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Evaluation of the Cuprizone Model

INAUGURAL - DISSERTATION

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Tag der mündlichen Prüfung:

ABREVIATIONS

antibody…….Ab

antigen……..……Ag

blood brain barrier……...…..……BBB

central nervous system….…….……CNS

deoxyribonucleic acid……..……..……DNA

electron microscope.....….....EM

experimental allergic encephalitis…….…….....EAE

glial fibrillary acidic protein.…….…….....GFAP

immunohistochemistry…….....IHC

in situ hybridization.…….……..ISH

luxol fast blue-periodic acid schiff's…….LFB-PAS

microglia/macrophage…….……..Mi/Ma

myelin basic protein……..……MBP

multiple sclerosis…….…….....MS

phosphate buffer solution……...…..…….….PBS

proteolipid protein…….…..……...….PLP

ribonucleic acid……...…...……..RNA

standard error of the mean…….SEM

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1. INTRODUCTION

1.1 MYELIN SHEATH

The *Myelin sheath* is a special type of the plasma membrane. It is a multilayered membrane which consists of lipids and proteins. The majority of those lipids and proteins are specific to the myelin sheath (de Vries *et al.*, 1997; Simons *et al.*, 2000).

Myelination of axons in the central nervous system (CNS) is a process of vital importance to men. Myelin formation is essential for rapid conduction of electric impulses through axons (Hildebrand *et al.*, 1993; Barkovich, 2000). It is important for the development and maturation of normal axons. Normal myelin formation is essential for the normal development, stability, size and structure of axons. Accordingly, abnormal myelin formation has adverse effects on axonal diameter, structure, maturation and survival (Yin *et al.*, 1998; Brady *et al.*, 1999; Baumann and Pham-Dinh, 2001; Taylor *et al.*, 2004).

Myelin sheath formation includes the coordinated production, transport and stability of different myelin components. Myelin formation is the function of oligodendrocytes (Ludwin, 1979; de Vries *et al.*, 1997; Nait-Oumesmar *et al.*, 2000). Although oligodendrocytes may play a principal role in myelin formation, their specific mode of action is still not fully understood (Levine *et al.*, 2001).

1.2 DEMYELINATING DISEASE

Myelin loss (demyelination) is the most frequent pathological change common to a large number of neurological diseases (Billinghurst *et al.*, 1998; Chitnis and Khoury, 2003; Oh *et al.*, 2003). Furthermore, the persistence of demyelination is a major factor in the pathogenesis of a number of neurological diseases such as multiple sclerosis, leukodystrophies and spinal cord trauma (Blakemore *et al.*, 2000).

The process of demyelination involves a broad panel of mechanisms. In principle, this includes all mechanisms that can cause oligodendrocyte and/or myelin sheath damage such as immunologic, toxic or viral insults (Graca *et al.,* 1988; Lucchinetti *et al.,* 1999; Brück *et al.,* 2003). Common causes of Demyelinating Disease (DD) involve the following main groups. *Immune mediated DD:* MS, acute disseminated encephalomyelitis and transverse myelitis (Damoiseaux and Tervaert, 2002; Bennetto and Scolding, 2004; Newswanger and Warren, 2004; Rocha *et al.,* 2004); *inherited DD:* adrenoleukodystrophy and metachromatic leukodystrophy (Spurek *et al.,* 2004; Singhal, 2005); *metabolic DD:* central pontine myelinolysis and vitamin B12 deficiency (Medana and Esiri, 2003); *virus induced DD:* progressive multifocal encephalopathy and subacute encephalitis (Kennedy, 2004, Matthews *et al.,* 2002) and DD caused by *systemic* inflammatory, vascular and malignant diseases (Scolding, 2001; Dale and Branson, 2005).

1.3 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is the prime example of human myelin-related disease. MS is the most common demyelinating disease of the CNS in young adults. MS is complicated by major neurological deficits (Althaus, 2004; Peireira *et al.,* 1996).

However, its *etiology* is still largely unknown, but there are significant predisposing factors (Lutton *et al.,* 2004). In general, a combination of genetics and environmental factors has been postulated to play a role in the etiology of MS.

A *genetic* effect is apparent in many epidemiological studies. MS is a familial disease (Tardieu and Mikaeloff, 2004). It has a striking geographical variation (Wasay *et al.,* 2006). Certain groups in high incidence areas have resistance to the disease. MS has a higher incidence in mono/zygotic twins than in di/zygotic twins. Half-siblings and adopted children in MS families have an incidence similar to that in the general population

(Dyment *et al.,* 1997). However, a non-genetic role is apparent in the influence of emigration on MS incidence. Another non-genetic effect is apparent in the possible clustering of some cases and the occurrence of sporadic cases and small epidemics (Dyment *et al.,* 1997; Christensen, 2006).

Although a number of genes were related to MS, there is still no confirmation of a direct causal role. Therefore, genetic factors might be directly involved in the higher susceptibility to MS in certain populations (Noseworthy *et al*., 2000).

A strong *environmental* influence is believed to play a role in the etiology of MS. Many researchers have studied possible roles of some infectious agents. They suggest that infectious agents, particularly viruses, may initiate a complex autoimmune response to some myelin components. A number of viruses are thought to be associated with MS pathogenesis; these include herpes simplex virus (HSV), Epstein Barr virus (EBV) and human herpes virus (HHV in particular HHV 6 & 8). Nevertheless, there was no evidence of a direct relationship (Simmons *et al.,* 2001; Lutton *et al.,* 2004, Merkler *et al.,* 2006).

Pathology of MS; Different immunological mechanisms are thought to induce the pathologic effect in MS. These include demyelinating antibodies, cytokines production (demyelinating cytokines) and direct interaction with cellular immune mediators' leukocytes and T lymphocytes (Brück *et al.,* 1995; Ruffini *et al.,* 2004; Huang *et al.,* 2005).

Well-documented *pathological features* of MS are "demyelination, inflammation, gliosis and axonal damage" (Brück and Stadelmann, 2003; Brück *et al.,* 2003). In *histopathologic studies*, the characteristic morphological changes in MS patients are the plaques. They consist of a variable degree of perivascular mononuclear cellular infiltrates (microglia/macrophage and T lymphocytes), demyelination, gliosis and axonal damage

(Brück et al., 1996; Jain *et al.*, 2006). The histology of the plaques is variable between acute and chronic plaques. Chronic plaques are characterized by demyelination, oligodendrocyte loss, axon damage, gliosis and a decrease in the mononuclear cellular infiltrate (Brück *et al.*, 1994; 1996). Detailed histopathology studies have found different types of plaques according to their activity. They were classified as early active lesion, late active lesions, inactive demyelinating lesions, early remyelinating lesions and late remyelinating lesions (shadow plaque) (Brück *et al.*,1995; 1996). This classification of the plaques could be correlated to the changes in the activity, pathology and radiology of the disease (Nijeholt *et al*., 1998; Bitsch *et al.*, 2001; Ukkonen *et al*., 2003).

However, many researchers stress that MS pathology is much more complex (Lucchinetti *et al.*, 2001). The histopathology of MS lesions is variable. This might be due the different pathogenic mechanisms in the different types of MS patients (Brück *et al.*, 2003, Lucchinetti *et al*., 2000). Different pathogenic mechanisms are involved in demyelination, oligodendrocytes damage and axonal damage (Lucchinetti *et al.*, 1996; Lucchinetti *et al.*, 2000; Lassmann *et al.*, 2001). This concept is also evident in the immunopathology of the disease. Different types of immunological mechanisms are involved in the pathology of MS. In addition, MS patients have variable response to the immune therapy (Lucchinetti *et al.*, 1999; Lucchinetti *et al.*, 2000; Wingerchuk *et al.*, 2001; Emerson *et al.*, 2001). Furthermore, genetic factors are different in MS subgroups. In conclusion, the disease has multiple pathological radiological and clinical variants (Nijeholt *et al.*, 1998; Ukkonen *et al.*, 2003).

A significant pathological change found in MS patients is *demyelination* resulting from myelin destruction and removal from their axons. Myelin damage is usually complicated

by recurrent failure of myelin repair (unsuccessful remyelination). Persistence of myelin damage (chronic demyelination) is associated with axonal damage. Demyelination and axonal damage are important causes of the deterioration in the CNS functions (Compston, 1996; Smith and McDonald, 1999; Trapp *et al.,* 1999; Brück and Stadelmann, 2003).

Inflammation of the CNS is a major exacerbating factor for many neurodegenerative and demyelinating diseases. *Inflammation* is thought to be a major contributing factor in the pathology of MS (Brück *et al.,* 1995, 1996; Raine, 1997; Huang *et al.,* 2005). Important effectors cells in MS are Mi/Ma and T-lymphocytes. Their role is complex. Mi/Ma are the main cells involved in removing damaged myelin during an inflammatory process (Brück *et al.,* 1995; Kuhlmann *et al.,* 2002). They appear early in demyelinating lesions. This may involve disruption of BBB, and suggest an early participation in lesion formation. Their role is seen through out the recurrent relapses and remissions in MS. A protective role of an inflammatory environment (inflammatory mediators and inflammatory cytokines) has emerged recently in models of demyelination and traumatic brain injury (Arnett *et al.,* 2003). The evidence for a protective role is based on the ability of Mi/Ma to secrete different inflammatory mediators during the pathological process. These mediators are involved in different and sometimes opposing actions. This includes an impact on damage and/or repair (Brück *et al.,* 1995; Bitsch *et al.,* 1997; Ruffini *et al.,* 2004). Inflammation seems to participate in demyelination, axonal damage, BBB breakdown and in remyelination.

Axonal damage in MS is associated with significant neurological defects. The extent of axonal damage is more evident in MRI studies (Miller *et al.,* 2002; Ukkonen *et al.,* 2003). Although the detailed mechanisms of axonal damage are not clear, possible contributing

factors might be chronic demyelination, severe inflammation and primary axon degeneration (Bitsch *et al.,* 2001; Medana and Esiri, 2003; Schneider *et al.,* 2004).

Breakdown of BBB is part of the pathological change in MS. It was shown that BBB is disrupted in MS patients during early lesion formation and during old lesion extension (Engelhardt *et al.,* 1997; Piccio *et al.,* 2002; Lapointe *et al.,* 2004).

1.4 REMYELINATION

An important aspect in the pathology of demyelinating diseases and MS is the recurrent failure of adequate remyelination. It is likely that failure of remyelination has adverse consequences for the patients (Keirstead and Blakemore, 1999; Smith and McDonald, 1999; Franklin, 2002).

Therefore studying the mechanisms of successful remyelination is critical to understand the pathology and improve the management of demyelinating diseases. Successful remyelination is present in a number of situations. Spontaneous myelin repair is a normal physiological response to myelin injury that could occur without application of any extrinsic measures (Lucchinetti *et al.,* 1997; Rodriguez and Miller, 1994; Miller *et al.,* 1996). Demyelinating lesions in MS patients show focal remyelination on histological examination (Brück *et al.,* 2003). Similarly, in experimental models remyelination shows promising results. Remyelination was induced by promoting endogenous or exogenous repair mechanisms (Billinghurst *et al.,* 1998; Stangel and Hartung, 2002; Brück *et al.,* 2003).

However, spontaneous remyelination has a limited role in many human demyelinating diseases with a high rate of failure (Ludwin, 1984; Lucchinetti *et al.,* 1997; Smith and McDonald, 1999; Franklin, 2002).

1.5. EXPERIMENTAL RESEARCH

1.5.1. Experimental models in neuropathology

The practice of experimental research in the study of neuropathology is a result of the following important points:

1) The widespread occurrence and unexplained etiology of human demyelinating disease (Ludwin, 1978).
2) The relative inability of the CNS to undergo significant remyelination (Ludwin, 1979b; Lucchinetti *et al.*, 2000).
3) The limitations encountered in collecting human material for research studies (Peireira *et al*., 1996).
4) The similarity with many aspects of human DD.
5) The ability to establish and evaluate therapeutic trials (Stanislaus *et al.*, 2005; Van Epps, 2005).

Those experiments are intended to provide a better understanding of the molecular mechanisms involved in myelin damage and repair (Lucchinetti *et al.*, 1997). However, experimental animal models encounter some difficulties.

1.5.2 Experimental models in multiple sclerosis

Different animal models are used to study the pathology of MS. Those models closely simulate many aspects of MS pathology and constitute the bases for extensive research work. However, there is a continuous need for new designs and models.

Experimental allergic encephalitis (EAE) is widely used because of its close similarity to MS (Van Epps, 2005). EAE is characterized by a diffuse type of inflammatory demyelination, with axonal damage and BBB breakdown. EAE is suggested to be a T-cell

mediated autoimmune disease (Battistini *et al.*, 2003; Schneider *et al.*, 2004; Guo *et al.*, 2004). It is used for studying immunopathological mechanisms responsible for the disease (Huang *et al.*, 2005). It is also employed to study the correlation between pathological and radiological features of the disease and it is also helpful in the therapeutic trials (Degaonkar *et al.*, 2002; Stanislaus *et al.*, 2005).

EAE pathology depends on the sensitization procedure and on the susceptibility of the animal strain. Thus, EAE pathology is of variable severity and of diffuse pattern. This makes the evaluation of certain parameters complex. It is difficult to correlate structural damage with specific functional abnormality (Kerschensteiner *et al.*, 2004a; Guo L *et al.*, 2004). Therefore, many modifications were established in the EAE model. Important modifications are intended to produce focal and reproducible lesions. Modified models that target specific anatomical sites should facilitate this goal. The use of stereotaxic surgical procedures to induce targeted EAE lesions has many advantages. The lesions are at specific locations. They occur in predictable intervals. They are easily reproducible in acute, chronic or recurrent patterns. A correlation between structural damage and functional abnormality might be easier to establish with the targeted EAE-model. The application of specific behavioral tests was promising and could be related to specific structural changes (Degaonkar *et al.*, 2002; Kerschensteiner *et al.*, 2004a; 2004b).

Toxin induced models; models of toxin-induced focal demyelinating lesions are intended to study repeated episodes of demyelination. Stereotaxic focal injection of toxins has advantages similar to that of targeted EAE (Penderis *et al.*, 2003). This model is designed to avoid damage to small size fibers, which might occur when repeating injections in the same location. It is used to evaluate age effect on remyelination. The model is also applied

to study cellular and molecular factors involved in remyelination (Sim *et al.,* 2000; Sim *et al.,* 2002; Fushimi and Shirabe, 2002).

Viral models are also used to simulate the pathology and etiology of MS. Theiler's virus infection produces a diffuse inflammatory demyelinating disease in susceptible strains. It has an interesting similarity to MS. However, the lesions are extensive and associated with extensive axonal loss (Rodriguez *et al.,* 1994, Oleszak *et al.,* 2004). Mouse hepatitis virus produces encephalitis that is followed by an inflammatory demyelinating disease. It is similar to human demyelinating diseases. It is used to study the role of the immune system. Again, the difficulties include a diffuse pattern of demyelinating lesions and a complex immune-mediated pathology (Kristensson *et al.,* 1986, Matthews *et al.,* 2002).

An important group of *genetically modified models* is now available for experimental research. They include both naturally occurring (spontaneous) and targeted gene mutations. They involve many genetic parameters that are related to inflammation, demyelination and remyelination (Yool *et al.,* 2000; Mathis *et al.,* 2000, Woodruff *et al.,* 2004). These genetic models are very useful in understanding the pathological changes in function, structure, morphology and biochemical molecules in demyelinating diseases (Yin *et al.,* 1998; Brady *et al.,* 1999). They could be used in combination with other demyelinating models and for in-vivo evaluation of many important parameters (Uschkureit *et al.,* 2000; Mathis *et al.,* 2000; Woodruff *et al.,* 2004).

However, important limitations are seen. Genetic damage is irreversible. The severity of genetically induced damage is variable. Experimental animals may suffer from severe neurological dysfunction. They may die before they reach their normal lifespan (Uschkureit *et al.,* 2000; Yool *et al.,* 2000). In addition, the pathogenic mechanisms are

multiple and complex (Mathis *et al.,* 2000). There is also a design of genetic models with some control over lesion severity and time course (Mathis *et al.,* 2000; Yool *et al.,* 2000).

1.6 The cuprizone model

More recently, due to the development of many new technologies, the interest for the cuprizone model has emerged again (Hiremath *et al.,* 1998; Masson *et al.,* 2001b). The cuprizone model was repeatedly shown to induce reliable demyelination (Blakemore, 1973a). Demyelinating lesions were induced by the inclusion of the copper chelator, cuprizone (bis-cyclohexanone oxalyldihydrazone), in the diet of young adult mice. This intoxication produces a massive and consistent demyelination of specific brain regions, mainly of the corpus callosum (Suzuki and Kikkawa, 1969; Blakemore, 1973a; Hiremath *et al.,* 1998; Morell *et al.,* 1998).

Important features of the cuprizone model include the following points:

1.6.1 Cuprizone toxicity

Dietary administration of cuprizone (bis-cyclohexanone oxalydihydrazone) is neurotoxic to rodents (Carlton, 1971). At the beginning of this experimental design, cuprizone feeding was used to establish a model of status spongiosis of the brain and of a chemically induced brain edema (Suzuki and Kikkawa, 1969; Kesterson and Carlton, 1971; Cammer, 1999). At high doses, experimental animals encountered a spongiform encephalopathy and general systemic effects as weakness, lethargy, weight loss, variable forms of hydrocephalus and liver toxicity (Kesterson and Carlton, 1971; Ludwin, 1978). At the same time, the myelin sheath developed edematous vacuoles, degenerative changes and consequently demyelination. This sequence formed the basis for the cuprizone model (Blakemore, 1973b; Cammer, 1999; Matsushima and Morell, 2001). While it is generally accepted that cuprizone exposure is a metabolic insult that adversely affects mitochondrial energy

metabolism, detailed biochemical changes have not yet been well established (Suzuki and Kikkawa, 1969; Johnson and Ludwin, 1981; Cammer, 1999; Gao *et al.*, 2000; Morell, 2001).

Cuprizone is a copper chelator in clinical chemistry. Copper is an important cofactor for a number of catalytic and metabolic pathways. Thus, copper deficiency resulting from cuprizone administration might form the basis for its neurotoxic effect (Hiremath et al., 1998; Matsushima and Morell, 2001).

In brief, the toxic effect of cuprizone might be explained according to the following suggestions. An inhibition of monoamine oxidase due to deficiency of copper requiring cytochrome oxidase results in abnormal mitochondrial energy metabolism. The abnormal metabolic pathways cause damage to the mitochondrial membrane, accumulation of abnormal mitochondrial DNA dimmers and giant mitochondria formation. All of this eventually leads to cell destruction (Suzuki, 1969; Kesterson and Carlton, 1971; Ludwin and Johnson, 1981; Fujita *et al.*, 1990; Cammer, 1999).

Why specific targeting of *oligodendrocytes*? This might be because the oligodendrocytes need large amounts of energy for the synthesis and maintenance of the myelin sheath. In addition, they have high copper content. These two reasons could explain their high susceptibility to damage in the cuprizone model (Levine *et al*., 2001).

1.6.2. Cuprizone dose

Researchers tested variable cuprizone doses on different strains of rat and mice. The low doses, feeding 0.1% cuprizone diet for 6 weeks, have resulted in an incomplete demyelination. The high doses, giving 0.3%, 0.4% or 0.5% cuprizone diet for 6 and sometimes fewer weeks, showed significant side effects like weight loss, lethargy and systemic toxicity.

Therefore adverse systemic effects must be avoided by strictly limiting the use to 0.2% cuprizone (Hiremath *et al.,* 1998; Levine *et al.,* 2001; Stidworthy *et al.,* 2003).

1.6.3. Mice strain

In this model, different animal strains and species showed variable susceptibility and response to the cuprizone-induced intoxication (Ludwin, 1978; love, 1988).

Later on, the use of the C75BL/6 mice in the cuprizone model has offered many advantages: First, high susceptibility to the cuprizone treatment. Second, the absence of significant liver toxicity. Third, the presence of numerous knock-out mice (genetically altered on the C75BL/6 genetic background). The last point allows the performance of specific tests for cellular and sub-cellular functions in vivo within different knock out mice (Hiremath *et al.,* 1998; Matsushima and Morell, 2001).

1.6.4 Age

The most proper animal age for the induction of demyelination is a matter of significant concern. At first it was suggested that age may have a negative effect on proper remyelination (Blakemore, 1973b), but the following experimental trials showed that age is not a major limiting factor for successful remyelination (Blakemore, 1974).

However, many researchers accept that the most optimal demyelination and remyelination occurs in weanling rats or young adult mice and within a specific time course (Blakemore, 1973b; Tansey *et al.,* 1996; Hiremath *et al.,* 1998; Matsushima and Morell, 2001).

1.6.5. Duration of cuprizone administration

In the acute model, mice were fed with cuprizone diet for a short period of time (6 weeks). A significant demyelination occurs within few weeks following oral intake of cuprizone. Total demyelination occurs around week 5 to week 6, with no further changes

during the administration of cuprizone diet for longer periods (Blakemore, 1973a). Furthermore, terminating the cuprizone diet at this time point and returning the experimental animals to normal chow, allowed most of the demyelinated axons to remyelinate (Blakemore, 1973b).

A chronic model, with a longer intoxication period of 6-7 months, showed that the axons capacity for remyelination is decreased in comparison with the acute model. Despite this, the axons were still able to remyelinate to a certain degree. In the chronic model the severe depletion of oligodendrocytes rather than age is an important limiting factor for remyelination (Ludwin, 1980; Ludwin, 1994).

A model of recurrent demyelination and remyelination is conducted by recurrent feeding on cuprizone diet-normal chow. This design had an adverse effect on experimental animals during the second time of cuprizone feeding. Those adverse effects include: protracted demyelination, decreased glial and inflammatory reaction, variable remyelination pattern and remyelination takes longer time to occur (Johnson and Ludwin, 1981).

1.6.6 Site reproducibility

A reproducible lesion site should have a well-defined anatomical area and a size large enough to perform the measurements. This easily reproducible site has the possibility of performing more reliable qualitative and quantitative morphometric and molecular measurements (Matsushima and Morell, 2001).

In the cuprizone model, specific demyelination occurs in the cerebral white matter, particularly the CC and superior cerebral peduncles (Blakemore, 1973a; Ludwin, 1978; Hiremath *et al.,* 1998; Morell *et al.,* 1998).

Still few researchers argue that demyelination does not occur as consistently as desired and not as completely as expected in all white matter tracts. However, they agree that CC

and dorsal hippocampal commisure show the most consistent demyelination and the most severe cellular reaction (Stidworthy *et al.,* 2003). This argument increases the importance of clearly defining the anatomical area of the lesion. Thus, restricting the analysis to the CC will overcome the variability found if studying the cuprizone effect on all white tracts (Blakemore, 1973a; Hiremath *et al.,* 1998; Stidworthy *et al.,* 2003).

1.7 SPECIFIC CHARACTERISTICS OF THE CUPRIZONE MODEL

These are the specific characteristics of the cuprizone model.

1.7.1 Demyelination and Remyelination

The demyelinated area is well defined in the CC. It is large enough to permit the induction of highly reproducible and easily detectable demyelinating lesions (Ludwin, 1978; Ludwin, 1994). In addition a very efficient and spontaneous remyelination follows removal of the cuprizone from the diet (Ludwin, 1978; 1994).

In this model, demyelination is primary in type. The primary demyelination follows the degeneration of the oligodendrocytes and their processes (Ludwin, 1978; Matsushima and Morell, 2001). The primary type of demyelination might have a degree of simplicity during data interpretation in contrast to demyelination complicated by variable degrees of axonal damage (Ludwin, 1978).

1.7.2. Cellular Response

Oligodendrocytes: Cuprizone diet at a proper dose produces a selective damage to oligodendrocytes with minimal effect on other cell types. This specific damage seems to result from the failure of the oligodendrocytes to sustain the huge metabolic demand needed to stabilize and maintain sufficient amounts of myelin (Blakemore, 1973a; Hiremath *et al.,* 1998). The sequence of oligodendrocyte degeneration and regeneration in

this model should allow a detailed examination on the origin, development and proliferation capacity of oligodendrocytes (Ludwin, 1979b).

Microglia/Macrophage: Resident and infiltrating Microglia/macrophages (Mi/Ma) are present in a variety of CNS disorders and their experimental models (Hiremath *et al.,* 1998; Matsushima and Morell, 2001). In the cuprizone model, a large number of Mi/Ma accumulates during demyelination, followed by a marked decline in their numbers upon remyelination. Although, Mi/Ma response is severe and huge, they are still located specifically with in the demyelinated white tracts and the CC (Blakemore, 1973a, Hiremath *et al.,* 1998; Morell *et al.,* 1998). Even in a more recent study providing detailed evaluation of the types of recruited cells into the demyelinated CC, it was found that a small percentage of the peripheral macrophages are present among the recruited cells, and they were specifically located in demyelinating areas (McMahon *et al.,* 2002). They confirmed the presence of those peripheral macrophages by flow cytometry and in situ hybridization (ISH) techniques. However, those peripheral macrophages still form only a very small percentage of a much larger number of locally recruited Mi/Ma (McMahon *et al.,* 2002).

Astrogliosis is a well documented constituent of the cellular response to cuprizone intoxication (Blakemore, 1973a; Ludwin, 1978; Hiremath *et al.,* 1998).

1.7.3. Biochemical molecular parameters

Quantitative biochemical and metabolic measures of myelin-related parameters, other than morphometric studies, were also applicable to this model. An interesting finding was that temporal and spatial changes in the myelin-related biochemical parameters during cuprizone administration and withdrawal were profound and preferential to the demyelinated CC when compared to other brain tissue (Jurevics *et al.,* 2001; 2002).

1.8 Other important characteristics

1.8.1 T lymphocytes

T lymphocytes are almost completely absent from cellular recruitment during the cuprizone intoxication (Matsushima and Morell, 2001). Even when T lymphocytes are detected, they are present in very small numbers and are not localized in areas of demyelination. On the contrary, they were scattered diffusely through both white and gray matters of brain. Furthermore, they were not found in perivascular areas within the demyelinated lesions (Komoly *et al.,* 1992; Hiremath *et al.,* 1998; McMahon *et al.,* 2002).

More interesting, is the finding of increased expression of the major histocompatibilty complex (MHC class 2) that has a signaling function in macrophages independent of T lymphocytes (Arnett *et al.,* 2003).

The absence of a significant role of T lymphocytes in this model should allow the evaluation of the remyelination process in the absence of complex immune-mediated damage (Matsushima and Morell, 2001).

1.8.2 Blood brain barrier

The blood brain barrier (BBB) is largely different from all other vascular barriers in the human body. BBB has a highly selective control over leukocyte passage into the CNS during both health and disease (Engelhardt *et al.,* 1997; Carvalho-Tavares *et al.,* 2000, Kivisakk *et al.,* 2003).

Although a marked cellular reaction is a constant feature of the cuprizone induced demyelinating lesion, still a normal morphology of the BBB is evident in this model. Many studies provide support for this concept. First, the BBB remained intact during exposure to horseradish peroxidase (Kondo *et al.,* 1987). Second, there is no evidence of significant protein leakage into the sub-endothelial basement membrane or extra-vascular space

during demyelination, when tested by specific antibodies against extravasated serum proteins (Bakker and Ludwin, 1987). Finally, the ultrastructural examination revealed the presence of normal blood vessels in the demyelinated superior cerebellar peduncle (Bakker and Ludwin, 1987).

A normal BBB in the cuprizone model is in contrast to the early increase in BBB permeability during immune-mediated demyelinating diseases (Komoly *et al.,* 1987). Furthermore, in MS and its animal model EAE, a defect in BBB and subsequent peripheral leukocyte leakage precedes the appearance of clinical symptoms and correlates with the severity of the lesions (Bakker and Ludwin, 1987; Brück *et al.,* 1996; McMahon *et al.,* 2002).

In fact, there are many points about the role of the BBB that remain unclear. First, what is the effect of an intact BBB on the movement of circulating leukocytes in and out of the brain during the demyelination process (Matsushima and Morell, 2001)? Second, inflammatory demyelinating disease may show peripheral leukocyte infiltration in the absence of BBB defect (Kondo *et al.,* 1987). Third, activated leukocytes (activated T lymphocytes) are capable of movement in and out of the CNS during normal immunological functions (Piccio *et al.,* 2002; Kivisakk *et al.,* 2003). Fourth, many areas of the CNS like meningeal peri-vascular areas, the cerebro-spinal-fluid and the choroid plexuses stroma in tissue sections from autopsy cases of normal non-inflamed CNS contain normal resident leukocyte populations (Engelhardt *et al.,* 1997; Kivisakk *et al.,* 2003). Fifth, leukocyte infiltration into the CNS is documented in diseases in which barrier defect is minimal (McMahon *et al.,* 2002).

Nevertheless, it could be concluded that in the cuprizone model there is no histological evidence of BBB disruption (Bakker and Ludwin, 1987; Kondo *et al.,* 1987; McMahon *et*

al., 2002). The intact BBB in the cuprizone model would give opportunity to study Mi/Ma response during the demyelinating process while the barrier is still largely intact (Hiremath *et al.,* 1998).

1.8.3 Axons

Normal axons are critical for successful myelination. Myelin formation depends on functional interaction between normal axons and oligodendrocytes (Brück *et al.,* 2003). This bi-directional interaction promotes oligodendrocyte movement toward axons. It allows oligodendrocytes processes to surround axons and proceed into myelin formation (Ludwin, 1994). Furthermore, it is suggested that the number of oligodendrocytes is ultimately determined by axonal interaction (Armstrong *et al.,* 2002).

In the acute cuprizone model, axons are relatively well preserved, without any histological change in their morphology (Blakemore, 1973a; Ludwin, 1980; Komoly *et al.,* 1992), and the aberrations that occurred were transient and minor (Johnson and Ludwin, 1981; Mason *et al.,* 2001).

In chronic cuprizone toxicity, there is no significant axonal loss or disruption of architecture even after a year of demyelination (Ludwin, 1994). Although the axons retained their capacity for remyelination, it was greatly reduced when compared to acutely demyelinated axons. The axons themselves were normal morphologically, but electron microscopy (EM) studies showed a decrease in the average of their diameter, which later on during remyelination returned to near normal (Ludwin, 1980; 1994; Mason *et al.,* 2001b).

Numerous abnormalities of the myelin sheath appear in recurrent nerve fiber demyelination. Nevertheless, even in the face of repeated demyelination, induced by

recurrent cuprizone diet-normal chow feeding, axons in the CNS are still able to remyelinate (Johnson and Ludwin, 1981).

Although most of morphological studies have found normal axons during cuprizone intoxication, some authors still argue that axonal pathology receives little attention in recent reviews. They found minor axonal damage during demyelination which was still present in some axons during early remyelination. Thus, they suggested that axonal pathology constitutes a subcomponent of the response to cuprizone intoxication (Mason *et al.*, 2001b; Stidworthy *et al.*, 2003).

To understand this argument, one should consider the complex oligodendrocyte-axonal interaction and proper interpretation of EM measurements. These include the following important points: First, the interaction between oligodendrocytes and axons directly alter the axonal diameter. Second, a complex relationship exists between axon diameter and myelin sheath thickness, especially during the early stages of remyelination. Third, when interpreting small-sized axons, difficulties were encountered when differentiating between normal small-size axons and remyelinated axons that were still small in size (Mason *et al.*, 2001b, Stidworthy *et al.*, 2003).

Lastly, many researchers suggest that the formation of chronically demyelinated lesions induced by cuprizone appears to be the result of oligodendrocyte depletion within the lesion and not due to the inability of the chronically demyelinated axons to be remyelinated (Blakemore, 1974; Ludwin, 1980; Mason *et al.*, 2004).

1.9 Experimental Aim and Design

The aims of this thesis are

1- Characterize the myelin staining patterns during demyelination and remyelination, by quantifying and comparing conventional (LFB-PAS) and IHC (MBP & PLP) myelin stain scores.

2- Study the role of oligodendrocytes during demyelination and remyelination.

3- Quantify the response of Mi/Ma during demyelination and remyelination.

4- Study the role of astrocytes.

Experimental design

Our design is focused on two main parts of the cuprizone model. First, myelin sheath morphology during demyelination and remyelination. Second the cellular response during demyelination and remyelination. These parts contain many subheadings that were studied as follows.

1.9.1 Myelin sheath stain

1.9.1.1 Conventional stains

Myelin sheath is routinely examined in neuropathology practice on the light microscopic level by the histochemical stain luxol fast blue-periodic acid schiff (LFB-PAS). It is a widely used conventional myelin stain (Lucchinetti, *et al.,* 2000; Brück *et al.,* 2003). This stain is repeatedly used in the cuprizone model, as a standard semi-quantitative measurement for the degree of demyelination and remyelination of the CC in the C75BL/6 mice (Hiremath *et al.,* 1998; Morell *et al.,* 1998; Masson *et al.,* 2000).

1.9.1.2 Immunohistochemical stains

IHC techniques are used to stain and detect specific antigenic determinants of different cells and tissues. Thus, IHC staining can easily differentiate specific types of cells and

tissues according to their antigens. At the same time, some antigens are similar in both human tissue and the corresponding tissue in other mammalian species like rodents (Albrechtsen *et al.,* 1984; Pelc *et al.,* 1986).

PLP and MBP myelin proteins

IHC is largely used to identify specific myelin lipids and proteins which are constituents of the myelin sheath (Potter and Lees, 1988).

In this study two major constituent of the myelin sheath are used for the assessment of the myelin change during the time course of the experiment. Those are the integral (intrinsic) membrane protein, Proteolipid protein (PLP), (Stoffel *et al.,* 1984; Trifilieff *et al.,* 1986; Gow *et al.,* 1997), and the extrinsic membrane protein, myelin basic protein (MBP), (Hudson *et al.,* 1989; Griffiths *et al.,* 1998). Besides being major constituents of the myelin protein, they are differentially located on the myelin membrane (Konola *et al.,* 1991).

PLP is one of the major protein components of the CNS myelin (Yamada *et al.,* 1999; Jalabi *et al.,* 2003; Peyron *et al.,* 1997). It is located in the cytoplasm of the oligodendrocytes and in the compact myelin sheath (Nussbaum and Roussel, 1983; Nussbaum *et al.,* 1985; Schwob *et al.,* 1985).

MBP is also one of the major myelin protein constituents (Barbarese *et al.,* 1988; Zecevic *et al.,* 1998). MBP is located at the oligodendrocyte process and in the myelin sheath (Konola *et al.,* 1991; Gendelman *et al.,* 1985; Webster *et al.,* 1985).

Both PLP and MBP have important structural and functional roles in formation and stabilization of myelin sheath during and after development (Yamada *et al.,* 1999; Konola *et al.,* 1992; Greer and lees, 2002; Kruger *et al.,* 1999; Akiyama *et al.,* 2002; Zecevic *et al.,* 1998).

The MBP-IHC stain and the conventional LFB-PAS myelin stain are closely related so that MBP-IHC stain is decreased or lost in same areas of reduced or absent LFB stain from the myelin sheath (Gendelman *et al.,* 1985, Webster *et al.,* 1985).

1.9.2 Cellular response

1.9.2.1 Immunohistochemistry

- *Microglia/macrophages*

MAC 3 is a specific Mi/Ma marker. It is directed against the macrophage differentiation glycoprotein, which is located on the cell surface and in its cytoplasm (Backe *et al.,* 1991; Pulford *et al.,* 1992; Ho and Springer, 1983; Walker *et al.,* 1985)

- *Astrocytes*

Cells expressing glial fibrillary acidic protein *(GFAP)* are derived from an astrocytic differentiation. GFAP is used as a specific marker of astroglial elements in tissues and cell lines (Royds *et al.,* 1986; Sawa *et al.,* 1986; Hayashi *et al.,* 1987; Perentes *et al.,* 1987; Debus *et al.,* 1983).

1.9.2.2 In situ hybridization/PLP mRNA localization

PLP mRNA in situ localization

In situ hybridization (ISH) is widely used for detecting specific sequence of nucleic acid material (DNA or RNA) (Breitschopf *et al.,* 1992; Bessert and Skoff, 1999).

Its use in detecting oligodendrocytes has increased. The ISH technique can detect multiple oligodendrocyte-specific nucleic acid material. In this experiment, ISH is used to detect oligodendrocytes that express PLP mRNA (Jalabi *et al.,* 2003; Breitschopf *et al.,* 1992; Bessert and Skoff, 1999).

This technique has many advantages. It is highly sensitive technique and it has a very good resolution at light microscopic level. It is used with routinely formalin fixed tissue and no need for special fixation. It is practical for routine laboratory use especially with development of digoxigenin labeled probes, which is safer than handling radioactive labeled probes (Breitschopf *et al.,* 1992; Jalabi *et al.,* 2003; de Vries *et al.,* 1997; Bessert and Skoff, 1999).

2. MATERIALS and METHODS

2.1 EXPERMENTAL ANIMALS

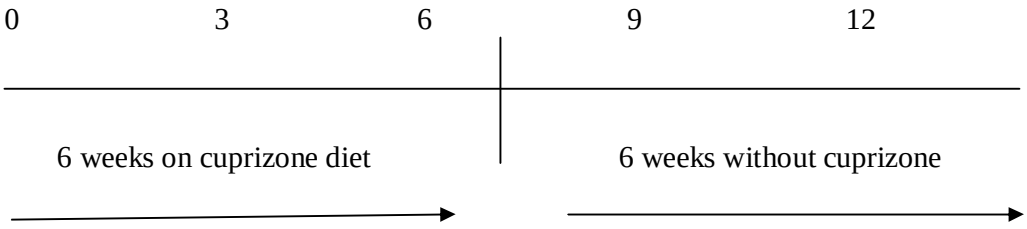
Wild-type C57Bl/6 male mice were purchased from the Jackson Laboratories. They were 7 to 8 weeks old. Their weight was at a range of 19.8 to 22 g (average 21.05 g).

2.2 CUPRIZONE DIET

The 0.2% cuprizone diet was prepared by mixing cuprizone into the ground chow weight by weight. Cuprizone, bis-cyclohexanone oxalyldihydrazone, was purchased from Sigma-Aldrich, Lot 102k0933, Steinheim, Germany.

Mice were put first on a ground chow (SSniff GmBH Soest, Germany) diet without cuprizone for a few days. Then the 8-week-old mice were put on the cuprizone diet (ground chow mixed with cuprizone).

Groups of mice were put on the cuprizone diet for 3 and 6 weeks ad libido to induce acute demyelination. Each group consisted of 7 to 10 mice per time point. Then the mice were returned to the normal diet (ground chow without cuprizone) for another 3 and 6 weeks to allow remyelination to occur (Hiremath *et al.,* 1998; Morell *et al.,* 1998). Mice groups were sacrificed at the following time points: at 3 weeks, at 6 weeks, at 9 weeks, and at 12 weeks from the beginning of the cuprizone diet feeding.



Groups of age matched control mice were fed on normal diet (ground chow without cuprizone). Control groups were used at all time points.

Experimental mice body weights and systemic changes were monitored regularly during the whole period of the experiment.

All animals were housed in pathogen-free cages, were maintained in accordance with the guidelines and protocols of German animal protection laws, and approved by the responsible governmental authority (Bezirksregierung Braunschweig).

2.3 TISSUE PREPRATION

At each time point, one group was sacrificed by an overdose of anesthesia (ketamine/medetomidine) injected into the intra-peritoneal space. Then the mice were perfused transcardially with 0.1 M phosphate buffer saline (PBS) for a few minutes. This was followed directly by the perfusion of 4% para-formaldehyde in phosphate buffer solution (PBS) (PH 7.2).

The brains were dissected, removed and post-fixed in the same fixative overnight. Then sections were put in PBS until tissue preparation for paraffin embedding.

The brains were coronally bisected to expose the CC at the level of the optic chiasma which is focused approximately to the corresponding figures 22-27 of the Mouse Brain in stereotaxic coordinates by KEITH B J and FRANKELIN GEORGE PAXIONS (1997), then sections were paraffin embedded en face.

Several 3-4 micrometer sections were made and mounted on glass slides for the use in the routine light microscope, histochemical, immuno-histochemical and in situ hybridization staining and quantification techniques.

2.4. STAINING METHODS

Hematoxylin and eosin

For routine histology, sections were deparaffinized, cleared through xylene, dehydrated in graded alcohols and stained in hematoxylin and eosin. Then they were rehydrated, permanently mounted with Depex mounting media (BDH England), and covered.

Conventional myelin stain

LUXOL FAST BLUE-PERIODIC ACID SCHIFFS STAIN (LFB-PAS):

LFB-Staining procedure:

- Sections were deparaffinized, and then dehydrated into 90% alcohol.
- Sections were incubated into LFB solution over night, at 60 c° oven.
- Differentiation of section was made by the following steps:
  -- immerse shortly in 0.05 lithium carbonate.
  -- Rinse in 70% alcohol.
  -- wash in distilled water.
  -- repeat the three steps until sharp contrast between blue white matter and colorless gray matter.
- sections were rehydrated mounted and covered.

PAS-stains procedure:

Sections were put in the following solutions:

- periodic acid for 5 minutes
- under running tap water for 5 minutes
- washed carefully in distilled water
- Schiff's reagent 20-30 minutes
- tap water 6 minutes

- counter stain in Myers hematoxylin 2- 3 minutes
- washed in distilled water
- differentiated in acid-alcohol
- bluing in running tap water for 10 minutes
- sections were rehydrated mounted and covered.

2.5 IMMUNOHISTOCHEMISTRY

The following primary antibodies were used: anti-myelin basic protein (anti-MBP) for myelin, anti proteolipid-protein (anti-PLP) also for myelin, as a double stain on the same slides used for in situ hybridization. anti-MAC 3 for Mi/Ma and anti glial fibrillary acid protein (anti-GFAP) a marker for astrocytes, for specification and dilutions (see table 1).

antibody	**isotype**	**Clone**	**code no**	**Source**
Monoclonal Mouse anti proteolipid protein Also recognizes DM20	IgG2a	Plpc1	BZL04476	BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany
Polyclonal RABBIT anti human myelin basic protein			A0623	DAKO Corporation, CA, USA
Monoclonal mouse anti-human glial fibrillary acid protein	IgG1, kappa	6F2	M06761	DAKO, Glostrup, Denmark
Monoclonal Rat anti-mouse MAC3	IgG1, K	M3184	553322	PharMingen, BD Biosciences, Europe

Figure (1) Table of antibody specifications and sources

STAINING PROCEDURE FOR IMMUNOHISTOCHEMISTRY

The avidin-biotin technique was used as follows:

- sections were deparaffinized and dehydrated in graded alcohols
- antigen retrieval, by heating the sections in the microwave 5 times, each for 3 minutes, sections was heated in citrate buffer
- blocking endogenous peroxidase, by putting the sections in a 3% hydrogen peroxide solution in PBS
- blocking nonspecific Ab binding, by incubate the section in 10 % fetal calf serum in PBS
- adding all primary antibodies on the sections and incubate overnight at 4 C°
- incubation with secondary biotinylated (biotin conjugated) antibodies which are specific to the primary antibodies at room temperature for one hour
- incubate with the avidin peroxidase complex at room temperature for one hour
- visualization using the chromogen diaminobenzidin (50 ml PBS + 1 ml DAB stock + 30% H2O2)
- counter stain with mayor's hematoxylin for 60 seconds
- differentiate in acid alcohol
- bluing in running tap water
- sections were rehydrated mounted and covered

2.6 IN SITU HYBRIDIZATION

The ISH technique was used for detecting PLP mRNA on oligodendrocytes. The technique was performed according to the detailed protocol as previously described (Breitschopf *et al.,* 1992).

In summary:

Pretreatment:

- paraffin sections were de-paraffinized
- post-fixed in paraformaldehyde in a 0.1 m phosphate buffer
- nonspecific stain was prevented by acetyliation
- proteinase K digestion was performed at 37 c and stopped at 40 c in a TRIS HCL buffer (PH 7.4) , Sigma St Louis MO, USA (p 0390)

CDNA probes:

- probes specifically encoded for PLP were digoxigenin labeled
- digoxigenin label, mouse monoclonal anti-digoxigenin, peroxidase-conjugated anti digoxigenin, labeling & detection kit Boehringer, Mannheim, FRG

Hybridization:

- after probe was de-nutrated

-Then adding hybridization solution on slides and incubated for 14-16 hr

Detection method:

- anti-digoxigenin mix (blocking reagent (boehringer kit) containing 10% fetal calf serum, 15 min)

Color reaction:

- NBT/BCIP as recommended by Boehringer, developed in refrigerator
- NBT/BCIP 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, Boehringer
- then use a dry mount

2.7 MYELIN SCORES AND CELLS COUNTING PROCEDURE

Myelin Score:

A semi-quantitative method was used to assess the degree of demyelination. A score of 0 to 3 was given to the intensity of LFB-PAS-stained sections (Hiremath *et al.* 1998; Morell *et al.*,1998). During the evaluation, we divided the CC into three different areas right lateral, central and left lateral. The score values are estimated as follows:

Score 0 = normal myelination (indicated by blue color) as seen in untreated controls.

Score 1 = 1/3 or less of the corpus callosum is demyelinated (loss of blue color of myelin).

Score 2 = more than 1/3 and less than 2/3 of the corpus callosum is demyelinated.

Score 3 = 2/3 or more of the corpus callosum is demyelinated.

Cell Counting Methods:

Mi/Ma and oligodendrocytes were counted according to the following procedure: Images of the stained sections were captured using an Olympus BX51 microscope combined with a 3 CCD digital camera. Images were taken for sections containing the CC area at the chiasma (fornix) level. Mi/Ma and oligodendrocytes were counted using the computer aided software analySIS from Soft Imaging System Gmbh Germany. Two sides of the CC were defined on the pictures by drawing a vertical line at the midline of the CC. This line identifies an area at the right side to the midline and an area at the left side of the midline. These areas were further divided into lateral and medial areas by drawing a vertical line at the level of medial comissures. This line identifies lateral and medial areas on the right side and on the left side of the CC. The surface area of each division was measured using the software. Then each single cell in the defined area was given a mark by the aid of the software. The software calculated the total number of cells. The counts per square

millimeter of tissue were calculated as follow: the total number of cells divided by the surface area = cell counts/mm^2.

Astrocyte density was determined by using an ocular morphometric grid, a 25-point Zeiss eyepiece. Counts were determined at 400-fold magnification. First, we counted the number of astrocyte processes meeting the randomly distributed points. Then we counted the total number of points at the same field. Astrocytes score was calculated by dividing the number of astrocytes by the total number of points. We evaluated at least six randomly selected fields at the CC area per animal.

2.8. STATISTICAL METHOD

All statistical comparisons were made by using the software NCSS/PASS Dawson edition. Statistically significant differences between the groups were calculated using one way ANOVA, followed by the Tukey-Kramar multiple comparisons tests.

3. RESULTS

3.1 LFB-PAS score of myelin

3.1.1 Demyelination:

Our study is restricted to demyelinating lesions in the CC area. During the demyelination period, the mice were exposed to cuprizone diet. The following demyelinating groups were evaluated: at week 3 and week 6 from the start of the cuprizone diet. At the same time, control mice were fed with normal chow. The cuprizone diet is a mixture of powder chow and 0.2% cuprizone.

LFB-PAS-stained brain sections were scored blindly for the intensity of LFB stain. Brain sections of untreated control mice, on normal chow, showed consistent blue staining of the whole CC (Figure 3). This indicates complete myelination of nerve fibers in light microscope scale. In contrast, brain sections from mice fed on cuprizone diet for 6 weeks showed total pink-color staining of the CC. This indicates complete demyelination in light microscope scale while the sections from the mice on cuprizone diet for 3 weeks showed a staining pattern intermediate between the control group and the totally demyelinated group at week 6. Brain sections from the week 3 group show a decrease in intensity to an absence of the blue staining in multiple areas of the CC. The occurrence of progressive demyelination upon cuprizone treatment is in accordance with many studies (Hiremath *et al.,* 1998; Morell *et al.,* 1998; Mason *et al.,* 2000a; Arnett *et al.,* 2001).

LFB-PAS score at weeks 3 and 6 showed significant demyelination ($P<0.05$) compared to the untreated control group. During the progress of demyelination, a continuous increase in the score was evident at week 3 and reached peak at week 6 (Figure 2). There is also a significant difference ($P<0.05$) in the score of demyelination between week 3 and week 6.

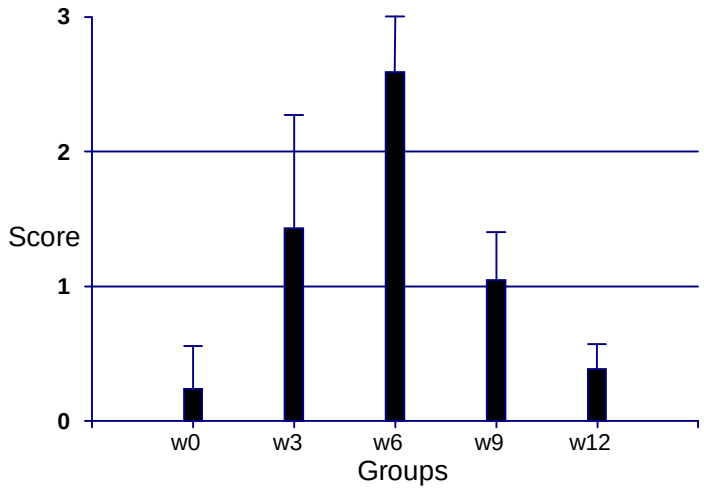


Figure (2) LFB-PAS score

LFB-PAS stained brain sections were scored during demyelination and remyelination. The scores are plotted as an average with SEM for all groups

A significant demyelination (P<0.005) was found during cuprizone feeding at week 3 and week 6 compared to week 0 (control group), another significant difference was present between week 3 and week 6. A significant remyelination (P<0.005) was found after termination of the cuprizone feeding and return to normal chow at week 9 and week 12 compared to week 6. n= 7 mice/ group

W0= control, w3= three weeks on cuprizone, w6= six weeks on cuprizone, w9= six weeks on cuprizone and three weeks on normal chow, 12w= six weeks on cuprizone and six weeks on normal chow

3.1.2 Remyelination:

Successful remyelination depends on termination of the cuprizone diet and feeding mice on normal chow. Therefore, in our design we expected remyelination to occur in the 9 week and 12 week groups, which correspond to feeding for 3 weeks and 6 weeks on normal chow after termination of the cuprizone diet. LFB-PAS scores for brain sections

from both groups confirmed the presence of extensive and statistically significant remyelination (figure 2). The result of the score indicates significant myelin formation in both remyelinating groups (week 9 and week 12) when compared to the most demyelinated group at week 6, which is also well documented in many studies (Morell *et al.,* 1998; Mason *et al.,* 2000b; Arnett *et al.,* 2001). This successful remyelination is present as a continuous decrease in the score, reflecting a continuous increase in the blue color stain. Eventually, the score reaches values near those of the control group (figure 2).

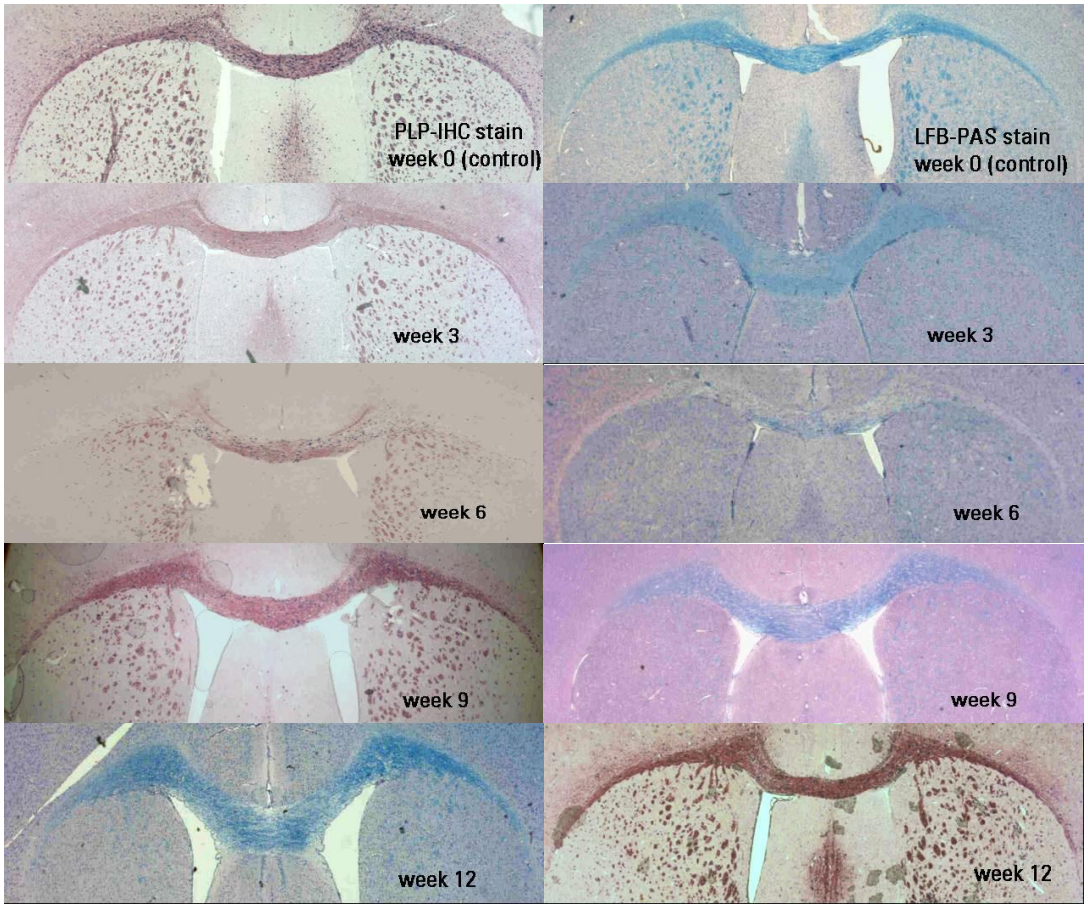


Figure (3) Histology of LFB and PLP-IHC myelin staining

Representative histological sections from the mice brains, at the selected time points to study the cuprizone effect. Both conventional and IHC staining methods show the course of demyelination and remyelination in the CC area. Compare LFB-PAS stain on the right side (LFB-blue color) to PLP-IHC stain on the left side (PLP-IHC brown color). NOTE that the nerve fibers at the CC area have a similar staining pattern at the corresponding time points.

3.2 IHC-staining score

3.2.1. Demyelination

PLP-IHC stained sections (figure 3) showed a continuous decrease in intensity of staining subsequent to demyelination, this is reflected in the score as a progressive increase in the mean value starting at week 3 and reaching peak value at week 6. However, only week 6 has a statistically significance difference ($p<0.05$) from the control group (figure 4).

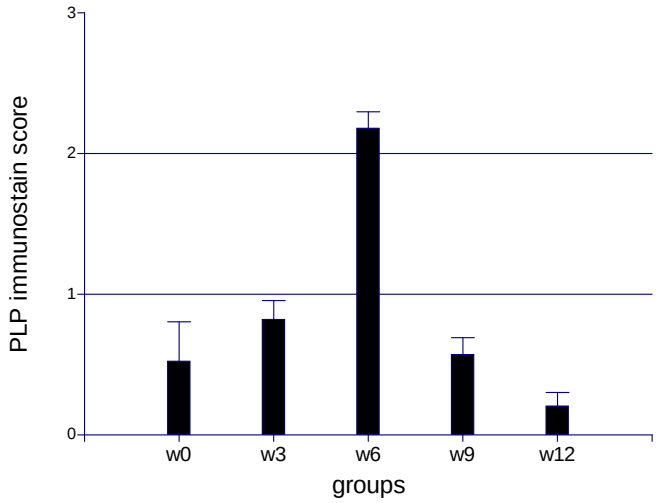


Figure (4) PLP-IHC stain score

Average and SEM for PLP-IHC-staining score.

PLP score shows a progressive demyelination during cuprizone feeding. A significant demyelination ($P<0.01$) was found at week 6 compared to week 3.

The score indicates a successful remyelination during the period of normal chow feeding. Both time points (week 9 and week 12) show significant ($P<0.01$) remyelination compared to maximum demyelination at week 6

MBP-IHC stain has a similar pattern to other myelin stain scores during demyelination course. Thus, a decrease in MBP-IHC score is directly associated with the progress of demyelination. On microscopically examination of the MBP-stained sections, there is a gradual and continuous loss of staining intensity. Again, only week 6 showed a statistically significant difference from control group ($p<0.05$) (figure 5).

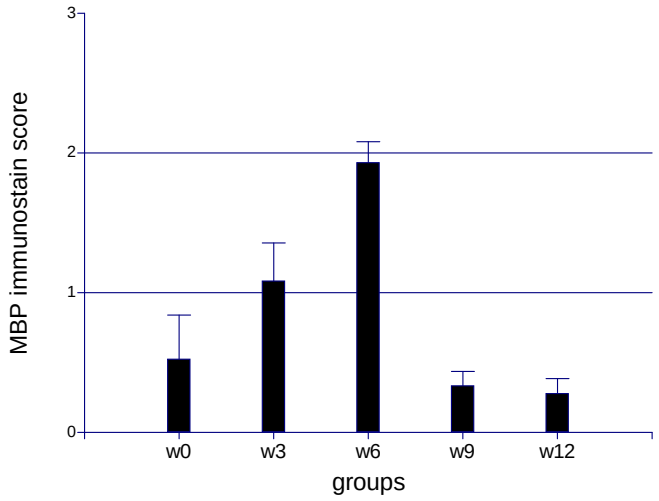


Figure (5) MBP-IHC stain score

Average and SEM for MBP-IHC staining score. In a similar pattern to the PLP-IHC score, a significant (P<0.01) demyelination was seen at week 6 compared to the control group. Upon removal of cuprizone from the diet, remyelination appears to be relatively faster at the MBP-IHC staining score. A statistically significant (P<0.01) remyelination was found at week 9 and week 12 when compared to week 6.

The results of MBP-IHC staining are in accordance with previous studies. At week 3, moderate loss of stain reactivity and organization was found on the CC fibers. At week 6, the CC was almost completely devoid of myelin (McMahon *et al.*, 2002). The course of demyelination in the cuprizone model has a characteristic pattern. It was described in

earlier cuprizone studies to start as vacuolization, swelling, and fragmentation of the myelin sheath. Then removal of degraded myelin proceeds until the tracts have no stainable myelin sheath after 5-6 week (Ludwin, 1984).

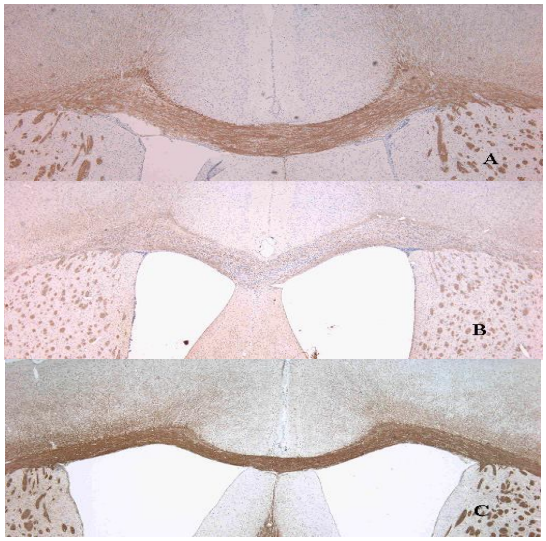


Figure (6) Histology of MBP-IHC staining

Anti-MBP stains the myelin fibers at the CC area in brown color. MBP-IHC staining has a clear ability to differentiate significant changes in myelination. The staining pattern and distribution is used to estimate the score. A) Sections from week 0 (control) show uniform distribution of the stain (normal myelination). B) Sections from week 6 show total loss of the stain at the CC area (total demyelination). C) Sections from week 12-6 weeks on cuprizone and returned on cuprizone free diet for additional 6 weeks- show total and uniform staining which indicate successful remyelination.

3.2.2 Remyelination

PLP-IHC staining of brain sections was evaluated during the remyelination period, when cuprizone treated mice were allowed to feed on normal chow. Our result shows a progressive decrease in the score (morphologically seen as a progressive increase in PLP stain intensity). This pattern indicates a successful remyelination. Both remyelinating

groups at week 9 and week 12 shows a lower score which is a statistically significant different ($p<0.05$) from mice groups at week 6 (figure 4).

MBP stain has similar staining pattern to the PLP stain, however, there is mild difference at week 9, where MBP score is slightly lower than that of PLP score for the same time point. This difference might be due to a more rapid accumulation of MBP during remyelination (see week 9 in figure 4 and figure 5).

In general, similar trends were observed for both PLP and MBP-IHC staining. These IHC stains are qualitative measure for myelin formation. Both proteins show consistent homogenous staining of nerve fibers in the CC of untreated control mice. In contrast, mice on cuprizone diet for 6 weeks showed rare and sparse staining patterns (Hiremath *et al.*, 1998).

3.3 Cellular count

3.3.1 Oligodendrocytes

Oligodendrocytes are identified as cells having positive staining for PLP mRNA using ISH technique.

Oligodendrocyte counts were performed according to the following criteria: Counting was restricted to the CC area of mice brain sections. Stain is typically located on the peri-nuclear region of the oligodendrocytes. Oligodendrocytes are distributed in characteristic interfascicular rows (de Vries *et al.*, 1997; Bessert and Skoff, 1999; Jalabi *et al.*, 2003). Characteristic oligodendrocytes staining are seen in (figure 8).

During the demyelination period (figure 7) a marked decrease in oligodendrocyte count was found, which is closely related to changes in conventional and IHC myelin stains. Oligodendrocyte disappearance is well documented by other markers in many studies

(Blakemore, 1973a; Ludwin, 1978; Masson *et al.*, 2000; Arnett *et al.*, 2002; Mason *et al.*, 2003).

An interesting finding is in the higher counts of oligodendrocytes at week 6 when compared to week 3. At week 3, the oligodendrocytes depletion rate was higher. This is interesting because this spontaneous regeneration occurred while mice were still undergoing cuprizone intoxication (Mason *et al.*, 2000a, 2001b).

Demyelinating mice at week 3 and week 6 have oligodendrocyte counts that are significantly ($P < 0.05$) lower than counts of the untreated control group (figure 7).

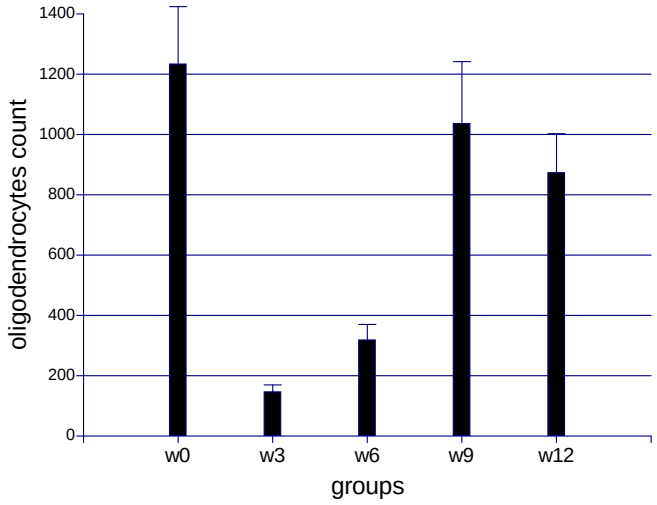


Figure (7) Oligodendrocyte counts

Absolute counts of the PLP mRNA + oligodendrocytes are plotted as means with SEM. Oligodendrocytes counts at week 3 and week 6 are significantly (P<0.01) lower than in untreated controls. This is followed by a marked oligodendrocyte recruitment and accumulation at the CC which starts at week 9 and reaches values near controls at week 12, by returning mice on normal diet without cuprizone. Counts/ mm^2

In contrast, upon removal of cuprizone from the diet, the remyelinating groups at week 9 and week 12 showed a marked increase in oligodendrocytes numbers (figure 7). This

increase is of statistical significance ($p < 0.05$) when compared to the initial spontaneous increase at week 6.

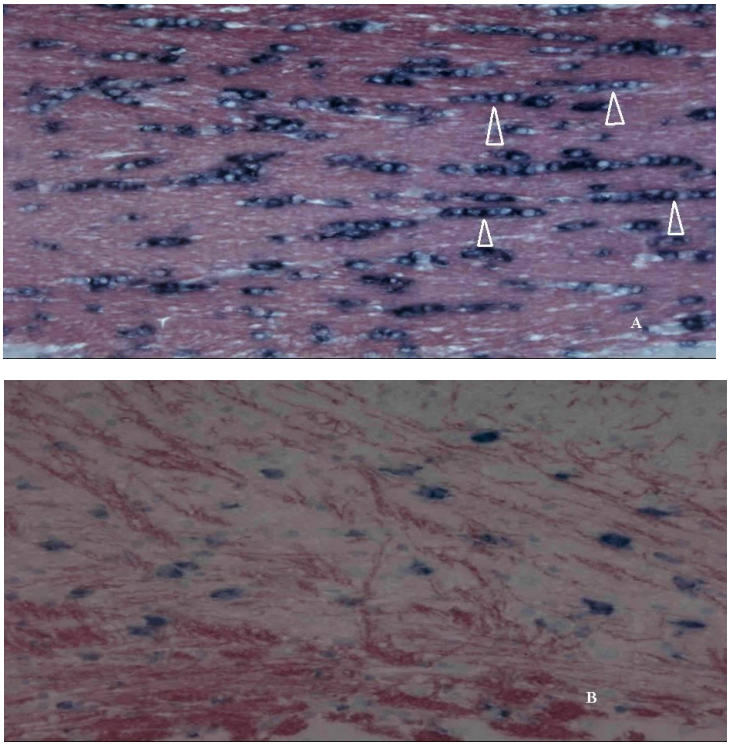


Figure (8) Histology of PLP mRNA + oligodendrocytes

ISH has a typical staining pattern for PLP mRNA + oligodendrocytes. They are typically seen in rows, and the stain is located in the perinuclear area (arrows). A) At week 0 (control) groups, normal distribution of + oligodendrocytes. B) Marked disappearance of oligodendrocytes from demyelinated CC area.

3.3.2 Microglia/macrophages

A marked increase in the counts of MAC 3 + Mi/Ma (figure 10) was found in demyelinating mice groups on cuprizone diet. The Mi/Ma recruitment was specifically located within the CC area (figure 9). There is a statistically significant increase ($p<0.05$)

in the counts of Mi/Ma for both groups on cuprizone (week 3 and week 6) compared to untreated control groups (week 0).

Mi/Ma recruitment is associated with the occurrence of significant demyelination and oligodendrocyte depletion, which are the major changes in the cuprizone model (Blakemore 1973a; Ludwin, 1978; Hiremath *et al*., 1998; Morell *et al*., 1998).

Our results show a reduction in Mi/Ma counts at week 6 compared to week 3. This finding was not expected and is different from studies (Hiremath *et al*., 1998; Morell *et al.*, 1998) suggesting peak accumulation of Mi/Ma at week 6. However, our finding coincides with the peak score for demyelination and at the same time with initial spontaneous oligodendrocyte regeneration.

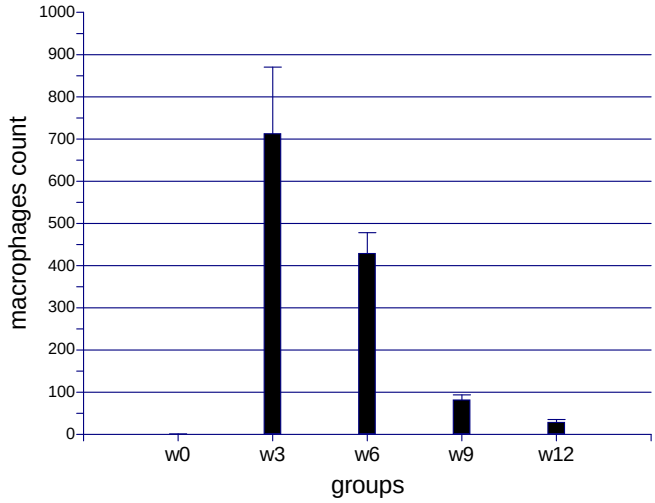


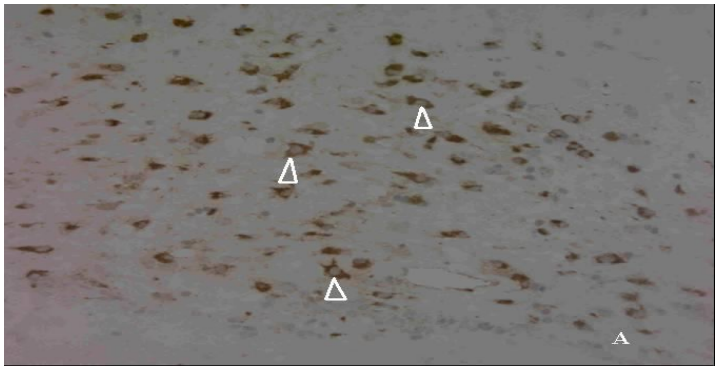
Figure (9) microglia/macrophage counts

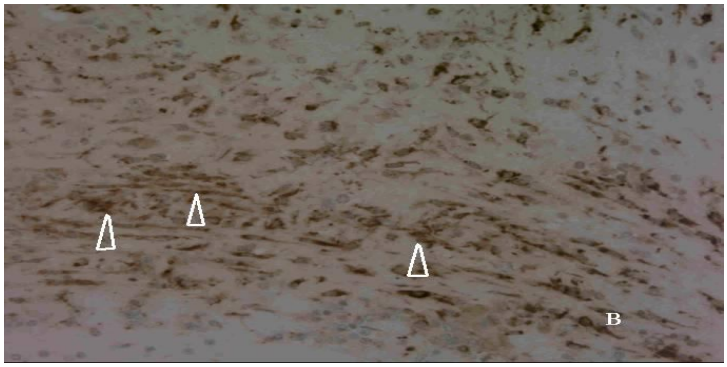
Counts are plotted as mean with SEM of MAC 3 + Mi/Ma.

A significant (P<0.01) accumulation of Mi/Ma occurred during demyelination at week 3 and week 6 and then Mi/Ma average counts markedly decreased to near control groups during remyelination.

The replacement of the cuprizone diet by normal chow caused a rapid disappearance of Mi/Ma from the remyelinating CC (figure 10). Mi/Ma counts continue to decrease upon remyelination and eventually reach numbers near the control group. A significant ($p<0.05$) decrease was found in both remyelinating groups at week 9 and week 12 when compared to demyelinated groups at week 6 (figure 9).

Another indicator for the activity of Mi/Ma was found at the LFB-PAS-stained sections. Here Mi/Ma cells have a PAS eosinophilic staining inside their cytoplasm. The PAS stains degraded myelin inside cytoplasm of Mi/Ma (Blakemore, 1973a; Brück *et al.*, 1996).





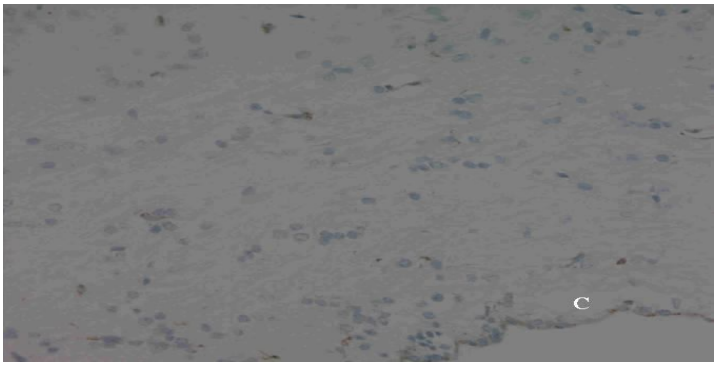


Figure (10) Histology of MAC 3 + microglia/macrophages

Huge accumulation of MAC 3 + Mi/Ma during time course of demyelination, A) at week 3 and B) at week 6. NOTE that Mi/Ma are largely accumulating at CC, arrows. C) Remyelination is associated with significant reduction in Mi/Ma counts to values of the control counts.

3.3.3 Astrocytes

GFAP + Astrocyte glial fiber density

A different method was used to evaluate the role of astrocytes (figure 12). The quantity of GFAP + astrocytes was estimated as a density by using a morphometric grid. First, we counted the number of + astrocytes that are in direct contact with the lines on the grid. Then we calculate the density by dividing the number of lines attached to Astrocytes by the total number of lines on the grid.

During cuprizone feeding there was a continuous increase in the density of astrocytes. This increase is associated with demyelination. Although a continuous increase in the density of astrocytes occurs during demyelination, only week 6 has a highly significant increase when compared to untreated control group (figure 11).

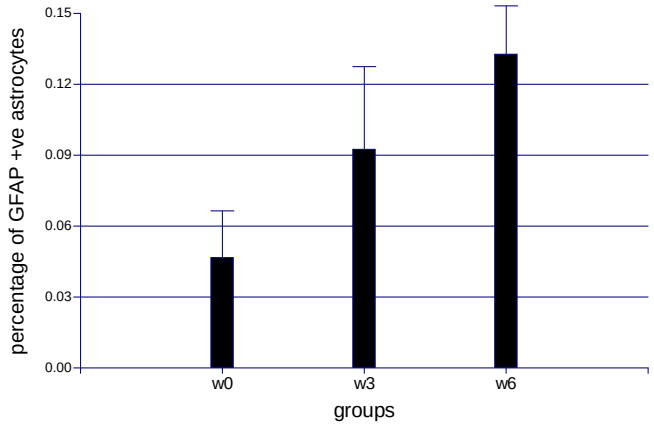


Figure (11) Density of the GFAP + astrocytes

Mean and SEM of the density of GFAP + astrocytes.

Astrocytosis is evident during demyelination. A significant ($P<0.05$) accumulation is found at week 6 compared to control at week 0.

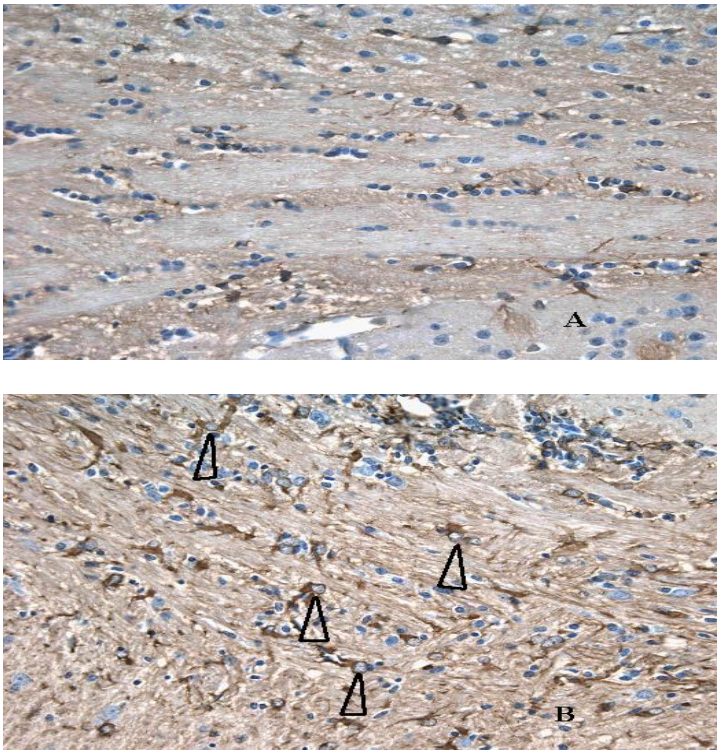


Figure (12) Histology of GFAP + astrocytes

Astrocyte density is increasing at the demyelinated CC during the progress of demyelination. A) Week 0 (control) shows normal astrocytes density. B) At week 6, an increase in astrocytes density is seen (arrows).

4. DISCUSSION

The cuprizone model is a useful addition to experimental models in neuropathology. It illustrates the possibility of conducting reproducible, well-localized and reversible types of demyelinating lesions. It gives the opportunity to make experimental-based predictions on some important aspects of myelin damage and repair.

Our results demonstrate that both conventional and IHC myelin scores clearly identify the time course of demyelination and remyelination. MBP appears to disappear faster during demyelination and accumulates earlier during remyelination when compared to PLP. But both display predictable myelin changes during demyelination and remyelination. We have examined the role of cellular response. Our results show important spontaneous oligodendrocyte regeneration and Mi/Ma disappearance at week 6, while mice were still on the cuprizone diet. This finding is interesting and gives us an opportunity to investigate cellular response in the face of a continuous insult. Evaluation of astrocytes showed a moderate increase in their density. Their specific role should be further evaluated.

4.1. GENERAL CHARACTERISTICS

The general characteristics of the cuprizone model are well documented in the literature. The design avoids systemic side effects on mice, but still produces efficient demyelination and successful remyelination. Mild weight fluctuations occur at the beginning of the cuprizone treatment. This might be due to changing the mice from solid chow to powdered chow needed for the preparation of the cuprizone diet (Hiremath *et al.*, 1998; Levine *et al.*, 2001; Stidworthy *et al.*, 2003).

Myelin assessment in this model and in our study showed unique and uniform changes within a specific *time course*. These changes follow the designed time points for an acute

type of demyelination (week 0, week 3, week 6, week 9 and week12). This time course is well reflected in the LFB-PAS score and the IHC score.

4.2 DEMYELINATION and REMYELINATION

4.2.1 Conventional myelin stain

LFB-PAS stain is used in routine histology laboratory for the examination of the myelin sheath changes (Matsushima and Morell, 2001; Brück *et al.,* 2003). The LFB-PAS scoring method in this study supports the advantages of the score. The score is simple, rapid and reproducible, which allow rapid screening of large areas.

Examination of the LFB-PAS stain showed a continuous increase in the score that is directly proportional to the degree of myelin loss. The reverse occurred during remyelination with continuous decrease in the score associated with the progress of myelin formation. This pattern is similar to other studies (Hiremath *et al.*, 1998; Morell *et al.,* 1998). The LFB-PAS score is in accordance with IHC staining for myelin proteins and with the more quantitative morphometric studies using an EM. (Masson *et al.,* 2001b; McMahon *et al.,* 2001; Arnett *et al.,* 2003). In LFB-PAS score the peak demyelination is delayed; in our study it is at week 6, and in literature the peak demyelination occurs after 5 or 6 weeks on cuprizone (Hiremath *et al.*, 1998; Arnett *et al.*, 2001). Later on, when mice return to normal chow, significant remyelination involve the majority of the CC (Mason *et al.*, 2000b; 2001a).

An accurate understanding and interpretation of the LFB-PAS score includes the consideration of the following points. LFB-PAS stain continues to give positive signal until the degraded myelin is completely cleared from the lesion area. On remyelination no positive stain is detected until enough myelin has been accumulated (Mason *et al.,* 2001a). Evaluation of the stain is subjective (may be affected by inter-observer and intra-observer

variation). The score is unable to detect early and small changes in myelin, as a result the score has a time delay in detecting myelin changes when compared to EM. This time delay has a significant impact on the score. It is important for proper interpretation of the score, for comparison of the score with other morphometric methods, and for correlation of the score with other molecular and cellular parameters. An additional important limitation is the biologic variability; there is some variation in myelination even in the untreated controls (Stidworthy *et al.,* 2003).

The result of LFB-PAS score should be validated and confirmed by other morphometric method. EM is more suitable for accurate quantitative measurements (Matsushima and Morell, 2001; Masson *et al.,* 2001b; Stidworthy *et al.,* 2003).

4.2.2 IHC stain for specific myelin proteins

IHC is widely used in detecting specific myelin proteins and large number of other antigens. Here we applied IHC to major myelin proteins, PLP and MBP. Qualitative measurement of these myelin-specific proteins was performed by assessment of IHC-stain intensity and applying a scoring method similar to that of LFB-PAS score.

The IHC score for myelin proteins has confirmed the sequence of events, which was reported by the LFB-PAS score.

Qualitative assessment of the PLP and MBP myelin staining has a clear capacity to differentiate between total myelination (total myelination includes: normal myelination in the control group or complete successful remyelination in groups returned to cuprizone-free diet for another 6 weeks) and total demyelination (groups on cuprizone diet for 6 weeks).

Our IHC stain score shows an earlier loss of MBP compared to PLP during demyelination (see figure 2 and figure 5 in result section). Again, during remyelination

MBP accumulated faster than PLP. This might be due to the assumption that MBP is more sensitive to damage and appears as an early marker for myelin damage. Successful remyelination is thought to be similar to normal embryonic myelination with early accumulation of MBP (Schwob *et al.,* 1985, Jurevics *et al.,* 2001).

Although the IHC-stain score confirms a specific pattern of myelin pathology during the cuprizone treatment, still many important points are required for the proper use and interpretation of the score.

The score depend on subjective evaluation of the stain intensity. It has a limited ability to detect small difference in myelination occurring over short time points (Matsushima and Morell, 2001; Masson *et al.,* 2001b; Stidworthy *et al.,* 2003). This lead to a time delay similar to that of the LFB-PAS score. There is also a variation in the IHC stain between experimental animals within the same group, as indicated by the high SEM in some groups. These variations occur in both MBP and PLP IHC-stains (figure 2 and Figure 5). One explanation for this variation in staining pattern is that MBP is produced in large amount when cells return to their normal development state and then MBP undergoes continuous anabolism and catabolism (Ludwin and Sternberger, 1984).

Therefore, some researchers have applied more quantitative methods for the evaluation of the myelin proteins. Still they found it difficult to obtain reliable quantitative results because of the high variability in staining within the examined groups (Armstrong *et al.,* 2002).

Other researchers have studied demyelination and remyelination by a number of myelin specific biochemical markers. Myelin associated glycoprotein (MAG) (Ludwin and Sternberger, 1984) 2-3-cyclicnucleotide 3-phosphodiesterase (CNPase) (Arnett *et al.,* 2001) Rip (early marker of remyelination) and myelin oligodendrocyte glycoprotein

(MOG) (Armstrong *et al.,* 2002) or measuring the quantity of MBP (western blot technique) (Jurevics *et al.,* 2001; 2002) and all confirmed the pattern of demyelination and remyelination in the cuprizone model).

4.3 CELLULAR RESPONSE

4.3.1 Oligodendrocytes

Oligodendrocyte degeneration and regeneration is directly associated with demyelination and successful remyelination.

Our data from the morphometric analysis (counting positive cells by ISH for PLP mRNA) show a highly significant disappearance of oligodendrocytes from the CC upon cuprizone intoxication for 3 weeks. Oligodendrocyte disappearance is associated with demyelination. This relation is lost at week 6. At this time point, myelin score shows the highest demyelination while oligodendrocyte counts show a spontaneous initial mild regeneration of PLP mRNA +ve oligodendrocytes. This regeneration occurred while mice were still on cuprizone diet, a finding that was also present in many studies (Blakemore, 1973a; Ludwin, 1978; Cammer and Zhang, 1999; Mason *et al.,* 2004). Oligodendrocyte recovery at week 6 is associated with recovery in many myelin-related m RNA species (Armstrong *et al.,* 2002; Jurevics *et al.,* 2002) and in genes regulating oligodendrocytes and myelin (Jurevics *et al.,* 2001; Jurevics *et al.,* 2002) while mice are still on the cuprizone diet.

This initial recovery is a rapidly progressive process if cuprizone was removed from the diet, which allows for restoration of high numbers of oligodendrocytes reaching levels near those of the control group at the 12-week time point (6 weeks on normal chow).

Oligodendrocyte degeneration; Oligodendrocyte damage in this model is an important primary event, which is followed by disintegration and removal of myelin proteins

(Cammer and Zhang, 1999). In addition, genes and mRNA species related to the myelin sheath underwent a rapid down-regulation prior to myelin protein degeneration (Jurevics *et al.,* 2001).

Fate of oligodendrocytes; Many assumptions were made in the literature. However, the main hypothesis is that the oligodendrocytes are specifically insulted during cuprizone intoxication and appear to be largely depleted by apoptosis. This view is based on the following points: *first* no apoptotic oligodendrocytes in the untreated control group (Mason *et al.,* 2000a; 2000b), *second* no apoptotic death of Mi/Ma or astrocytes during cuprizone intoxication (Arnett *et al.,* 2002). Beside the suggestion of the disappearance of the majority of oligodendrocytes by apoptosis, there are other possibilities for their disappearance, which include death by necrosis (Ludwin and Johnson, 1981; Mason *et al.,* 2000b) or dedifferentiation (Mason *et al.,* 2000b).

Source of remyelinating oligodendrocyte: The source of the remyelinating mature oligodendrocytes is not clear. An earlier assumption was that remyelinating oligodendrocytes are derived from proliferating dedifferentiated or residual surviving oligodendrocytes. However, many studies (Ludwin, 1994; Keirstead and Blakemore, 1999; Matsushima and Morell, 2001) suggest that remyelinating oligodendrocytes are derived from oligodendrocyte progenitor cells, which are either locally resident or recruited from other areas of the brain (Ludwin, 1979; Morel *et al.,* 2001).

Morphological and IHC evidence from many studies support the view that oligodendrocyte progenitors could be the main source for the remyelinating mature oligodendrocytes (Blakemore 1973b; Ludwin, 1978; Matsushima and Morell, 2001; Brück *et al.,* 2003). Those progenitor cells have multi-potential stem-cell-like properties (Ludwin, 1979b; Cammer and Zhang, 1999; Armstrong *et al.,* 2002). Thus they migrate, differentiate

and ultimately accumulate at specific lesion sites (Mason *et al.*, 2000a; 2000b). An additional support to the role of progenitors is the relative inability of residual surviving mature oligodendrocytes to proliferate (Mason *et al.*, 2000b).

Inflammatory mediators and oligodendrocytes; Accumulating evidence in literature indicates that control of oligodendrocyte proliferation, survival and regeneration depends on two important factors. One is the growth factor in the surrounding environment. The other is the bidirectional signal transfer and interaction between oligodendrocytes and axons (McMorris and McKinnon, 1996; Arnett *et al.*, 2001; Althaus, 2004).

4.3.2 Microglia/macrophages

Mi/Ma are the main cells recruited during cuprizone intoxication (Hiremath *et al.*, 1998; Morell et al., 1998; McMahon *et al*., 2001).

In this study a large number of MAC-3 +ve Mi/Ma accumulated at the lesion site. These results clearly support a major role for the Mi/Ma in the cellular response to the cuprizone intoxication. Furthermore, during cuprizone intoxication, Mi/Ma recruitment has a temporal and spatial correlation to other main changes, which include demyelination and oligodendrocyte disappearance.

Their role is also evident during examination of the LFB-PAS stain. The finding of an intra-cytoplasmic eosinophilic deposit within the Mi/Ma indicates an increase in phagocytic activity to remove myelin degradation products (Bitsch et al., 1997; Arnett et al., 2002, Brück et al., 2003).

An increase in phagocytic activity is also evident in ultrastructural studies (Blakemore, 1973a; Ludwin, 1978; Komoly *et al*., 1987) and by the increase in lysozymes which are markers of phagocytosis (Jurevics *et al.*, 2001; 2002).

Peak accumulation;, During the demyelination period, our data showed a moderate decrease in Mi/Ma average counts at week 6 when compared to week 3, which is not a statistically significant decrease. This finding is different from many studies reporting a peak value of Mi/Ma recruitment to occur at week 6.

On review, most studies report that Mi/Ma gradually accumulates between week 1 and week 6. Week 6 should have the highest count and it should be higher than the week 3 count (Hiremath *et al.*, 1998; Morel *et al.,* 1998; Masson *et al.,* 2004). This point is further complicated by studies referring to the peak accumulation as a range occurring from week 4 to week 6 (Matsushima and Morell, 2001; Masson *et al.,* 2004), or reducing the range to occur between week 4 and week 5 (Masson *et al.,* 2000a) or limiting it to single point at week 5 (Arnett *et al.,* 2001).

This difference raises important points. First, a limitation of the counting method, especially when counting largely crowded Mi/Ma population. Second, there is a need to improve efficiency and reproducibility of the counting methods. Third, there is a need for the evaluation of Mi/Ma and oligodendrocyte counts at smaller time intervals.

It is still interesting that the beginning of the decrease in the Mi/Ma counts occur simultaneously with the beginning of spontaneous oligodendrocytes recruitment into a totally demyelinated CC by conventional and IHC stains.

Source of recruited Mi/Ma; Many studies found that Microglia/ macrophages are proliferating (undergoing mitotic activity) (Ludwin, 1979 b; McMahon *et al.,* 2002). This finding has strengthened the view that their main source is the recruitment and proliferation of local brain-resident microglia (Matsushima and Morell, 2001).

In addition, one detailed study confirms the presence of few peripheral macrophages among large numbers of local microglia (Jurevics *et al.,* 2002, McMahon *et al.,* 2002).

Mi/Ma count is directly related to all major changes in the cuprizone model and is an important subject for further evaluation.

4.3.3 Astrogliosis

The density of GFAP-positive astrocytes demonstrates that astrogliosis is an important element among the cellular response to cuprizone intoxication (Komoly *et al.,* 1987). However, its role is not as pronounced as the role of Mi/Ma (Hiremath *et al.,* 1998; Morell *et al.,* 1998). Astrocytes are proliferating. They show an increase in mitotic activity during response to cuprizone treatment (Ludwin, 1978; 1979b). Apart from mild morphological change (swelling), they were well preserved (Ludwin, 1978).

Growth factors and astrocytes seem to play a complex role during demyelination and remyelination. They are interacting with oligodendrocytes. They affect oligodendrocyte function; they may have a direct stimulating role on myelin regeneration by oligodendrocytes (Komoly *et al.,* 1992).

These reactive astrocytes in the cuprizone model are suggested to come from the resident astrocytes. They were not derived from bone marrow (McMahon *et al.,* 2002)

4.4 EFFICENCY OF THE COUNTING METHOD

Manual counts are widely used in the cuprizone model (Morell *et al*., 1998, Hiremath *et al*., 1998; Arnett *et al*., 2002; Masson *et al*., 2003).

Manual counts are intended to reflect absolute values of cells under investigation. Manual counts confirm a uniform pattern of changes occurring at a specific time points during this experiment. Nevertheless, counting methods encounter certain limitations. Some degree of variability is present at two levels.

One is the variation between experimental animals of the same group. This might result from biological variability. Some researchers have reported that there are anatomical

variations in the counts in different regions of the CC (Masson *et al.,* 2003). They also noticed a variable counts occurring at different time points. A variable count is prominent at week 4. Later on, less variable count is seen at week 9 (Masson *et al.,* 2003). They suggested that there is variability in remyelination in different areas of the CC and between different time points.

The second level is the variation in the average values reported from different counting methods. Counting techniques are well characterized in literature. Thus, only the positive signal, which is associated with a counterstained nucleus, is counted. Then the average for all mice in each group was calculated. The only difference is in defining the part of the CC used for performing the counts. In this study, we included the whole area of the CC on both sides of the midline. Many studies have limited their counts in the medial region of the CC. They choose the area from the midline of the CC to a line dividing the fornix area (Morell *et al*., 1998; Arnett *et al*., 2002; Masson *et al*., 2004). However, all methods gave a similar average values.

Lastly, the most important factor remains in the reported difficulty when performing manual counts in areas containing very high populations of cells. This is due to the high stain density resulting from the closely packed cells (McMahon *et al*., 2002; Masson *et al*., 2003).

Many studies used automated methods to overcome this limitation such as signal measurement with stereologer or densitometry system. All resulted in the confirmation of the general trends of manual counts and still facing some limitations (Armstrong *et al*., 2002)

Despite these limitations, the manual count is still the most representative way for reporting absolute values of cellular response (Hiremath *et al.,* 1998).

4.5 POTENTIAL FOR REMYELINTION IN THE CUPRIZONE MODEL and MS

4.5.1 Conventional and IHC myelin scores

Both conventional and IHC myelin stains reflect a stable histological picture of demyelination and remyelination occurring in a predictable pattern.

In general, remyelination in experimental animals should be similar to developmental myelination (Cedric *et al.*, 1997). The regenerated myelin sheaths are similar to the normal myelin sheath in the content and the distribution of many myelin components (Ludwin and Sternberger, 1984; Matsushima and Morell, 2001). Therefore it is possible to investigate cellular and sub-cellular environment permitting successful remyelination in the cuprizone model that might be relevant to inflammatory demyelinating diseases in humans, including MS. Although remyelination is present in MS, it is different from remyelination in the cuprizone model. It has a relapsing remitting course, the site and time of remyelination is still unpredictable and remyelination in MS markedly decreases in late chronic stages of the disease.

4.5.2 Oligodendrocytes

The availability of oligodendrocytes and their progenitors is an important factor for successful remyelination in experimental models and in MS. Moreover, the depletion of oligodendrocytes and their progenitors is a major limiting factor for successful remyelination (Blakemore, 1974; Ludwin, 1980; Johnson and Ludwin, 1981; Miller *et al.*, 1996; Mason *et al.*, 2001a; Matsushima and Morell, 2001).

Although rapid regeneration of oligodendrocyte progenitors is detrimental to successful remyelination, there are still other contributing factors. An optimal well-balanced environment is needed (i.e. remyelination permissive environment). This environment includes gene products, growth factors, immune modulators and metabolic enzymes. In

addition, reactive axons must be available at lesion site (Ravi *et al.,* 1994; Billinghurst *et al.,* 1998; Mason *et al.,* 2001a; 2004).

4.5.3 Microglia/macrophages

The increased interest in Mi/Ma is due to their major contribution in the cellular response during the cuprizone intoxication. This rapid and huge accumulation during demyelination suggests that Mi/Ma may have a damaging effect, which exacerbates demyelination (Ludwin, 1994; Hiremath *et al.,* 1998; Mason *et al.,* 2001b; Arnett *et al.,* 2003).They play a significant role in clearing myelin debris, but their role is not that simple. They appear to be key mediators for inflammatory changes in this model. They are related to complex overriding and sometimes opposing functions (Matsushima and Morell, 2001; Armstrong *et al*., 2002).

Beneficial functions are also reported. Mi/Ma recruitment and accumulation is directly associated with the up-regulation of myelin genes and inflammatory genes. Both play important roles as myelinogenic factors (facilitate and enhance myelin formation) (Arnett *et al.,* 2001; McMahon *et al.,* 2001; Jurevics *et al*., 2002; Arnett *et al.,* 2003). Furthermore, these factors might have a direct interaction with oligodendrocyte. This relation may have an effect on oligodendrocyte recovery (Arnett *et al.,* 2001; Mason *et al.,* 2001a; Armstrong *et al.,* 2002). There is spontaneous remyelination in Ms Lesions which are still having an ongoing inflammatory process.

4.5.4. Axonal interaction

The process of remyelination is largely dependent on the functional interaction between myelin forming cells and axons (Brück *et al.,* 2003; Ludwin, 1994).

In the acute model of intoxication, a significant number of axons were almost totally remyelinated in a predictable time pattern (Ludwin, 1979b; Johnson and Ludwin, 1981;

Mason *et al*., 2001a). In contrast, axonal capacity for remyelination was decreased in chronic demyelination when compared to acute demyelination (Ludwin, 1980).

The interaction between oligodendrocytes and axons is thought to cause many important changes. *First*, remyelinating oligodendrocyte numbers may be determined by axonal interaction (Armstrong *et al*., 2002). *Second* a decrease in axonal remyelination response, without any histologically obvious abnormality might be caused by demyelination (Blakemore 1973a; Ludwin, 1980; Mason *et al.*, 2001a). *Third*, the formation of chronically demyelinated lesions induced by cuprizone appears to be the result of oligodendrocyte depletion within the lesion and not due to the inability of chronically demyelinated axons to be remyelinated (Mason *et al.*, 2004).

4.6. CONCLUSION

The cuprizone model could be used for the examination of some important pathological changes observed in human demyelinating diseases. Our data confirm the occurrence of a totally reversible demyelinating lesion. This lesion is reproducibly localized within the CC area.

There are some limitations. Myelin scores are reliable and simple but only in a qualitative approach. Different studies report similar cellular counts. Still variations exist in the absolute values. This depends on the method used, measuring absolute counts or measuring density of cells.

Therefore, careful attention must be paid to the criteria used in the myelin scores and the manual counts. It is also important to correlate all major changes with more quantitative EM measurements. In addition, evaluating demyelination parameters at more time points might be important for further studies.

This model gives us new possibilities to study the factors that are in favor of remyelination.

5. ABSTRACT

This work on the cuprizone model was intended to establish the cuprizone mouse model and to characterize pathologic changes that are relevant to human demyelinating diseases.

This animal model is characterized by extensive remyelination after cuprizone removal from the diet. Histological analysis of various myelin-related components and cells was correlated to demyelinating and remyelinating lesions in the corpus callosum. Extensive demyelination was induced by cuprizone administration for 6 weeks. Demyelination was directly associated with disappearance of oligodendrocytes and strong microglia/macrophage accumulation. Successful remyelination follows termination of the cuprizone diet and feeding on normal chow for additional 6 weeks. Remyelination was significantly associated with oligodendrocyte repopulation and reduction of microglia/macrophages.

Our results reveal several points: One is the initial spontaneous oligodendrocyte regeneration at week 6. More interesting is the occurrence of both oligodendrocyte regeneration and microglia/macrophage disappearance at week 6 while mice are still on the cuprizone diet. The last point is that the qualitative myelin scores are less sensitive than the EM in detecting early and small changes in remyelination.

In conclusion, the model is useful for a wide variety of scientific questions addressing important aspects of the pathology of human demyelinating disease such as multiple sclerosis.

6. REFERENCES

Akiyama K, Lehinose S, Omori A, Sakurai Y, Asou H (2002): Study of expression of myelin basic proteins (MBPs) in developing rat brain using a novel Ab reacting with four major isosforms of MBP. J Neurosci Res 68(1), 19-28

Albrechtsen M, von Gerstenberg AC, Bock E (1984): Mouse monoclonal antibodies reacting with human brain glial fibrillary acidic protein. J Neurochem 42(1), 86-93

Althaus HH (2004): Remyelination in MS: a new role for neurotrophins? Prog Brain Res 146, 415-32

Armstrong RC, Le TQ, Frost EE, Borke RC and Vana AC (2002): Absence of Fibroblast Growth Factor 2 Promotes Oligodendroglial Repopulation of Demyelinated White Matter. J Neurosci 22(19), 8574-8585

Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK and Ting JPY (2001): TNFα promotes proliferation of oligodendrocyte progenitors and remyelination. Nat Neurosci 4(11), 1116-1122

Arnett HA, Hellendall RP, Matsushima GK, Suzuki K, Laubach VE, Sherman P and Ting JPY (2002): The Protective Role of Nitric Oxide in a Neurotoxicant-Induced Demyelinating Model. J Immunol 168, 427-433

Arnett HA, Wang Y, Matsushima GK, Suzuki K and Ting JPY (2003): Functional Genomic Analysis of Remyelination Reveals Importance of Inflammation in Oligodendrocyte Regeneration. J Neurosci 23(30), 9824-9832

Arnett HA, Fancy SPJ, Alberta JA, Zhao C, Plant SR, Kaing S, Raine CS, Rowitch DH, Franklin RJM, Stiles CD (2004): bHLH Transcription Factor Olig1 Is Required to Repair Demyelinated Lesions in the CNS. SCIENCE 306, 2111-2115

Backe E, Schwartinng R, Gerdes J, Ernst M, Stein H (1991): Ber-MAC3: new monoclonal antibody that defines human monocyte/macrophage differentiation antigen. J Clin Pathol 44(11), 936-45

Bakker DA and Ludwin SK (1987): Blood-brain barrier permiability during Cuprizone-induced demyelination implications for the pathogenesis of immune-mediated demyelinated diseases. J Neurol Sci 78, 125-137

Barbarese E, Barry C, Chou CH, Goldstein DJ, Nakos GA, Hyde-DeRuyscher R, Scheld K, Cerson JH (1988): expression and localization of myelin basic protein in oligodendrocyte and transfected fibroblasts. J Neurochem 51(6), 1737-45

Barkovich AJ (2000): Concepts of Myelin and Myelination in Neuroradiology. American Journal of Neuroradiology 21, 1099-1109

Battistini L, Piccio L, Rossi B, Bach S, Galgani S, Gasperini C, Ottoboni L, Ciabini D, Caramia MD, Bernardi G, Laudanna C, Scarpini E, McEver RP, Butcher EC, Borsellino G, Constantin G (2003): CD8+ T cells from patients with acute MS display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1. Blood 101(12), 4775-82

BAUMANN N AND PHAM-DINH D (2001): Biology of Oligodendrocyte and Myelin in the Mammalian Central Nervous System. PHYSIOLOGICAL REVIEWS 81(2), 871-910

Bennetto L and Scolding N (2004): INFLAMMATORY/POST-INFECTIOUS ENCEPHALOMYELITIS. J Neurol Neurosurg Psychiatry 75, i22-8

Bessert DA and Skoff RP (1999): High-resolution In situ Hybridization and TUNEL staining with free-floating Brain Sections. J Histochem Cytochem 47(5), 693-701

Billinghurst LL, Taylor RM, Snyder EY (1998): Remyelination: cellular and gene therapy. Semin Pediatr Neurol 5(3), 211-28

Bitsch A, da Costa C, Bunkowski S, Weber F, Rieckmann P, Brück W (1997): Identification of macrophage populations expressing tumor necrosis factor-alpha mRNA in acute MS. Acta Neuropathol (Berl) 95(4), 373-7

Bitsch A, Kuhlmann T, Stadelmann C, Lassmann H, Lucchinetti C, Brück W (2001): A longitudinal MRI study of histopathologically defined hypointense MS lesions. Ann Neurol 49(6), 793-6

Blakemore WF (1973a): Demyelination of the Superior Cerebellar Peduncle in the Mouse Induced by Cuprizone. J Neurol Sci 20, 63-72

Blakemore WF (1973b): Remyelination of the Superior Cerebellar Peduncle in the Mouse Following Demyelination Induced By Feeding Cuprizone. J Neurol Sci 20, 73-83

Blakemore WF (1974): Remyelination in the Superior Cerbellar Peduncle in Old Mice Following Demyelination induced by Cuprizone. J Neurol Sci 22, 121-126

Blakemore WF, Smith PM, Franklin RJ (2000): Remyelinating the demyelinated CNS. Novartis Found Symp 231, 289-98 discussion 298-306

Brady ST, Witt AS, Kirkpatrick LL, de Waegh SM, Readhead C, Tu P, and Virginia M.-Y. Lee VMY (1999): Formation of Compact Myelin Is Required for Maturation of the Axonal Cytoskeleton. The Journal of Neuroscience 19(17), 7278-7288

Breitschopf H, Suchanek J, Gould RM, Coulman DR, and Lassmann H (1992): In situ hybridization with digoxigenin-labelled probes: sensitive and reliable detection method applied to myelinating rat brain. Acta Neuropathol 84, 581-587

Brück W, Schmied M, Suchanek G, Brück Y, Breitschopf H, Poser S, Pidddlesden S, and Lassmann H (1994): Oligodendrocytes in the Early Course of MS. Ann Neurol 35, 65-73

Brück W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA and lassman H (1995): Monocyte/Macrophages differentiation in Early MS Lesions. Ann Neurol 38, 788-796

Brück W, Sommermeier N, Bergmann M, Zettl U, Goebel HH, Kretzshmar HA, and Lassmann H (1996): Macrophages in MS. Immunobiol 159, 588-600

Brück W, Stadelmann C (2003): Inflammation and degeneration in MS. Neurol Sci 5, S265-7

Brück W, Kuhlmann T, Stadelmann C (2003): Remyelination in MS. J Neurol Sci 206, 181-185

Cammer W (1999): the neurotoxicant, cuprizone, retards the differentiation of oligodendrocytes in vitro. J Neurol Sci 168, 116-120

Cammer W and Zhang H (1999): Atypical glial cells in demyelinated and hypomyelinated mouse brains. Brain Res 837, 188-192

Carvalho-Tavares J, Hickey MJ, Hutchison J, Michaud J, Sutcliffe IT, Kubes P (2000): A Role for Platelets and Endothelial Selectins in Tumor Necrosis Factor-α –Induced Leukocyte Recruitment in the Brain Microvasculature. Circ Res 87(12), 1141-8

Chitnis T, Khoury SJ (2003): 20. Immunologic neuromuscular disorders. J Allergy Clin Immunol 111(2 suppl), 659-68

Christensen T (2006): The Role of EBV in MS Pathogenesis. The International MS Journal 13, 52–57

Compston A (1996): Remyelination of the central nervous system. Mult Scler 1(6), 388-92

Dale RC, Branson JA. (2005): Acute disseminated encephalomyelitis or MS: can the initial presentation help in establishing a correct diagnosis? Arch Dis Child 90(6), 636-9

Damoiseaux JG and Tervaert JW (2002): The definition of autoimmune disease: are Koch's postulates applicable? Neth J Med 60(7), 266-8

de Vries H, de Jonge JC, Schrage C, van der Haar ME (1997): Differential and Cell Development-Dependent localization of Myelin mRNAs in oligodendrocytes. J Neurosci Res 47, 479-488

Debus E, weber K, Osborn M (1983): Monoclonal antibodies specific for glial fibrillary acidic (GFA) protein and for each of the neurofilament triplet polypeptides. Differentiation 25(2), 193-203

Degaonkar MN, Jayasundar R, Jagannathan NR (2002): Sequential diffusion-weighted magnetic resonance imaging study of lysophosphatidyl choline-induced experimental demyelinating lesion: an animal model of MS. J Magn Reson Imaging 16(2), 153-9

Dyment DA, Sadnovich AD and Ebers GC (1997): Genetics of MS. Hum Mol Genet 6(10), 1693-1698

Emerson MR, Biswas S, Le Vine SM (2001): cuprizone and piperonyl butoxide, propsed inhibitors of T-cellfunction, attenuate experimental allergic encephalomyelitis in SJL mice. J Neuroimmunol 119, 205-213

Engelhardt B, Vestweber D, and Hallmann R, Schulz M (1997): E- and P-selectin are not involved in the recruitment of inflammatory cells across the blood-brain barrier in experimental autoimmune encephalomyelitis. Blood 90(11), 4459-72

Franklin RJ (2002): Why does remyelination fail in MS? Nat Rev Neurosci 3(9), 705-14

Frohman EM, Filippi M, Stuve O, Waxman SG, Corboy J, Phillips JT, Lucchinetti C, Wilken J, Karandikar N, Hemmer B, Monson N, De Keyser J, Hartung H, Steinman L, Oksenberg JR, Cree BA, Hauser S, Racke MK (2005) : Characterizing the mechanisms

of progression in MS: evidence and new hypotheses for future directions. Arch Neurol 62(9), 1345-56

Fujita N, Ishiguro H, Sato S, Kurihara T, Kuwano R, Sakimura K, Takahashi Y and Miyatake T (1990): Induction of myelin-associated glycoprotein mRNA in experimental remyelination. Brain Res 513(1), 152-155

Fushimi S and Shirabe T (2002): The reaction of glial progenitor cells in remyelination following ethidium bromide-induced demyelination in the mouse spinal cord. Neuropathology 22(4), 233-42

Gendelman HE, Pezeshkpour GH, Pressman NJ, Wolinsky JS, Quarles RH, Dobersen MJ, Trapp BD, Kitt CA, Aksamit A, Johnson RT (1985): A quntitation of myelin-associated glycoprotein and myelin basic protein loss in different demyelinating disease. Ann Neurol 18(3), 324-8

Gow A, Gragerov A, Gard A, Colman DR and Lazzarini RA (1997): Conservation of Topology, But Not Confirmation, of the proteolipid Proteins of the Myelin Sheath. J Neurosci 17(1), 181-189

Graca DL (1988): Multiple, demyelination and re-myelination in the central nervous system. Arq Neuropsiquiatr 46(3), 292-7

Greer JM, Lees MB (2002): Myelin proteolipid protein--the first 50 years. Int J Biochem Cell Biol 34(3), 211-5

Griffiths I, Klugmann M, Anderson T, Thomson C, Vouyiouklis D, Nave KA (1998): Current concepts of PLP and its role in the nervous system. Microsc Res Tech 41(5), 344-58

Guo L, Li Y, Lin H, Ji X, Li J, Que L, Zhang Y, Rong Y, Wang J (2004): Evaluation of a rat model of experimental autoimmune encephalomyelitis with human MBP as antigen. Cell Mol Immunol 1(5), 387-91

Hayashi K, Motoi M, Nose S, Horie Y, Akagi T, Ogawa K, Taguchi K, Mizobuchi K, Nishimoto A (1987): An immunohistochemical study on the distribution of glial fibrillary acidic protein, S-100 protein, neuron-specific enolase, and neurofilament in meduloblastoma. Acta Pathol Japn 37(1), 85-96

Hildebrand C, Remahl S, Persson H, Bjartmar C. (1993): Myelinated nerve fibers in the CNS. Prog Neurobiology 40(3), 319-84

Hiremath MM, suitor Y, Knapp GW, Ting JPY, Suzuki K, Matsushima GK (1998): Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice. J Neuroimmuno l 92, 38-49

Ho MK and Springer TA (1983): Tissue distribution, structural characterization, and biosynthesis of Mac-3, a macrophage surface glycoprotein exhibiting molecular weight heterogeneity. J Biol Chem 258(1), 636-42

Huang Y, Erdmann N, Peng H, and Zhao Y, Zheng J. (2005): The role of TNF related apoptosis-inducing ligand in neurodegenerative diseases. Cell Mol Immunol 2(2), 113-22

Hudson LD, Friedrich VL, Jr., Behar T, Dubois-Dalcq M and Lazzarini RA (1989): The Initial Events in Myelin Synthesis: Orientation of Proteolipid Protein in the Plasma membrane of cultured Oligodendrocytes. J Cell Biol 109, 717-727.

Jain D, Rajesh LS, Vasishta RK, Radotra BD, Banerjee AK (2006): Demyelinating disease simulating brain tumours: a histopathologic assessment of seven cases. Indian J Med Sci. 60(2), 47-52.

Jalabi W, Cerghet M, Skoff RP and Ghandour MS (2003): detection of oligodendrocytes in Tissue Sections Using PCR Synthesis of Digoxigenin-labeled Probes. J Histochem Cytochem 51(7), 913-919.

Johnson ES and Ludwin SK (1981): The Demonstration of recurrent Demyelination and Remyelination of axons in the central Nervous System. Acta Neuropathol (Berl) 53, 93-98

Jurevics H, Hostettler G, Muse ED, Sammond DW, Matsushima GK, Toews AD and Morell P (2001): Cerebroside synthesis as a measure of the rate of remyelination following cuprizone-induced demyelination in brain. J Neurochem 77, 1067-1076

Jurevics H, Largent C, Hostettler J, Sammond DW, Matsushima GK, Kleindienst A, Toews AD and Morell P (2002): Alterations in metabolism and gene expression in brain regions during cuprizone-induced demyelination & remyelination. J Neurochem 82, 126-136

Keirstead HS and Blakemore WF (1999): The role of oligodendrocytes and oligodendrocyte progenitors in CNS remyelination. Adv Exp Med Biol 468, 183-97

Kennedy PG (2004): Viral encephalitis: causes, differential diagnosis, and management. J Neurol Neurosurg Psychiatry 75 Suppl 1, i10-5

Kerschensteiner M, Stadelmann C, Buddeberg BS, Merkler D, Bareyre FM, Anthony DC, Linington C, Brück W, Schwab ME (2004a):Targeting Experimental Autoimmune Encephalomyelitis Lesions to a Predetermined Axonal Tract System Allows for Refined Behavioral Testing in an Animal Model of MS. Am J Pathol 164(4), 1455-69

Kerschensteiner M, Bareyre FM, Buddeberg BS, Merkler D, Stadelmann C, Brück W, Misgeld T, and Schwab ME (2004b): Remodeling of Axonal Connections Contributes to Recovery in an Animal Model of MS. JEM 200(8), 1027-1038

Kesterson JW and Carlton WW (1971): Histopathological and Enzyme Histochemical Observations of the Cuprizone-induced Brain Edema. Exp Mol Pathol 15, 82-96.

Kivisakk P, Mahad DJ, Callahan MK, Trebst C, Tucky B, Wei T, Wu L, Baekkevold ES, Lassmann H, Staugaitis SM, Campbell JJ, Ransohoff RM (2003): Human cerebrospinal fluid central memory CD4+ T cells: Evidence for trafficking through choroid plexus and meninges via P-selectin. Proc Natl Acad Sci 100(14), 8389-94

Komoly S, Jeyasingham MD, Pratt OE and Lantos PL (1987): Decrease in oligodendrocyte carbonic anhydrase activity preceding myelin degeneration in cuprizone induced demyelination. J Neurol Sci 79, 141-148.

Komoly S, Hudson LD, Webster HD and Bondy CA (1992): Insulin-like growth factor 1 gene expression is induced in astrocytes during experimental demyelination. Proc Natl Acad Sci 89, 1894-1898.

Kondo A, Nakno T and Suzuki K (1987): Blood-Brain Barrier permiability to horseradish peroxidase in twitcher and cuprizone-intoxited mice. Brain Res 425, 186-190.

Konola LT, Tyler BM, and Yamamura T, Lees MB (1991): Distribution of proteolipid protein and myelin basic protein in cultured mouse oligodendrocytes: primary vs. secondary cultures. J Neurosci Res 28(1), 49-64

Konola JT, Yamamura T, Tyler B, Lees MB (1992): Orientation of the myelin proteolipid protein C-terminus in oligodendroglial membranes. Glia 5(2), 112-21

Kristensson K, Holmes KV, Duchala CS, Zeller NK, Lazzarini RA, Dubois-Dalcq M. (1986): Increased levels of myelin basic protein transcripts in virus-induced demyelination. Nature 322(6079):544-7. Erratum in: Nature 323(6083), 91

Kruger GM, Diemel LT, Copelman CA, Cuzner ML (1999): Myelin basic protein isoforms in myelinating & remyelinating rat brain aggregate cultures. J Neurosci Res 56(3), 241-7

Kuhlmann T, Wendling U, Nolte C, Zipp F, Maruschak B, Stadelmann C, Siebert H and Brück W (2002): Differential regulation Phagocytosis by Macrophages/Microglia, Involvement of target Myelin, Fc Receptors and Activation by Intravenous Immunoglobulins. J Neurosci Res 67, 185-190

Lapointe BM, Herx LM, Gill V, Metz LM and Kubes P (2004): IVIg therapy in brain inflammation: etiology-dependent differential effects on leucocyte recruitment. Brain 127(12), 2649-2656

Lassmann H, Brück W, Lucchinetti C (2001): Heterogeneity of MS Pathogenesis: implications for diagnosis and therapy. Trends Mol Med 7(3), 115-21

Levine JM, Reynolds R, Fawcett JW (2001): The oligodendrocyte precursor in health and disease. Trends Neurolsci 24(1), 39-47

Love S. (1988): Cuprizone neurotoxicity in the rat: morphologic observations. J Neurol Sci 84(2-3), 223-37

Luccinetti CF, Brück W, and Rodriguez M, Lassmann H (1996): Distinct patterns of MS pathology indicate heterogenity in pathogensis. Brain Pathol 6(3), 259-74

Lucchinetti CF, Noseworthy JH, Rodriguez M (1997): Promotion of endogeneous remyelination in MS. Mult Scler 3(2), 71-5

Lucchinetti CF, Brueck W, Rodriguez M, Lassmann H (1998): MS: lessons from neuropathology. Semin Neurol 8(3), 337-49

Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M and Lassmann H (1999): A quantitative analysis of oligodendrocytes in MS lesions A study of 113 cases. Brain 122, 2279-2295

Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M and Lassmann H (2000): Heterogeneity of MS Lesions: Implications for the Pathogenesis of Demyelination. Ann Neurol 47, 707-717

Lucchinetti C, Brück W, Noseworthy J (2001): MS: recent developments in neuropathology, pathogensis, magnitic resonance imaging studies and treatment. Curr Opin Neurol 14(3), 259-69

Ludwin SK (1978): Central Nervous System Demyelination and Remyelination in the Mouse An Ultrastructural Study in Cuprizone Toxicity. Lab Invest 39(6), 597-612

Ludwin SK (1979a): The perineuronal Satellite Oligodendrocyte. Acta Neuropathol 74, 49-53

Ludwin SK (1979b): An autoradiographic study of cellular proliferation in Remyelination of the Central Nervous System. Am J Pathol 95(3), 683-96

Ludwin SK (1980): Chronic Demyelination Inhibits Remyelination in the Central Nervous System. Lab Invest 43(4), 382-387

Ludwin SK and Johnson ES (1981): Evidence for a Dying-Back Gliopathy in Demyelinating Disease. Ann Neurol 9(3), 301-305

Ludwin SK, Sternberger NH (1984): An Immunohistochemical Study of Myelin Proteins during Remyelination in the Central Nervous System. Acta Neuropathol (Berl) 63(3), 240-8

Ludwin SK (1994): Central Nervous System Remyelination Studies in Chronically Damaged Tissue. Ann Neurol supp 36, s143-s145

Lutton JD, Winston R, Rodman TC (2004): MS: etiological mechanisms and future directions. Exp Biol Med (Maywood) 229(1), 12-20.

Mason JL, Ye P, Suzuki K, Creole AJD and Matsushima GK (2000a): Insulin-like Growth Factor-1 Inhibits Mature Oligodendrocyte Apoptosis during Primary Demyelination. J Neurosci 20(15), 5703-5708

Mason JL, Jones JJ, Taniike M, Morell P, Suzuki K and Matsushima GK (2000b): Mature Oligodendrocyte Apoptosis Precedes IGF-1 Production and Oligodendrocyte Progenitor Accumulation and Differentiation during Demyelination/Remyelination. J Neurosci Res 61, 251-262

Mason JL, Langaman C, Morell P, Suzuki K and Matsushima GK (2001a): Episodic demyelination and subsequent remyelination within the murine central nervous system: changes in axonal caliber. Neuropath App Neurobiol 27, 50-58

Mason JL, Suzuki K, Chaplin DD and Matsushima GK (2001b): Interlukin-1β Promotes Repair of the CNS. J Neurosci 21(18), 7046-7062

Mason JL, Xuan S, Dragatsis L, and Efstratiadis A, Goldman JE (2003): Insulin-Like Growth Factor (IGF) Signaling through Type 1 IGF Receptor Plays an Important Role in Remyelination. J Neurosci, 23(20), 7710 –7718

Mason JL, Toews A, Hostettler JD, Morell P, Suzuki K, Goldman JE, Matsushima GK (2004): Oligodendrocytes and progenitors become progressively depleted within chronically demyelinated lesions. Am J Pathol 164(5), 1673-82

Mathis C, Hindelang C, LeMeur M, Borrelli E. (2000): A transgenic mouse model for inducible and reversible dysmyelination. J Neurosci 20(20), 7698-705

Matsushima GK and Morell P (2001): The neurotoxicant, Cuprizone, as a model to Study Demyelination & Remyelination in the Central Nervous System. Brain Pathol 11, 107-116

Matthews AE, Weiss SR, and Paterson Y (2002): Murine hepatitis virus—A model for virus-induced CNS demyelination. Journal of NeuroVirology 8, 76± 85

McMahon EJ, Cook DN, Suzuki K and Matsushima GK (2001): absence of Macrophage-Inflammatory Protein1-α Delays Central Nervous System Demyelination in the Presence of an Intact Blood-brain Barrier. J Immunol 167, 2964-2971

McMahon EJ, Suzuki K, Matsushima GK (2002): Peripheral macrophages recruitment in cuprizone-induced CNS demyelination despite an intact blood-brain barrier. J Neuroimmunol 130, 32-45

McMorris FA and McKinnon RD (1996): Regulation of oligodendrocyte development and CNS myelination by growth factors: prospects for therapy of demyelinating disease. Brain Pathol 6(3), 313-29

Medana M and Esiri MM (2003): Axonal damage: a key predictor of outcome in human CNS diseases. Brain 126(3), 515-530

Merkler D, Oertle T, Buss A, Pinschewer DD, Schnell L, Bareyre FM, Kerschensteiner M, Buddeberg BS, Schwab ME (2003): Rapid induction of autoantibodies against Nogo-A and MOG in the absence of an encephalitogenic T cell response: implication for immunotherapeutic approaches in neurological diseases. FASEB J 17(15), 2275-7

Merkler D, Boretius S, Stadelmann C, Ernsting T, Michaelis T, Frahm J, Brück W. (2005): Multicontrast MRI of remyelination in the central nervous system. NMR Biomed 8(6),395-403

Merkler D, Horvath E, Brück W, Zinkernagel RM, Del la Torre JC, Pinschewer DD. (2006): "Viral deja vu" elicits organ-specific immune disease independent of reactivity to self. J Clin Invest 116(5), 1254-63

Miller DJ, Asakura K, Rodriguez M (1996): Central nervous system remyelination clinical application of basic neuroscience principles. Brain Pathol 6(3), 331-44

Miller DA, Barkhof F, Frank JA, Parker GJM and Thompson AJ (2002): Measurement of atrophy in MS: pathological basis, methodological aspects and clinical relevance. Brain 125(8), 1676-1695

Morell P, Barrett CV, Mason JL, Toews AD, Hostettler JD, Knapp GW and Matsushima GK (1998): Gene Expression in Brain during Cuprizone-induced Demyelination & remyelination. Mol Cell Neurosci 12, 220-227

Nait-Oumesmar B, Lachapelle F, Decker L, Baron-Van Evercooren A (2000): Do central nervous system remyelinate?. Pathol Biol (Paris) 48(1), 70-9

Newswanger DL and Warren CR (2004): Guillain-Barre syndrome. Am FAM Physician 69(10), 2405-10

Nijeholt GJ, van Walderveen MA, Castelijns JA, van Waesberghe JH, Polman C, Scheltens P, Rosier PF, Jongen PJ and Barkhof F (1998): Brain and spinal cord abnormalities in MS. Correlation between MRI parameters, clinical subtypes and symptoms. Brain 121(Pt 4), 687-97

Noseworthy JH, Lucchinetti C, Rodriguez M, and Weinshenker BG (2000): MS. NEJM 343, 938-952

Nussbaum JL, Roussel G (1983): immunocytochemical demonstration of the transport of myelin proteolipids through the Golgi apparatus. Cell Tissue Res 198(2), 235-9

Nussbaum JL, Roussel G, Wunsch E, Jolles P (1985): site-specific antibodies to rat myelin proteolipids directed against C-terminal hexapeptide. J Neurol Sci 68(1), 89-100

Oh Sj, Kurokawa K, de Almeida DF, Ryan HFJr, Claussen GC (2003): Subacute inflammatory demyelinating polyneuropathy. Neurology 61(11), 1507-12

Oleszak EL, Chang JR, Friedman H, Katsetos CD, Platsoucas CD (2004): Theiler's virus infection: a model for MS. Clin Microbiol Rev 17(1), 174-207

Peireira LA, Cruz-Hofling MA, and Dertkigil MS, Graca DL (1996): Biology of the repair of central nervous system demyelinated lesion: an appraisal. Arq Neuropsiquiatr 54(2), 331-4

Pelc S, Fondu P, Gompel C (1986): Immunohistochemical distribution of glial fibrillary acidic protein, neurofilament polypeptides and neuronal specific enolase in the human cerebellum. J Neurol Sci 73(3), 289-97

Penderis J, Shields SA, Franklin RJ (2003): Impaired remyelination and depletion of oligodendrocyte progenitors does not occur following repeated episodes of focal demyelination in the rat central nervous system. Brain 126(Pt 6), 1382-91

Perentes A, Herbort CP, Rubinstein LJ, Herman MM, Uffer S, Donoso LA, Collins VP (1987): Immunohistochemical characterization of human retinoblastomas in situ with multiple markers. Am J Ophthalmol 103(5), 647-58

Peyron F, Timsit S, Thomas JL, Kagawa T, Ikenaka K and Zalc B (1997): In Situ Expression of PLP/DM-20, MBP, and CNP During Embryonic and Postnatal

Development of the jimpy Mutant and of Transgenic Mice Overexpressing PLP. J Neurosci Res 50, 190-201

Piccio L, Rossi B, Scarpini E, Laudanna C, Giagulli C, Issekutz AC, Vestweber D, Butcher EC and Constantin G (2002): Molecular Mechanisms Involved in Lymphocyte Recruitment in Inflamed Brain Microvessels: Critical Roles for P-Selectin Glycoprotein Ligand-1 and Heterotrimeric Gi-Linked Receptors. The Journal of Immunology 168, 1940-1949

Potter NT, Lees MB (1988): Immunochemical characterization of antibodies to the myelin proteolipid protein (PLP). J Neuroimmunol 18(1), 49-60

Pulford K, Micklem K, McCarthy S, Cordell J, Jones M, Mason DY (1992): A monocyte/macrophage antigen recognized by the four antibodies GHI/61, Ber-MAC3, Ki-M8 and SM4. Immunology 75(4), 588-95

Raine CS (1997): The Norton Lecture: a review of the oligodendrocyte in the MS Lesions. J Neuroimmunol 77, 135-152

Rocha MS, Brücki SM, Carvalho AA, Lima UW. (2004): Epidemiologic features of Guillain-Barre syndrome in Sao Paulo, Brazil. Arq Neuropsiquiatr 62(1), 33-7

Rodriguez M and Miller DJ (1994): Immune promotion of central nervous system remyelination. Prog Brain Res 103, 343-55

Royds JA, Ironside JW, Taylor CB, Graham DI, Timperley WR (1986): An immunohistochemical study of a glial and neuronal makers in primary neoplasms of the central nervous system. Acta Neuropathol 70(3-4), 320-6

Ruffini F, Kennedy TE and Antel JP (2004): Inflammation and Remyelination in the Central Nervous System A Tale of Two Systems American Journal of Pathology 164, 1519-1522

Sawa H, Takeshita I, Kuramitsu M, Mannoji H, Machi T, Fukui M, Kitamura K (1986): neuronal and glial proteins in medulloblastomas. I. Immunohistochemical study. Anticancer Res 6(5), 905-9

Schneider A, Arau jo GW, Trajkovic K, Herrmann MM, Merkler D, Mandelkow E, Weissert R, and Simons M (2004): Hyperphosphorylation and Aggregation of Tau in Experimental Autoimmune Encephalomyelitis. J Biol Chem 279(53), 55833–55839

Schwob VS, Clark HB, Agrawal D, Agrawal HC (1985): Electron microscopic immunocytochemical localization of myelin proteolipid protein and myelin basic protein to oligodendrocytes in rat brain during myelination. J Neurochem 45(2), 559-71

Scolding N (2001): The differential diagnosis of MS. J Neurol Neurosurg Psychiatry 71(Suppl 1), ii9-ii15

Siebert H, Engelke S, Maruschak B, Brück W (2001): Concentration-dependent effects of the estrase inhibitor BNPP on macrophage migration and myelin phagocytosis. Brain Res 916(1-2), 159-64

Sim FJ, Hinks GL, Franklin RJ (2000): The re-expression of the homeodomain transcription factor Gtx during remyelination of experimentally induced demyelinating lesions in young and old rat brain. Neuroscience 100(1), 131-9

Sim FJ, Zhao C, and Penderis J, Franklin RJ (2002): The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. J Neurosci 22(7), 2451-9

Simmons A (2001): Herpesvirus and MS. Herpes 8(3), 60-3

Simons M, Kramer EM, Thiele C, Stoffel W and Trotter J (2000): Assembly of Myelin by Association of Proteolipid Protein with Cholesterol- and Galactoasylceramide-rich Membrane Domains. J Cell Biol 151, 143-153

Singhal BS (2005): Leukodystrophies: Indian scenario. Indian J Pediatr 72(4), 315-8

Smith KJ and McDonald WI (1999): The pathophysiology of MS: the mechanism underlying the production of symptoms and the natural history of the disease. Philos Trans R Soc Lond B Biol Sci 354(1390), 1649-73

Spurek M, Taylor-Gjevre R, Van Uum S, Khandwala HM. (2004): Adrenomyeloneuropathy as a cause of primary adrenal insufficiency and spastic paraparesis. CMAJ 171(9), 1073-7

Stangel M and Hartung HP (2002): Remyelinating strategies for the treatment of MS. Prog Neurobiol 68(5), 361-76

Stanislaus R, Gilg AG, Singh AK, Singh I (2005): N-acetyl-L-cysteine ameliorates the inflammatory disease process in experimental autoimmune encephalomyelitis in Lewis rats. J Autoimmune Dis 2(1), 4

Steinman L (2003): Optic Neuritis, A New Variant of Experimental Encephalomyelitis, A Durable Model for All Seasons, Now In Its Seventieth Year. J. Exp. Med 197(9), 1065-1071

Stidworthy MF, Genoud S, Suter U, Mantei N, Franklin RJM (2003): Quantifying the Early Stages of Remyelination Following Cuprizone-induced Demyelination. Brain Pathol 13, 329-339

Stoffel W, Hillen H, Giersiefen H (1984): Structure and molecular arrangement of proteolipid protein of central nervous system myelin. Proc Natl Acad Sci U S A 81(16), 5012-6

Suzuki K (1969): Giant hepatic mitochondria production in mice fed with cuprizone. Science 163, 81-82

Suzuki K and Kikkawa Y (1969): Status Spongiosus of CNS and Hepatic Changes Induced by Cuprizone (Biscyclohexanone Oxalyldihydrazone). Am J Pathol 54(2), 307-325

Tansey FA, Zhang H, Cammer W (1996): Expression of Carbonic Anhydrase π mRNA and Protein in Oligodendrocytes During Toxic Demyelination in the Young Adult Mouse. Neurochem Res 21, 411-416

Tardieu M and Mikaeloff Y (2004): MS in children. Int MS J 11(2), 36-42

Taylor CM, Marta CB, Claycomb RJ, Han DK, Rasband MN, Coetzee T and Pfeiffer SE (2004): Neuroscience Proteomic mapping provides powerful insights into functional myelin biology. PNAS 101(13), 4643-4648

Trapp BD, Bo L, Mork S, Chang A (1999): Pathogenesis of tissue injury in MS lesion. J Neuroimmunol 98(1), 49-56

Trifilieff E, Luu B, Nussbaum JL, Roussel G, Espinosa de los Montero A, sabatier JM, Van Rietscoten J (1986): A specific immunological probe for the major myelin proteolipid. Conformation of a deletion in DM-20. FEBS Lett 198(2), 235-9

Ukkonen M, Dastidar P, Heinonen T, Laasonen E, Elovaara I (2003): Volumetric quantitation by MRI in primary progressive MS: volumes of plaques and atrophy correlated with neurological disability. Eur J Neurol 10(6), 663-9

Uschkureit T, Sporkel O, Stracke J, Bussow H, Stoffel W (2000): Early onset of axonal degeneration in double (plp-/-mag-/-) and hypomyelinosis in triple (plp-/-mbp-/-mag-/-) mutant mice. J Neurosci 20(14), 5225-33

Van Epps HL (2005): Thomas Rivers and the EAE model. JEM 202(1), 4-4

Walker EB, Akporiaye ET, Warner NL, Stewart CC (1985): Characterization of subsets of bone marrow-derived macrophages by flow cytometry analysis. J Leukoc Biol 37(2), 121-36

Wasay M, Khatri IA, Khealani B, Sheerani M (2006) : MS in Asian Countries, The International MS Journal 13, 58–65

Webster HD, Shii H, Lassmann H (1985): Immunocytochemical study of myelin-associated glycoprotein (MAG), Basic protein (BP), and Glial Fibrillary Acidic Protein (GFAP) in chronic relapsing allergic encephalomyelitis (EAE). Acta Neuropathol 65(3-4), 177-89

Wingerchuk DM, Lucchinetti CF, Noseworthy JH (2001): MS: current pathophysiological concepts. Lab Invest 81(3), 263-81

Woodruff RH, Fruttiger M, Richardson WD, and Franklin RJM (2004): bPlatelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination Mol. Cell. Neurosci Xx (2004) xxx–xxx YMCNE-01430 No of Pages 11 4C:3, 5, 6

Yamada M, Ivanova A, Yamaguchi Y, Lees MB and Ikenaka K (1999): Proteolipid Protein Gene Product Can Be Sectreted and Exhibit Biological Activity during early development. J Neurosci 19(6), 2143-2151

Yin X, Crawford TO, Griffin JW, Tu P, Lee VMY, Li C, Roder J, and Trapp BD (1998): Myelin-Associated Glycoprotein Is a Myelin Signal that Modulates the Caliber of Myelinated Axons. The Journal of Neuroscience 18(6), 1953-1962

Yool DA, Edgar JM, Montague P, Malcolm S (2000): The proteolipid protein gene and myelin disorders in man and animal models. Hum Mol Genet 9(6), 987-92.

Zanardo RCO, Bonder CS, Hwang JM, Andonegui G, Liu L, Vestweber D, Zbytnuik L and Kubes P (2004): A down-regulatable E-selectin ligand is functionally important for PSGL-1–independent leukocyte–endothelial cell interactions. Blood 104(12), 3766-3773

Zecevic N, Audjelkovic A, Matthieu JM, Tosic M (1998): Myelin basic protein immunoreactivity in the human embryonic CNS. Brain Res Dev Brain Res 105(1), 97-108

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