AMELIORATION OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE) BY PHASE 2 ENZYME INDUCER

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Saskatoon

By

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ABSTRACT

The pathology of multiple sclerosis (MS) is characterized by an inflammatory mononuclear infiltration in the white matter. There has been converging evidence of the oxidative stress playing a role in the onset and progression of MS. We postulated that the decreasing oxidative stress might help in the management of MS. We know that the induction of phase 2 enzymes decreases the oxidative stress. The experimental allergic encephalomyelitis (EAE) induced in the Lewis rats were used to test this hypothesis. The 24 animals were placed into two groups: 1) those on normal rat chow, 2) those on rat chow containing 7.5 g/kg of tetra-butylhydroxyanisole (BHA), a food preservative. All the animals were administered 100 µg of guinea pig myelin basic protein in their tails to induce EAE and examined daily in a double blinded fashion. On 29th day of the induction, the animals were sacrificed, blood collected for glutathione (GSH) measurements and tissues collected for histology. All the animals, regardless of their diet status, developed symptoms of EAE on different days ranging from tail weakness to hind limb paralysis and all of them reached remission of acute EAE before the 28^{th} day of induction. The non-BHA fed animals developed hind limb weakness in 8 animals and hind limb paralysis in 4 cases, while that of BHA fed group developed tail paralysis in 2, hind limb weakness in 2 and hind limb paralysis in 8 cases. The histology of the non-BHA group correlated well with the clinical symptoms of perivascular mononuclear infiltration. However, the BHA group revealed complete pathological recovery. Animals with BHA in the diet had significantly raised GSH, indicating the induction of phase 2 enzymes. We conclude that dietary phase 2 enzyme inducers show potential therapeutic benefits in EAE and should be examined for this role in MS.

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DEDICATION

To my loving family,

who offered me unconditional love and support throughout the course of this thesis. I thank and appreciate very well the support and help provided by my daughters Iram, Anam and Maryam in my endeavor in spite of all hardship including financial difficulties. This work could have not been possible without their selfless commitment and support.

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LIST OF ABBREVIATIONS

ADEM	Acute Disseminated Encephalomyelitis
ADM	Active Demyelinating
APC	Antigen Presenting Cell
APP	Amyloid Precursor Protein
BBB	Blood Brain Barrier
BCG	Bacillus Calmette Guerin
Bcl	B Cell Lymphoma
BHA	Butylhydroxyanisole
BM	Bone Marrow
BMT	Bone Marrow Transplantation
Cat	Catalase
CFA	Complete Freund's Adjuvant
CNDB	1-Chloro-2,4-dinitrobenzene
CNS	Central Nervous System
Cox	Cyclooxygenase
CTLA	Cytotoxic T Lymphocyte Antigen
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic Acid
DTH	Delayed-Type Hypersensitivity
EAE	Experimental Allergic Encephalomyelitis
EC	Endothelial Cell

EDTA	Ethylenediaminetetraacetic Acid
ESC	Embryonic Stem Cell
EtBr	Ethidium Bromide
Fe	Iron
GPX	Glutathione Peroxidase
GSH	Glutathione
GSSG	Oxidized form of GSH
GST	Glutathione-S-Transferase
H & E	Hematoxylin and Eosin
HLA	Human Leukocyte Antigen
H_2O	Water
H_2O_2	Hydrogen Peroxide
ICAM	Inter Cellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INO	Internuclear Ophthalmoplegia
IV	Intravenous
LFB	Luxol Fast Blue
LPC	Lysolecithin/Lisophosphatydilcholine
LT	Leukotrience
MAG	Myelin Associated Glycoprotein
MBP	Myelin Basic Protein

MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinases
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogenase
NAWM	Normal Appearing White Matter
ND4	NADH Dehydrogenase Subunit 4
NF-κβ	Nuclear Factor kappa Beta
NO	Nitric Oxide
NOS	Nitric Oxide Synthetase
O_{-2}	Superoxide
O_2	Oxygen
OH	Hydroxyl Radical
OLG	Oligodendrocyte
ONOO	Peroxynitrite
OPN	Osteopontin
P53	Protein 53 /TP53 – Tumor Protein 53; Tumor Suppressor Gene/Protein
PAMP	Pathogen Associated Molecular Pattern
PAS	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
PLP	Phospholipid Protein

PPMS	Primary Progressive Multiple Sclerosis
PPWM	Periplaque White Matter
PT	Pertussis Toxin
RM	Remyelinating
RNA	Ribonucleic Acid
ROI	Reactive Oxygen Intermediate
ROS	Reactive Oxygen Species
RRMS	Relapsing Remitting Multiple Sclerosis
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
SPMS	Secondary Progressive Multiple Sclerosis
TCA	Tri-Chloro-Acetic Acid
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th1/TH1	Helper T Cell Subset 1
Th2/TH2	Helper T Cell Subset 2
TLR	Toll Like Receptor
TMEV	Theiler's Murine Encephalitis Virus
TNF	Tumor Necrosis Factor
VLA	Very Late Antigen
VCAM	Vascular Cell Adhesion Molecule

1.0 REVIEW OF THE LITERATURE

1.1 Introduction

Multiple Sclerosis (MS) is still the major disabling neurological disease in young adults, affecting 0.05-0.15% of the Caucasian population. It usually starts in young adulthood with a clinical bout encompassing symptoms like visual disturbances, numbness and/or tingling, difficulties in walking or coordination, and sometimes unspecific complaints, like fatigue, attention, or endurance problems [1]. Possible causes of this disease are viral and/or autoimmune in nature. MS is influenced by genetic and environmental factors, but the root cause of the disease remains unknown. The distinguishing features of MS include demyelination zones with T-cell, predominantly perivascular, inflammation in the white matter of the spinal cord and brain. These features may not impact all axons. MS is relatively benign in a majority of patients and is marked by a few attacks which become increasingly worse and more common with time. This stage of the disease is known as relapse-remitting MS (RRMS). Subsequently, one is never able to acquire complete functionality again upon remission of the disease, with disability occurring in later stages. This last stage is known as secondary progressive MS (SPMS) [2-3].

MS does not necessarily exhibit the same series of events in all patients. Primary progressive MS is more common among females. Furthermore, the axons are less damaged than in SPMS [4]. The disease also does not make a transition from one stage to the next as RRMS does prior to SPMS [3]. Consequently, primary progressive multiple sclerosis (PPMS) can seem distinct in its development. There are MS variants that progress quickly, including acute or Marburg's MS, neruomyelitis optica (Devic's

disease), and Balo's concentric sclerosis. Benign disease patterns or those characterized by single attacks can also be diagnosed as MS [3, 5].

One study that includes biopsy and autopsy analyses conducted primarily by a multicenter collaborative group show a heterogeneous pathology behind clinical MS. Four pathologies can be identified based on what has been learnt so far [6]. Two immune-inflammatory pathologies which demonstrate focal, perivascular lesions of T-cells and macrophages (Type I), and antibody and complement (Type II), are the most common of the four pathologies. The lesions are due to demyelination and damage of axons in the white matter. Rarer pathologies include those that exhibit damage caused by viruses or toxins, or those that are the result of ischemia with diffuse white matter involvement (Type III), and what seems to be primary oligodendrocyte dystrophy (Type IV) [6-7]. More analysis is needed to determine whether these pathologies can occur simultaneously in the same patient [8-9]. From these studies, one can conclude that the pathology of MS is quite heterogeneous. Other studies have demonstrated that pathology in some MS variants include more characteristic features than the classical demyelinated plaque in white matter. Demyelinated plaque is a consequence of acute and RRMS. Chronic disease, on the other hand, is marked by cortical involvement, greater microglial activation, and diffuse myelin and axonal loss [10-11]. Acute MS commonly does not exhibit intrathecal immunoglobulin synthesis or oligoclonal immunoglobulin bands in cerebrospinal fluid (CSF). These features are hallmarks of MS and were used

for diagnosing the disease before the advent of MRIs. Several cases of SPMS show less immune involvement than other forms of MS, such as decreased T-lymphocyte infiltration. However, activated macrophages or microglia is present throughout [3]. The graphic representation of possible pathology is in fig.1.1

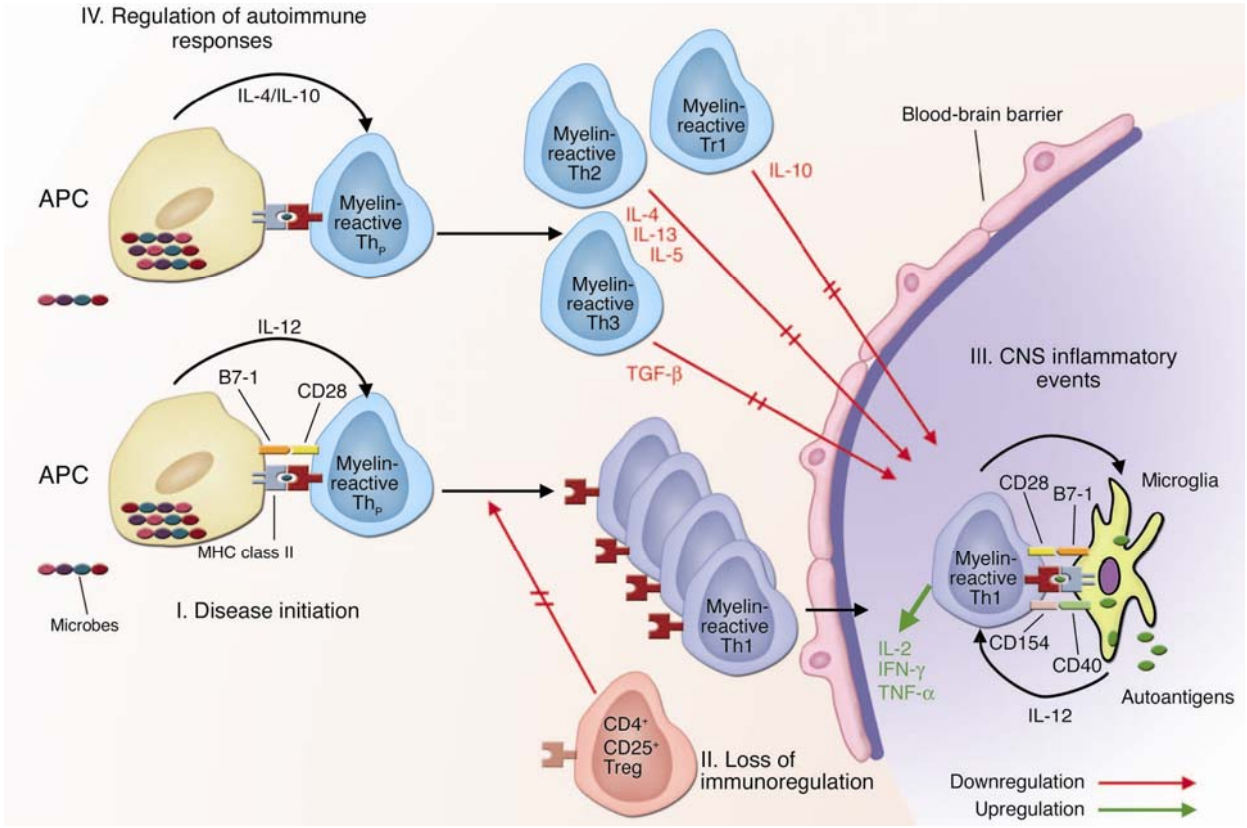


Fig. 1.1: MS Pathology (*Courtesy: JCI* [12])
Working hypothesis as to the cause of MS. (I) In a genetically susceptible host, common microbes both activate the APCs through toll receptors and contain protein sequences cross-reactive with self myelin antigens. This leads to what can be defined as the minimal requirement for inducing an autoimmune, inflammatory CNS disease in mammals. (II) Underlying immunoregulatory defects, such as decreases of regulatory T-cells in the circulation of patients with MS, allow the further pathologic activation of autoreactive T-cells (96). (III) Activated myelin-reactive T cells migrate into the CNS and recognize antigen presented by microglia, local APCs. Th1 cytokines are secreted and an inflammatory cascade is initiated. (IV) Regulation of autoimmune responses. Naturally occurring mechanisms may exist to regulate autoimmune responses including the induction of autoreactive Th2 (IL-4, IL-5, IL-13), Th3 (TGF-β), or Tr1 (IL-10) cytokine–secreting T-cells that migrate to the CNS and downregulate (red arrow) inflammatory Th1 autoreactive T cells (green arrow). Therapies may attempt to induce Th2 (Copaxone, altered peptide ligands), Th3 (mucosal antigen), or Tr1 (β-IFNs, steroids). ThP, precursor T-cell.

The roles of cytokines in MS pathology are very important. However, there have been various studies that sometimes contain conflicting expressions of different cytokines. The cytokines that are generally found to be increased during the disease process are TNF-α, $sTNF_{RI}$, LT, IFN-γ, IL_2, sIL_{2R}, IL_6, IL_{23}, Osteopontin, and Oncostatin M [13-18]. The cytokines that are generally decreased during the disease process are IL_4, IL_{10}, IL_{12}, TGF_β, IL_{12p40}, IL_{13}, IL_{15}, IL_{18}, and IL_{17} [19-21]. In conclusion, the polymorphism in the expression of various cytokines at different stages or clinical setups of MS makes the well demarcated association difficult at all the times. The complicated interactions of the various cytokines are graphically expressed in fig. 1.2 [22].

Oxidative stress has been blamed in numerous pathologies including MS [23-27]. The role of oxidative stress and effectiveness of antioxidants of various kinds have been studied in various demyelinating conditions of the brain. It has been established in animal models like experimental allergic encephalomyelitis (EAE), an excellent model for multiple sclerosis (MS), especially expressing the possible autoimmune pathogenesis of MS.

The possibly predominant role of oxidative stress in the pathogenesis of multiple sclerosis has been considered as a target for therapeutic intervention. Various efforts have been made at the experimental animal levels using different chemical antioxidants. We would like to exploit the same pathway using the dietary antioxidant tetra-butylhydroxyanisole (BHA), commonly used in food preservation, in our EAE models.

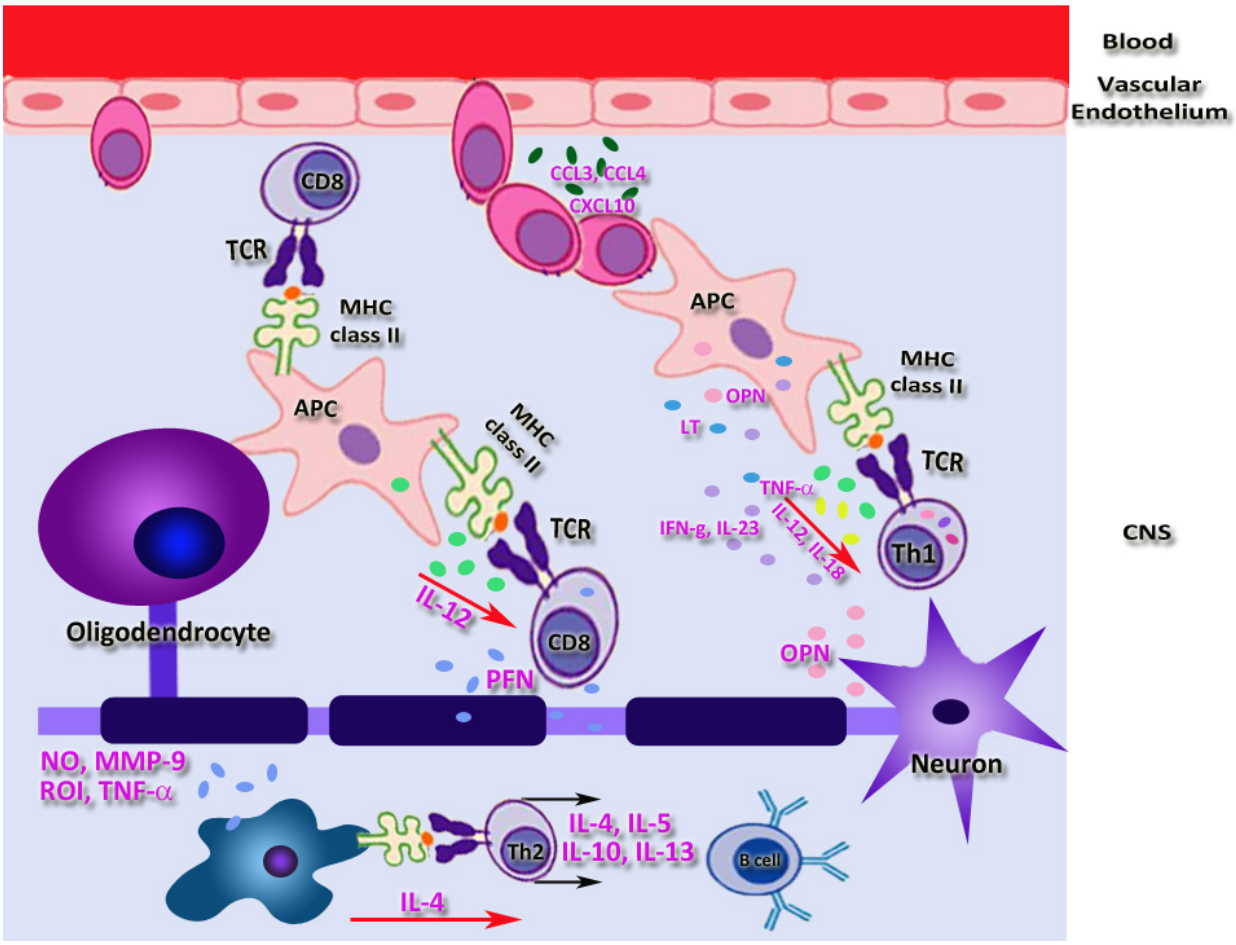


Fig. 1.2: Cytokines control mechanisms of structural damage to myelin and axons. Immune cells (CD_4, CD_8, T-cells, and B lymphocytes) are directed by chemokines to encounter APC (microglia or DC), which present their cognate autoantigen for reactivation. Pro-inflammatory cytokines (e.g. IFN-γ, IL_{23}, osteopontin OPN) are secreted by T-cells and either directly affect myelin structures or activate macrophages to release nitric oxide (NO), reactive oxygen intermediates (ROI), matrix metalloproteinases (MMP), or TNF-α. CD8 cells may directly attack axons by release of the cytotoxic mediator perform (PFN). B lymphocytes are induced to terminal plasma cell differentiation by Th_2 cytokines, and upon activation release myelin-specific antibodies, which can induce complement (C) mediated demyelination. Plasma cell Macrophages factor in the blood, CSF, or destructive lesion of MS patients could be a hint for a causal relationship, but it could also represent an endogenous regulatory process to reduce tissue damage or indicate an innocent epiphenomenon. Considering these three options, any therapeutic approach counteracting a specific factor may result in different outcomes depending on its temporal and spatial role during inflammation or tissue destruction. These problems are best exemplified by the story of TNF-α in MS.

The various beneficial pathways of dietary antioxidants are expressed in fig. 1.3.

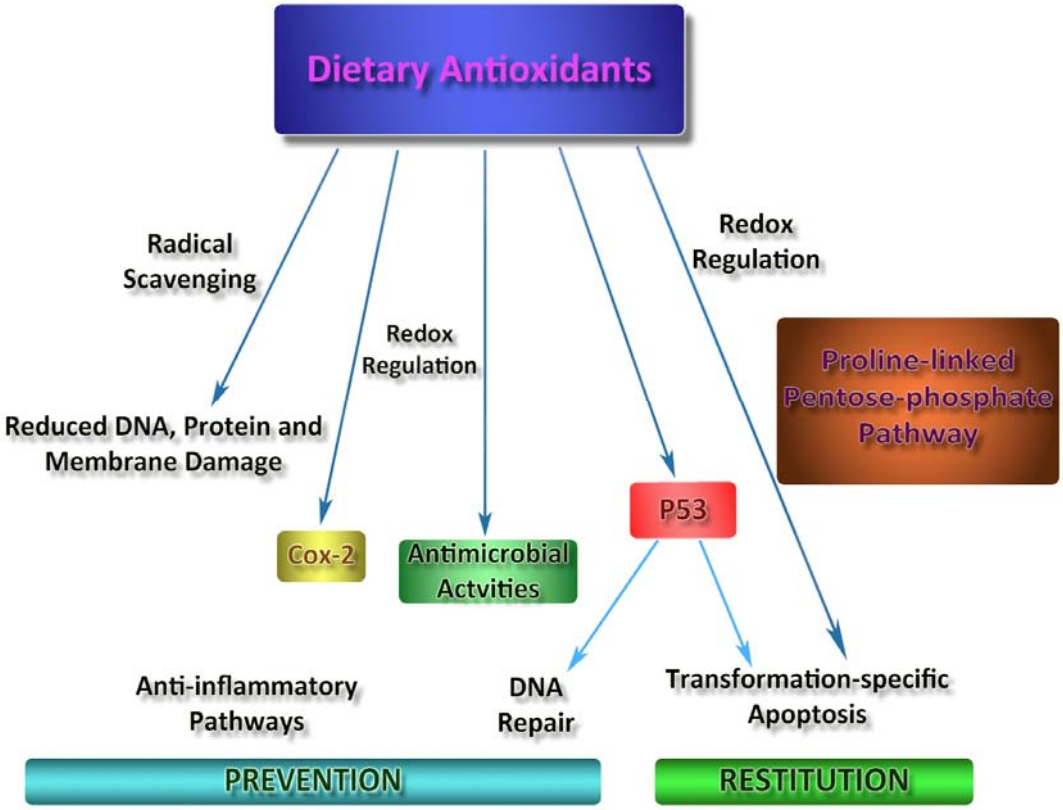


Fig. 1.3: Beneficial pathways of dietary antioxidants {Model for metabolic biology and chemopreventive activities of phenolic antioxidants. (*Adopted with courtesy from Brash and Have, PNAS 2002; 99, 13969*)}

1.1.1 Historical Perspective

The first case of MS was found in 1824 in a young man as myelitis [28]. MS cases came to be known as *sclerose en plaque dissemine*, a name that Vulpian christened to the disease in 1866 [29]. The name, *disseminated sclerosis,* was subsequently replaced by *multiple sclerosis* which was obtained from its German name [30].

It was in the 19th century that the pathologic characteristics of MS were studied. In 1838 [31] and 1841 [32] respectively, Carswell and Cruveilier described the morphological characteristic in MS patients: sharply circumscribed translucent gray

sclerotic plaques in the CNS white matter. Rindficisch in 1863 and Charcot in 1868 described the microscopic attributes of the MS plaque including perivascular inflammation which occurred due to demyelination, axonal damage, and astrocytic scar formation [33-35]. The laboratory markers of the disease were determined in 1948 by Kabat [36-37]. These included an increase in oligoclonal immunoglobulin in the cerebrospinal fluid (CSF) of MS patients [12, 38]. Between 1999 and 2001, Lucchinetti et al. delineated the structural and immunopathological subtypes of MS [39-40]. Interindividual heterogeneity and intraindividual homogeneity was found in terms of the immunological basis of demyelination, what happens to oligodendrocytes, and the range of axonal damage and/or remyelination.

1.1.2 Epidemiology

One million people are impacted by MS globally. North America is witness to 350,000 cases. The ratio of women to men affected by MS is 2:1. MS generally strikes patients in young adulthood and is characterized by frequent inflammatory attacks against the white matter of the spinal cord and brain. Blindness, loss of sensation, lack of coordination, loss of bowel and bladder control, and difficulty in walking are all features of MS. MS pathology is characterized by a plaque which is a region of myelin that has been exposed due to inflammation and scarring by a non-neuronal cell in the brain, such as microglia that are derived from BM, and the star-shaped astroglia which are derived from the brain. The specific cause remains unclear. Two theories have been postulated to explain why this happens: either it is due to an autoimmune reaction (post-inflammatory) or a neurodegenerative process followed by inflammatory response (pre-inflammatory).

Europeans or the descendants of European ancestors make up the majority of patients affected by MS [41]. MS is quite rare in certain races and is well-studied [42]. Such groups include American Indians, African blacks, Hutterites [43], and Asians. Demyelinating diseases are also found in Asians but their pattern and laboratory results are different from those of MS [44-45]. The world-wide distribution of MS is described in fig. 1.4.

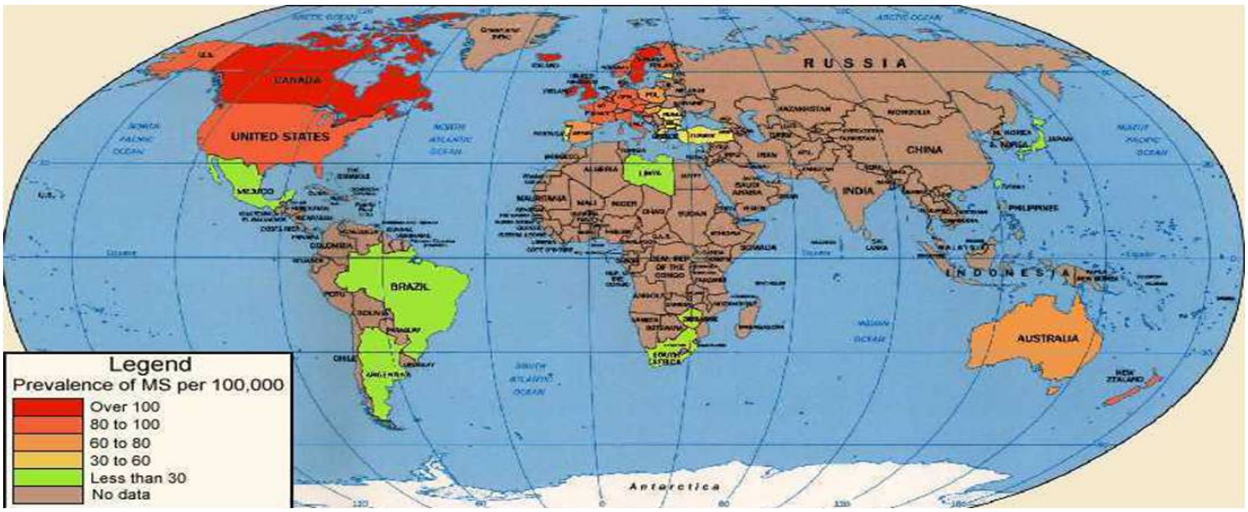


Fig. 1.4: The world-wide distribution of MS

1.1.3 Genetics of MS

The role played by genes and the environment in the etiology, susceptibility, onset and prognosis of MS needs to be clearly outlined.

It was in the 1800s when Gowers [46] declared that occurrence of MS within families was extraordinary and Eichhorst described MS as an "inherited, transmissible" disease [47]. From 1921-1948, medical literature described a total of 64 sibling pairs, 13 parent-child pairs, and 15 second and/or third-degree relative pairs as having MS [48]. These were single case reports as opposed to large series. Research on familial MS was impeded by the lack of consensus diagnostic criteria until 1965 [49], which have

been updated to include technological advances and understanding about the natural history and heterogeneity of the disease [50-51].

"Multiplex families" was a name given to families with greater than one case of MS, had hereditary cerebello-pyramidal disease or hereditary spastic ataxia [52-53]. One of these types of families with 12 affected patients [54] actually had the Pelizaeus-Merzbacher disease [55] instead of MS. Irrespective of this, multiplex, multigenerational MS families do exist and the evidence for this is quite substantial [56]. It is unknown whether these families exhibit a rare, autosomal dominant form of MS which was found in 5% of cases of Alzheimer's disease [57] and which had been hypothesized for MS several years ago [58].

1.1.4 MS Susceptibility and Genes

The HLA class I antigens A3 and B7 [59-61] were the first genetics links to MS. These were later subtyped into a strong and consistent link with the HLA DR_{15}, DQ_{6}, and Dw_{2} haplotype [62-65]. Recent research has cast doubts as to whether this link truly is as simplistic as described here [66-67].

In the search to determine a genetic link to MS, association studies, whole genome scans, SNP analyses, HapMap approaches and a variety of other experiments have been conducted but to no avail. To date, no specific gene has been identified for MS. The University of California in San Francisco has an in-depth listing of the MS candidates (positive and negative) [68]. Our inability to identify the genes responsible for MS can be thought of as an indicator of the disease's intricacy instead of assuming that a genetic link is to blame. Other disease such as diabetes and Alzheimer's disease also exhibit a

difficulty in determining a genetic link. These diseases, along with MS, are known as common complex disorders.

Longitudinal, population-based, genetic epidemiological studies are used to identify the relative roles of genes and environment in MS.

1.1.5 Familial Recurrence Risks

Employing the classical genetic epidemiological approach meant that the initial step was to determine whether relatives of MS patients were at a higher risk of coming down with the disease compared to the rest of the population. The Canadian Network of Multiple Sclerosis Clinics [69] allows one to study age-adjusted familial recurrence risks with first-degree relatives of MS patients and compare them to the rest of the population. The age-correction methodology adopted in these studies has been verified by the Vancouver Multiple Sclerosis Clinic which first stated these risks [70] and by the longitudinal follow-up of Canadian MS patients [71]. Additional studies coupled with these Canadian studies [72-74] have shown that first-degree relatives of MS patients are at greater risk of developing MS.

This is due to the biological sharing of DNA rather than a common family environment which promotes the increased frequency of MS within biologically related family members [75-78].

Majority of the individuals affected with MS are females. Evidence has been mounting, however, that there may be a parent-of-origin effect in MS whereby a maternal effect may be at work [76-77, 79].

A recent study of four northern MS populations [80] discovered a May:November birth ratio in living MS patients from Scotland (1.89), Denmark (1.22), Sweden (1.18),

and Canada (1.13). This means that there is a peak in May and a nadir in November. Intriguingly, the birth month ratios declined in the order of the prevalence of the population. A potential parent-of-origin effect is seen with this data. Furthermore, one can compare this data with earlier observations of links between higher latitudes and risk of MS. This suggests that sun exposure may be the reason for the geographical variation of MS [81-82]. Exposure to ultraviolet radiation produces most biologically active vitamin D [82]. The ascending risk of MS in relation to the birth month may be due to seasonal shortage of vitamin D in the mother [83]. Vitamin D receptors are found in the brain, as recent literature shows, and the gestational shortage of vitamin D has a major impact on brain development in experimental animals [84].

The precise impact of genes and the environment on MS is still unclear. The indisputable facts up until now include the following, however:

- MS is the product of an interaction between genes and the environment.
- The sharing of genetic material is the cause for the phenomenon of increased frequency of MS within families compared to the general population.
- The environmental impact of MS acts at the population level as opposed to in the family environment.
- Transmission of susceptibility to the disease is variable based on several factors such as the gender and age at which the disease developed in family members.
- Several genes are at work here. Therefore, etiologic heterogeneity is a possibility. The link between MS and MHC may be more complicated than

it seems.

- MS can not be transmitted at any point in an individual's lifetime.

The information from genetic epidemiological studies is being used in genetic and reproductive counseling for MS families. This shows how research and clinical practice can be linked.

1.2 Pathology of MS

1.2.1 Demyelination

MS is a dynamic disease and is marked by several levels of demyelination. The MS plaque may show the different levels of demyelination such as early and late active demyelination along with completely demyelinated regions that may or may not be remyelinated, in one lesion. The non-remyelinating regions are inactive areas. Actively demyelinating lesions, on the other hand, are characterized by a variable inflammatory infiltrate comprised primarily of macrophages, reactive astrocytes, and different lymphocytic cuffs. Different immunocytochemical stains can be used to identify the different levels of demyelination [80, 85]. These include antibodies to minor myelin proteins, such as myelin-associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG). Antibodies to major myelin proteins such as myelin basic protein (MBP) and phospholipid protein (PLP) along with the Luxol fast blue (LFB) myelin stain are also other examples of immunocytochemical stains that can be used to demarcate the level of demyelination. The early active demyelinating lesion is defined by reactive astrocytes and macrophages carrying granules that are positive for LFB and which contain both minor and major myelin proteins. Due to the quick degradation of minor myelin proteins, macrophages in the late active lesions only carry the more slowly digested major myelin proteins, namely, PLP and MBP. Due to their declining activity, these PAS-positive, granule-carrying macrophages begin to aggregate in large numbers to perivascular areas instead of being interspersed throughout the lesion. In the later levels of demyelination, macrophages do not have any more myelin peptides or partially digested glycoproteins which means that the granules are neither LFB- nor PAS-

positive. Inflammation subsequently decreases. However, glial reactivity remains. Hypocellularity defines the inactive, chronic MS lesion, which only carries astrocytes and has scattered perivascular chronic infiltrate. The general pathological process in MS is expressed below in Fig. 1.5.

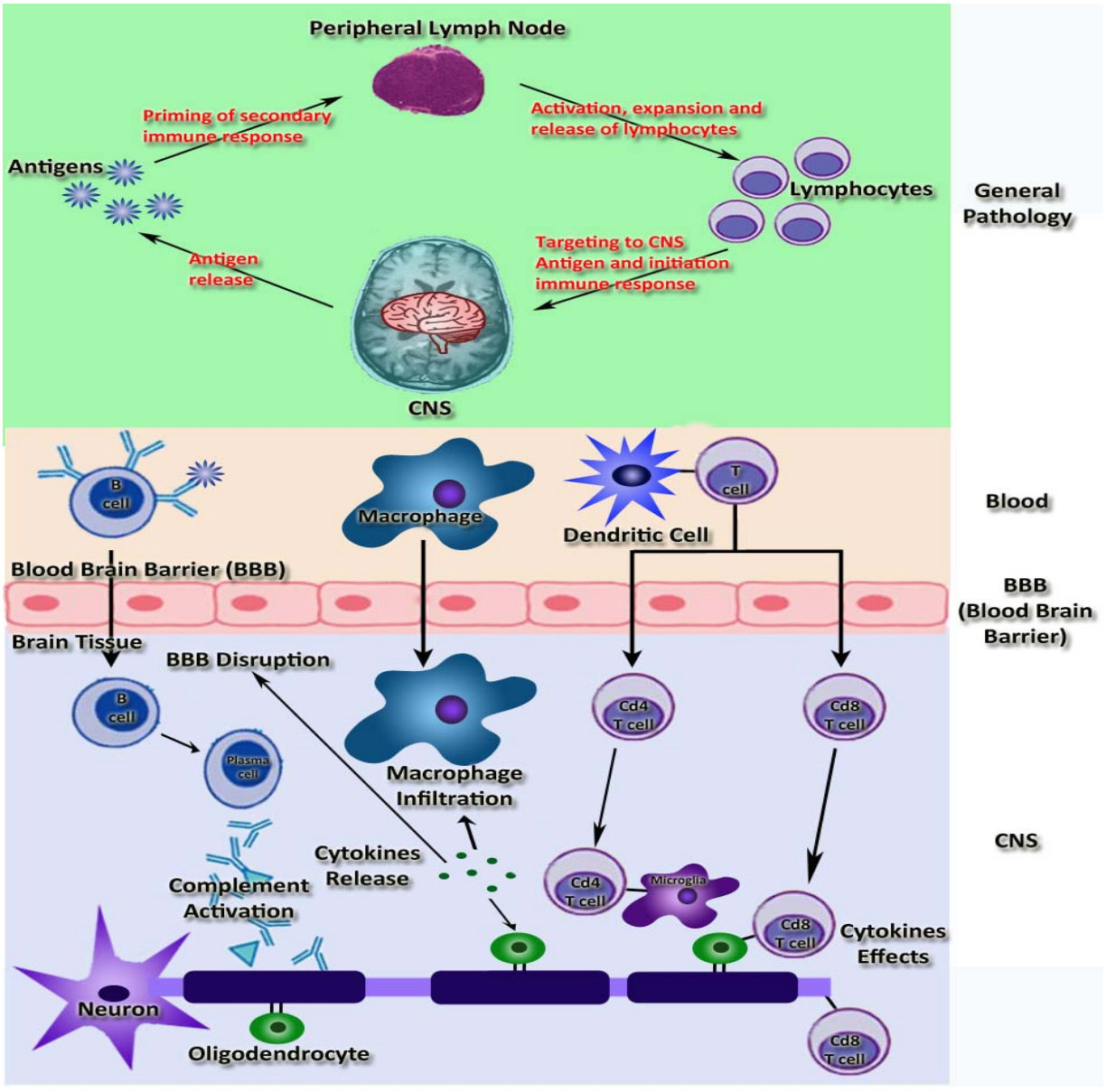


Fig. 1.5: A pathological representation of the disease process. Cross-reactive viral antigen or CNS antigen is processed in peripheral lymph nodes and presented to lymphocytes resulting in their clonal expansion, release and migration into the CNS where they are reactivated upon recognition of the myelin bound antigen. CD_{8+} T-cells interact directly with oligodendrocytes and neurons inducing apoptosis. CD_{4+} T-cells recognize antigen presented by CNS resident microglia and secrete cytokines required for maintenance of the inflammatory environment. These cytokines induce MHC expression on CNS resident cells, disrupt the blood brain barrier to allow further cellular infiltration, and attract peripheral macrophages, further enhancing the immune response.

In early active MS lesions, there are four histopathological patterns of demyelination. The factors involved in distinguishing these patterns include plaque geography, extent of oligodendrocyte survival, levels of remyelination, presence of complement activation, and loss of myelin proteins [86]. When a biopsy and autopsy was performed on 82 patients with early MS, about 70% of the lesions had demyelinating patterns with primary myelin destruction which described patterns I and II. Both of these patterns showed sharp macrophage borders on the plaque edge and the surrounding periplaque white matter (PPWM). Furthermore, perivascular myelin loss and infiltration by macrophages and CD_8 T-lymphocytes were also evident. Immunoglobulin and complement deposition within macrophages and at sites of active myelin destruction are found only in pattern II lesions. A primary oligodendrocyte (OLG) injury is found in the last 30% of MS cases and this is characteristic of patterns III and IV. Pattern III lesions are marked by a great deal of macrophage and T-cell infiltration but have poorly defined macrophage rims, perivascular sparring of myelin, absence of complement activation, and a loss of myelin associated glycoprotein (MAG) on the plaque edge, along with prevalent OLG apoptosis with little remyelination. Pattern IV lesions show degeneration of oligodendrocytes in the normal-appearing periplaque white matter (PPWM), no MAG loss and no complement activation [86-87]. After analyzing several active lesions at autopsy or of several biopsies of the same patient, it has been demonstrated that the immunopathological pattern has remained consistent. Interindividual heterogeneity implies that the targets and immunopathogenic mechanisms which lead to tissue injury in MS may be heterogeneous [86].

The cellular make-up of tissue damage and repair found in MS lesions is

heterogeneous among MS patients. Oligodendrocytes within MS plaques are prone to injury by several immune and toxic agents. For example, both myelin and oligodendrocytes can be injured by tumor necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ), reactive oxygen and nitrogen species, T-cell products (i.e., perforin or lymphotoxin) and CD_8 cytotoxicity, and other inflammatory products. Other examples of toxins that can cause harm within MS plaques are excitatory amino acids (i.e., glutamate), complement cascade proteins, proteolytic and lipolytic enzymes, or viral infections [88-89].

Dysregulation of T-lymphocyte and/or oligodendrocyte apoptosis, namely the Fas-Fas ligand pro- and bcl_2 anti-apoptosis cascades, were all implicated in the MS pathogenesis. These stimuli may lead to different degrees of demyelination, oligodendrocyte loss, or axonal injury in MS plaques.

Demyelinating MS lesions are classified by structure based on oligodendrocyte density and extent of remyelination along with how much of the axon is preserved relative to the periplaque white matter. About 70% of MS patients have plaques with preserved oligodendrocytes and a great deal of remyelination which is known as *OLG category I lesions*. The oligodendrocytes in these MS plaques are present because they either survived the injury from the demyelination or were taken from OLG progenitor pools after the acute phase of demyelination [89-90]. The last one-third of MS patients has *OLG category II lesions* which have decreased oligodendrocyte survival and little or no remyelination. The density of macrophages, CD_8 (cytotoxic) T-cells, and density of oligodendrocytes in MS lesions at all levels show a major negative correlation. This inverse correlation suggests that macrophages and cytotoxic T-cells have a very

important pathogenic role to play in decreased oligodendrocyte survival and remyelination [40]. OLG category I lesions are more commonly linked with patterns I or II, but diminished oligodendrocyte preservation (OLG category II lesions) is seen in patterns III or IV of active demyelination [87, 91]. Oligodendrocytes are preserved or lost differently in acute MS lesions which support the hypothesis of a heterogeneous pathology of demyelination and oligodendrocyte damage or preservation in lesions [88-89].

The occurrence of remyelination in early MS lesions correlates with oligodendrocyte survival. But the narrow range of remyelination in chronic MS lesions is not only due to an absence of these cells. Oligodendrocytes may be preserved to varying extents in chronic MS plaques with no remyelination [92]. These sources of "premyelinating" oligodendrocytes can be used as substrates for remyelination. These chronic lesions do not have the necessary pro-remyelinating environment. Remyelination needs healthy oligodendrocytes but also intact interactions between the axons and oligodendrocytes, growth-promoting cytokine and chemokine profiles, along with limited fibrillary gliosis for extending the myelin processes within the MS plaque [87, 92-95].

A high level of interindividual heterogeneity is seen within the structure and immunopathology of MS lesions [86]. The histopathology of lesions and the clinical course and outcomes in prototypic MS show no correlation. Acute, relapse-remitting, secondary progressive, primary progressive, or progressive-relapsing courses of MS, all fail to correlate with the immunopathological patterns of demyelination. All lesions from one patient show only one pattern of demyelination, similar oligodendrocyte density, and

degree of remyelination along with comparable degrees of axonal injury [40]. This shows that different "mechanisms and targets" of demyelination and tissue destruction and repair are behind the pathological subgroups, irrespective of clinical characteristics [86, 91]. MS therapy can be focused in future on searching for less-invasive clinical or paraclinical surrogate markers to accurately distinguish between pathological subtypes [86, 89, 91, 96].

1.2.2 Remyelination

Remyelination, unlike demyelination, restores structural and functional integrity in denuded axons. Clinically, early remyelination, which precedes or prevents more axonal damage, is crucial in improving or slowing neurological disability in MS patients. No conclusions can be drawn from information on the frequency, course, or extent of remyelination in MS lesions, derived either by pathological or radiological methods. Remyelination is partially limited by the degree of oligodendrocyte and axon preservation after the injury [86]. Intraindividual heterogeneity of MS lesions is stark obvious in terms of the character of the inflammatory infiltrates in the early demyelination lesions and in terms of oligodendrocyte survival and remyelination at all stages of demyelination [40, 86].

Remyelination is partially limited by the degree of oligodendrocyte and axon preservation. How much remyelination has occurred in MS lesions varies from none to partial, which is limited to the lesion edge, and rarely, reaching throughout the whole of the previously demyelinated lesion.

The ultrastructural characteristics of remyelination include shortened internodes and decreased myelin thickness to an axonal diameter ratio, relative to the surrounding normal-appearing (or periplaque) white matter (NAWM or PPWM) [89, 97-98). Early

remyelination is marked by patches of thin, short, and irregularly ordered myelin sheaths or groups of preserved (or recruited progenitor) oligodendrocytes as well as short myelin processes, mixed in with the macrophage-rich infiltrate [89, 99).

Macrophages in remyelinating regions within inactive (completely) demyelinated plaques lack myelin degradation products, which mean that they are negative for LFB, PAS or myelin protein stains. On the contrary, remyelinating lesions have little or no inflammation within regions of relatively thin, yet more densely packed and regularly organized myelin sheaths that may cover all of the previously-naked axons. These sharply demarcated regions of myelin pallor may show widespread fibrillary gliosis and axonal loss, relative to the surrounding PPWM [85, 89]. Remyelination can be difficult to identify microscopically from active MS lesions with incomplete demyelination. Even though both have thin, truncated, and irregularly arranged myelin sheaths, the active demyelinating lesion is defined by T-cells and macrophages which contain granules bearing myelin degradation products [89].

Remyelination can also be mistaken for secondary (Wallerian) degeneration. Both are defined by areas of myelin loss. However, Wallerian degeneration displays a decrease in the axonal number and an absence of T-cell infiltration which is present in MS lesions [89]. Active remyelination can also happen in lesions with concurrent demyelination which further complicates the process of identifying MS lesions. These active demyelinating and remyelinating (ADM/RM) plaques are similar to the typical remyelinating MS lesions with T-lymphocytes and macrophages bearing myelin degradation products and which also exhibit diverse immunopathological patterns which are described in nonremyelinating early active demyelination lesions. Remyelination is

more often seen with the early active autoimmune patterns of demyelination (patterns I and II) and rarely in patterns III and IV which are normally linked with major oligodendrocyte loss [89, 100-101).

1.2.3 Pathology of Axons

Oligodendrocytes wrap around approximately 40 adjacent nerve axons in the CNS [102]. Myelin is necessary for salutatory axonal conduction to occur. Therefore, demyelination destroys nerve conduction that results in a number of neurological signs and symptoms and neurophysiological findings that is often found in multiple sclerosis. Along with delays in conduction of evoked potentials, action potentials travel at much lower velocities in demyelinated axons [102]. Additionally, neural firing is a consequence of hyperexcitability of demyelinated axons and this may explain the flashes of light that occur with eye movement *(phosphenes)* and the electrical sensation that occurs running down the spine or limbs responsible for neck flexion *(L'hermitte sign)* [102]. Even though the frequency of firing increases, demyelinated neurons experience problems in sustaining an action potential along the axonal length. As a result, the nerve fatigues with repeated usage causing transient neurological deficits especially after exercise or a hot bath *(Uhthoff phenomenon).* MS patients thus grow tired upon engaging in physical activity or tasks requiring mental prowess and recover slowly [102]. Neighboring axons are isolated physically and electrically by myelin sheaths. Cross-talk between adjacent axons *(ephaptic transmission)*, causing paroxysmal symptoms like trigeminal neuralgia, ataxia, dysarthria, or painful tetanic posturing of the limbs, which lasts for a few minutes and occurs after a touch or movement [102]. After reorganizing at the cellular and systems level of the surviving functional pathways that are affected by the early

demyelination and inflammation [102], we observe clinical remission.

Toxicity due to the inflammatory process of active lesions causes more injury to the participating tissue. Nitric oxide that is released by macrophages results in irreversible damage to axons and glia which promote the disintegration of the blood-brain barrier [103]. Transected axons in acute inflammatory MS plaques may experience Wallerian degeneration during the later 18 months without extending the lesion size or clinical deficit [102]. This is shown by both histology and imaging. Reactive astrocytes and microglia produce cytokines and growth factors throughout the acute inflammatory phase. These growth factors promote remyelination by surviving oligodendrocytes. Astrocytic proliferation (gliosis), however, may shield the chronic lesion, producing a physical barrier to further remyelination [102].

Axonal injury may be worsened by the loss of trophic support by myelin or glia with no further axonal degeneration and accumulating clinical deficits [102]. Along with the large oligodendrocyte loss, the degree of acute axonal injury which is measured by the expression of amyloid precursor protein (APP), along with the extent of chronic axonal loss relative to the PPWM, correlate with the degree of demyelination and nature of the inflammation [93, 104]. Acute axonal injury shows a positive correlation to the density of macrophages and CD_8 cytotoxic T-lymphocytes within MS lesions, whether demyelinating, inactive, or remyelinating. No correlation exists with the expression of TNF-α or inducible nitric oxide synthetase (iNOS). Acute axonal injury is the worst in secondary progressive MS compared to those with primary and the most severe in secondary progressive MS compared to those with primary progressive (PPMS), or relapsing-remitting MS (RRMS) [104]. Acute axonal injury may occur without myelin

degradation, which suggests that an ischemic factor contributes to pathogenesis of the lesion [93, 104].

It is believed that disability occurs only after reaching a threshold of axonal loss after which compensatory CNS resources are used up [105-106]. Therefore, chronic progressive axonal loss may cause progressive neurological decline which is typical of primary progressive MS [93, 104]. Along with the variable patterns of demyelination and cellular composition, heterogeneity occurs amongst MS lesions with respect to degree of axonal damage.

1.3 Cellular Biology of Remyelination in MS

Literature suggests that remyelination occurs in some demyelinated areas. Therefore, it should be possible to myelinate myelin-deficient areas by transplanting glial cells in experimental models of MS [107-110]. It is important to understand the background behind the success or failure of endogenous remyelination in MS because some areas may show remyelination while others may not. Understanding the molecular basis of remyelination is imperative to design possible remyelinating transplantation. For remyelinating transplantation, it is important to answer a few important questions such as i) which cell leads to the production of remyelinating oligodendrocytes, ii) over what distance can these cells be recruited into a demyelinated region, and iii) can the cells move freely within the adult CNS and within demyelinated areas.

Two major differences exist in myelin sheaths in remyelinated areas: i) they are thinner than normal [111] and ii) internodal length is greatly reduced [112]. It has been insinuated that the large oligodendrocyte number is needed for remyelination since it is stated that oligodendrocytes are greater in remyelinated areas in EAE and MS lesions [113-114]. These extra cells may have emerged from a number of sources. It is being suggested that oligodendrocytes may survive myelin sheath loss in acute MS lesions [115-116] which means that it could directly remyelinate the demyelinating axons or proliferate itself with or without dedifferentiating quality to produce new oligodendrocytes [117-119]. Oligodendrocytes from the surrounding demyelinated areas may be induced to proliferate [120-121]. Another explanation is that the oligodendrocyte that has already generated myelin sheaths can not undergo further proliferation. Consequently, fresh

oligodendrocytes from progenitor cells are needed for remyelination to occur. Progenitor (neural stem) cells are present in the adult nervous system [122-123]. These cells arise from demyelinated areas where they may survive or migrate into the required region from the nearby normal tissue. However, studies have found that both precursors and differentiated oligodendrocytes act as potential cells for remyelination [124].

1.3.1 Mitosis and Remyelination

Remyelinating demyelinated areas is produced by cells undergoing mitosis [124-127]. Preventing mitosis via experiments involving irradiation has shown that mitosis is a prerequisite for any remyelination [107, 128].

1.3.2 Proliferation of Oligodendrocytes

No conclusive evidence of remyelination triggered by demyelinating axons presently exists [129]. Proliferation of oligodendrocytes can not begin by demyelinating axons. Therefore, there is no remyelinating potential.

Contrasting with the above results, transplanting the embryonic human brain fragments into newborn mice causes myelin sheath formation [130-131] while the adult human brain does not cause myelin sheath formation even though the transplanted cells do survive [132].

In conclusion, mitosis is a requirement for remyelination [118, 124-127] and the majority of remyelinating oligodendrocytes are derived from progenitor cells [130-131].

1.4 Clinical Presentation

- ✓ Relapsing – 85-90% at onset
- ✓ Primary progressive – 10% and older age at onset
- ✓ Secondary progressive:
    - ➢ Develops in up to 80% of relapsing patients
    - ➢ 5-15 years after onset

1.4.1 Initial Clinical Symptoms in Relapsing-Remitting MS

Symptoms are categorized in neurologic terms (numbness, weakness, and tremors) and functional changes like increased difficulty with handwriting or buttoning clothes, increased difficulty with walking, running, or leg coordination, or difficulty tracking with reading. Some of the most common symptoms are changes in visual function which can take the form of monocular (rarely binocular) decreased visual acuity or blurred vision, which is described as “looking through a fogged window or smudged eyeglasses,” often involving central vision more than peripheral vision. Loss of color intensity where everything appears “bleached” or lighter is less common. In some cases, loss of vision occurs due to inflammation of the optic nerve as opposed to optic chiasmal lesion. There may be a sharp or dull pain behind and over the eye which is worse with eye movement, and at times, preceding the onset of visual difficulty by days to weeks. In MS, these visual difficulties are most likely due to optic nerve inflammation (optic neuritis) and can occur over a few hours or days. It may subside without treatment over weeks to months.

Sensory symptoms in MS also occur commonly. Their onset, in contrast to

sensory changes of compressive neuropathies or radiculopathies, is usually painless, with a few exceptions [133]. Patterns of sensory change differ widely. In some cases, they involve particular dermatomes, such as the C_7-T_2 dermatomes, hands, feet, and legs. Gradually, ascending sensory symptoms are common and which involve tightness in the abdomen or thorax ("like a girdle or rope"). Facial and tongue numbness and tingling are less frequently noted. Symptoms may be unilateral or bilateral and can be made worse with fatigue or increased body temperature. The nature of the sensory changes vary and can range from numbness, to numbness and tingling, to a dysesthetic, uncomfortable, prickling, and often burning sensation (causalgia). These sensory changes are not MS-specific and if present bilaterally and associated with a buzzing, electric, shock-like sensation into the back and/or limbs upon neck flexion (L'hermitte sign), indicate the presence of spinal cord dysfunction. Along with problems with vision, symptoms may spontaneously resolve partially or completely over a period of days to weeks. Vertigo or feelings of imbalance or dizziness are among the most common symptoms in any clinical practice of neurology and can be related to medication, blood pressure changes, inner ear (vestibular) dysfunction, musculo-skeletal abnormalities of the neck (cervical vertigo) and, of course, brainstem dysfunction in MS. The dizziness and vertigo of CNS origin can be positioned and made worse with rapid eye movements but it is unusual for tinnitus and decreased hearing to be present. Since inflammation of the brain stem in MS often involves other nuclei, the vertigo associated with MS is often associated with other symptoms such as diplopia, oscillopsia, dysarthria, and/or numbness. Vertigo occurs in some people with MS and is most often due to inner ear dysfunction instead of central nervous system inflammation

[134], and deciding whether a previous episode of vertigo was of central or peripheral origin can be difficult.

Double vision with or without oscillopsia (the symptom of nystagmus, with bouncing or jumping vision) can be a symptom of MS. Pupillary or eyelid changes are not usually associated with diplopia but is often present on gaze to either side and vertically.

MS patients also often exhibit weakness which is seen after extended exertion ("My right leg begins to drag only after I've walked five miles"), or with elevations of core body temperature like with infections or hot weather. Weakness may involve any body part but most commonly affects the lower extremities, either proximally or distally. Muscle cramping or myoclonic jerks are the muscular symptoms that occur, especially when lying down at night.

Fatigue occurs most commonly with MS. This type of fatigue is the kind one feels after a period of flu or other viral infection, namely, a type of fatigue that rest alone does not undo and which is best described as a generalized feeling of weakness. It can be mild or severe and can be a major symptom during MS.

1.4.2 Symptoms of Primary Progressive MS

The symptoms in people with primary progressive MS may appear in such a way that determining the time at which the disease occurred becomes difficult and can only be narrowed to months or years. Additionally, visual or cranial nerve symptoms occur at the beginning of primary progressive MS and are most unusual in keeping with the pathophysiology of the disease, which mainly involves the spinal cord. Most common symptoms include increasing imbalance, extremity weakness, limb tightness, numbness

and tingling of either a limb or limbs (primarily the legs), and often the lower trunk, with changes in bowel, bladder and sexual function, similar to those noted with relapsing-remitting MS. This pattern of neurologic change is not unique to people with primary progressive MS.

1.4.3 Signs in Multiple Sclerosis

In general, neurologic exam results are appropriate for the symptoms of an individual.

Visual difficulties of an optic neuritis – Visual acuity declines and not corrected with glasses, defects in the visual field, loss or decrease in color intensity, edema of the optic nerve (less common).

Sensory changes in MS often will not be sharply defined: Diplopia – internuclear ophthalmoplegia (INO), or an abducens weakness.

Limb weakness – muscle weakness, with or without changes in tone may be with spasticity, increased reflexes, and Babinksi responses.

The biologic onset of MS occurs before the initial clinical presentation of symptoms. The MRI shows a much more silent disease than is associated with clear clinical symptoms and signs. 1 in 10 new T_2 or contrast-enhancing lesion is associated with the appearance or worsening of a symptom. As a result, there may be findings on a neurologic exam that were observed by the individual and were not linked to any symptom.

The presence of certain symptoms and signs may suggest a diagnosis of MS, however their absence can make the diagnosis doubtful. It has been suggested that a

set of so-called red flag negatives that should cause a diagnostician pause [136]. These include the absence of objective optic nerve or oculomotor findings, the absence of clinical remissions, the absence of sensory findings, the absence of bowel or bladder difficulties, and normal or atypical MRI and CSF findings. None of these completely excludes MS, especially early on during the MS disease or in persons with variants of MS, such as those with primary progressive MS. A patient's disease history, apart of MS, should be considered if the patient has had a long neurologic history and several of the above negatives [135].

1.5 Diagnosis and Management

1.5.1 Magnetic Resonance Imaging (MRI)

Using MRIs have made diagnosing MS both simpler and more complex. Since MS is an inflammatory disease of the CNS, MRIs are able to visualize changes in a patient. However, the changes seen in MRIs are changes in proton density (water content) and therefore are nonspecific.

MRI changes should therefore never be solely used to diagnose MS, irrespective of the claims of the radiology report. However, certain changes in MRI are very indicative of MS. MRI changes seen associated with the disease correlate with the gross and histologic pathology of the disease [136-137].

1.5.2 CSF Analysis

Up to 90% of people with clinically definite MS have spinal fluid that exhibits changes of low-grade inflammation. These changes are both measured directly in CSF, such as the immunoglobulin G (IgG) concentration and oligoclonal bandings, are calculated from measuring CSF and serum IgG, and albumin. Using certain formulae, calculated values include the IgG index and IgG synthesis rate. The presence of these changes is influenced by both the progression of the disease and the anatomic location of the inflammatory lesions. Those near ventricular surfaces will result in spinal fluid problems. However, with greater involvement of the blood-brain barrier, the specificity of the calculated changes seen in MS CSF (like IgG synthesis rate) decreases [138]. Early during the progression of the disease, the spinal fluid is often normal [139] but can deteriorate over time [140]. Spinal fluid changes at this stage are imperative in diagnosing MS. In up to 20% of patients with clinically definite MS, the spinal fluid is normal [141],

probably due to differences in the disease pathogenesis in these individuals [142]. However, the presence of a low-grade inflammation in CSF without the occurrence of other diseases could cause similar changes which would support an MS diagnosis.

1.5.3 Management

The following is the treatment modality employed by the Mayo Clinic and other centers in USA.

1. **Symptomatic treatment**
    i. **Corticosteroids**
    ii. **Spasticity:** Mild spasticity can be managed by stretching and exercise like water therapy, yoga and physical therapy. Medication like baclofen (lioresal), tizanidine (zanaflex), gabapentin (neurontin), and benzodiazepines are effective antispastic agents for stiffness that interferes function or sleep [143]
    iii. **Paroxysmal Disorders:** Carbamazepine (Tegretol) [144], and for paroxysmal pain anticonvulsants or amitriptyline (elavil) are effective [145]
    iv. **Bladder urgency:** Oxybutynin (Ditropan) and Tolterodine (Detrol) [146]
    v. **Fatigue-reducing medications:** Amantadine (Symmetrel) [147] and for narcolepsy – Modafinil (Provigil) [148]
    vi. **Depression:** Selective Serotonin Reuptake Inhibitors (SSRIs) like Amitriptyline [149]
    vii. **Sexual Inadequacy:** Sildenafil (Viagra) [150]
2. **Disease-Modifying Therapies:**
    a. Interferon β-1a (Avonex), Interferon β-1a (Rebif), and Interferon β-1b (Betaseron) [151]

b. Glatiramer (Copaxone) for β interferon intolerant patients [152]

c. Mitoxantrone [153]

3. **New and Other Drugs:**

   i. Natalizumab (Antegren), a monoclonal antibody against VLA_4 [154]

   ii. Other drugs: IV IgG, azathioprine, methotrexate, and cyclophosphamide [155-159]

4. **BMT:** Autologous Bone Marrow Transplantation has been tried by Dr. George Kraft's group [160] from Seattle, USA and by the cohort study of Canadian MS BMT Study Group [161]. The interim results have been encouraging

5. **Statins:** Atorvastatin has been found promising in their animal (EAE) study by Scott Zamvil and his colleagues [162] from University of California, USA and in-vitro human study from the German and Austrian group [163]

6. **Experimental:** Plasma exchange therapy for sudden and severe attacks of MS that are not responding to high doses of corticosteroid.

1.6 Animal Model of MS

1.6.1 Experimental Allergic (Autoimmune) Encephalomyelitis (EAE)

Animal models for the disease are necessary to understand the complexity of the human disease. Since we are unable to go inside the human disease process, the animal models enable us to study the disease progression. Animal models to study disease have been used since ancient times. Sacrificial animals were first used to observe the internal organ pathology. Autoimmune diseases were best characterized in animal models.

Multiple sclerosis (MS) is a difficult organ-specific autoimmune disease to model. Autopsies are usually limited and confirm the existence of a long-established disease. Biopsy and autopsy examinations show that an autoimmune process had occurred but since the disease process is found in the central nervous system (CNS), it is difficult to obtain or analyze biopsies or draining fluids that allow detailed monitoring of the disease progression. Only recently did imaging of live patients become possible, which allowed us to learn about previously inaccessible stages of the disease.

Experimental Autoimmune Encephalomyelitis (EAE), also known as Experimental Allergic Encephalomyelitis, is an animal model of Multiple Sclerosis. Animal models of human diseases are diseases that occur in non-human species (especially rodents) which are similar to the human form of the disease. This allows us to study and gain a better understanding in treating the human form. EAE is not multiple sclerosis. It is also not a single disease specific to only one species, but its different forms are very similar to the stages of MS.

EAE is an acute or chronic-relapsing, acquired, inflammatory autoimmune disease which is also characterized by demyelination. Animals are injected with whole or parts of different proteins that make up myelin, the insulating sheath which surrounds nerve cells (neurons). An autoimmune response results from the proteins in these animals. In other words, the animal's immune system attacks its own myelin due to exposure of the injection. The animals develop a disease process that is very similar to MS in humans. This process is expressed in fig. 1.6.

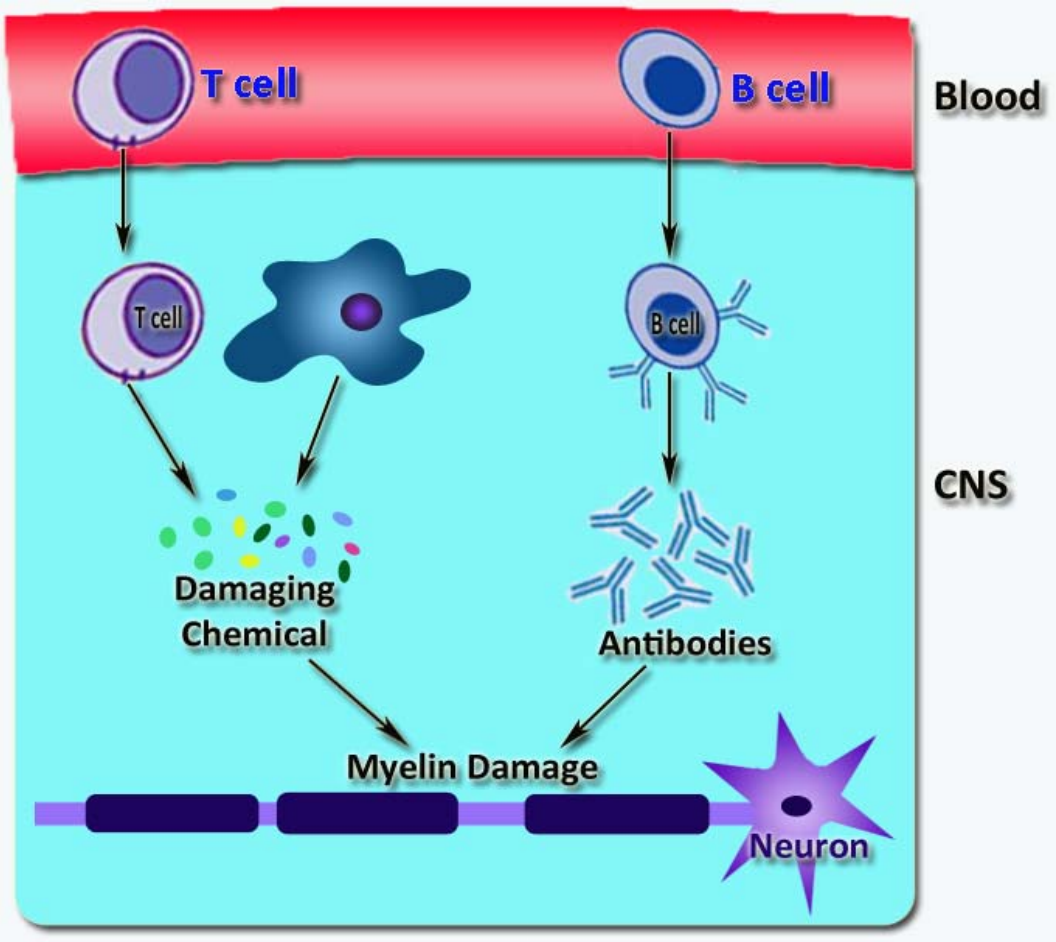


Fig. 1.6: EAE pathogenesis causing myelin damage. The inoculated myelin antigen is processed in peripheral lymph nodes and presented to lymphocytes resulting in their clonal expansion of B-cells and T-cells. These cells migrate into the CNS where they are reactivated upon recognition of the myelin antigen. B-cells secrete antibodies directed against myelin antigen. The autoreactive T-cells recognize antigen presented by CNS resident microglia and secrete cytokines that. These cytokines induce MHC expression on CNS resident cells, disrupt the blood brain barrier to allow further cellular infiltration, and attract peripheral macrophages, further enhancing the immune response.

1.6.2 Historical Perspective

EAE was initially discovered as a side-effect of the rabies vaccination in the beginning of the 20th century [164]. EAE was induced in mice by Olitsky in 1949 [165]. In 1947, Kabat stated that self antigens found in CNS white matter were the cause of EAE [166]. In 1962, Einstein discovered myelin basic protein (MBP), which makes up 30% of CNS myelin, as an encephalotogenic antigen in CNS tissue [167].

In 1933, Thomas Rivers identified experimental autoimmune encephalomyelitis (EAE), an autoimmune inflammatory demyelinating disease, as a model for ADEM [168-169].

EAE has been found in several animal species such as mice, rats, guinea pigs, rabbits, macaques, rhesus monkeys and marmosets. For several reasons including the number of immunological tools, the availability, lifespan and animal fecundity and the similarity of the induced disease to MS, mice and rats are the most commonly used species.

The animals are in-bred to induce susceptibility to EAE. Such as with humans and MS, not all mice or rats will have a natural tendency to acquire EAE. However, different breeds will develop different forms of EAE, some of which act as good models for the different human forms of MS. Different EAE forms are also used as models for the different stages in MS.

Many proteins or protein parts (antigens) are used to induce EAE such as: myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG).

1.6.3 EAE Pathogenesis

To produce EAE, four integrins must be expressed on activated T-lymphocytes [170]: MBP-specific T-lymphocytes produce a tumor necrosis factor (TNF) and interferon-γ (IFN-γ) - cytokines of the helper T-cell subset 1 (TH_1) type [171]. These cytokines are responsible for the upregulation of VCAM expression on CNS endothelial cells which promotes the binding of four integrins expressing T-lymphocytes to endothelium and upregulation of expression of MHC class II molecules on APCs within CNS [172-173]. T-lymphocytes interact with ICAM-1 and VCAM-1 and become attached to the ECs, then migrate in [170].

Interferon-α (IFN-α) induces interleukin-12 (IL_{12}) [174-175]**.** IL_{12} treatment makes EAE worse and antibody to IL_{12} improves the disease [174-176]. IL_4 and IL_{10} (TH_2) improve MBP-induced EAE, where TH_2 downregulates TH_1 [177-179].

1.6.3.1 T-lymphocytes Require Two Signals

- ✓ TCR recognize antigenic peptides within MHC complexes on APCs.
- ✓ Other receptors (costimulatory) on T-cells that bind to their respective ligands on APCs.

As a result, B7-1 and B7-2 ligands on APCs with CD_{28} and CTLA-4 receptors on T-lymphocytes play a role in EAE [180-182].

Upregulated Chemokines at the EAE lesions are the following [183-186]:

- ➢ macrophage inflammatory protein-1
- ➢ monocyte chemotactic protein-1
- ➢ neurotactin

Peripheral tolerance of myelin-specific T-lymphocytes experiences a break during relapsing EAE [183]. In lymphoid tissues, myelin-specific T-lymphocytes are activated in vivo. Therefore, different interactions in the pathogenesis are potential sites of therapeutic interventions.

1.6.3.2 Nuclear Factor kappa B (NFκB) and Oxidative Stress

NFκB is a DNA binding transcriptional factor complex that interacts with promoter areas in pro-inflammatory genes [184]. Oxidative stress causes an activation of the transcriptional factor NFκB, that in turn upregulates pro-inflammatory gene expression [185]. Strong inducers of NFκB activation are the gram-negative bacterial endotoxin lipopolysaccharide (LPS), and the cytokines tumour necrosis factor – α (TNF-α) and interleukin-1β (IL-1 β). Most of the identified stimuli for NFκB activation, including LPS, TNF-α, and IL-1β, manufacture oxidative stress in cells [186,187]. The over expression of glutathione peroxidase [188] inhibits the cytokine-induced activation of NFκB. The TNFα-induced NFκB activation is attenuated by over expression of γ-glutamylcysteine synthase, which is the rate-limiting enzyme for GSH synthesis [189].

1.6.3.3 NFκB and Pro-Inflammatory Gene Expression

Activation of NFκB promotes transcription of pro-inflammatory genes that include cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and VCAM-1, enzymes such as inducible nitric oxide synthase (iNOS) and cycloxygenase-2 (COX-2), cytokines such as IL-1β, interleukin-6 (IL-6) and TNFα, and chemokines such as regulated upon normal T-cell expressed and secreted protein (RANTES), monocyte chemoattractant protein-1(MCP-1), interleukin-8 [190-195].

One should be able to inhibit inflammatory changes by decreasing the activation

of NFκB. Lessening the chances of the activation of NFκB should be achievable by promoting the scavenging of strong oxidants formed by normal and abnormal cellular metabolism. To do this rationally requires an understanding of the significant components of the cellular anti-oxidant defense systems.

1.6.3.4 EAE and Oxidative Stress

The inflammatory infiltrate in the lesions of EAE that comprises mononuclear infiltrate in the parenchyma of brain and spinal cord and also around blood vessels as perivascular cuffing plays an important role in causing the damage to myelin and oligodendrocyte. The mononuclear infiltrate is attributed with enzymes that produce reactive oxygen species (ROS) [196-198]. Thereby it is being said that oxidative stress contributes significantly in the pathogenesis of MS [199, 200]. The activated microglia and/or macrophages generate ROS through actions of NADPH oxidase enzyme. [201]. Damaging effects of superoxide most likely contributes to the injury of oligodendrocytes *in vitro* [202]. The cyclo-oxygenase 2 (COX-2) enzyme also produces ROS [203]. The nitric oxide (NO) is generated by inducible nitric oxide synthetase (iNOS) enzyme using arginine, oxygen and NADPH [204]. Nitric oxide acts by various means. It can induce COX-2 enzyme [205, 206]. It reacts with superoxide to form the highly toxic species peroxynitrite that can damage proteins, cell membrane and nucleic acids [207, 208]. The peroxynitrite can react with tyrosine to produce nitrotyrosine. The nitrotyrosine is found in the demyelinating lesions of EAE. This attributes the damaging effects of peroxynitrite [209].

The white matter is rich in iron and it can convert hydrogen peroxide to hydroxyl radicals that may contribute to demyelination [198]. The reduced form of iron (ferrous ion

[Fe2+]) is used for this reaction, which is called the Fenton reaction [210]. The oxidised ferric ion (Fe3+) formed in this reaction can be reduced back to form ferrous ion (Fe2+) by vitamin C, thereby a continuous reaction with hydrogen peroxide to generate hydroxyl radical [210]. Therefore, the large doses of vitamin C may be harmful in MS [211]. Vitamin C does not give protection against EAE, and it may increase lipid peroxidation in the presence of Fe3+ [212]. Lipids make nearly 70% of myelin. This contributes in lipid peroxidation leading to damage to myelin [213-215].

The oxidative stress in different demyelinating diseases is expressed below in fig. 1.7 [216].

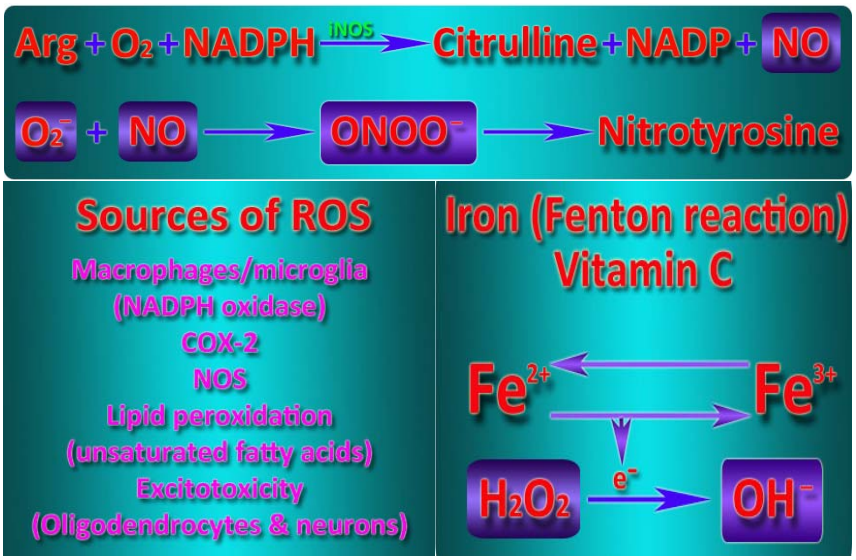


Fig. 1.7: Oxidative stress in a demyelinating disease. The interaction between NO and ROS to form peroxynitrite catalysed by iNOS. In Fenton reaction, the iron contributes to produce hydroxyl radical, and vitamin C in reducing ferric ions to ferrous ions required in the Fenton reaction. **Arg** = arginine; **COX-2** = cyclo-oxygenase 2; **H_2O_2** = hydrogen peroxide; **iNOS** = inducible nitric oxide synthase; **NADP** = nicotinamide adenine dinucleotide phosphate; **NADPH** = reduced NADP; **NO** = nitric oxide; **NOS** = nitric oxide synthase; **O_{2-}** = superoxide; **OH_-** = hydroxyl radical; **ONOO**– = peroxynitrite; **ROS** = reactive oxygen species.

As literature on EAE suggests, there have been various attempts to use chemical antioxidant for therapeutic intervention in animal experimentation with generally encouraging and successful results of various grades. The following table 1.1 presents

the various antioxidant used by different research groups.

Table 1.1: Effects of different antioxidants in EAE

Antioxidant	Effects in EAE
Caffeic acid phenethyl ester (CAPE) [217]	Decreased severity of disease and decreased levels of reactive oxygen species
Lipoic acid [218-219]	Decreased inflammation, demyelination, and axonal loss
Phenidone (cyclo-oxygenase/lipoxygenase inhibitor) [220]	Decreased incidence and severity of disease
N-acetylcysteine amide (AD4) [221]	Decreased inflammation, demyelination, axonal loss and clinical symptoms
Trichostatin A (TSA) [increases levels of antioxidant enzymes] [222]	Decreased inflammation, demyelination, axonal loss and clinical symptoms
Bilirubin [223]	Prevented acute and chronic EAE (protected permeability of blood-brain barrier)
Thymoquinone [224]	Decreased incidence and severity of disease
t-Butylhydroxyanisole (BHA) [225-226]	Decreased incidence and severity of disease
Uric acid [227-228]	Prevented disease symptoms
Hemin (inducer of haeme oxygenase-1) [229]	Inhibited EAE
Epigallocatechin-3-gallate (EGCG) [green tea constituent] [230]	Inhibited EAE
Metallothionein [231]	Prevented demyelination and axonal damage, promoted tissue repair
Flavonoids [232]	Delayed recovery of clinical symptoms

The modes of damage to myelin and axons are thought to be an autoimmune injury followed by apoptotic damage. This injury is possibly caused by reactive oxidative

species (ROS)-induced apoptosis in CNS and peripheral nervous system in addition to the injury caused by autoimmunity. This mode of damage is illustrated in fig. 1.8 [233]

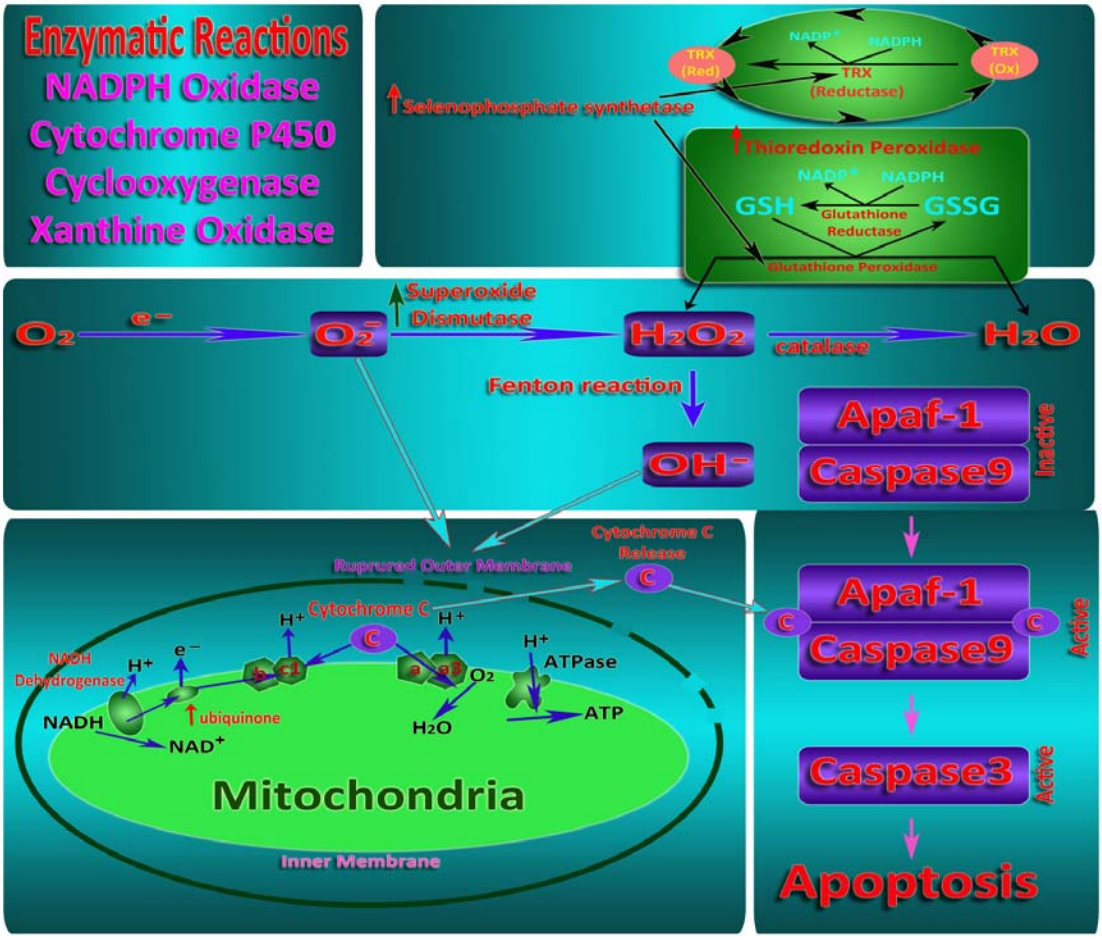


Fig. 1.8: Pathways and mechanisms of Ras-mediated protection from reactive oxidative species (ROS)-induced apoptosis. Transformation of human ovarian epithelial cells by oncogene *Ras* leads to the enhanced expression of several antioxidant proteins involved in major cellular pathways for ROS metabolism. Protein targets up-regulated in transformed HOSE are highlighted with an arrow. These proteins include thioredoxin peroxidase, peroxiredoxin 3 (a mitochondrial member of thioredoxin peroxidases), NADH dehydrogenase ubiquinone Fe/S protein, selenophosphate synthetase, and mitochondrial superoxide dismutase. In conjunction, these proteins significantly increase the overall antioxidant capacity and protect cells from apoptosis under high level of ROS.

1.6.3.5 Phase 1 and Phase 2 Enzymes

Phase 1 and phase 2 enzymes are involved in xenobiotic metabolism. Xenobiotics are chemicals that are alien to the body or to live organisms. These xenobiotics are metabolized by enzymes placed into phase 1 (mono-oxygenases such as cytochrome P450s) and phase 2 categories of enzymes [234]. The phase 1 enzymes

are electrophiles (electron receiver/lover). These phase 1 enzymes generally oxidize or reduce xenobiotics, though they usually give rise to potentially harmful secondary products [235]. The products of phase 1 enzymes are acted upon by phase 2 enzymes that cause a conjugation reaction. These phase 2 enzyme conjugates are comparatively easy for excretion. The enzymes like γ-glutamyl-cysteine synthase, quinone reductase, glutathione transferase, epoxide hydrolase, UDP-glucoronosyltransferase are very important phase 2 enzymes [235], including the selenoprotein family of thioredoxin reductases [236]. Directly or indirectly, the phase 2 enzymes have an essential role in inactivating xenobiotics, often by forming conjugates, as glutathiyl-xenobiotic conjugates. In contrast, there has been broad research on phase 2 enzymes and their induction (particularly quinone reductase and glutathione S-transferases) in cancer prevention [235,237,238].

1.6.3.6 BHA and BHT

Two commonly used food additives are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). The dietary intake of BHA [239-241] and BHT increases tissue phase 2 enzyme activities [242]. BHA is apparently mediating its phase 2 enzyme induction by its metabolite BHT [243]. The glutathione peroxidase is one of the phase 2 enzyme can be induced by tetra-butylhydroxyanisole (BHA). BHA is a food preservative, which also increases the glutathione (GSH) [225-226]. This reiterates again that EAE can be improved by antioxidants [217-232], thereby having a useful potentials in the management of MS.

1.6.3.7 Phase 2 Enzyme Inducers, GST, and Peroxide Scavenging

The green tea (polyphenolics) has been shown to induce glutathione S-transferase and quinone reductase in a variety of tissues in mice [244]. The soy ingestion also increases quinone reductase and glutathione S-transferase in various tissues [245]. It is apparent that there must be efficient mechanisms that scavenge hydrogen peroxide and organic peroxides. Two defined enzymatic mechanisms, whereby the cell can scavenge hydrogen peroxide, are known as the catalase and the glutathione peroxidase (GPx) systems. Of the two, the GPx system appears to be the most important [246, 247].

The GPx family of proteins is selenoproteins that can scavenge hydrogen peroxide and organic peroxides, as well as lipid peroxides. Contrasting with catalase, these enzymes have a high affinity for their substrate [248-250]. GPx uses the tripeptide GSH (glutathione) as the electron donor in the scavenging of peroxides [250, 251]. The GSH is made up of three amino acids: glutamic acid, cysteine, and glycine. With the GSH-dependent GPx's, because of the sequential reactions of two GSH molecules with glutathione peroxidase in the scavenging of peroxide, increasing GSH concentrations clearly increases the peroxide scavenging efficiency [252]. Many lipid peroxides as well as peroxide breakdown products, such as 4-hydroxyalkenals, are scavenged by glutathione S-transferase which causes the creation of inactive glutathiyl adducts [253-255].

Thus, the GSH plays several critical roles in minimizing oxidative stress; it is essential for: i) peroxide scavenging by GPx, ii) scavenging of 4-hydroxyalkenals and other oxidants by glutathione S-transferase, and iii) for the ultimate regeneration of vitamin E.

The advanced glycation end products (AGEs) seen in diabetes mellitus affect cellular metabolism by the inactivation of affected proteins; for example, glycation of glutathione reductase inactivates this enzyme [256] which plays a significant role in decreasing oxidized-glutathione. Undeniably, a negative relationship exists between the extent of diabetic complications and erythrocyte GSH level [257], which is perhaps due, in part, to glutathione reductase inactivation. GSH plays a chief role in preventing the formation of AGE. The decreasing red blood cells GSH increases hemoglobin glycation [258]. Therefore, GSH inhibits the formation of AGEs most likely through the glyoxalase pathway [259]. This is just another example of the many important roles that GSH plays in reducing and minimizing oxidative stress.

In combination with the increased inefficiencies of mitochondrial respiration are reduced abilities by ageing cells to synthesize GSH, to reduce GSSG to GSH [260], as well as decreased activities of GPx [261-263], superoxide dismutase and catalase [262, 263].

1.6.3.8 Anti-Oxidant Defense System

The important components of the anti-oxidant defense system are the following:

a) Scavenging of peroxides, as peroxides can give rise to strong oxidants such as hydroxyl/peroxyl radicals, 4-hydroxyalkenals and pro-inflammatory isoprostanoids

b) Regeneration of vitamin E, since vitamin E plays a necessary role in preventing lipid peroxidation chain reactions

c) Scavenging α-oxo-aldehydes, as these strong oxidants can form AGEs

d) Scavenging 4-hydroxyalkenals, because these strong oxidants can also form AGEs and inactivate protein function via the formation of protein carbonyls;

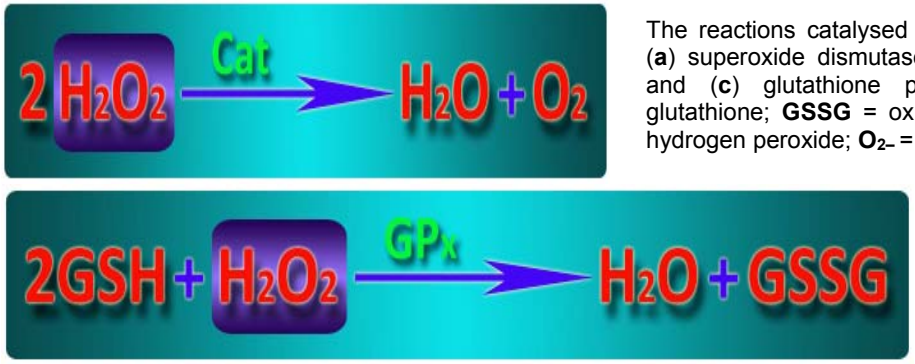
e) Scavenging of quinone radicals

Thioredoxin-dependent peroxidases, which utilize thioredoxin as the electron donor, may also play an imperative role in scavenging peroxides [264]. Glutathione S-transferases eliminate many electrophiles by catalyzing the development of the glutathiyl adducts [253]. Additionally, quinone reductase plays an important role in reducing quinones, such aminochrome [265]. GSH plays vital roles in numerous scavenging activities, either as being the electron donor in the reduction of oxidants or by forming glutathiyl adducts with the oxidants [266].

How do cells manage oxidative stress? There are anti-oxidant defense enzymes that play major roles. Superoxide anions are converted into H_2O_2 by superoxide dismutases (SOD). H_2O_2 is turned into water (H_2O) and oxygen using catalases or glutathione peroxidases (GPX). GPX activity requires the presence of glutathione (GSH). The biochemical reactions that lead to the detoxifying role of antioxidant are expressed below in fig. 1.9 [216].

Fig. 1.9: Antioxidant activity

Oxidation states of oxygen. Oxygen can be reduced to form water by the addition of four electrons. The intermediates (reactive oxygen species) are free radicals and are highly reactive with other molecules. **H_2O_2** = hydrogen peroxide; **O_{2-}** = superoxide; **OH_{-}** = hydroxyl radical.



The reactions catalysed by the antioxidant enzymes: (**a**) superoxide dismutase (SOD), (**b**) catalase (Cat); and (**c**) glutathione peroxidase (GPx). **GSH** = glutathione; **GSSG** = oxidized form of GSH; **H_2O_2** = hydrogen peroxide; **O_{2-}** = superoxide.

1.6.3.9 Inflammatory Demyelination

MS is not a monolithic disease. One aspect that is recurring with most MS variants is immune inflammation with demyelination and almost all animal models involve inflammatory demyelination as a prerequisite. The three most common inflammatory diseases of the CNS which exhibit differing levels of demyelination and axonal damage are MS, viral infection, and acute disseminated encephalomyelitis (ADEM). Etiological links between these diseases are hypothesized. A great deal of anecdotal and pathological evidence exist which uses viral infection to act as a trigger for MS, and ADEM often uses a postviral or postvaccination encephalomyelitis [267]. It is not surprising, therefore that the two most commonly studied genres of animal models for MS are infection with a neurotropic virus and a broad range of autoimmune diseases that are grouped together as experimental autoimmune encephalomyelitis (EAE). Thomas Rivers established this autoimmune inflammatory demyelinating disease as EAE in 1933 as a model for ADEM [41].

Generically, though varying by strain and protocol, EAE and neurotropic viral models show inflammation, demyelination, and axonal damage. Using such models has improved our understanding of MS but may have also biased it.

1.6.4 Molecular Immunopathology of EAE

1.6.4.1 Innate Immunity

Immune stimulation is needed to model an inflammatory disease. Innate immunity is important to initiating immune responses. The term “innate immunity” refers to a wide spectrum of preprogrammed responses to infection or ligands specific to infectious agents. An evolutionarily ancient recognition and signaling system forms the foundation of pathogen recognition by innate components. The Toll-like receptors (TLRs), eleven of which have been described thus far, are called this because of their homology to Toll receptors, which were first described as being important in the development of the nervous system in Drosophila. TLRs bind ligands that are uniquely expressed by microbes, fungi, and viruses. Pathogen-derived epitopes are called pathogen-associated molecular patterns (PAMPs). Production of cytokines is induced by TLR signaling, especially the type I interferons, which are responsible for early cellular responses to viral infection and chemokines which leads to leukocyte entry to the CNS. TLR-induced cytokines are responsible for an adaptive or antigen-specific lymphocyte response and direct the quality of these responses. Therefore, TLR-expressing cells in mucosae, epithelia, and endothelia are both found and appropriately equipped to serve as a frontline defense against pathogens. TLR-expressing cells are also found in the CNS and have been shown to exhibit early responses to PAMPs

1.6.4.2 Adjuvant and Its Effects

Initiating T-cell immune responses and thus of adaptive immune responses generally is dependent on adequate provision of co-stimulatory signals. These are provided through ligating signaling receptors on T-cells by

co-stimulator ligands expressed on antigen-presenting cells (APCs). Expressing adequate levels of co-stimulator ligands by APC is induced through TLR signals along with other signals including cytokines, several of which are TLR-inducible.

Using adjuvants is grounded in this immunological basis. Administering a potential antigen along with an adjuvant aids in its recognition in a co-stimulator adequate milieu, thereby promoting an immune response. It is important to recognize that adjuvants are needed for experimental initiation of immune responses, unless the immunogen is itself a TLR ligand (e.g. bacteria, viruses). The quality of the immune response induced is impacted by the nature of the TLR ligand. Therefore, the choice of adjuvants directs the nature of the immune response. Moreover, it is essential to recognize that choices are limited based on what works. This allows the discussion to address the issue of what facet of MS one wishes to model. Rodent models have been used to complete Freund's adjuvant (CFA) with an approach that focuses on inflammatory pathology along with the need for aggressive immunization to overcome self-tolerance. This oil-based emulsion utilizes heat-killed *M. tuberculosis* as its active ingredient. The issue of the appropriateness of using the delayed-type hypersensitivity (DTH) reaction in furthering our understanding of MS remains unresolved.

Pertussis toxin (PT), or heat-killed *Bordetella pertussis*¸ is often used as a co-adjuvant, especially because the move toward gene-deficient "knockout" mice in EAE studies. A large majority of ESC lines used for the in vitro first step of homologous recombination in generating a gene knockout are from mice of the 129/J background. Most knocks are 129/J initially, and are most frequently back-crossed to C57B1/6. Mice

on the C57B1/6 genetic background are resistant to EAE induction. Inducing EAE in C57B1/6 mice always requires the use of PT along with CFA. This is now a popular model since the emergence of gene-deficient mice.

Adjuvants are important for generating a sufficiently potent T-cell response to produce CNS inflammation. Neurotropic viruses act as their own adjuvants. Similarities shared between the inflammatory response to virus and that in EAE are used to support physiological relevance of the adjuvant-induced EAE model. The relevance of virus infection to MS might be questioned initially. This should always be kept in mind even though commentators would agree that this is not unreasonable.

1.6.4.3 Immune Responses

The CD_{4+} T-cell response can be divided based on cytokine production. The Th_1 versus Th_2 division is one of the most long-lasting paradigms of modern immunology. Inflammatory CD_{4+} T-cells secrete Th_1 cytokines, which is defined by interferon gamma (IFNγ) and tumor necrosis factor alpha (TNF-α). More importantly, Th_1 T-cells express profiles of adhesion ligands and chemokine receptors that direct movement to tissues, and together with their cytokine profile, this is likely why Th_1 T-cells were originally defined as cells that induced DTH responses. Both CFA and neurotropic viruses such as Theiler's murine encephalitis virus (TMEV) steer the immune response towards a Th_1 profile. Excluding experimentally immunosuppressed or immunomodulated animals, Th_2 responses (which lack IFNγ and TNF-α, are dominated primarily by interleukins 4, 5, and 10, and are linked to antibody responses) fail to induce a MS-like disease in mice. It would not be wise to use this information to suggest that MS is a Th_1 disease. MS may have some grounding in immunoregulation deficiency [268] and this can be seen from the

fact that myelin-specific Th_2 cells can induce CNS inflammation in mice which are deficient in appropriate immunoregulatory systems [268]. Antibodies play a major role in MS, whether or not a Th_2 response would be necessary for them to be produced [3, 269]. Recent reports suggest that antibodies may influence the quality of disease in MS, rather than play an initiating role; this is broadly consistent with experimental demonstrations of synergy between antibodies against myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) specific T-cells for inducing demyelinating EAE in rats [270-272]. The fact that CFA-based immunizations that are used to induce EAE can themselves induce antibody responses is often neglected. Whether such anti-myelin antibodies play an important role in EAE has been an important issue; most recent studies suggest that the nature of the myelin protein (peptide versus polypeptide, autologous versus xenogeneic) may influence their requirement [273-274].

1.6.4.4 CD_4 & CD_8 T- Cells

In EAE models, the main infiltrating T-cell is the CD_4 T-cell. The evidence from MS shows that CD_8 T-cells may be more important in human disease [275] and are therefore implicated as cytotoxic effectors. Earlier studies showed that CD_8 T-cells were either unnecessary for or played an immunoregulatory role in EAE [276-277]. Three recent papers showed the ability of CD_8 T-cells to induce EAE [278-280]. However, only a minority of the views are represented in these papers. Therefore, the CD_4 versus CD_8 division is maintained as being an additional difference between MS and EAE. Therefore, viral models such as TMEV encephalomyelitis may be more reliable models for MS inflammation.

1.6.4.5 Selecting a Species

The previous analysis has furthered the theme that one must choose which facet to model when designing animal models for a disease with a heterogeneous pathology. Experimental variables such as adjuvants may limit immunological models in ways that need careful consideration. A third consideration is whether the choice of animal species influences the behavior of the model. Rodents, especially mice and rats, are the most commonly studied experimental animals. These two species have replaced guinea pigs, hamsters, rabbits, and other species which were once routinely studied. Primate usage has gone up which is a reflection of earlier times when monkeys served as ADEM models [281]. Primates have several advantages as MS models. Like humans, their colonies are out-bred and there exists a high degree of homology between TCR and MHC which allows prediction of peptide specificities. The pathway to clinical application is therefore shorter than from rodents. The primate model of MOG_{p14-36} induced EAE in the common marmoset *(Callithrix jacchus)* is likely to replace other primate models including Cynomolgus *(Macaca fascicularis)* and rhesus monkeys *(M. mulatta)*. Complete prevalence is evident in the marmoset model, an animal which can be imaged by MRI. It has a relapse-remitting or primary-progressive clinical course, MS-like pathology including axonal pathology, evidence for B-cell/antibody involvement, and the possibility of adoptive transfer between chimeric twins. There is not much major histocompatibility complex (MHC) II polymorphism of DR and DQ equivalent regions and both MBP and proteolipid protein (PLP) can induce disease. The EAE model is being used more and more in testing therapeutics [282]. Mouse models are becoming increasingly important in studying MS and are replacing primates because of the high cost of using the latter. Furthermore, mouse

genetics has been further developed than that of other species. Producing transgenic and gene-deficient animals often relies on using mice which are crucial for understanding the role of specific genes in many studies. The current concentration of systems and questions through one species must be kept in mind when evaluating the information that comes from animal studies. Two specific examples where the variables discussed have influenced interpretation of animal studies for MS are studies on the role of IFNγ and of TNFα in EAE. The strain of animals also has a major influence. EAE may be demyelinating or nondemyelinating, monophasic, or relapse-remitting [270] based on the strain of rat chosen. Specific to the strain of mouse and adjuvant chosen, the same encephalitogenic peptide can induce either an RRMS-like or a PPMS-like EAE [283].

Table 1.2: *Use of selected species of animal in studies of EAE since 1950*

Decade	Mouse	Rat	Rabbit	Guinea pig	Monkey
1950-1960	0	0	0	0	0
1960-1970	0	4	1	3	0
1970-1980	30	111	27	140	10
1980-1990	174	416	50	320	21
1990-2000	775	727	52	234	32
2000-2004	517 (147)	274 (78)	24 (69)	54 (15)	38 (10)

Data are listings of numbers found in PubMed searching for Species (as per column headings) and EAE. The numbers in parantheses are for 2000-2004 over a 10-year period based on approximately 3.5 years already listed.

When EAE first appeared in the PubMed service of the National Library of Medicine in 1962 [284], the number of studies employing rat and guinea pig were approximately the same until the 1990s when the mouse became more widely used than the guinea pig. From 2000 onwards, studies that were under the search title of "mouse + EAE" were greater than all other "species + EAE" results (Table). Throughout the decades, there has been a triplicate increase in "monkey + EAE" studies from the 1990s to this decade (projected), which translates into the possibility of increased primate usage in future.

1.6.4.6 Interferon-γ Controversy

As stated previously, IFNγ is the paradigmatic Th_1 cytokine. Pro-inflammatory cytokine is responsible for inducing the expression of MHC, adhesion ligand, and co-

stimulator ligand. Additionally, other cytokines, like TNF-α are also induced to be expressed. Levels of IFNγ protein and ribonucleic acid (RNA) went up in target tissue in many autoimmune diseases, and in the CNS in MS and EAE [285]. These levels were linked to the severity of disease and they fell in remission. Naturally, it was logical to draw a causative relationship between the IFNγ and CNS inflammatory disease. Due to the fact that IFNs are described as antiviral mediators and there is an assumed link between viral infection and MS, the IFNγ was delivered via intravenous injection to RRMS patients with the belief that the viral cause of disease would be for the patients' advantage. Unexpectedly, the attack rate increased during the one month of weekly administration, causing the study to halt in light of safety concerns [286]. Regardless of certain perceived shortcomings in the study design [287], it was found that IFNγ facilitated or even worsened MS. In another study, antibodies were given against the IFNγ-receptor to patients with SPMS. After progression, improvement was evident which suggests a disease-worsening role for IFNγ in MS [288].

1.6.4.7 IFNγ in EAE

Surprisingly, analogous interventions in EAE models have produced the exact opposite effects [285]. Some of these interventions have been more invasive than would be possible for clinical studies, using viral vectors [289] or directly injecting the CNS with antibodies. The same experimental design employed in MS has never been repeated in animals. Overall, it was found that completely opposite results were produced in MS and EAE. In MS, IFNγ makes the disease worse unlike in EAE, where IFNγ ameliorated the disease or prevented it from occurring. The question that emerges is whether the EAE model is the incorrect choice to use for MS and whether there are major

differences between animals and humans regarding the role of IFNγ, or something else.

1.6.4.7.1 IFNγ & CD_8 T- Cells

That "something else" may be that EAE is inducible by CD_{8+} T-cells [278-280]. In one of these studies, anti-IFNγ antibodies were needed to prevent disease [279]. This is probably the only point where an IFNγ blockade has inhibited EAE, and so produced a great deal of interest [290]. There was a suggestion that the discrepancy between EAE and MS as to the effects of an IFNγ blockade may be suggestive of an artefactual predominance of CD_{4+} T-cells in EAE. However, inducing EAE with CD_{8+} T-cells has never been replicated after the initial publication and these two papers are the only ones that describe CD_8 induced EAE. Another perspective is that the demyelinating disease that occurs in mice over-expressing the co-stimulator ligand B7.2/CD86 on microglia. CD_{8+} T-cells also mediate this disease [291]. These cells also produce IFNγ which causes adjuvant-independent encephalomyelitis, suggesting that this is more preferable to EAE as an animal model of inflammatory events in MS [291]. It is uncertain whether IFNγ blockades inhibit disease.

1.6.4.7.2 IFNγ in Viral Encephalomyelitis

One way to answer these questions is to determine whether IFNγ has similar roles in viral inflammation in the CNS. CD_{4+} and CD_{8+} T-cells are trigged as part of the immune response to the TMEV infection and subsequent demyelinating disease, and therefore, offers another system for evaluating the role of IFNγ. The cytotoxic and humoral immune response to viral in the CNS does not usually use IFNγ [292-293], even though the decreased expression of immune markers was not found in the CNS of IFNγ-deficient, TMEV-infected mice of susceptible background. The disease occurred more

swiftly when IFNγ was delivered intracerebrally [292]. There was a correlation between motor dysfunction and axonal damage [294], and both viral persistence and neuronal damage were greatly increased by an IFNγ blockade or deficiency [293]. The lack of IFNγ or its blockade did not inhibit the demyelination that normally occurs with a TMEV infection [293], suggesting that there is consistency between viral and autoimmune animal models. In IFNγ-deficient mice, demyelination was worse, and an IFNγ deficiency or blockade both overcame strain-dependent resistance to TMEV demyelination [293]. Neuronal damage worsened in mice that were normally susceptible to TMEV encephalomyelitis [293].

These observations also suggest another point: intra-strain variation within a species can have effects that are as dramatic as intra-species differences in some cases. This is important when interpreting findings (e.g. from EAE studies where effects of a similar immunization may produce varying results from no demyelination to major demyelination. This also depends on the mouse strain).

1.6.4.7.3 IFNγ Expression in CNS

1.6.4.7.3.1 Transgenic or Hypodermic

An alternative method involves looking at results from transgenic mice. All of the transgenic mice with only one exception had an IFNγ over-expression in the CNS and used oligodendrocyte (OLG)-specific promoters, and all showed pro-inflammatory effects, from spontaneous demyelination and inflammation to a more severe disease where EAE was induced with myelin protein and adjuvant immunization [285, 295-296]. Transgenic models exhibit the effects of IFNγ which are different from those that occur after the IFNγ or IFNγR blockade, or a genetic deficiency of IFNγ. Directly injecting IFNγ

into the CNS using a hypodermic needle induced inflammation in rats [297-298] (mice have not shown the same results so far). Consequently, direct injection and transgenesis exhibit similar results. Only one difference exists between these experiments and those in which IFNγ had an immunoregulatory role where an absence of adjuvant in transgenic or hypodermic IFNγ models. Despite the fact that this can be easily criticized, is not as clear as it may initially appear. For example, transgenic mice that expressed IFNγR in OLG showed worsening severity of EAE as induced by myelin basic protein with CFA [295].

These data which were obtained from several studies show a lot of material for discussion and debate. Both transgenic and hypodermic administration of IFNγ to rodent CNS have qualities that are also found in those of two studies in which IFNγ was marked in MS, which is that IFNγ was found to be pro-inflammatory. Animal experiments in which IFNγ was targeted or administered during EAE show an effect that is opposite and protective instead. IFNγ had a protective effect against virus infection and CNS damage in Theiler's virus encephalomyelitis (TMEV) [292]. Two points must be emphasized which are that animal models are not monolithic, like MS, and the immune-regulatory effects of IFNγ on CNS inflammation have been seen only in animals. Therefore, depending on the animal model chosen for MS, one can find results that corroborate or counter the MS experience. Rodents model the inflammatory role of IFNγ well. However, whether EAE is a good model for MS is not what one should be focusing on. The question that should be asked is whether one can get answers by asking specific questions about certain aspects of MS by studying certain forms of EAE.

1.6.4.8 Role of Adjuvants

The effects of IFNγ are greatly different from EAE to MS. Despite the imperfect correspondence, there is some ground to suspect that adjuvants (or TLR signaling, as in the case of viral infection) may be an important difference between the MS-like and MS-unlike systems. This is supported by the observation that immune-suppression in bacilli Calmette-Guerin (BCG, *M. tuberculosis)*-infected mice was overcome in the absence of IFNγ [299], with findings from the same and other groups showing an IFNγ-dependent nitric oxide (NO)-mediated T-cell suppressive mechanism [300-301]. This type of observation is often made in CFA or pathogen-involving experimental systems. Simultaneously, disease-improving IFNγ was given to mice by a herpes virus vector [289], and IFNγ deficiency showed a protective role for this cytokine against TMEV encephalomyelitis in mice. Both viruses would act as TLR ligands. If a certain effect of bacterial PAMPs is evident (e.g. from *M. tuberculosis*) in directing a protective role for IFNγ, then incidental infections may influence the course of MS. This shows an interesting and probable explanation for some.

It is interesting to note that Chlamydia pneumonia has been isolated from a very high number of MS cases [302]. However the evidence to connect MS with C. pneumonia has not been found so far.

1.6.5 EAE Pathology

1.6.5.1 Demyelination - Chemical or Inflammatory

Demyelinating pathology is also seen in animals by using myelin-specific toxins. The most commonly used toxins include copper chelator cuprizone [303-304], the chromatin-disrupting agent ethidium bromide (EtBr) [305-306], and the lipid toxin lysolecithin/lysophosphatidylcholine (LPC) [307, 308]. These have been used to model certain facets of demyelinating pathology, their advantage since local administration or intrinsic characteristics allow prediction of the site of effect, unlike in EAE where lesion occurrence is always uncertain. Cuprizone and EtBr have been used to study remyelination and these studies have furthered our understanding of oligodendrocyte precursor cells and their dynamics [304, 306]. It can be said that toxin-induced demyelination is not a reliable model for MS. These models are not used with an established causal relationship between a toxin and the disease but to understand the mechanism underlying this. Therefore, they further our understanding of demyelination and remyelination. There is a downstream, inflammatory component to toxin-induced demyelination and study of cellular and cytokine/chemokine responses have contributed to a better understanding of injury-reactive inflammation in the CNS, which may occur in MS [308-309]. Another method that can be used is to transgenically promote demyelination by over-expressing the DM-20 myelin proteolipid [310]. This produced a mouse (the ND4 trangenic) which myelinates normally but develops spontaneous demyelination with attendant immune sequelae as it ages [311]. This would seem to produce a useful model for Type IV MS pathology [6].

1.6.5.2 Different Effects in Humans and Mice

Generally, one can learn from parallel situations in which the players are different

but the same principles apply. If IFNγ-induced, NO-mediated immune-regulation turns out not to be a main feature of immune-regulation in humans, then one can expect that another cytokine-driven system will work in its place. Whether human macrophages/microglia uses an equivalent source of NO as their murine counterparts may be one critical species-specific difference [312]. Reports of elevated iNOS expression and NO levels in MS tissue are interpreted as showing a cytopathic effect for NO [313], but it should be remembered that the same correlations were seen in EAE. A number of contradictory inhibitor studies along with unexpected findings for iNOS-deficient mice moved our thinking towards the possibility of a neuroprotective role for NO [312]. Some interest has been shown in IL_{17} as a potential alternative inflammatory cytokine to IFNγ in facilitating inflammatory responses in mice [314]. We are familiar with other species-specific differences, such as that there is no IL_8 in mice and the preferential role of GM-CSF over IL_3 in hematopoietic stem cell growth in humans. Therefore, we should not expect exact trans-species homology in the mechanism of effect rather look for the similar regulatory systems.

1.6.5.3 Effects of Interventions

With animal models, one can tell whether a reagent directly accesses the CNS. In MS, however, this is not possible and too often, the only thing we know for certain is that the drug or reagent accesses the periphery. The increased attack rate in the intravenous IFNγ study was suggested to reflect intra-CNS action of IFNγ because of the likelihood of a blood-brain barrier in these patients [286]. It is also possible that the cytokine and the anti-receptor antibody in a later study acted purely on the peripheral immune response. Administering IFNγ to mice or rats using the viral infection of

ependymal cells [289], or of cytokine or antibodies using the hypodermic route [297-298, 315], places IFNγ or antibodies directly in the CNS, and it is possible that the discrepant results between animals and MS patients reflect that difference in site of action. This may be due to aid in explaining the discrepancy between effects of TNF-α-targeting reagents in EAE versus in MS [316]. EAE was inhibited by anti-TNF-α but drugs that were based on similar principles did not ameliorate MS.

It would be crucial to know whether there was differential access to the CNS in MS patients versus in animals. The fact that some patients showed worsening symptoms is probably due to the differential effects of TNF-α acting through either of the two receptors for this cytokine, one of which signals for oligodendrocyte precursor cell survival [317]. The same drugs are effective against rheumatoid arthritis, but some patients exhibited neurological problems [318]. It would be difficult to explain how this may occur if the anti-TNF-α drugs did not access the CNS, although these questions remain unanswered.

1.6.6 Summary

As there is no specific pathology which defines MS, there is no single animal model for MS. Choice of animal species is critical and it may be important to maintain studies of less-popular models to maintain a broad perspective. The most commonly used animal model of EAE demonstrates differences in immune regulation and CNS pathology from that of MS. It is still a useful model for autoimmune inflammation (Table 1). Viral encephalomyelitis models exhibit some of the same immune-regulatory distinctions as EAE, possibly due to being focused on mice. Transgenic models permit the study of specific aspects of disease and its induction even though

they are inevitably mouse models. Toxin-induced demyelination has been useful in examining regenerative processes, with the obvious disclaimer that there is little evidence supporting a role for toxins in MS. Finally, one has to choose whatever model is important for the question one wishes to address. The greatest risk is to over-interpret results from any one model as being representative of what is a globally heterogeneous disease.

2.0 HYPOTHESIS

We hypothesize that the phase 2 enzyme inducers can improve EAE.

The phase 2 enzymes like glutathione peroxidase with the help of glutathione (GSH) can convert reactive oxygen intermediates like hydrogen peroxide into water and oxygen molecule. The oxidative stress has been attributed in the mechanism of damage in EAE and MS. [216, 233] Therefore, those chemicals that increase the phase 2 enzyme can benefit EAE through antioxidation. [225, 226]

2.1 EAE - Animal Model

At the turn of the century, EAE was identified as an "allergic" side effect seen in those individuals who had been administered the Pasteur rabies vaccine. This vaccine is comprised of the fixed rabies virus that had been grown in rabbit brain tissue. Acute disseminated encephalomyelitis [ADEM] which is a monophasic paralytic illness was seen in about 0.1% of vaccine recipients [166]. Perivascular mononuclear cell infiltrate and focal areas of demyelination in CNS tissue from these individuals was also observed. ADEM was not a result of rabies vaccine. It was due to CNS tissue that was used to develop rabies vaccine. In 1947, introducing adjuvants as well as homologous brain tissue led to the first reproducible induction of "allergic" encephalomyelitis in experimental animals [166]. Therefore, the murine EAE was identified as a model for demyelinating disease of CNS. To date, EAE has been successfully induced in many animal species, such as mice and rats.

3.0 MATERIAL AND METHOD

3.1 Experimental Model

24 Lewis rats between the ages of 10-12 weeks were purchased. The animals were housed at standard condition of a 12-hour light/dark cycle and fed in the animal facility. The animals were inoculated with 100 μg of Myelin Basic Protein (MBP) with Complete Freund's Adjuvant (CFA) at their tail base.

3.1.1 Experimental Technique

All the rats were inoculated with MBP + adjuvant (CFA) at the beginning of the experiment in a batch of 3 or 4 rats a day so that each one of them can be sacrificed exactly after the 28^{th} day of the inoculation.

The animals were separated into two groups of 12 each after inoculation. The 1st group was fed their normal diet chow (without added BHA) for all of the 28 days from the induction of EAE. The 2nd group consumed a diet chow containing a 7.5 g/kg concentration of BHA from induction of EAE until the 28^{th} day.

The animals underwent clinical evaluation (behavioral examination) by two people that were blinded to the experiment, in each animal group, twice daily for 28 days. The grading system were ranging from 0 (no overt symptoms) to 3 (hind limb paralysis). On the 29th day of post-induction of EAE, the animals were anesthetized with halothane inhalation. The blood samples in EDTA were collected by cardiac puncture. Then the animals were sacrificed by the perfusion of saline through the left cardiac ventricle. The animals were dissected to obtain the spinal cord and the brain. These tissues were fixed in 10% formaldehyde.

3.1.2 Clinical Scoring (Grades of Symptoms)

The tail, forelimb, and hind limb of the animals were examined for weakness as described in Pender [319] in a double blinded fashion. The clinical scoring is as follows:

0 No overt symptoms

0.5 Tail weakness only

1 Tail paralysis

1.5 Tail paralysis with some hind limb weakness

2 Hind limb weakness

3 Hind limb paralysis

All the animals were examined twice daily (12 hourly) and the grades were recorded from the day of induction until the 28th day.

3.1.3 Histological Studies

The multiple 10 mm sections of the formalin fixed spinal cord and brain were taken. The spinal cord samples were from cervical, thoracic and lumbosacral regions. These tissue samples were processed in an automated tissue processor for dehydration. The processed tissues (dehydrated) were embedded in paraffin blocks. Then the serial sections of the brain and spinal cords were made using microtome. These sections were taken on the slides that are coated with tissue fixative and rehydrated to stain with water soluble hematoxylin and eosin. The sections are first stained by hematoxylin for 4 minutes. Then 0.3% acid alcohol is used for differentiation to the point that after blueing up, the background is colorless. This is followed by staining with eosin for 2 minutes. Over staining by eosin is removed by washing in running water. Then the sections are dehydrated and cleared followed by mounting with

a cover slip.

The 10 sections of the spinal cord (of cervical, thoracic & lumbosacral segments) and the brain were examined for the presence or absence of inflammation. The presence of mononuclear infiltrate around the vessels and/or parenchymal mononuclear infiltration is considered as presence (positive) of inflammation. These findings were documented in both the groups of rats as positive and negative for inflammation.

3.1.4 RBC Glutathione (GSH) Estimation [320]

The whole blood samples of all the animals that were collected in an EDTA container and stored in 4º C are used for the estimation of RBC glutathione (GSH).

To prepare the samples for GSH estimation, the equal volumes of the whole blood and 30% Tri-chloro-acetic acid (TCA) containing 2mM of Ethylene diamine tetra-acetic acid (EDTA) are mixed in a test tube to deproteinize it. The final concentration of TCA becomes 15% which is essential to preserve the GSH. This mixture is centrifuged at 15000G for 5 minutes at 4 ºC. The acidic supernatant is removed in a separate test tube and stored at –80ºC until it is used for GSH estimation.

The stock solution of reagents is made by adding 0.5M potassium phosphate in 1mM of EDTA to make a buffer with pH 7. Then 2 mg of 1-Chloro-2,4-dinitrobenzene (CDNB) is mixed in 1 ml of ethanol and is placed in water bath at 37 ºC. Finally, the 500U of enzyme Glutathione-S-transferase (GST) is dissolved in 1 ml of 0.01 M of phosphate buffer (PBS).

The frozen acidic supernatant sample is thawed. The 25 μL of samples and the standards are placed in separately labeled microcuvettes. Then 825 μL of potassium phosphate is added in each microcuvettes followed by 10 μL of CDNB and GST each.

The CDNB is well shaken before adding it into microcuvettes. The GSH-CDNB conjugate is catalyzed by GST enzyme. The reaction takes 5 minutes to reach the end point. The absorbance of the mixture is measured by a spectrophotometer at 340 nm. The GSH is estimated by using the Beer and Lambert Equation; C = A/E. *(C = Concentration; A = Absorbance; E = Extinction coefficient, which is 9,600 M^{-1} cm^{-1})*

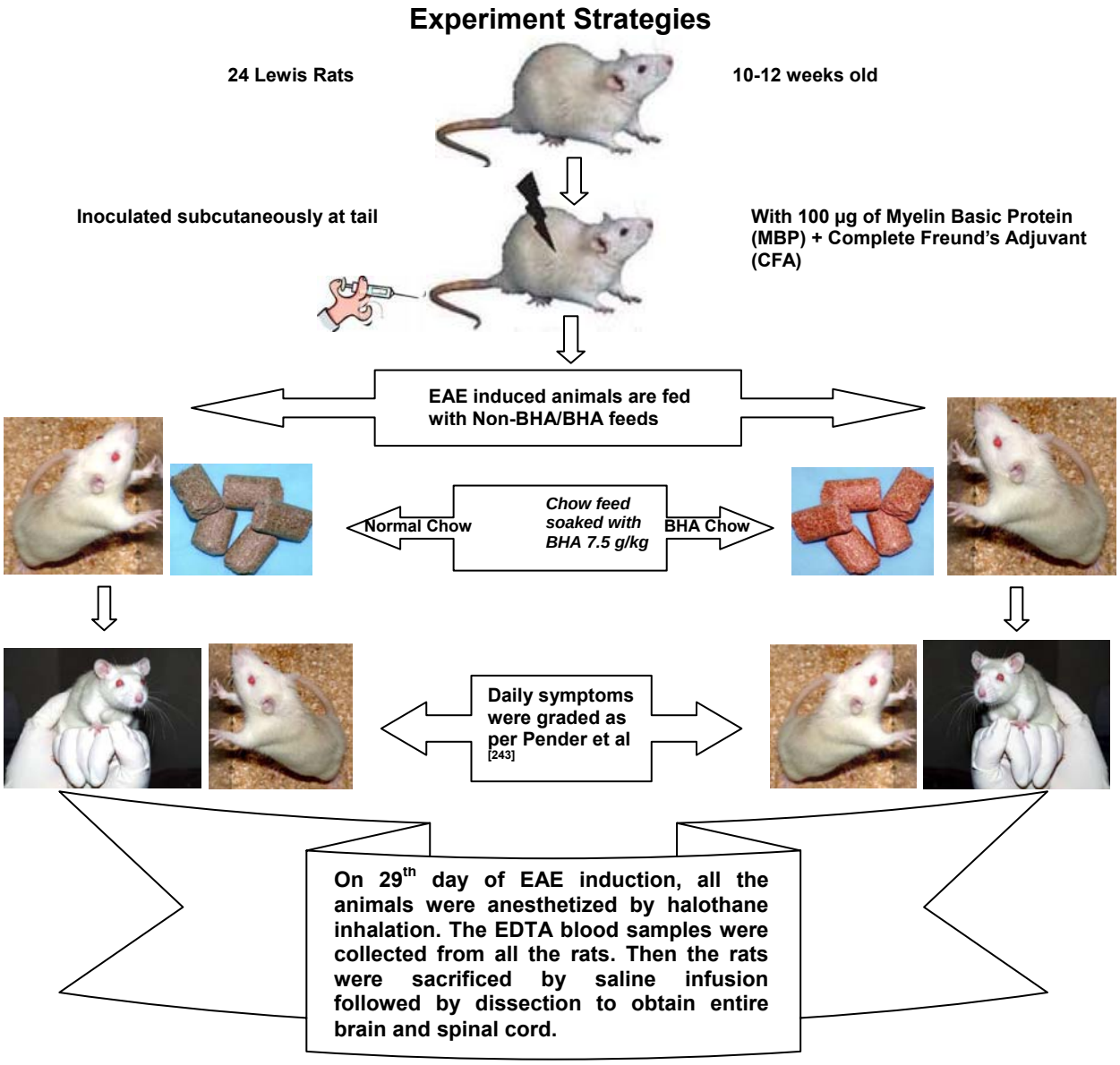


Fig. 3.1.. : Experiment Strategy

4.0 RESULTS

4.1 Clinical Scoring

The following tables show the clinical scoring of non-BHA and BHA groups.

Table 4.1.1: non-BHA fed EAE – Grades of symptoms

EAE IN LEWIS RATS non-BHA fed - Grades of Symptoms												
Dates / Rats	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**
Feb. 22, '06	0-In	0-In	0-In	0	0	0	0	0	0	0	0	0
Feb. 23, '06	0	0	0	0-In	0-In	0-In	0-In	0	0	0	0	0
Feb. 24, '06	0	0	0	0	0	0	0	0-In	0-In	0-In	0-In	0
Feb. 25, '06	0	0	0	0	0	0	0	0	0	0	0	0-In
Feb. 26, '06	0	0	0	0	0	0	0	0	0	0	0	0
Feb. 27, '06	0	0	0	0	0	0	0	0	0	0	0	0
Feb. 28, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 1, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 2, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 3, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 4, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 5, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 6, '06	0.5	0	0.5	0.5	0	0	0	0.5	0.5	0.5	0	0
Mar. 7, '06	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5
Mar. 8, '06	1	0.5	0.5	1	0.5	1	0.5	1	1	1	0.5	0.5
Mar. 9, '06	1	0.5	1	1	1	1	1	1	1	1	0.5	1
Mar. 10, '06	1	1	1	3	1	2	1	2	1	2	1	1
Mar. 11, '06	2	1	1	2	1	3	1	3	2	3	1	1
Mar. 12, '06	1	1	2	1.5	2	3	1.5	3	1	3	1.5	2
Mar. 13, '06	1	1	1	1.5	1	3	2	3	1	3	2	1.5
Mar. 14, '06	1	2	1	1	1	2	2	2	1	3	1.5	1.5
Mar. 15, '06	1	1	1	1	1	1	1	1	1	2	1.5	1
Mar. 16, '06	1	1	1	1	1	1	1	1	1	1	1	1
Mar. 17, '06	1	1	1	1	1	1	1	1	1	1	1	1
Mar. 18, '06	1	1	1	1	1	1	1	1	1	1	1	0.5
Mar. 19, '06	0.5	0.5	1	0.5	1	1	0	1	1	1	0.5	0.5
Mar. 20, '06	0	0.5	0	0.5	1	1	0	0.5	1	1	0.5	0.5
Mar. 21, '06	0	0	0	0	0	0.5	0	0	1	1	0	0
Mar. 22, '06	S	S	S	0	0	0	0	0	0	0	0	0
Mar. 23, '06				S	S	S	S	0	0	0	0	0
Mar. 24, '06								S	S	S	S	0
Mar. 25, '06												S

Table 4.1.2: BHA fed EAE – Grades of symptoms

EAE IN LEWIS RATS BHA fed - Grades of Symptoms												
Dates / Rats	**H13**	**H14**	**H15**	**H16**	**H17**	**H18**	**H19**	**H20**	**H21**	**H22**	**H23**	**H24**
Feb. 22, '06	0	0	0	0	0	0	0	0	0	0	0	0
Feb. 23, '06	0	0	0	0	0	0	0	0	0	0	0	0
Feb. 24, '06	0	0	0	0	0	0	0	0	0	0	0	0
Feb. 25, '06	0-In	0-In	0-In	0	0	0	0	0	0	0	0	0
Feb. 26, '06	0	0	0	0-In	0-In	0-In	0-In	0	0	0	0	0
Feb. 27, '06	0	0	0	0	0	0	0	0-In	0-In	0-In	0	0
Feb. 28, '06	0	0	0	0	0	0	0	0	0	0	0-In	0-In
Mar. 1, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 2, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 3, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 4, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 5, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 6, '06	0	0	0	0	0	0	0	0	0	0	0	0
Mar. 7, '06	0.5	0	0	0	0	0	0	0	0	0	0	0
Mar. 8, '06	0.5	0.5	0.5	0.5	0.5	0	0.5	0	0.5	0.5	0	0.5
Mar. 9, '06	1	1	1	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mar. 10, '06	1	1	1	1	1	1	1	0.5	0.5	1	1	1
Mar. 11, '06	2	3	3	3	1	1	1	0.5	1	1	1	1
Mar. 12, '06	2	3	2	2	1.5	1	1	1	1	1	1	3
Mar. 13, '06	3	3	3	3	3	2	2	1	1	3	3	3
Mar. 14, '06	3	2	3	2	3	2	2	1	1	3	3	2
Mar. 15, '06	2	1.5	2	1.5	2	1.5	1.5	1	1	2	2	1.5
Mar. 16, '06	1	1.5	1.5	1.5	1	1.5	1.5	1	1	1.5	1.5	1.5
Mar. 17, '06	1	1	1	1	1	1	1	1	1	1	1	1
Mar. 18, '06	1	1	1	1	1	1	1	1	1	1	1	1
Mar. 19, '06	1	1	1	1	1	1	1	1	1	1	1	1
Mar. 20, '06	0.5	1	1	1	1	1	1	0.5	1	1	1	1
Mar. 21, '06	0.5	0.5	1	0.5	1	1	0.5	0.5	0.5	1	1	1
Mar. 22, '06	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5	1
Mar. 23, '06	0	0	0	0	0	0.5	0	0	0.5	0.5	0.5	1
Mar. 24, '06	0	0	0	0	0	0	0	0	0.5	0.5	0	0.5
Mar. 25, '06	S	S	S	0	0	0	0	0	0	0	0	0.5
Mar. 26, '06				S	S	S	S	0	0	0	0	0
Mar. 27, '06								S	S	S	0	0
Mar. 28, '06											S	S

Legends	
	In - Induction of EAE by MBP + adjuvant
	S - Rats were sacrificed by perfusing saline under halothane

Table 4.1.3: BHA and non-BHA fed EAE – Clinical symptoms

Clinical Scoring in non-BHA & BHA fed Animals			
Symptoms ⇨ / Chow fed ⇩	**Tail paralysis**	**Hind limb weakness**	**Hind limb paralysis**
non-BHA	0	8	4
BHA	2	2	8

4.1.1 Statistical Analysis – Mean of Grades of Symptoms in Rats

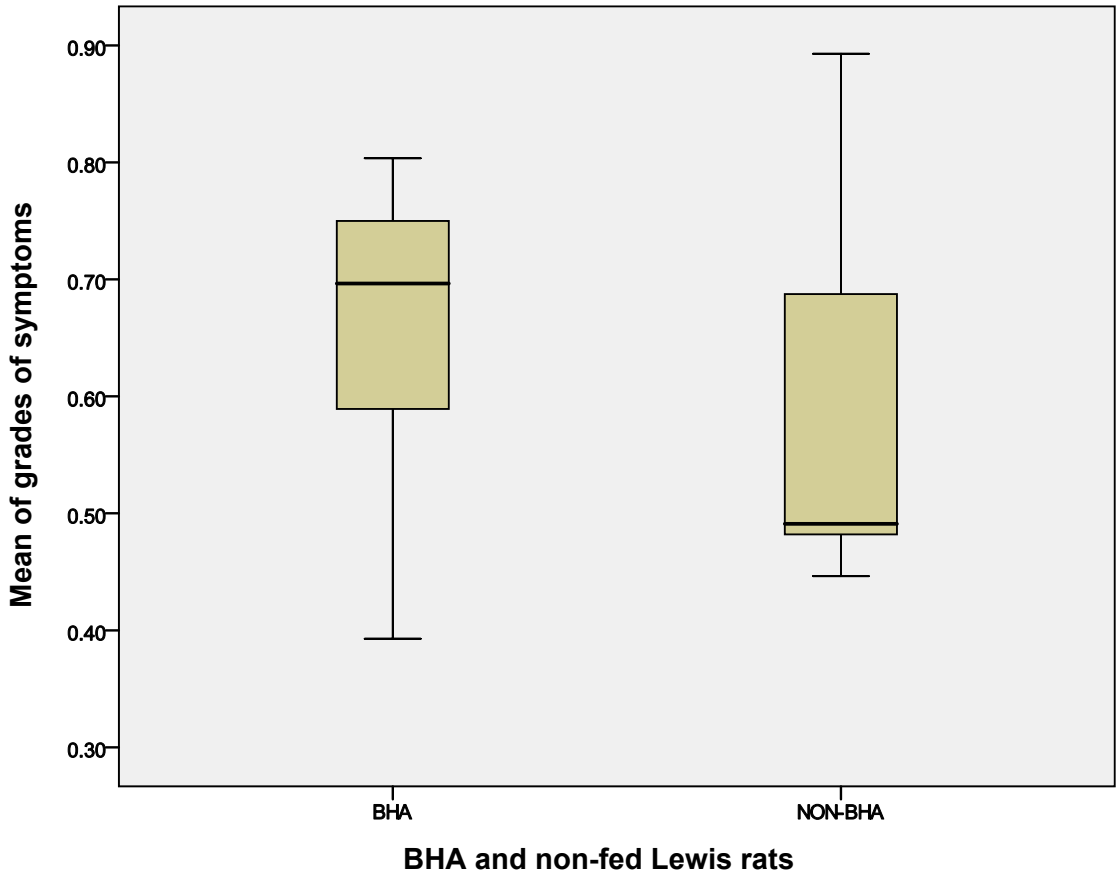


Fig. 4.1: The mean of grades of symptoms in BHA and non-BHA fed Lewis rats, 12 animals in each group. The dark black lines in box plot indicate the mean in each group. The x-axis indicates the two groups of animals; BHA and non-BHA fed rat. The y-axis is mean of grades of symptoms of each animal. The mean in BHA fed animals is 0.6622 and that of non-BHA is 0.5818. The range of grades of symptoms in BHA is lower than that of non-BHA. The independent t-test shows that there is no statistically significant difference between these two groups because the *p* value is 0.170, which is more than 0.05

4.1.2 Comparative Graphs of Grades of Symptoms of Each Rat (H1 to H24)

The grades of symptoms are compared in a pair of two animals in each figure below. The 24 animals are labeled as H1, H2…H24. The H1 to H12 are non-BHA group of Lewis rats and H13 to H24 are BHA fed group of Lewis rats. Statistically there is no significant difference between the groups as the *p* value is 0.17 which is more than 0.05.

Each Lewis rat is inoculated to induce EAE on a particular date then examined daily for grades of symptoms until 28 days later. Each animal is sacrificed on the following day.

The x – axis is the dates starting from inoculation for induction of EAE in each Lewis rat until 28 days.

The y – axis (values) are grades of symptoms.

The comparative analysis of fig. 4.2(a) to fig. 4.2(l) is given at the end of the graphs.

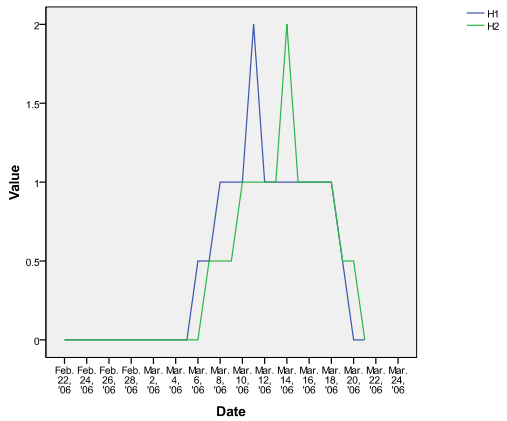


Fig. 4.2(a)

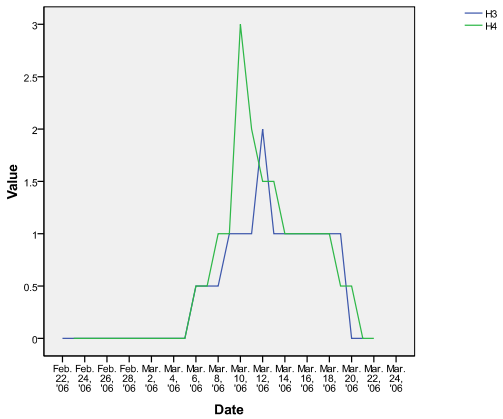


Fig. 4.2(b)

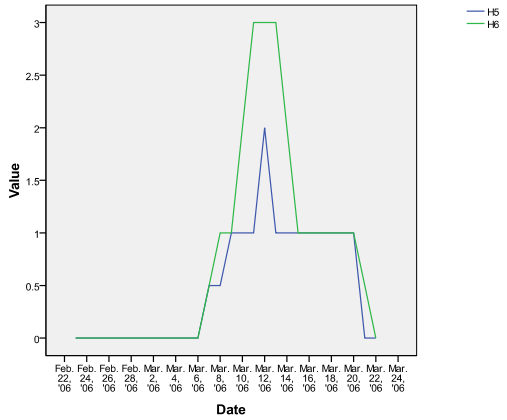


Fig. 4.2(c)

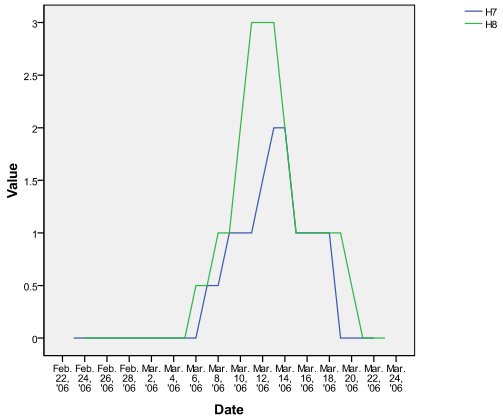


Fig. 4.2(d)

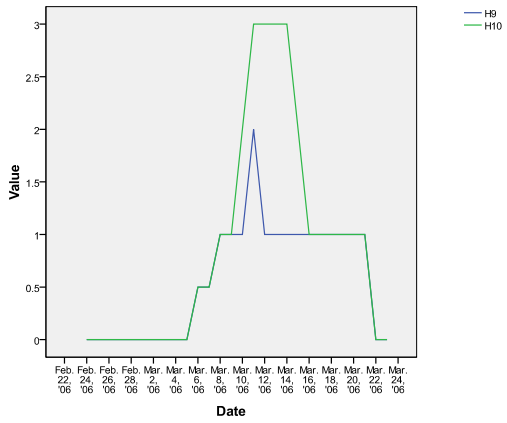


Fig. 4.2(e)

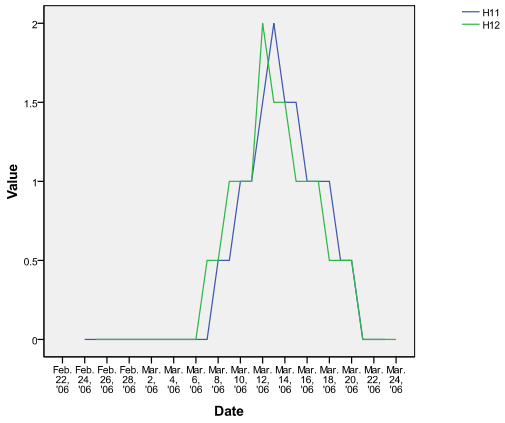


Fig. 4.2(f)

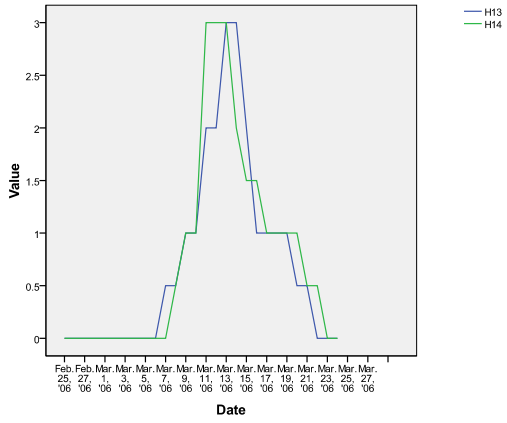


Fig. 4.2(g)

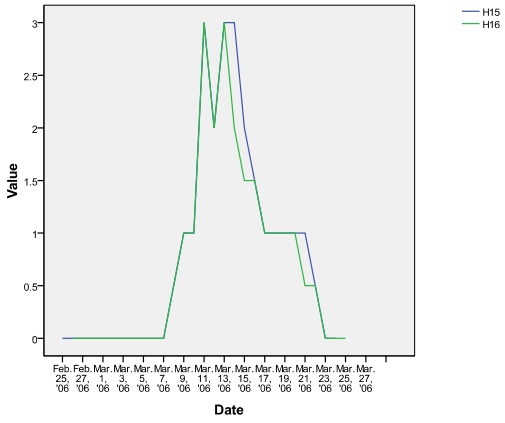


Fig. 4.2(h)

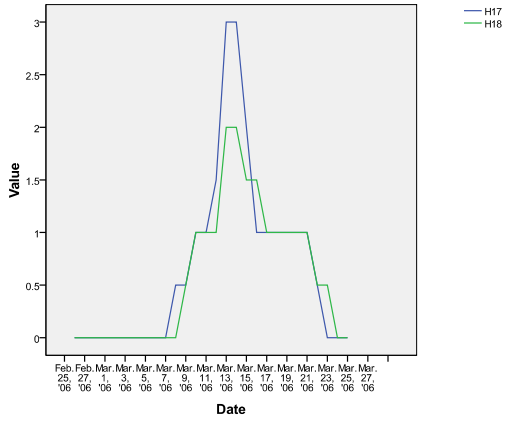


Fig. 4.2(i)

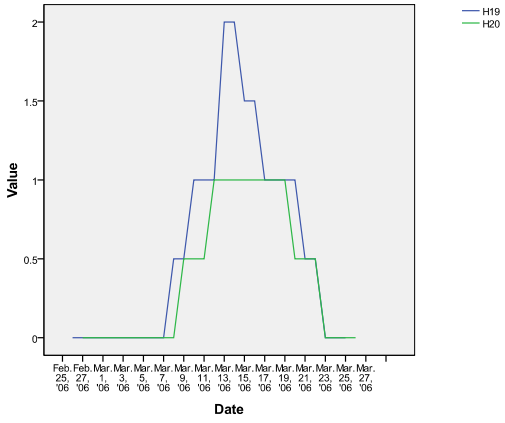


Fig. 4.2(j)

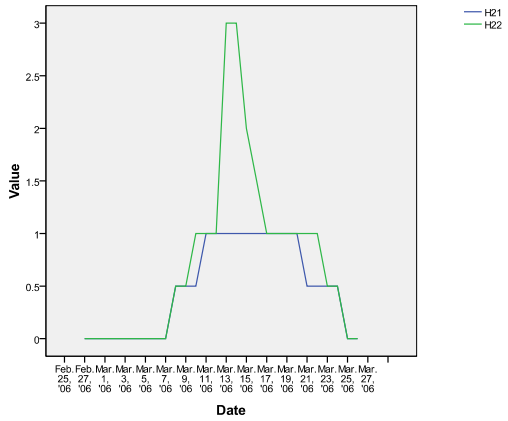


Fig. 4.2(k)

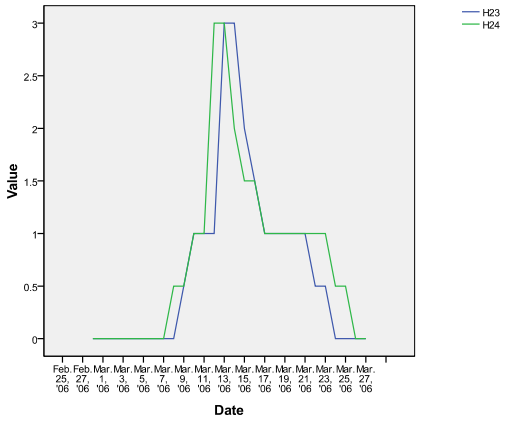


Fig. 4.2(l)

Non-BHA: **Acute attack**

No symptoms – 10 to 13 days
Tail weakness – 2 to 3 days
Tail paralysis – 2 to 4 days
Tail paralysis with some hind limb weakness – 1 day *(2 cases)*
Hind limb weakness – 1 day *(11 cases)*
Hind limb paralysis – 1 to 4 days *(4 cases)*

Remission

Hind limb weakness – 1 day *(4 cases)*
Tail paralysis with some hind limb weakness – 2 days *(3 cases)*
Tail paralysis – 3 to 10 days
Tail weakness – 1 to 3 days *(7 cases)*
No symptoms – 1 to 4 days

BHA: **Acute attack**

No symptoms – 8 to 11 days
Tail weakness – 1 to 2 days
Tail paralysis – 2 to 3 days *(except 2 cases, that had only this symptoms for 8 and 10 days each before remission as tail weakness started)*
Tail paralysis with some hind limb weakness – 1 day *(1 case)*
Hind limb weakness – 2 days *(3 cases)*
Hind limb paralysis – 1 to 3 days *(8 cases; 7 cases had no hind limb weakness)*

Remission

Hind limb weakness – 1 day *(8 out of 10 cases)*
Tail paralysis with some hind limb weakness – 1 to 2 days *(8 out of 10 cases)*
Tail paralysis – 4 to 7 days *(8 out of 10 cases)*
Tail weakness – 1 to 4 days
No symptoms – 2 to 4 days

There are variations in symptoms of acute attacks and remission in non-BHA and BHA groups. However these differences are statistically insignificant as the *p* value is 0.17.

4.2 Histology Screening

The light microscopy examination of the serial sections of the spinal cords from cervical, thoracic, lumbosacral regions including sections of cerebrum, cerebellum, and brain stem were undertaken. The predominant histological findings were mononuclear cell infiltration composed of lymphocytes, macrophages, and microglia. These infiltrates were arranged generally like perivascular cuffing (around the blood vessels) with few focal collections and sparsely scattered infiltration. Group-1 (non-BHA fed) showed the typical histological findings in all except in three (H6, H9, and H11) where there has been mainly focal collections and scattered mononuclear infiltrations with mild perivascular cuffing. In group-2 (BHA fed), there have been no findings that suggest any inflammatory response. The results are presented in the following table 4.2.1

Table 4.2.1: Histology changes in non-BHA fed and BHA-fed EAE animals

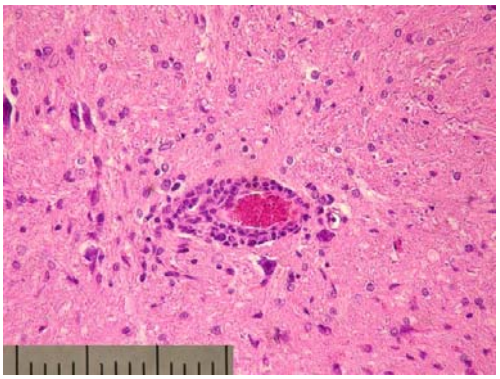
EAE IN LEWIS RATS **non-BHA fed - Histology - I**												
Rats	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**
Histology	P	P	P	P	P	MP	P	P	MP	P	MP	P

EAE IN LEWIS RATS **BHA fed - Histology - II**												
Rats	**H13**	**H14**	**H15**	**H16**	**H17**	**H18**	**H19**	**H20**	**H21**	**H22**	**H23**	**H24**
Histology	N	N	N	N	N	N	N	N	N	N	N	N

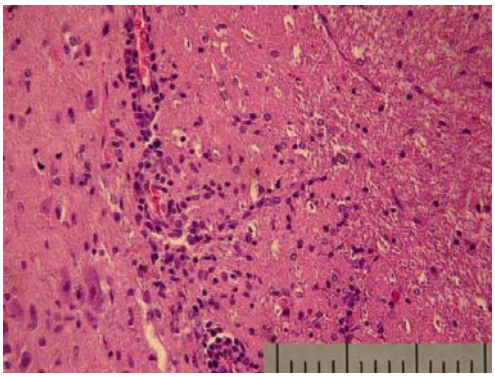
Legends	
	P - *Perivascular infiltration by mononuclear cells*
	N - *No perivascular infiltration by mononuclear cells*
	MP - *Mild perivascular infiltration by mononuclear cells*

The histology of the sections of the spinal cord and the brain were consistent with the pathological changes of perivascular mononuclear cuffing with focal collections and scattered mononuclear infiltrates. As anticipated, not all the sections of the brain and/or the spinal cord of non-BHA revealed inflammation. There have also been sections that show perivascular cuffing and normal blood vessels indicating the patchy nature of inflammation in the brain and the spinal cord in acute EAE. This acute EAE leads to relapsing remitting EAE or chronic EAE where the patchy nature of pathology turns to extensive damage manifested by an increasing degree of neurological deficit. The histology slides of non-BHA and BHA fed animal are presented below in fig. 4.3 (non-BHA) and 4.4 (BHA).

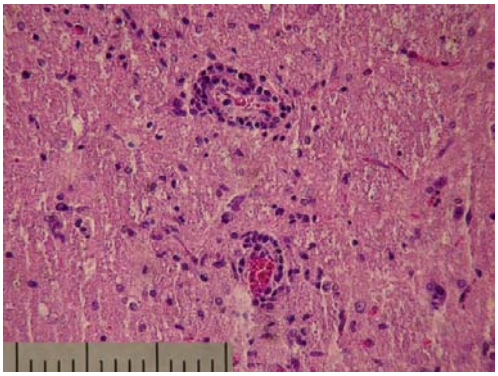
Fig. 4.3: Histology of non-BHA fed animals (group 1); H1 to H12
SC = Spinal cord and Br = Brain



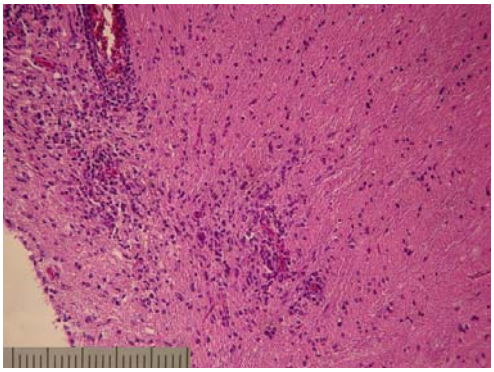
H1- SC – perivascular infiltrate



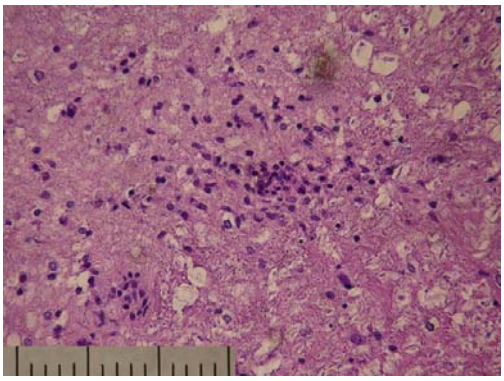
H1- SC – perivascular and scattered parenchymal infiltrate



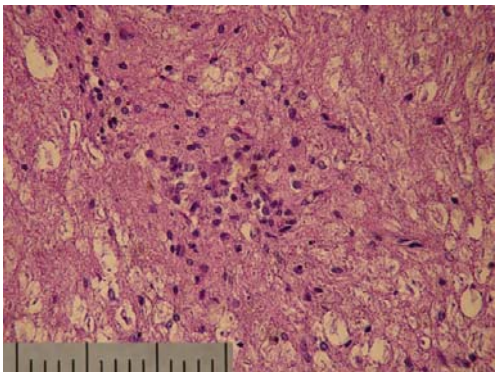
H1- Br – mild perivascular infiltrate



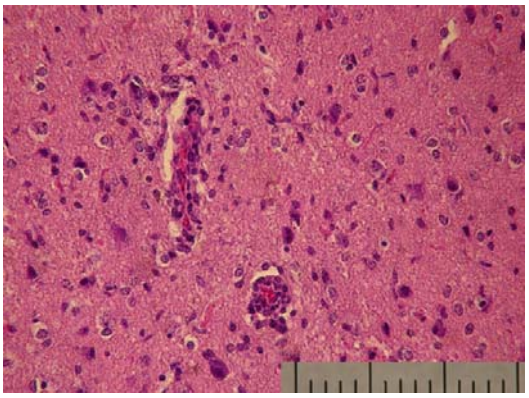
H1- Br – perivascular infiltrate



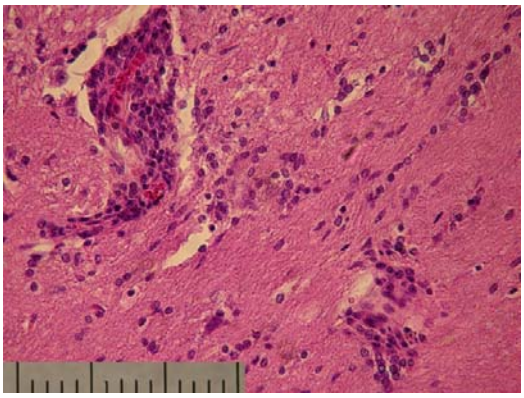
H2- SC – mild perivascular infiltrate



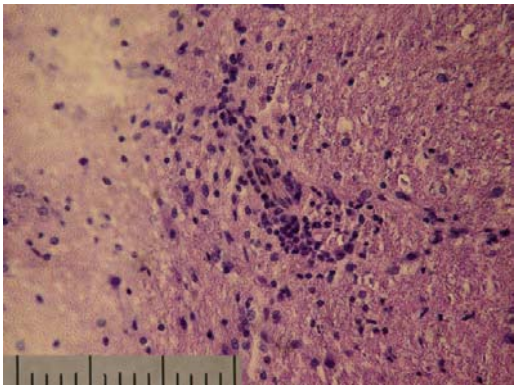
H2- SC – mild perivascular and scattered parenchymal infiltrate



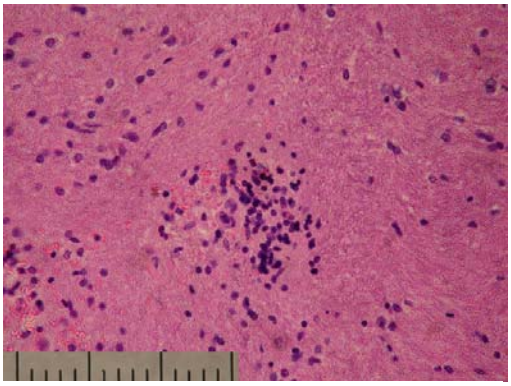
H2- Br – mild perivascular infiltrate



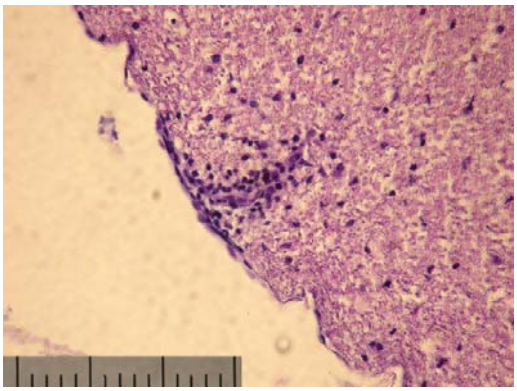
H2- Br – perivascular infiltrate



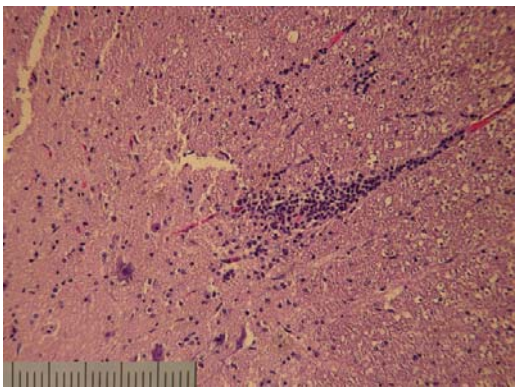
H3- SC – perivascular and scattered parenchymal infiltrate



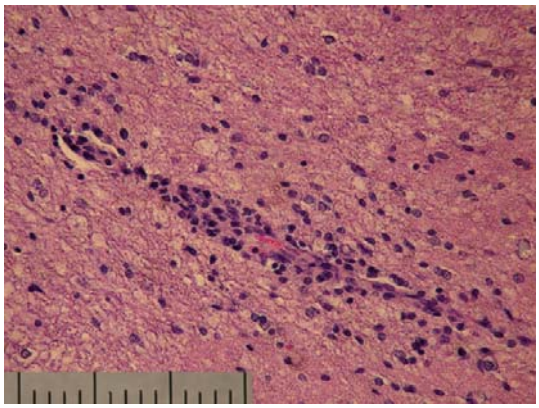
H3- Br – parencymal infiltrate



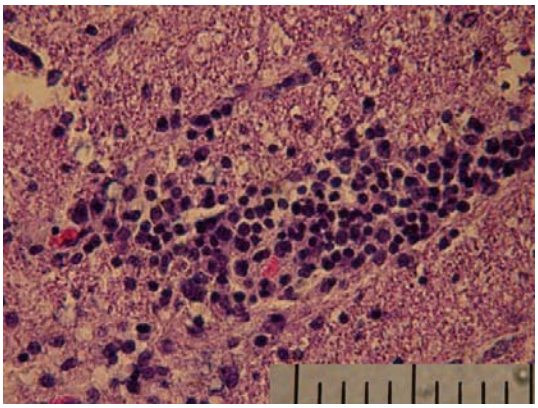
H4- SC – perivascular and subpial infiltrate



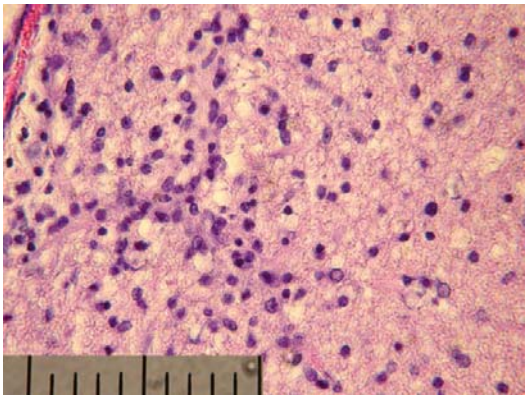
H4- SC – perivascular and scattered parenchymal infiltrate



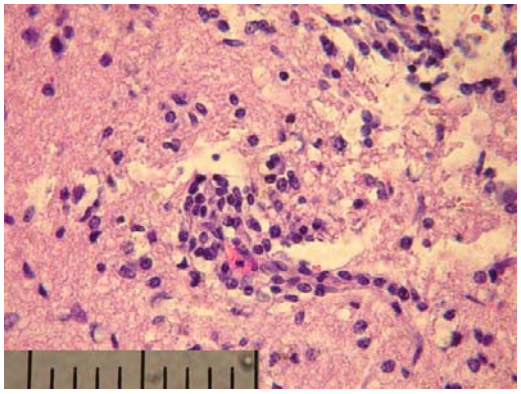
H4- Br – perivascular and scattered parenchymal infiltrate



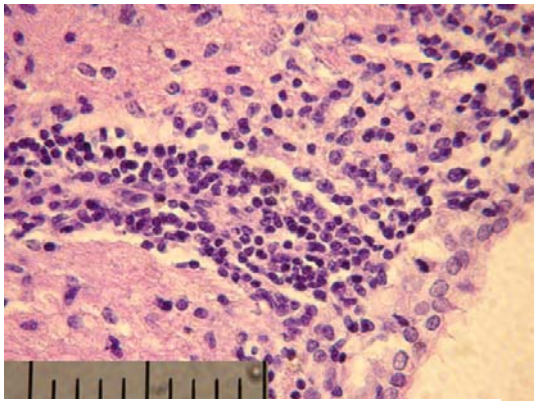
H4- Br – perivascular infiltrate



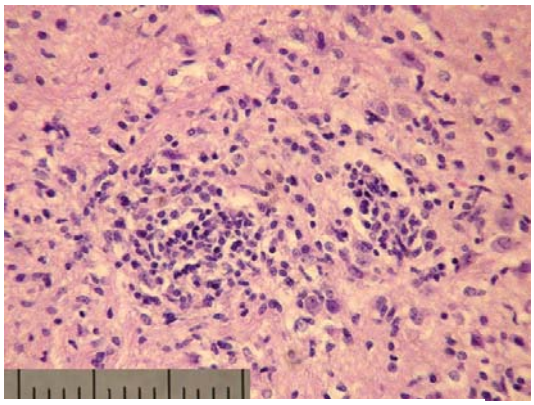
H5- SC – mild perivascular and scattered parenchymal infiltrate



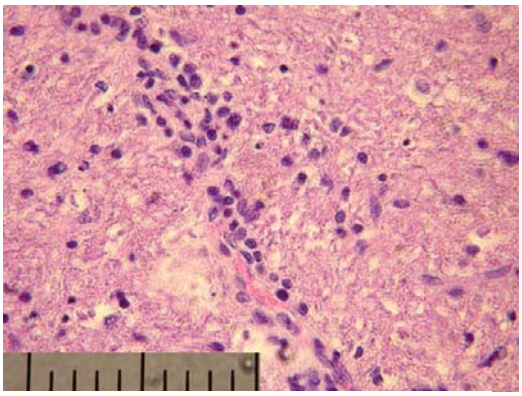
H5- SC – mild perivascular infiltrate



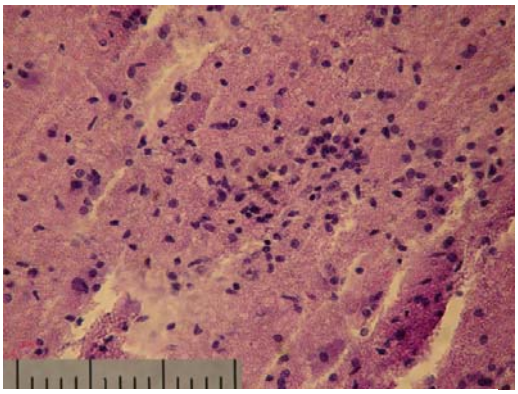
H5- Br – perivascular and parenchymal infiltrate



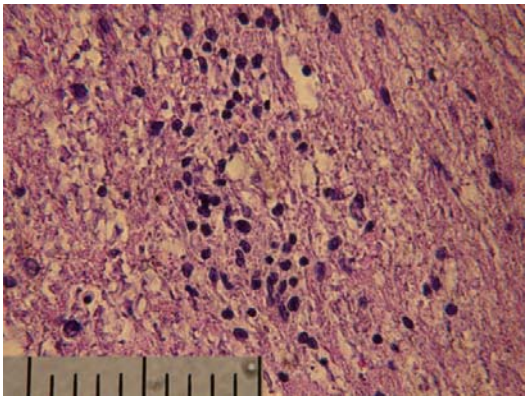
H5- Br – perivascular and scattered parenchymal infiltrate



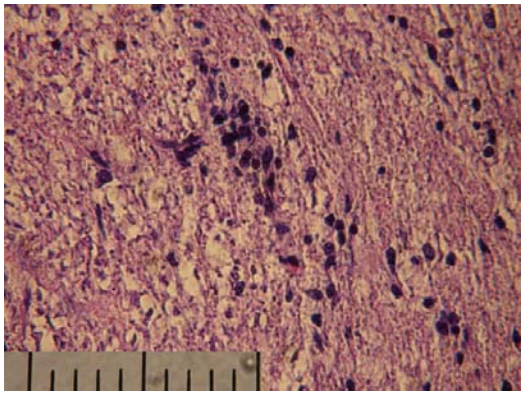
H6- SC – mild perivascular infiltrate



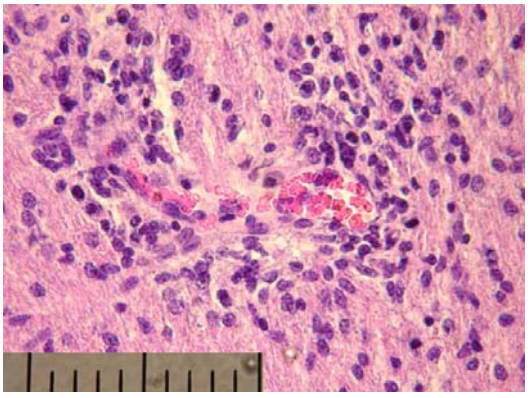
H6- Br – parenchymal infiltrate



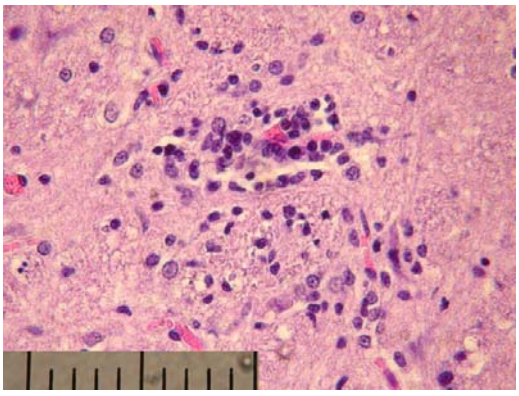
H7- SC – mild parenchymal infiltrate



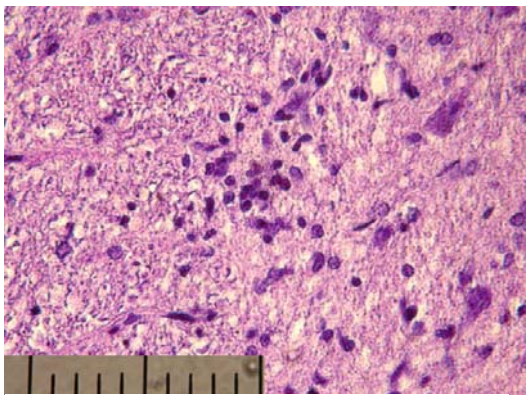
H7- SC – mild parivascular infiltrate



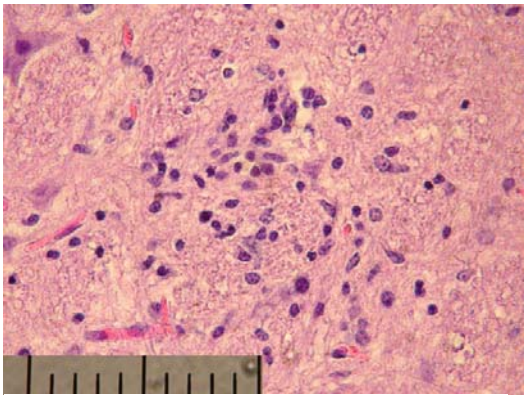
H7- Br – perivascular infiltrate



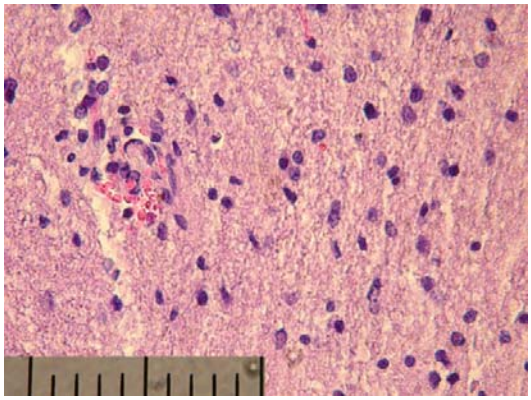
H7- Br – perivascular infiltrate



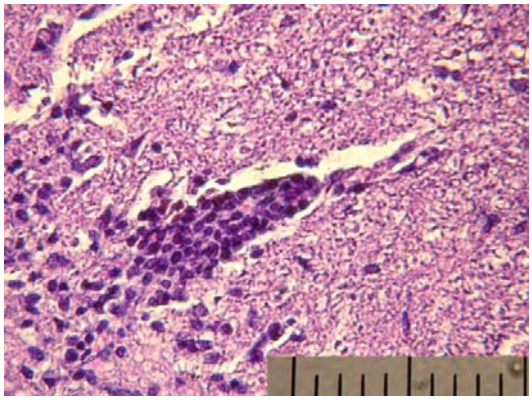
H8- SC – mild parenchymal infiltrate



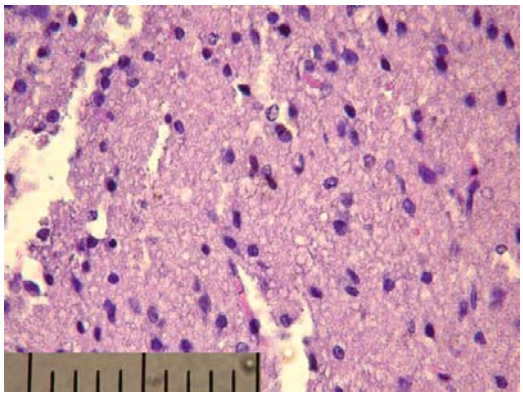
H8- SC – mild parenchymal infiltrate



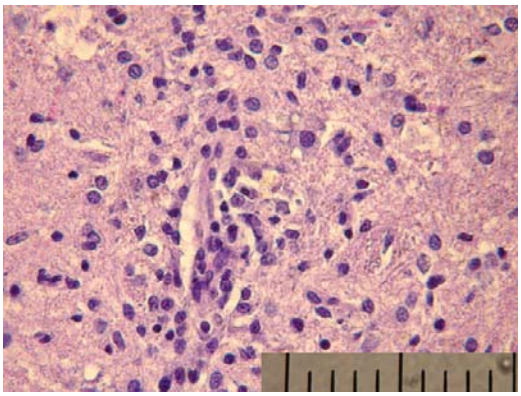
H8- Br – mild perivascular infiltrate



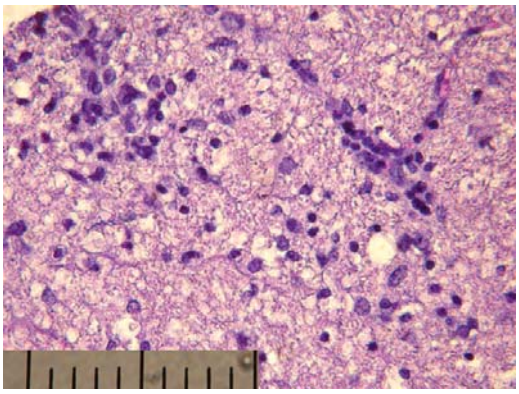
H8- Br – perivascular infiltrate



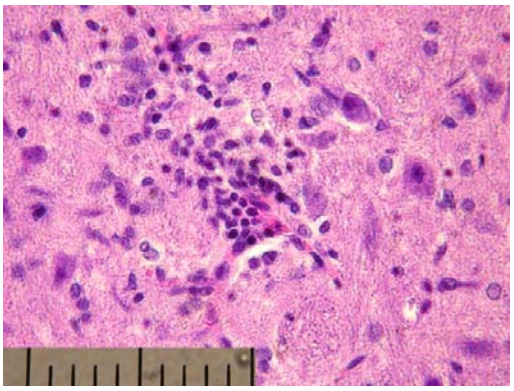
H9- SC – mild parenchymal infiltrate



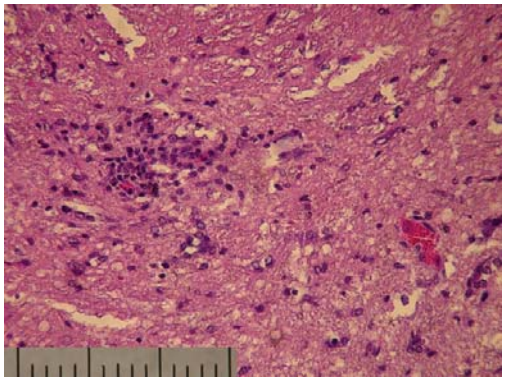
H9- Br – perivascular infiltrate



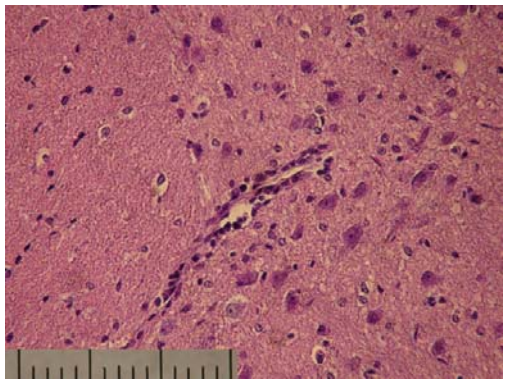
H10- SC – mild perivascular infiltrate



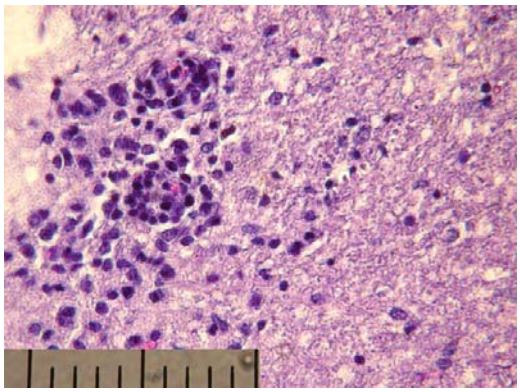
H10- Br – perivascular infiltrate



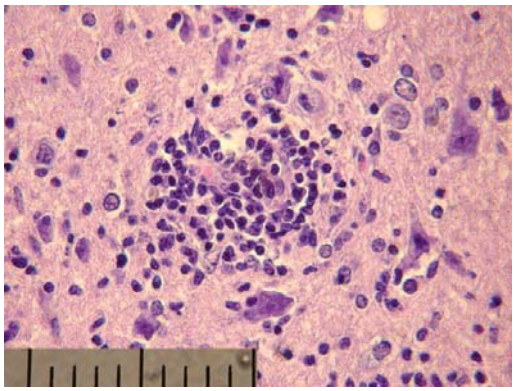
H11- SC – perivascular infiltrate



H11- Br – mild perivascular infiltrate

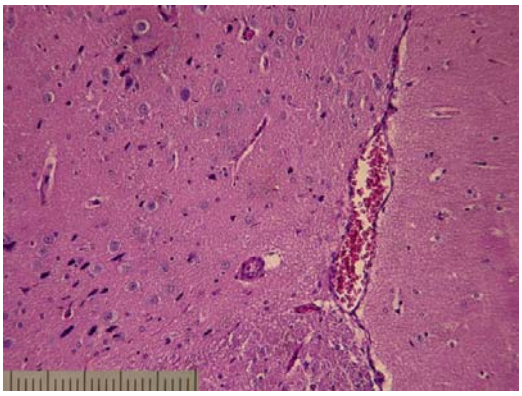


H12- SC – perivascular infiltrate

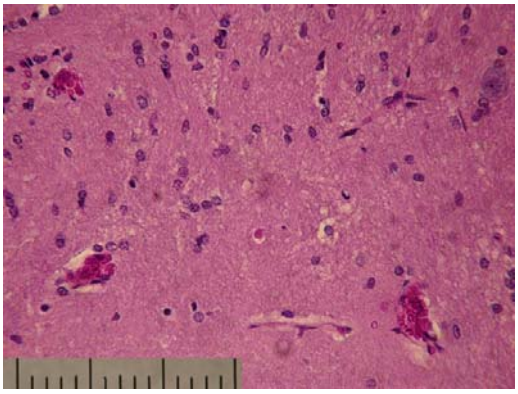


H12- Br – perivascular infiltrate

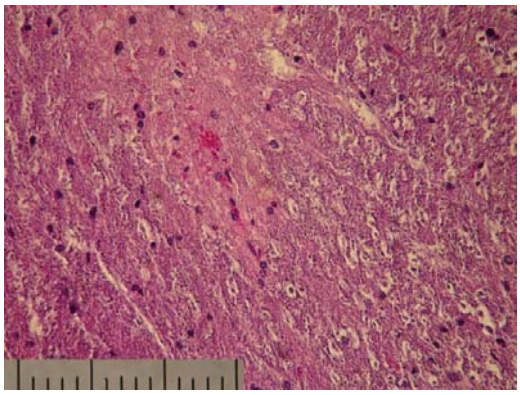
Fig. 4.4: Histology of BHA fed animals (group 2); H13 to H24
SC = Spinal cord and Br = Brain



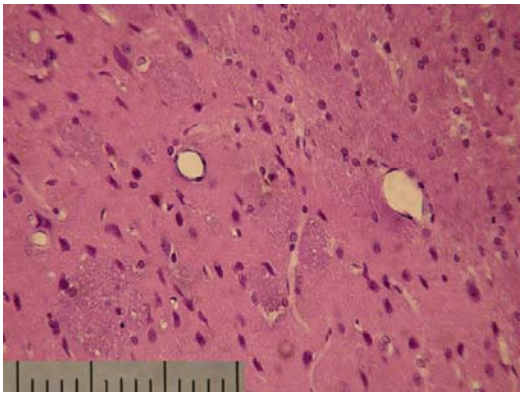
H13- SC – no perivascular infiltrate



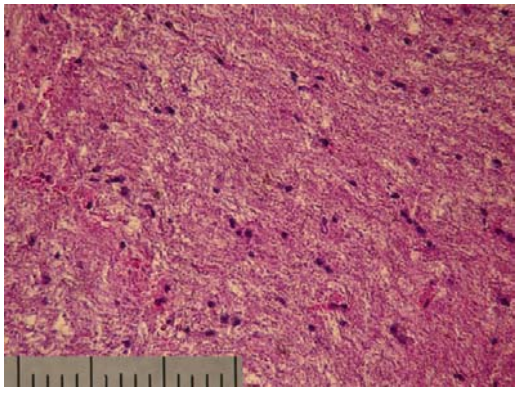
H13- Br – no perivascular infiltrate



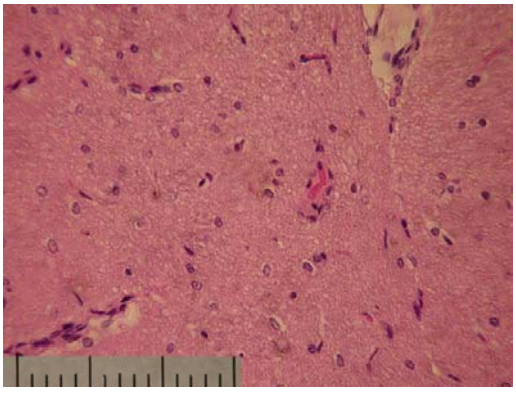
H14- SC – no perivascular infiltrate



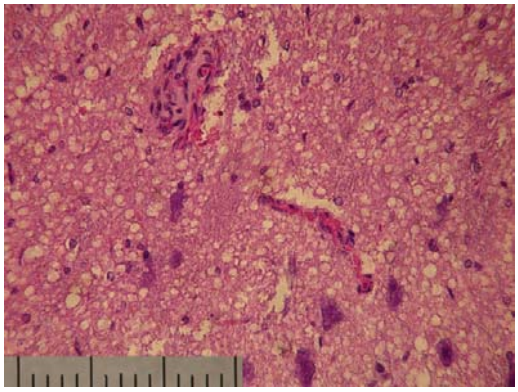
H14- Br – no perivascular infiltrate



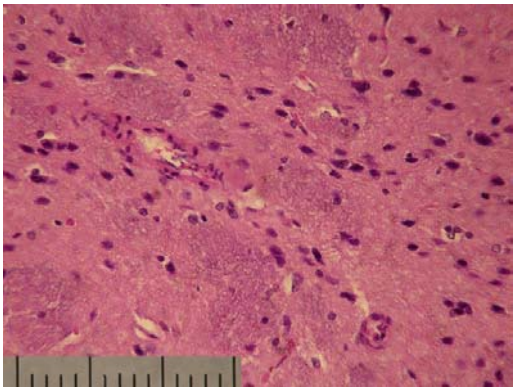
H15- SC – no perivascular infiltrate



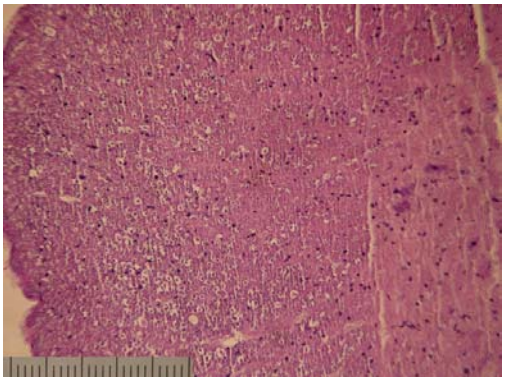
H15- Br – no perivascular infiltrate



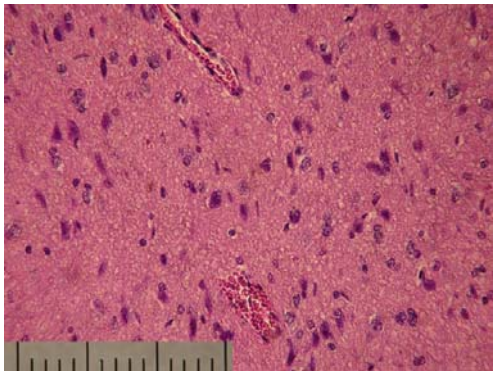
H16- SC – no perivascular infiltrate



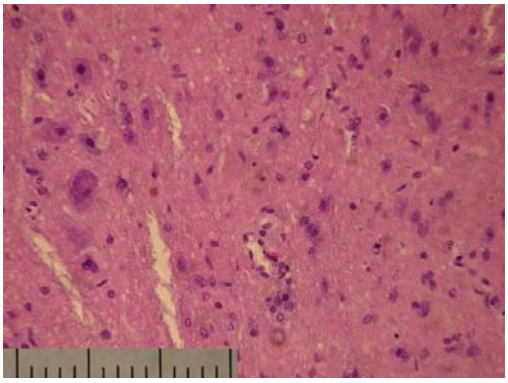
H16- Br – no perivascular infiltrate



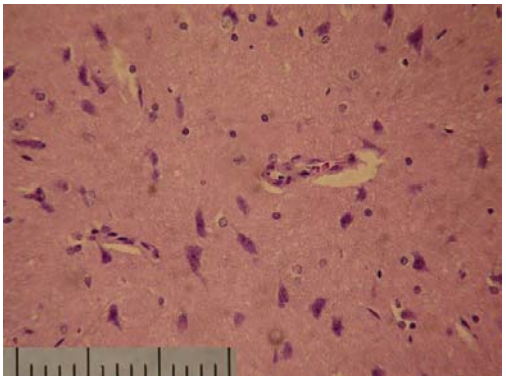
H17- SC – no perivascular infiltrate



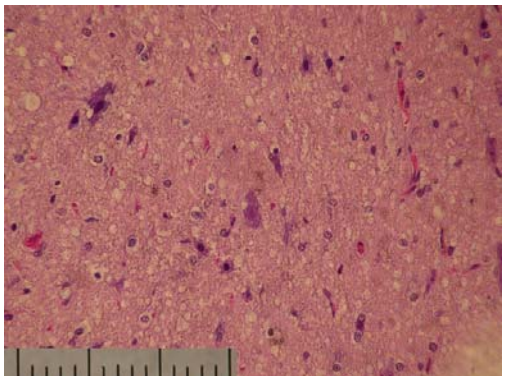
H17- Br – no perivascular infiltrate



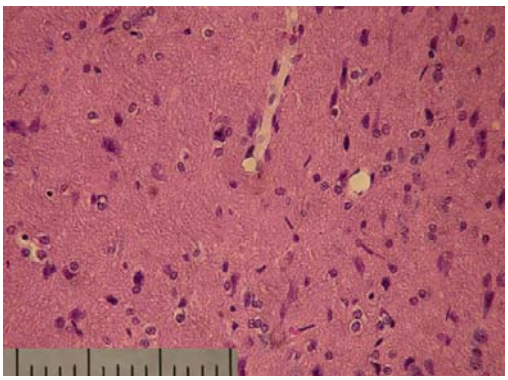
H18- SC – no perivascular infiltrate



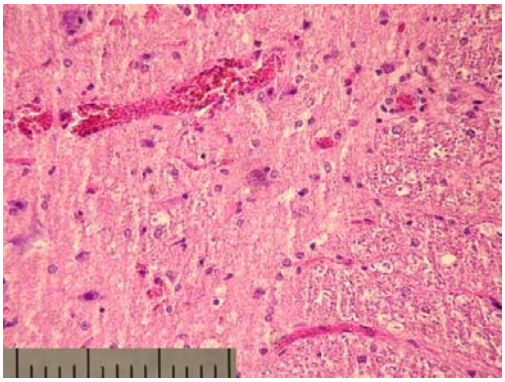
H18- Br – no perivascular infiltrate



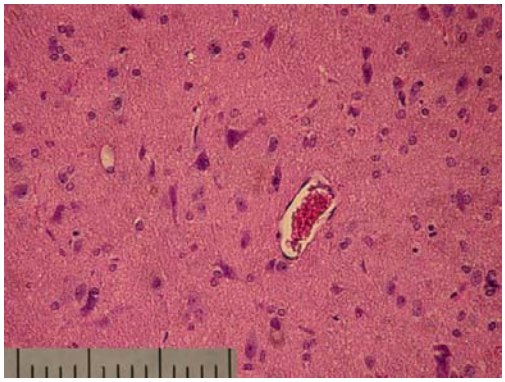
H19- SC – no perivascular infiltrate



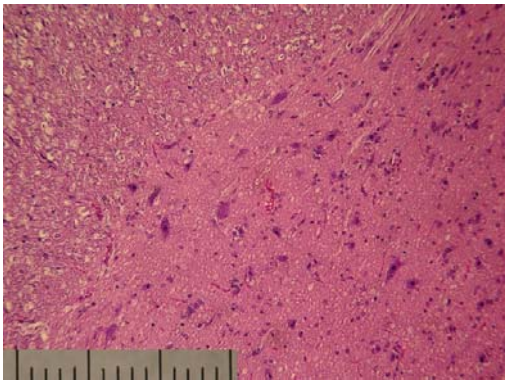
H19- Br – no perivascular infiltrate



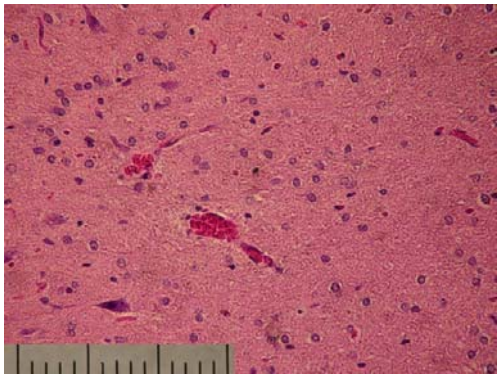
H20- SC – no perivascular infiltrate



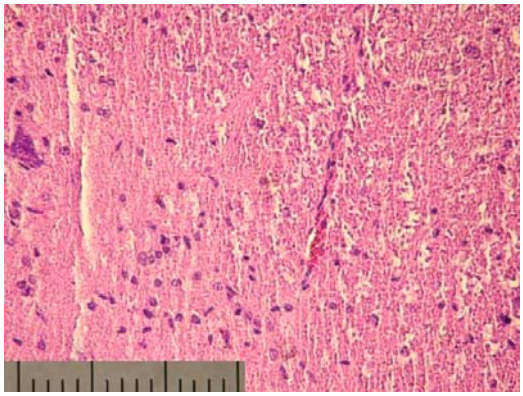
H20- Br – no perivascular infiltrate



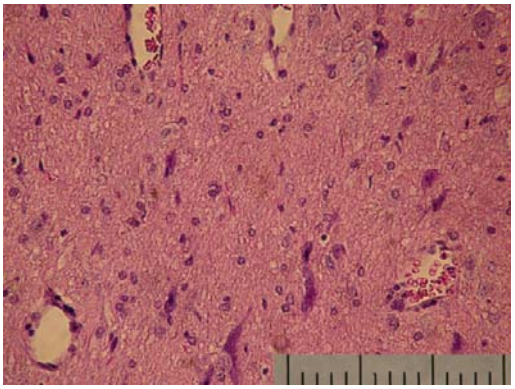
H21- SC – no perivascular infiltrate



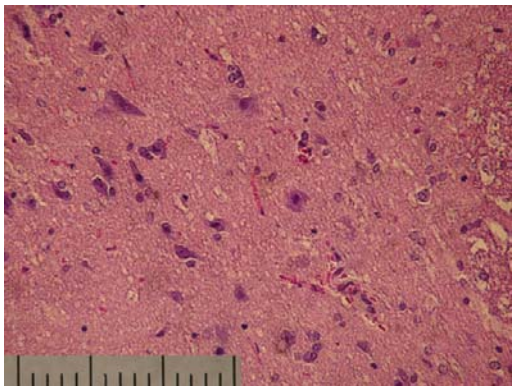
H21- Br – no perivascular infiltrate



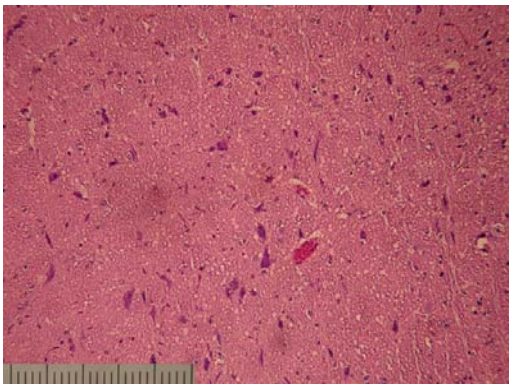
H22- SC – no perivascular infiltrate



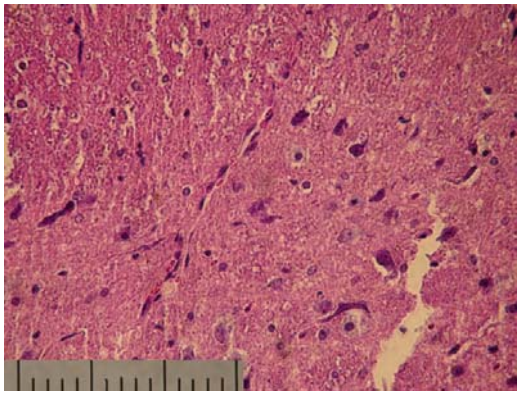
H22- Br – no perivascular infiltrate



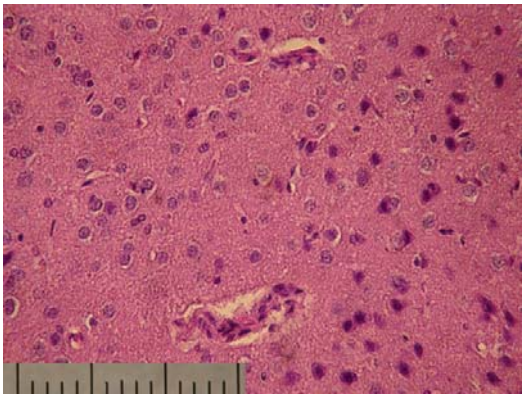
H23- SC – no perivascular infiltrate



H23- Br – no perivascular infiltrate



H24- SC – no perivascular infiltrate



H24- Br – no perivascular infiltrate

4.3 RBC Glutathione (GSH) Estimation

Table 4.3: Levels of Glutathione in non-BHA and BHA fed EAE animals

EAE IN LEWIS RATS non-BHA fed - Glutathione - I												
Rats	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**
Glutathione	11.5	13	12	12	13	14	12.5	11	13.5	14.5	13.5	13

EAE IN LEWIS RATS BHA fed - Glutathione - II												
Rats	**H13**	**H14**	**H15**	**H16**	**H17**	**H18**	**H19**	**H20**	**H21**	**H22**	**H23**	**H24**
Glutathione	24.5	27	28.5	26	26	30	27.5	29	23.5	29.5	25	26

4.3.1 Statistical Analysis – GSH levels in BHA and non-BHA fed rats

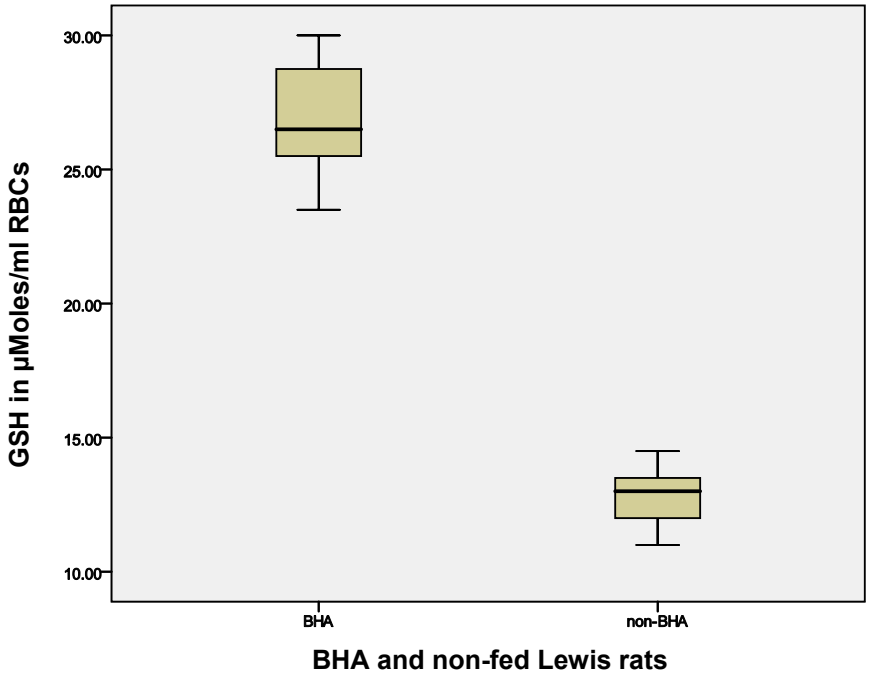


Fig. 4.5: The GSH levels have been shown in BHA fed and non-BHA fed Lewis rats, 12 animals in each group. The x-axis corresponds to two groups of animal studied; BHA and non-BHA fed rats. The y–axis is the level of GSH in each animal. The dark black lines in box plot indicate the mean in each group. The mean in BHA fed animals is 26.88 µMoles/ml RBCs and that of non-BHA is 12.79 µMoles/ml RBCs. The range of GSH levels in non-BHA is 11 to 14 and that of BHA is 23.5 to 30. The independent t-test shows that there is statistically significant difference between these two groups because the *p* value is 0.000 which is less than 0.05.

5.0 DISCUSSION

The behavioral study for clinical scoring has been performed as stated earlier in a double-blinded fashion to avoid bias. The findings ranged from no changes to hind limb paralysis at various days after induction until the 28th day. The rats were sacrificed on the 29th day of the induction of EAE. In the first group of non-BHA fed animals, all eventually showed the clinical symptoms. However, all recovered from it by showing no symptoms of weakness or paralysis by the 28th day. The histology of the non-BHA fed animals revealed an inflammatory disease (EAE) in the spinal cord and the brain of each animal.

In the 2nd group of BHA-fed animals, there has been no noticeable difference in symptoms from the 1st non-BHA fed group of animals. However, the histology of the BHA fed animals did not show any evidence of inflammatory disease (EAE). An inflammatory reaction has been attributed to the clinical symptoms ranging from weakness to paralysis [225, 226, 321]. The statistical analysis was performed using the means of grades of each animal (sum of grades of each animal/28 days) and compared with non-BHA and BHA groups of animal. The independent t-test shows that there is no statistically significant difference between these two groups of animals as the *p* value has been 0.17. These similar clinical presentations are different from earlier studies [226], where the BHA fed animals show minimal to no symptoms [226]. Even though histologically, there has been a clear difference in the inflammatory process in this study. BHA fed animals were free of inflammation as noticed in earlier studies where the use of BHA has improved EAE [225, 226]. How does one explain this apparent contradiction in clinical symptoms of all animals showing acute EAE followed by

remission and the histology where BHA fed animals were free from inflammation whereas non-BHA fed animals have revealed consistent features of inflammation in multiple sections of the brain and the spinal cord? The beneficial effects of BHA has been shown in acute EAE [226], where the animals were fed with a BHA and a non-BHA diet in both the groups of Lewis rats that were administered two weeks prior to the induction of EAE [226]. Therefore, the increased phase 2 enzyme induction prior to the acute EAE has most likely prevented oxidative stress before it actually caused inflammation. So, there has been no or minimal clinical symptoms. However, in this study, the animals were fed with BHA starting from the day of the inoculation of the animals for EAE induction. That is probably why the oxidative stress has caused clinical symptoms following the inflammation of the brain and the spinal cord. All the animals reached the remission clinically but the BHA fed animals could recover completely from the inflammatory process in contrast to the non-BHA fed animals due to the sufficient phase 2 enzyme that has been generated by the BHA. These findings give evidence in support of the beneficial effects of the phase 2 enzyme inducers in EAE [225, 226]. The histological findings have been complimented and confirmed by the raised glutathione (GSH) level in the BHA fed animals [226], thereby confirming the antioxidative role of phase 2 enzyme inducers like BHA [225-226]. The statistical test was performed on the level of GSH in each animal. The independent t-test reveals that there is a significant difference in the levels of non-BHA and BHA fed animal where the *p* value is 0.00. This also reiterates again that EAE can be improved by antioxidants [217-232] and thereby have potential use in the management of MS. Atorvastatin has been found promising in EAE models [162] and *in-vitro* human study has been found promising [163]. The analytical

explanation of the experiment can be summarized in the following. However, nothing excludes the extensive investigation needed to confirm these findings to know the role of various antioxidants in EAE and MS.

5.1 Analysis of Data

The clinical scoring (behavioral studies) of the Lewis rat throughout the 28 day duration show that all the induced EAE rats exhibited the same patterns of symptoms of weaknesses, partial and complete paralysis of the limbs before remission of the symptoms corresponding with the remission of the acute EAE.

However, the histological observations were different. The rats (group 1) that had consumed a normal (not containing BHA) diet exhibited variable grades of perivascular mononuclear cell infiltration. The rats that were on a BHA-containing diet exhibited no perivascular infiltration.

Correlation of the histological and the clinical findings show that all the rats had an acute EAE that ended with remission. Those rats which were on a BHA diet did show pathological recovery from the disease.

The RBC glutathione levels have shown to be correlating with the histological findings of pathological recovery through antioxidant activity. Thereby, group 1 has shown no increased glutathione, however, group 2 reveals increased glutathione levels, confirming our inference that the ameliorating effects of BHA in diet is due to induction of phase 2 enzyme (antioxidant role).

The earlier studies demonstrated the preventive potential of the phase 2 enzyme inducers (antioxidants), BHA. [252-253] But this study is suggestive of a possible pathological recovery by phase 2 enzyme inducers (BHA).

It is highly possible that phase 2 enzyme inducers have double potentials: preventive (if used well before the disease induction) and therapeutic if used during the disease or at the onset of the disease.

5.2 Conclusions

1. The phase 2 enzyme inducer (BHA) has been found to cause anti-oxidation in the brain and the spinal cord tissues of the EAE model.

2. The phase 2 enzyme inducer (BHA) brings about pathological recovery in acute EAE.

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