. The samples on the Clontech blot were normalized by the manufacturer with a beta-actin probe. A sequence hybridizing with the ya 10.3 probe was identified in adrenal, brain, colon, and testis. The intensities of the bands were very similar in each of these samples, indicating similar expression of the testican transcript in each of these samples. The remaining samples, cervix, liver, spleen, and thyroid, showed much weaker bands, indicative of lower testican expression, if any. A sequence hybridizing with the 58.4 probe was found to be strongly expressed in testis, with negligible expression in adrenal, brain, cervix, colon, liver, spleen, and thyroid. A sequence hybridizing with the 7.0 probe was found to be weakly expressed in all of the tissues tested, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas.

The sizes of the transcripts detected on the Northern blots were estimated by using the standard plot described in the section "Capillary Transfer of RNA". The transcript to which ya 10.3 bound is estimated to be 1.1 Kb, for 58.4 it is estimated to be 3.7 Kb, and for 7.0 it is estimated to be 0.9 Kb, Figures 3-3 and 3-4.

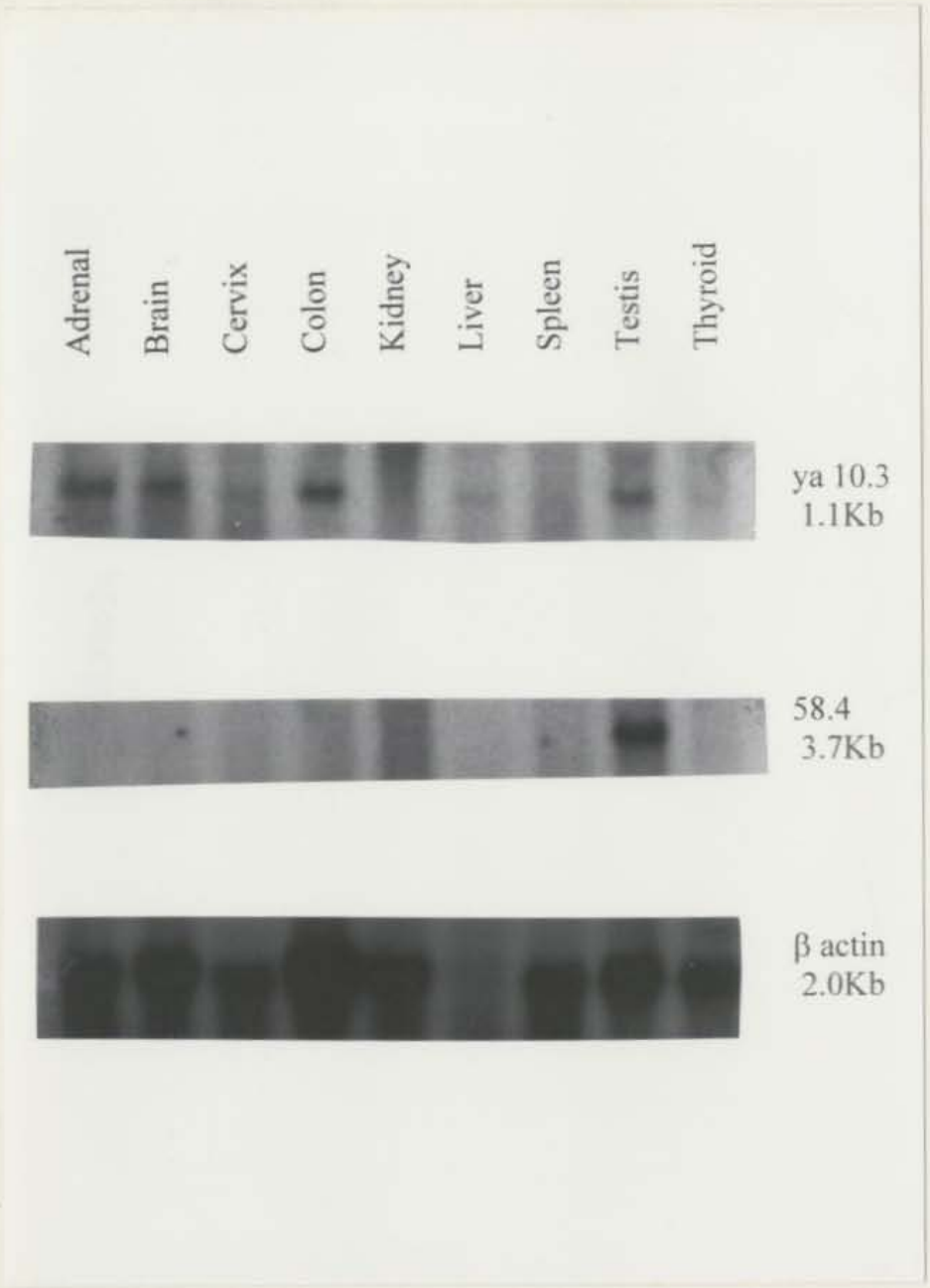

Figure 3-3 Northern blot of beta-actin, ya 10.3, and 58.4. The probes were prepared with the rediprime™ II random prime labelling system. Hybridization was carried out with ExpressHyb™ Hybridization Solution at 65°C for 1 hour. The blots were washed with 1X SSC / 1% SDS at room temperature for 15 minutes, 0.5X SSC / 0.5% SDS at room temperature for 15 minutes, 0.1X SSC / 0.1% SDS at room temperature for 15 minutes, and finally with 0.1X SSC / 0.1% SDS at 42°C for 15 minutes. The sizes written below each clone represent the estimated size of the transcript to which it is bound. These sizes were estimated using a standard plot as described in the section "Capillary Transfer of RNA".

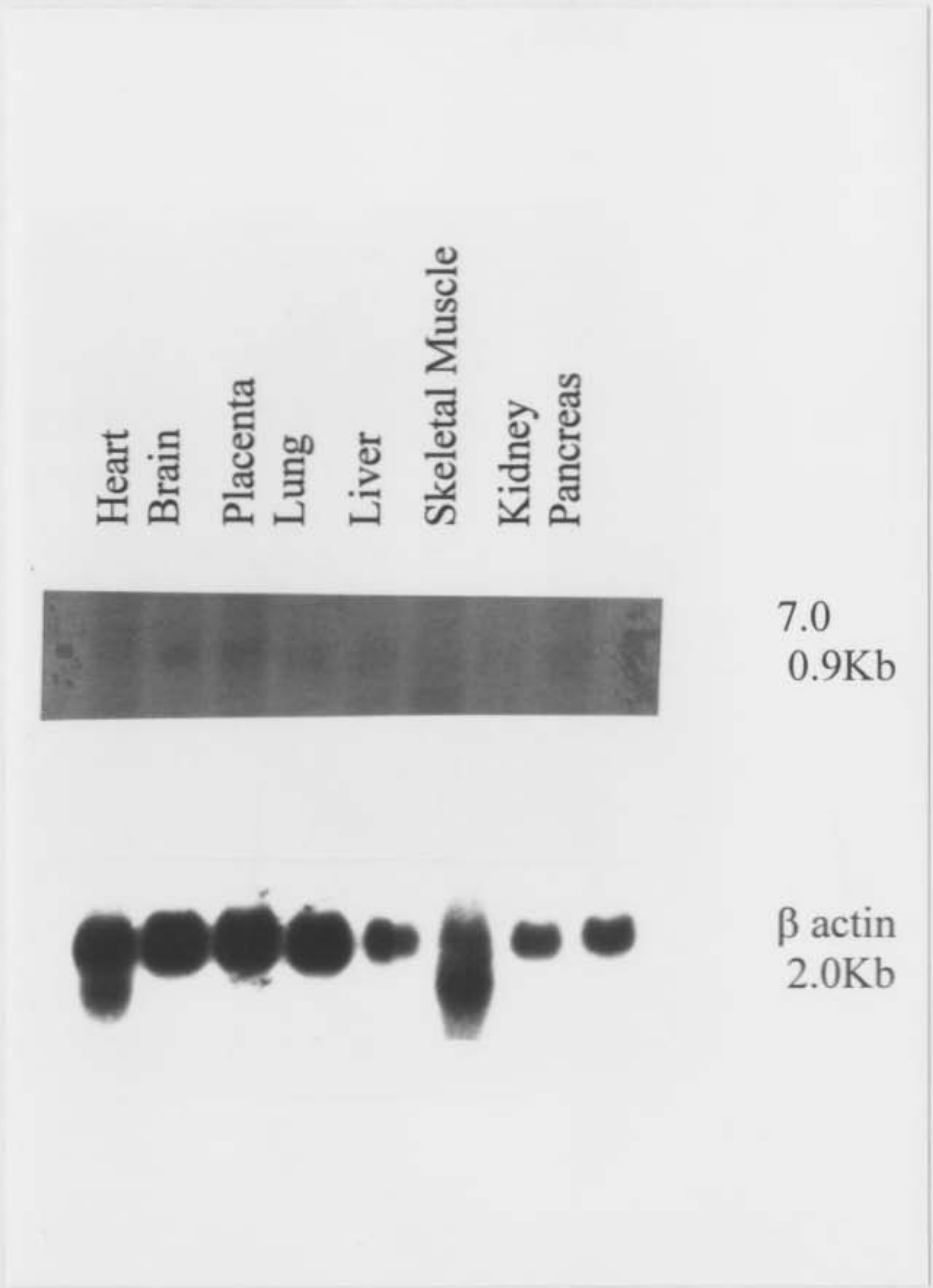

Figure 3-4 Northern blot (Clontech) of beta-actin and 7.0. The probes were prepared with the rediprime™ II random prime labelling system. Hybridization was carried out with ExpressHyb™ Hybridization Solution at 65°C for 1 hour. The blots were washed with 1X SSC / 1% SDS at room temperature for 15 minutes, 0.5X SSC / 0.5% SDS at room temperature for 15 minutes, 0.1X SSC / 0.1% SDS at room temperature for 15 minutes, and finally with 0.1X SSC / 0.1% SDS at 42°C for 15 minutes. The sizes written below each clone represent the estimated size of the transcript to which it is bound. These sizes were estimated using a standard plot as described in the section "Capillary Transfer of RNA".

Chapter Four Discussion

4.0 Multiple Sclerosis Autoantigens

Multiple sclerosis is believed by many investigators to be an autoimmune disease (Stinissen et al, 1997; Ewing and Bernard, 1998). In the context of autoimmunity, much work in multiple sclerosis has focused upon the identification of an autoantigen(s) responsible for the initiation and/or perpetuation of the disease. To date it is not possible to say with complete confidence that such an autoantigen(s) has been found (see however, a review by Weiner, Can. J. Neurol Sci., 1998). A number of myelin constituents have been investigated as possible autoantigens in MS, including MBP, PLP, MAG, MOG and others. T and B lymphocyte responses to these proteins have been demonstrated in patients with MS. As well, antibodies specific for these proteins have been isolated from MS patients (Panitch et al., 1980; Gorny et al., 1983; Wajgt and Gorny, 1983; Cruz et al., 1987; Link et al., 1989,1990; Moller et al., 1989; Ota et al., 1990; Olsson et al., 1990a,1992; Baig et al., 1991; Sun et al., 1991a,b; Chou et al., 1992; Kerlero de Rosbo et al., 1993,1997; Gerritse et al., 1994). Perhaps the strongest evidence implicating these proteins as autoantigens in MS is that they can induce EAE in animal models (Panitch and Ciccone, 1981; Yamamura et al., 1986; Satoh et al., 1987; Pender, 1988; Johns et al., 1995).

Although there is no definitive evidence to support these myelin components as being responsible for the myelin damage observed in multiple sclerosis, there is evidence which questions their importance in this process. First, T and B lymphocyte responses, and antibody responses, to these autoantigens are not specific to MS. In each of the

studies cited above these responses were also observed in healthy controls and patients with other neurologic disorders. Second, in the majority of MS patients the oligoclonal bands in the CSF do not contain significant amounts of anti-myelin antibodies (Nobile-Orazio et al., 1987; Kaiser et al., 1997). It is possible that the immune responses to these myelin proteins observed in MS patients are the result of previously sequestered autoantigens being released after myelin injury.

Since the lesions in multiple sclerosis typically involve the white matter of the CNS, the optic nerve, and the retina (Antel and Arnason, 1991; Boccaccio and Steinman, 1996; Kerrison, et al., 1994; Lucchinetti and Rodriguez, 1997; Martin, 1997; Stinissen et al., 1997), one would expect that the expression of autoantigen(s) important in MS would also be mainly limited to these areas. In particular, they should be limited to the myelin and/or the myelin producing cells of the CNS, the oligodendrocytes, or to some other component of the CNS such as astrocytes or the endothelial cells of the BBB. The fact that lesions are identified in the retina of MS patients is unexpected as the retina does not possess myelinated fibers, however, it does express CNP (Walsh and Murray, 1998). If MS is indeed an autoimmune disease, then it is also important that the autoantigen(s) be accessible to the immune system. Both MOG and PLP, unlike MBP and MAG, are found only in CNS myelin, and MOG is found on the extracellular surface of oligodendrocytes and the myelin sheath. Although PLP is an integral hydrophobic membrane protein, it too is somewhat accessible to an immune response.

A number of autoantigens have been identified in MS. However, the question still remains as to whether these autoantigens actually initiate the immune response which

leads to myelin damage or whether they become immune targets as a result of bystander activation. The importance of the immune response to these autoantigens in the destruction of myelin is also still under investigation.

The majority of studies which have identified myelin constituents as autoantigens relied on methods which required the investigator to examine the immune response to a particular autoantigen, for example solid-phase radioimmunoassays, immunoadsorbent assays, ELISA, immunoblot assays, the use of monoclonal antibodies to myelin constituents, and antigen-induced secretion of IFN-γ. These techniques are necessarily "biased" with respect to the autoantigen which they are able to identify. As there is no concrete evidence implicating an immune response to myelin components in the initiation of multiple sclerosis, I thought that a more unorthodox approach to identify potential autoantigens in MS was warranted.

4.1 The SEREX Technique

The work described in this thesis was based on the hypothesis that one or more previously unidentified autoantigens may be targets of an antibody response in MS. The approach was designed to identify autoantigens without any *a priori* assumptions as to what these autoantigens would be. The SEREX technique developed by Sahin and Tureci (Sahin et al., 1995) had previously been used to identify novel tumor antigens in a number of different cancers and so was adapted for use with multiple sclerosis. The SEREX method identifies autoantigens which have elicited a high titer antibody response (Chen et al., 1997). This implies that the antigens identified are not only B lymphocyte

antigens, but are also T lymphocyte antigens, as the production of high antibody titers requires T lymphocyte help. Therefore, even though the actual identification of autoantigens through SEREX relies upon antibodies in the patients' sera, these autoantigens could subsequently be examined for a role in the cellular immune response of the patient. In fact, the SEREX technique has identified tumor autoantigens, which had previously been identified by cytotoxicity assays (Chen et al. 1995). This is an important point, as multiple sclerosis is believed to be primarily a T lymphocyte mediated disease (Stinissen et al., 1997), see also the section "The Role of the T Lymphocyte".

The SEREX technique adds a potentially new dimension to serological screening. The identification of a putative autoantigen can be easily and quickly followed by cDNA sequencing and investigation of mRNA expression patterns. In addition, SEREX is able to recognize both intracellular and cell surface antigens (Chen et al., 1997).

When considering the antigens which can be recognized by the SEREX technique it is important to differentiate between conformational and linear epitopes. Conformational epitopes can be defined as epitopes in which the amino acids are not close together in the primary structure of the protein, but are brought closer together through protein folding. Although the conformation of the protein is integral to the immune response of such as epitope, certain parts of conformational epitopes can be mimicked by peptides, known as mimotopes (Morris, 1996). In contrast, linear epitopes are simply a series of adjacent amino acids. Such epitopes can easily be mimicked by peptide sequences. B lymphocytes are able to recognize both conformational and linear epitopes, while T lymphocytes recognize peptides presented by the MHC molecules.

Since a novel autoantigen in MS could represent a previously unknown molecule, there is no way of predicting if its recognition by the immune system will be due to conformational or linear epitopes. If the autoantigens in multiple sclerosis are recognized through conformational epitopes, they may be difficult or even impossible to identify through the antibody screening of an MS cDNA expression library. The reasons for this are as follows: (1) the correct folding of the protein, or protein fragment, may not occur in the bacterial expression system, (2) the expression of the full transcript may be necessary to produce the native form of the protein, and therefore the conformational epitope (in general the cDNA inserted into the phage vector does not contain a full transcript), (3) the post-translational modifications which may be required for the recognition of such epitopes do not occur in the bacterial system which expresses the protein, and (4) it is also possible that certain conformational changes of the epitopes may occur when they are expressed in the bacterial system (Tureci et al., 1997). However, if the antibodies produced against the conformational epitopes are able to recognize mimotopes, then they would be expected to be recognized by SEREX. Of course, antibodies produced in response to linear epitopes should also be detected with this method of screening.

Although it is generally believed that the majority of humoral responses are directed against conformational epitopes, Genain et al. (1999) were able to demonstrate that specific autoantibodies bound to myelin can be identified using gold-conjugated peptide antigens. Therefore, it is possible that even if the antibodies in the patients' sera

are produced against conformational epitopes they may recognize the proteins expressed in the MS2 expression library.

4.2 Studies Using Libraries to Identify Multiple Sclerosis Antigens

Studies similar to the one described here have been reported, but did not identify autoantigen(s) specific to multiple sclerosis. Owens et al. (1996) used mRNA isolated from chronic and acute multiple sclerosis plaques and from periplaque white matter to construct a directional cDNA expression library. The resulting libraries were screened with CSF from MS patients, or with IgG extracted from MS sera or CSF. These authors also used subtracted cDNA libraries in order to enrich mRNA sequences which were unique or over-represented in the MS plaque as compared with normal white matter. Screening of these libraries did not detect any sequences unique to MS, nor did it detect MBP or any other myelin specific proteins.

Cortese et al. (1996) screened random peptide libraries displayed on phage with sera and CSF of MS patients and with sera of controls. Although screening this library with two CSF samples did not reveal any MS specific antigens, it did identify certain common motifs. More importantly, this study and a similar study by this group in 1998 led to the following conclusions: (1) "antibodies displaying the same binding specificity are present in the CSF and serum of the same patient, (2) these antibodies may be directed against ubiquitous antigens, (3) some of these antibodies are specifically enriched in the CSF", (4) it seems that each MS patient has a different set of intrathecally

produced antibodies in the CSF, and (5) anti-phagotope (phage displayed epitope) antibodies are found in the sera of healthy individuals as well as those of MS patients .

Becker et al. (1997) constructed a normalized cDNA library from plaques of a chronic progressive MS patient. The majority of cDNAs in the library represented unknown or uncharacterized genes. However, 54 cDNAs corresponded to genes known to be involved in immune activation, such as those encoding MHC molecules, cell adhesion molecules, and cytokines and cytokine receptors. In addition, 19 cDNAs encoded known or suspected autoantigens, including MBP and PLP. This study did not identify any cDNA sequences identical to those of any infectious agents.

Archelos et al. (1998) used an oligodendrocyte-precursor cell line to construct two expression libraries, which were subsequently screened with pooled CSF from MS patients and patients with other neurologic diseases. This screening identified only 6 positive clones, none of which corresponded to a known protein. However, 5 of the 6 clones contained a common 7 amino acid sequence (EKPCLEK) which is highly homologous to the translated consensus *Alu* repeat epitope. These investigators were able to show that approximately 44 percent of relapsing/remitting MS patients possess antibodies specific for this motif. Although a lower percentage of primary and secondary progressive MS patients, healthy controls, and patients with other neurologic disorders also possess these antibodies, the titers are highest among the relapsing/remitting subjects. This group of investigators have come closest to identifying MS specific autoantigens.

Alu repeats are short, repetitive elements found in the introns of almost every known protein-encoding gene of humans and other primates (Makalowski et al., 1994). There are a number of mechanisms by which these repeats may lead to disease, these are discussed in the section "Studies Relevant to the Master's Project Discussed in this Thesis". The *Alu* peptides discovered by Archelos et al. (1998) may represent translated *Alu* repeat epitopes, epitopes of an unknown oligodendrocyte protein, or regulatory molecules.

In summary, it seems clear that even though use of expression libraries has potential to identify autoantigens in MS, the method has led to few findings of significance to date.

4.3 Recognition of Self-proteins or Self–peptides as Autoantigens

Why would self-proteins or -peptides be identified as autoantigens by the immune system? There are a number of possibilities to explain this:

1. Failure of central tolerance (negative selection). The process of negative selection, which occurs in the thymus, is responsible for the "elimination" of self-reactive T lymphocytes, either through anergy or deletion (Abbas et al., 1994). If this process fails then potentially self-reactive T lymphocytes are released into the peripheral circulation.
2. Failure of peripheral tolerance. Peripheral tolerance is the process which maintains tolerance when self-reactive T lymphocytes escape negative selection, or when tissue-specific antigens are not present in the thymus

(Abbas et al., 1994). Peripheral tolerance is believed to result from anergy or deletion. For example, when self-antigens are presented to T lymphocytes by antigen-presenting cells which do not express the proper co-stimulatory molecules the T lymphocytes are rendered anergic (Brown et al., 1998). However, if these antigen-presenting cells are activated, by certain cytokines for instance, and the expression of the co-stimulatory molecules is up-regulated, then tolerance may be lost.

3. The autoantigens may be released following injury to the myelin sheath. The myelin sheath is located in an immunologically privileged site. Therefore the release of myelin constituents, such as MBP, might be expected to elicit an immune response (Martin et al., 1992a; Poser, 1993; Hohlfeld et al., 1995).
4. Defects in immune regulation. A number of immunological abnormalities have been demonstrated in multiple sclerosis patients. For example, Neighbour (1984) found that lymphocytes isolated from MS patients and subsequently stimulated, produced lower levels of IFN-γ than lymphocytes from control subjects. In addition, a significant proportion of the MS patients had reduced levels of endogenous and augmented NK cell activity. Chofflon et al. (1988) demonstrated that patients with progressive multiple sclerosis had decreased functional suppression and decreased autologous mixed lymphocyte reactions as compared with controls.
5. The epitopes of the self-protein may be processed and presented differently by the antigen-presenting cells of multiple sclerosis patients as compared with

normal individuals, or the post-translational modifications of the protein may differ in MS patients as compared with normal individuals.

The identification of a potential autoantigen using the SEREX technique must be followed by a study of: (1) the tissue specificity of the antigen, (2) the antibody responses to this antigen in both the sera and CSF of healthy controls, patients with other neurologic disorders, and a larger number of MS patients, and (3) the T lymphocyte response to the antigen *in vitro*.

4.4 The Serological Screening of the MS2 cDNA Expression Library

The serological screening of a cDNA expression library constructed with mRNA extracted from MS plaques yielded a total of 6 positive clones. In the interest of time, it was decided that only 3 of these clones would be investigated, those corresponding most closely in sequence to testican, clone F4 transmembrane protein, and KIAA0530. The other 3 clones, a ribosomal protein and two unknown proteins, were excluded because they did not react with more than 2 sera (the clone which encoded the ribosomal protein) or because time did not permit the characterization of the proteins (the clones which encoded unknown proteins). However, these clones are still worthy of further study. If time had permitted, sequences from these clones would also have been amplified and used as probes in Northern blotting experiments, as were ya 10.3, 58.4, and 7.0. As well, the sequences of these clones could have been extended 5', the proteins isolated, and used in ELISAs and Western blots to study antibody reactivity to these proteins using a large number of sera and CSF samples of MS patients and healthy controls.

4.4.1 Clone Ya 10.3

The sequence obtained for the ya 10.3 clone corresponded to part of the open reading frame encoded by the testican gene. Testican was identified by Alliel et al. in 1993, using a probe which corresponded to the first 13 amino acids of a glycosaminoglycan-bearing peptide from human seminal plasma to probe a human testicular DNA library. In 1996, Bonnet et al. identified testican in mouse brain. They found that the protein core of their protein was 95% homologous with its human testicular counterpart. In the mouse brain, testican appeared to be present mostly in the postsynaptic regions of a subpopulation of pyramidal neurons in a specific area of the hippocampus. Although the function of testican has yet to be elucidated, this multidomain protein shares similarities with proteins involved in adhesion, migration, cell proliferation, neural development, synaptogenesis, and synaptic transmission (Bonnet et al., 1996). A more recent study established the location of the testican gene, now known as the SPOCK (an acronym for: SPARC/osteonectin, CWCV, and Kazal-like domains of proteoglycan) gene, to be 5q31 (Charbonnier et al., 1998). This is particularly interesting as this region contains the genes encoding growth factors, cytokines, and neurotransmitter and hormone receptors. The SPOCK gene borders the smallest commonly deleted region of chromosome 5. To date there is no information regarding the investigation of this region of chromosome 5 in relation to multiple sclerosis. However, a Finnish study provided evidence for an MS susceptibility locus on 5p14-p12 (Kuokkanen et al., 1996).

The BLAST dbEST database identified the molecule corresponding most closely to clone ya 10.3 as being expressed in brain and retina. This suggests that ya 10.3 may be an important antigen in multiple sclerosis, as brain and retina are two areas in which lesions are observed in MS patients (see however, the next paragraph). The fact that part of the ya 10.3 amino acid sequence is homologous with amino acids 255 to 411 of the testican protein indicates that ya 10.3 likely encodes for part of the testican molecule. However, the homology between the two sequences terminates at amino acid 411 of the testican gene. This unexpected result can be explained by the fact that the FASTA program inserted a "gap" into the testican sequence at position 1663 in order to achieve maximal alignment. There are two possible explanations for the non-homologous region between clone ya 10.3 and testican. First, cycle sequencing is not 100 percent accurate (Alphey, 1997) and therefore, a nucleotide may have been erroneously inserted in the clone ya 10.3 sequence leading to a frame shift compared to testican. Second, the ya 10.3 clone and testican are related genes. To resolve this issue it is necessary to extend the ya 10.3 sequence both 5' and 3' and compare the derived amino acid sequence with the amino acid sequence of testican.

Northern blotting experiments carried out as part of the work for this thesis, found transcripts hybridizing to the putative testican probe to be present in 4 out of 8 tissues tested, adrenal, brain, colon, and testis, Figure 3-3. In contrast, a study by Marr et al. (1997), demonstrated that testican mRNA was present in high levels in a human vascular endothelial cell line. This suggests that testican mRNA should be present in most human organs. Northern blotting experiments performed by this group detected testican mRNA

in samples of a number of human organs. However, they did not detect mRNA specific for testican in the other cell lines they tested. This led them to conclude that testican is differentially expressed. If this is true, then the expression of testican may differ in patients with multiple sclerosis as compared with normal healthy individuals.

In the section "Multiple Sclerosis Autoantigens", it was hypothesized that a relevant autoantigen in MS would be restricted to the central nervous system. However, the study by Marr et al. (1997) demonstrated that testican mRNA is present in most human organs. This creates a dilemma. If testican is not a CNS-restricted protein, how can it be a relevant autoantigen in MS? The most plausible explanation is the bystander effect. According to Ebers (1998), "... tissue injury may occur as part of a bystander effect and that myelin could be injured as the result of an immune reaction to a nonmyelin antigen". Perhaps, the primary target is present in the CNS, but is neither a myelin nor an oligodendrocyte antigen, for example it could be an endothelial antigen, such as testican. An immune response against a specific endothelial antigen may lead to major changes in the BBB, including a breakdown of the BBB similar to that observed in MS patients. Why such changes would be localized to the CNS is uncertain since endothelial cells are almost ubiquitous in the body.

The estimated size of the testican transcript is 1.1 Kb. This is approximately the same size as the clone ya 10.3 which was isolated from the MS2 cDNA library, Table 3-1. Knowing that ya 10.3 does not encode for the entire testican protein it is not expected that the clone and the transcript to which it hybridizes would be the same size. Perhaps this is due to the imprecise method of estimating the sizes of the bands on the gels (Dr. D

Healey, personal communication). If the sequence for testican submitted to BLAST represents the entire testican gene, then the transcript would be expected to be approximately 3.5 Kb, as this is the size of the sequence submitted by Alliel et al. (1993). However, Marr et al. (1997) reported that although the sequence published by Alliel et al. (1993) represents the entire open reading frame, the testican mRNA is in fact closer to 5 Kb. They also state that more than 60 percent of this mRNA is 3' untranslated sequence. The fact that the expected and estimated transcript sizes are different may be due to the ya 10.3 clone being homologous with the testican transcript only at the sequences that were compared. Perhaps if the ya 10.3 clone were compared with the testican sequence outside this region the two would not be as similar or may not share any homology at all. It is also possible that the ya 10.3 clone is the product of a related gene and/or that the testican and ya 10.3 gene products belong to the same gene family.

4.4.2 Clone 7.0

A FASTA search of the sequence obtained for clone 7.0 found it to correspond most closely with KIAA0530 (97% homology). However, a comparison of the amino acid sequence of KIAA0530 with the putative amino acid sequence of clone 7.0 found that only 67 of the 79 amino acids encoded by clone 7.0 were homologous with the sequence of KIAA0530. This unexpected result can be explained by the fact that the FASTA program inserted two "gaps" into the nucleotide sequence of clone 7.0 at positions 200 and 221 in order to achieve maximal sequence alignment. As with clone ya 10.3, two possible explanations may be suggested for the non-homologous regions of

clone 7.0 and KIAA0530. First, cycle sequencing does lead to sequencing errors (Alphey, 1997) and therefore nucleotides could have been erroneously omitted from clone 7.0 leading to a frame shift compared to KIAA0530. Second, the molecule encoded by 7.0 could be the product of a gene related to the KIAA0530 gene. Further work, including extending the sequence 5' and 3' and once again comparing the derived amino acid sequence with the amino acid sequence of KIAA0530 and with a database of other known amino acid sequences is needed to resolve this issue.

The KIAA0530 protein has not been extensively studied. KIAA0530 was isolated from a human brain cDNA library by Nagase et al. (1998). This protein has been identified as a zinc finger protein and is encoded on chromosome 6. The zinc finger proteins are a group of transcription factors (Mathews and van Holde, 1990). Antibodies specific for such proteins could inhibit the transcription of certain mRNA molecules, thereby preventing the production of the proteins encoded by the particular mRNA. In the case of multiple sclerosis such proteins may include those necessary for the production and/or maintenance of myelin.

A search of the BLAST dbEST database identified the molecule corresponding most closely to clone 7.0 as being expressed by germinal center B lymphocytes and in normal kidney. The Northern blotting experiments carried out as part of the work for this thesis, using clone 7.0 as a probe, detected transcripts in heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, Figure 3-4. Based on the original hypothesis for this thesis, i.e. that relevant autoantigens would be relatively restricted in their tissue distribution, detection of putative transcripts corresponding to clone 7.0 in all of the

tissues tested, strongly suggests that clone 7.0 does not encode an important autoantigen in multiple sclerosis. The study by Nagase et al. (1998) used reverse transcriptase polymerase chain reaction (RT-PCR) to examine the tissue expression of KIAA0530. They did not detect KIAA0530 in any of the 13 tissues, heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, and small intestine, which were tested. It is surprising that this group isolated KIAA0530 from a human brain cDNA library, but were unable to detect it by RT-PCR of brain tissue (the authors do not address this discrepancy in the paper). Perhaps this protein is only expressed in certain areas of the brain. Maybe if the investigators had used the same tissue samples, or samples from the same area of the brain, for both the construction of the cDNA library and the RT-PCR they would have detected KIAA0530 by RT-PCR. In contrast to the results of Nagase et al. (1998), the Northern blotting experiments performed as part of this project, which used clone 7.0 as a probe, detected transcripts in a number of tissues. This may indicate that clone 7.0 does not encode for KIAA0530, but rather encodes for a related gene product. Of course, it is also possible that the Northern blotting experiments were not sufficiently stringent. If this is the case, then the Northern blots may have cross-hybridized with other transcripts related to KIAA0530, and not hybridized to the KIAA0530 transcript itself. Of course, if time had permitted RT-PCR could have been carried out to test this using the same samples which I had used for the Northern blotting experiments.

The estimated size of the transcript obtained from the Northern blot is 0.9 Kb, this is much smaller than the expected size of the KIAA0530 transcript, approximately 6.5

Kb, assuming that the sequence submitted by Nagase et al. (1998) represents the entire KIAA0530 gene. Perhaps this is another indication that the product encoded by clone 7.0 is not KIAA0530, but may be a related gene. This 0.9 Kb transcript is also smaller than the 2.0 Kb (Table 3-1) clone 7.0 isolated from the MS2 cDNA library. Perhaps the clone 7.0 is derived from heterogeneous nuclear RNA (hnRNA), while the transcript detected on the Northern blot is cytoplasmic mRNA. Since hnRNA is the precursor of mRNA and is typically much larger than mRNA (Lewin, 1997), the clone 7.0 would therefore be larger than the transcript on the Northern blot.

4.4.3 Clone 58.4

A FASTA search of the nucleotide sequence of clone 58.4 identified it as corresponding most closely to the nucleotide sequence encoding clone F4 transmembrane protein. Translation of the clone 58.4 sequence in 6 frames revealed that the sequence encoded for at least 85 amino acids. The amino acid sequence of the clone F4 transmembrane protein has not been reported. However, I did submit the 1.6 Kb nucleotide sequence of this protein to the Baylor College of Medicine Human Genome Sequencing Center website for translation in 6 reading frames. This did not identify a nucleotide sequence capable of coding for a protein. Only 64 amino acids were translated before a stop codon was identified, and all 6 reading frames identified multiple stop codons. Perhaps Andrews (1993, unpublished) did not submit or obtain the entire sequence for the clone F4 transmembrane protein, or perhaps only the non-coding sequence was obtained by this investigator. In either case, it is not easy to understand

how Andrews was able to identify a transmembrane protein from this sequence. Therefore, it is not possible to speculate on the relation between the product of clone 58.4 and the clone F4 transmembrane protein. Instead it seems that the clone 58.4 may be the 3' end of another sequence that shares homology to the putative clone F4 transmembrane protein. The BLAST dbEST database was unable to identify any matches for the nucleotide sequence of the 58.4 clone with a score greater than 100. Thus, tissue distribution cannot be further commented upon based on searches in BLAST and FASTA databases.

To date there is no published literature on the clone F4 transmembrane protein. This protein was originally isolated from a cDNA library constructed from human bone marrow stromal cells (Andrews, 1993, unpublished). The Northern blotting experiments, showed that clone 58.4 hybridized with a transcript present in testis. Transcripts were either absent or expressed at very low levels in adrenal, brain, cervix, colon, liver, spleen, and thyroid, Figure 3-3. The fact that the clone F4 transmembrane protein was identified from an MS brain cDNA library, but I could not detect a corresponding transcript in a normal brain sample deserves further discussion. If one assumes that clone F4 transmembrane protein and clone 58.4 are identical, the clone F4 transmembrane protein may only be expressed in MS tissues, for example in regenerating or proliferating tissues, or it may be very weakly expressed in normal brain and up-regulated in MS tissues. As a second possibility, the clone F4 transmembrane protein could be irrelevant to MS but be expressed in proliferating astrocytes or in oligodendrocytes associated with regeneration. If so, this protein could well be detected at the margins of cerebral infarcts or other

cerebral lesions. Of course, to test this hypothesis it would be necessary to perform a Northern blotting experiment using several normal and multiple sclerosis brain samples, as well as samples of other cerebral lesions. This was not done as part of this work due to time constraints and the difficulty of getting MS brain samples. A third possibility is that the 58.4 clone cross-hybridized with a molecular species similar to but not identical to clone F4 transmembrane protein in the Northern blot experiments.

The estimated size of the transcript hybridizing with clone 58.4 from the Northern blot is 3.7 Kb. In contrast, the sequence of the nucleotides encoding the clone F4 transmembrane protein submitted by Andrews (1993, unpublished) gave the expected size of the transcript as approximately 1.6 Kb. However, as this sequence does not contain an open reading frame and therefore, does not encode a protein it cannot be used to predict the size of the clone F4 transmembrane protein transcript. In addition, this 3.7 Kb transcript is larger than the 1.1 Kb (Table 3-1) clone 58.4 isolated from the MS2 cDNA library. This size difference may be due to the full length transcript not being represented by the clone 58.4 and/or the detection of heterogeneous nuclear RNA on the Northern blot.

4.4.4 Failure to Detect Myelin Components

It is surprising that screening of the MS2 library did not detect MBP or other myelin components. There may be several possible explanations for this:

1. The IgG antibodies for these particular proteins were not present in the sera used for screening. Perhaps such antibodies are only present during certain

phases or stages of the disease or lesion development and were not present when the samples used in this study were taken (Tureci et al., 1997). Another possibility is that the antibodies specific for myelin constituents were not present at high enough titers to be detected by the SEREX technique (Owens et al., 1996).

2. Anti-myelin antibodies may have been present but of a different isotype, specifically IgM. The methodology used in this project assumes that the antibodies relevant to multiple sclerosis are IgG, however there are studies which have shown IgM antibodies may also be important in MS. For example, intrathecal fractions isolated from MS patients are 20 percent IgM (Reiber et al., 1998), 33 percent of relapsing/remitting MS patients were shown to have elevated levels of IgM antibodies reactive with MBP in CSF (Annunziata et al., 1997), and IgM antibodies specific for CNP have been detected in high titer in the sera of 74 percent of MS patients (Walsh and Murray, 1998).
3. The MS2 library was not large enough to include clones for proteins which are not expressed at high levels in the brain. In order to "represent all of the different mRNAs in a human cell type, a cDNA library must contain more than 10^6 independent clones" (Burton and Kaguni, 1997). The MS2 library contained approximately 850,000 clones in the primary cDNA library. The MS2 library was the result of a number of attempts to construct such a library. The size of this library is almost certainly due in part to the fact that the

tissues used for its construction were obtained at autopsy. It is known that remyelination occurs in MS plaques (Perier and Gregoire, 1965; Suzuki et al., 1969; Gledhill and McDonald, 1977; Lassmann, 1983; Prineas et al., 1993a; Rodriguez et al., 1993; Bruck et al., 1994; Rodriguez and Scheithauer, 1994). Therefore, one would expect mRNA encoding myelin constituents to be abundant in these plaques. However, mRNA is metabolically unstable (Mathews and van Holde, 1990) and degraded mRNA cannot be used to construct a cDNA library (Stratagene, La Jolla, CA). For these reasons, if a particular tissue is to be removed during an autopsy and subsequently used to construct a cDNA library, it is imperative that the interval between the death of the patient and the time of autopsy be short. As well, it is also important that once the tissue has been removed it is properly stored (under liquid nitrogen) within minutes. Such measures reduce the amount of degradation of the mRNA. As the tissues were provided by an outside source, I cannot be certain as to the times between death and autopsy, and between autopsy and the freezing of the tissues. Therefore, degradation of the mRNA may have contributed to the small size of the MS2 library. It is important to remember that in a directional λ ZAP cDNA library only one in three clones is expected to place the insert cDNA in frame with the Lac Z gene (Burton and Kaguni, 1997). This means that only one in three clones is expected to produce the correct fusion protein. Unlike a nucleotide probe which simply binds to the correct sequence, an antibody probe requires that the correct protein, or a part

thereof, be expressed. Therefore, even though the cDNA insert for a particular protein may be present, only one in three insertions of that cDNA gives a protein which has the potential to be recognized by an antibody. Perhaps screening the MS2 library with a nucleotide probe for MBP, MOG, MAG, or PLP would identify one or more of these constituents. As the purpose of this project was to screen a cDNA expression library without any *a priori* assumptions as to the identity of the autoantigens, no specific nucleotide probes were used.

An additional issue is that the cDNA library was screened after amplification of the library and this could have led to some distortion of the primary library with the loss of rare clones (Snead et al., 1997).

4. The mRNA encoding these myelin components may not have been present in the plaques used to construct the library, as the cells which express these proteins may not have been present in these lesions. In order to determine if this is a valid explanation, the plaque histology would have to be determined. If an acute plaque was used to construct the MS2 library, then myelin components should have been detected as acute MS lesions are known to contain myelin (Ebers, 1998). If a chronic active plaque had been used then it is possible that myelin components would have been detected as this type of plaque typically undergoes remyelination at its borders (Prineas and Graham, 1981; Raine et al., 1981). Therefore, if the border of the plaque had been included in the sample used to construct the library then myelin components

should be present. However, if a chronic silent plaque had been used then it is less likely that myelin components would have been detected, as the chronic silent plaque contains large number of demyelinated axons and does not typically undergo remyelination (Raine, 1991b).

5. The method used to construct the cDNA library used oligo (dT) priming. This means that the 3' ends of the mRNA are preferentially included in the library and therefore the 5' ends are less likely to be transcribed into cDNA. For this reason it is possible that the immunodominant epitopes of MBP, MOG and other myelin proteins were not expressed in the MS2 library. Without knowing the length of the insert of each protein in the MS2 library, there is no way to predict whether or not these proteins or their immunodominant epitopes would be expressed in the library. For example, the immunodominant epitope of MBP occurs between amino acids 84 and 106. On first consideration, an MBP cDNA insert greater than 350 bases would be expected to include the immunodominant epitope. Indeed, the vast majority of inserts in the MS2 library are much larger than 350 bases. However, it is possible that a large cDNA molecule may be mainly non-coding and include only a small part of an open reading frame. Thus, the immunodominant epitope of MBP, as an example, may well not have been included in the library.
6. Myelin constituents may not be important autoantigens in multiple sclerosis, or they may not be important autoantigens in the patients studied in this work.

It is possible that the autoantigens important in multiple sclerosis differ with the patient examined or with the type of MS diagnosed. There are a few investigators who have also voiced this opinion. For example, Lassmann et al. (1998) pointed out that different demyelinating mechanisms may result in different forms of MS. As well, McFarland et al. (1999) suggest that "Responsiveness (in MS patients) to multiple antigens may indicate that the inciting antigen could differ in different individuals....". Archelos et al (1998) found that a higher percentage of relapsing/remitting patients were positive for antibodies against the *Alu-6* peptide than primary and secondary progressive patients and that these antibody titers were higher in the relapsing/remitting patients as well. Although, this does not prove that the different forms of MS are the result of an immune response against different autoantigens, it certainly does raise the question. In light of this, it may be advantageous to screen cDNA expression libraries with autologous serum (Owens et al., 1996). Such autologous screening was not possible in this study because I did not have access to autologous serum. In addition, such a project would necessitate the construction of a large number of libraries and even more screening.

Overall, the most likely explanations for the inability to detect clones expressing myelin components is that nucleotide sequences encoding immunodominant myelin epitopes were underrepresented in the library. One cannot, of course, exclude

alternatives such as the sera having been used at too high dilutions for screening or that the antigenic targets are more likely to react with IgM than IgG.

4.4.5 Detection of Immunoglobulin

It was expected that immunoglobulin would be detected in the screening of the MS2 library, since B lymphocytes and plasma cells are often present in MS lesions (Prineas and Wright, 1978; Ozawa et al., 1994; Bruck et al., 1995) and therefore the clones would contain immunoglobulin fragments. However, it was thought that during secondary screening these clones would be identified as encoding IgG and then eliminated. The fact that some of these clones were sequenced and only then identified as immunoglobulin is easily explicable since the secondary antibody (goat anti-human IgG) used in the screening process is specific for the Fc portion of human IgG. Therefore, if the clone encoded determinants within the Fab fragment of IgG or the hinge region of IgG the clone would not have been detected by incubation with the secondary antibody alone. Indeed, the majority of the clones which were found upon sequencing to be immunoglobulin did encode the hinge regions of the antibody.

4.5 Northern Blotting Experiments

Northern blotting experiments were performed in this study to examine the tissue expression of transcripts that hybridize with three clones, ya 10.3, 7.0, and 58.4, and to estimate the sizes of the transcripts. Although both Northern blotting and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) can be used for such studies, only

Northern blotting allows the determination of the size of the transcript (Walker and Gaastra, 1983). RT-PCR does have several advantages over Northern blotting. It is more sensitive, and can even be performed on less than 1 ng of total RNA. It is easier than Northern blotting, and has a rapid turn-around time (Larrick and Siebert, 1995). However, the sensitivity of RT-PCR can be a hindrance when used in studies to determine the expression of proteins in different tissues. For example, Scanlan et al. (1998) used both RT-PCR and Northern blotting to investigate the tissue expression of a particular tumor antigen which they had isolated from a colon cancer using the SEREX technique. The results of the RT-PCR showed the antigen to be universally present, while the results of the Northern blotting experiments showed a gastrointestinal-restricted expression. This finding suggests that the RT-PCR method may have detected tiny amounts of transcript in many tissues.

The quantitation of mRNA levels in a number of tissues is more difficult with RT-PCR than with Northern blotting (Larrick and Siebert, 1995). The quantitation of mRNA levels in Northern blots requires normalizing the amount of RNA with a β-actin probe. In contrast, the quantitation of mRNA using RT-PCR requires estimating when the exponential accumulation plateaus so that the products are analyzed only during the exponential phase of PCR, thereby eliminating the differences in amplification efficiencies (Larrick and Siebert, 1995). Since Northern blotting is able to identify the size of the transcript and is an easier method of quantifying mRNA levels, it was used in this project.

4.6 Colorimetric Versus Radiolabel Methods of Detecting Positive Clones

The SEREX technique as originally reported (Sahin et al., 1995) uses an alkaline phosphatase method for the detection of positive clones. This is also the method which I chose to use. Radiolabel methods would have been more sensitive, detecting 10 pg or less of membrane bound protein (BioRad, Hercules, CA), but would certainly have made the task of screening 10^6 clones, followed by secondary and tertiary screening of the clones a much more laborious and expensive task. In addition, the sensitivity of radiolabel methods may have dramatically increased the number of false positives. As well, radioisotopes have a certain half-life, in the case of ^{32}P, 14 days. Therefore, a radiolabel method can only be used for several weeks before a new radioisotope must be purchased (Burton and Kaguni, 1997), this is expensive and a waste of reagents. Of course, a radioisotope with a longer half-life, such as ^{125}I, could be used but this also increases the risk to the experimenter. In contrast, colorimetric reagents can be stored for much longer periods of time (BioRad). The colorimetric method also allows easier identification of positive plaques on the agar plate by lining up the nitrocellulose filter with the agar plate. Such a technique would not be possible with a radiolabel method. From the sheer volume of screening which must be done when using the SEREX method, colorimetric methods seem to be the best option. The experiments performed to date by the Sahin and Tureci groups, using the SEREX method to identify tumor antigens have not discussed the use of alkaline phosphatase as being a potential problem. We have no reason to suspect that it may be so in this study.

4.7 Summary

The study described in this thesis was undertaken to search for potentially novel and also known autoantigens in multiple sclerosis. It was hypothesized that screening a cDNA expression library constructed from the mRNA extracted from MS plaques with patients' sera would allow the identification of novel autoantigens. The advantages of this method were that no assumptions would be made as to the identity of the autoantigens and that such screening might be able to recognize the majority of autoantigens in multiple sclerosis. Three novel autoantigens have been identified using this method. It seems unlikely based on the antibody screening, that any of these are really important in MS as only a minority of sera reacted with the clonal products. Also, no control sera were used and thus no real conclusions can be made as to whether these are important antigens in multiple sclerosis. At present we can only say that they represent, at most, potential autoantigens in multiple sclerosis. Also, at this stage it is still unknown if immune reactivity to these proteins actually represents a primary humoral response, if the immune targets are cross-reactive species, or if they are targets as the result of epitope spreading.

The relevance of the identification of the three potential autoantigens to the oligoclonal bands in the CSF of MS patients remains to be elucidated. Perhaps these purified proteins could be examined in adsorption assays to see if any of them is capable of binding to CSF oligoclonal bands or these proteins could be isolated and used in ELISAs with CSF from patients with MS. As well, one could re-screen the library with CSF isolated from MS patients to see if the same autoantigens are identified.

This is the first report of clones hybridizing with transcripts having sequence homologies to testican, KIAA0530, and the clone F4 transmembrane protein being identified as possible autoantigens in multiple sclerosis. As we did not detect the "conventional" autoantigens, MBP, MOG, etc., it is difficult to make conclusions about the usefulness of this method in the search for autoantigens in multiple sclerosis. It is important that in investigating this disease one does not become "caught up" in trying to reproduce the work of others by using a different method. That is to say that maybe we should not have expected to detect MBP, or MOG or other myelin constituents in this library as there is no solid evidence that these autoantigens are important in MS. Rather the goal should be to identify new possibilities so that they too can be investigated. The hypothesis of this project was that the SEREX method could be used to identify novel potential autoantigens in multiple sclerosis. We have succeeded in doing just that.

4.8 Future Work

1. The three clones isolated in this study have only been screened with a small number of MS patients' sera and have not been screened with any control sera. In order to determine their potential importance as autoantigens in multiple sclerosis it would be particularly important to screen these clones with a large number of sera from healthy controls as well as patients. Obviously, a clone which reacts with equal numbers of patients' and control sera is unlikely to be an important autoantigen in MS, while a clone which reacts with a greater number of sera from MS patients than from controls may have relevance to the disease.
2. The sequences of the clones 7.0 and 58.4 could be extended 5' and 3' and analyzed for translation in 6 frames. The derived amino acid sequences would be compared with those of KIAA0530 and clone F4 transmembrane protein, respectively, and would also be submitted to a protein database (BLAST) for identification.
3. As MS is typically believed to be a T lymphocyte mediated disease (Stinissen et al., 1997) it would be interesting to test the three clones, 7.0, ya 10.3, and 58.4, in T lymphocyte proliferation assays, to determine if they are capable of activating T lymphocytes. The three clones which were not chosen for further study could also be used in such proliferation assays.
4. One of the possible explanations for not detecting myelin components in the MS2 cDNA library was that the anti-myelin antibodies may have been IgM and not IgG. There are studies which indicate that IgM antibodies may be important in multiple sclerosis (Annunziata et al., 1997; Reiber et al., 1998; Walsh and Murray, 1998). As

the secondary antibody used in the screening of the MS2 cDNA library was specific for IgG, IgM was not detected in this study. Therefore, the MS2 library, or a commercially available library, should be screened using a secondary antibody which detects IgM.

5. Antibodies are known to be present in the CSF of multiple sclerosis patients (Link and Tibbling, 1977; Tourtellotte, 1985). The MS2 library could be screened with CSF from MS patients.
6. Another possible approach of significant future potential is to screen a brain library with T lymphocytes isolated from MS patients. This could be accomplished by incubating a certain number of clones per well in 96 well plates, adding T lymphocytes and performing proliferation assays. The wells which lead to proliferation of the T lymphocytes could be plated to allow isolation of individual plaques. Next single clones would be added to wells of the 96 well plate and the proliferation assays repeated (Dr. John Webb, personal communication). In this way single clones which activate T lymphocytes from MS patients could be isolated. Of course, this would be followed by nucleotide sequencing, analysis for translation in 6 reading frames, and mRNA tissue expression studies.
7. Perhaps a commercial library could be used for screening with patients' sera and/or CSF. Such libraries are typically optimized for large inserts. Using a commercial library would also avoid the problem of detecting sequences for proteins with epitopes at the C-terminus.

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