5.1 Focus of the Present Study

Specific validation of the selected myelin oligodendrocyte glycoprotein induced EAE in Wistar rats was carried out to confirm the reliability and reproduction for further studies. The present study was aimed at:

- Study of the cerebrospinal fluid changes following EAE
- Study of myelin specific antibodies in serum after EAE
- Evaluation of the breakdown of blood brain barrier following EAE
- Study of the demyelination after EAE

5.2 Experimentation

- Following immunization, cerebrospinal fluid was aspirated from the foramen of magnum of rats for the determination of total protein and oligoclonal bands using SDS PAGE after the first acute phase and the start of the first relapse.
- Blood was collected from orbital sinus and the serum was separated followed by the estimation of anti-MOG and anti-MBP antibodies using ELISA after the first acute phase and start of the first relapse.
- BBB disruption following EAE in Wistar rats was studied using macromolecular dye Evan's Blue which binds to the serum albumin and this complex extravasates into the CNS parenchyma at end of first acute phase and the start of the first relapse.
- Pathological studies following EAE induction in rats after the first acute phase by H&E and Toulidine blue histology.
- Immunohistochemistry was carried out to study MBP expression after first acute phase in periventricular white matter and cortex using monoclonal anti-MBP antibody.
- Oligodendrocyte surface marker CNP was estimated using a colorimetric assay after the first acute phase and the start of the first relapse.
5.3 Introduction

Neuroinflammation in multiple sclerosis presents with plethora of variations in clinical, pathological and immunological phenotypes (Sato et al., 2011) that might better be described as a syndrome rather than a single disease. The clinical heterogeneity of multiple sclerosis has been recognized for many years, but it is now apparent that this heterogeneity extends to both the genetics of the disease and the pathomechanisms (Graber et al., 2011).

The history of EAE dates back to the 1920s, when spinal cord inflammation in rabbits was induced by inoculation with human spinal cord (Koritschoner and Schweinburg, 1925). In the 1930s, researchers attempted to reproduce the encephalitic complications associated with rabies vaccination by repetitive immunization of rhesus monkeys with CNS tissue (Rivers et al., 1933). Since then EAE was elicited in many different species, including rodents and primates, and from these studies it became clear that EAE can reproduce many of the clinical, neuropathological and immunological aspects of human multiple sclerosis (Hohlfeld and Wekerle, 2001).

Clinically the M.S may present as a remitting-relapsing disease, or with steady progression of neurological disability signs (Moreira et al., 2000) which may be associated with the elevation of the total protein in CSF (Thompson and Freedman, 2006) along with the intrathecal synthesis of oligoclonal antibodies in 70% of the patients (Link et al., 2006). The subsequent course of disease is unpredictable with respect to the progression, the type of lesions found and the type of immunological response developed. Advances in molecular medicine have clearly demonstrated the heterogeneity of multiple sclerosis (Lassmann et al., 2001). Its pathology is, in part, reflected by the formation of focal inflammatory demyelinating lesions in the white matter formed due to the anti-myelin antigens and T-lymphocytes along with the disruption of the blood brain barrier mediated by the recruitment of the inflammatory cellular populations. The demyelination may be mediated by the phagocytosis of the myelin or the degeneration of the oligodendrocytes (Stys, 2010). These demyelinating lesions are the characteristic hallmarks in patients with acute and relapsing disease (Raine et al., 1997; Compston et al., 2005). In patients with progressive disease, the brain is affected in a more global sense, with diffuse but widespread damage in the white matter and demyelination can

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also be seen in the grey matter, particularly the cortex (Bo et al., 2003; Kutzelnigg et al., 2005). The mechanisms of tissue injury in focal white matter lesions are heterogeneous, resulting in patterns of demyelination that vary between patients or patient subgroups (Lassmann et al., 2001). Furthermore, there is a high inter-individual variability in the extent of axonal damage as well as remyelination and repair. The reason for this complex situation is largely unknown, although it is likely that genetic factors influencing immune-mediated inflammation as well as neuronal and glial survival may play a major role in modulating the phenotype of the disease (Compston, 2004).

For all these reasons, it very difficult to ascertain that these all variations can be covered in a single or even in several different models. Despite these limitations, most of our current knowledge regarding principal mechanisms of brain inflammation and the immunological patterns have been gathered from studies on EAE, and without this knowledge the understanding of the pathogenesis of multiple sclerosis or the development of new therapies would not be feasible.

Therefore this study was designed to study, whether the selected model viz. MOG induced EAE in Wistar rats can fulfill the basic criteria of the human M.S and can be used for other studies.
5.4 Results

5.4.1 Changes in cerebrospinal fluid (CSF)
Cerebrospinal fluid suffers various changes during autoimmune brain disease. Studies in CSF are of diagnostic value in human multiple sclerosis. Generally, the elevation of total protein in CSF and presence of oligoclonal bands in CSF corresponding to IgM molecules are evaluated to increase the diagnostic accuracy.

5.4.1.1 EAE elevated total protein in CSF
CSF was freshly collected in a phase wise manner from the EAE rats. Unconcentrated CSF was evaluated for total protein content after induction by MOG in Wistar rats corresponding to the first acute phase, 20 days p.i and first relapse, 31 days p.i. There was a significant increase ($P < 0.01$) in the total protein content in CSF of rats immunized with MOG (35-55) at the end of the first acute phase and first relapse ($P < 0.001$) when compared to adjuvant injected controls or control rats. Moreover, there was a significant ($P < 0.01$) increase in the protein concentration between first acute phase and the first relapse phase (Fig. 5.1). The elevation of total protein is a direct proof of intrathecal synthesis of some proteins or antibodies apart from the systemic synthesis after the induction of the disease which may be important in the pathological course followed by the MOG induced EAE in Wistar rats.

5.4.1.2 Oligoclonal bands were not present in MOG induced EAE.
Elevation of the total protein in CSF following EAE rats suggested intrathecal synthesis of either antibodies or some proteins. The presence of the oligoclonal bands in the CSF of MOG induced EAE rats was investigated at two different time intervals viz. after the first acute phase (20 days) and first relapse phase (31 days) and compared them with the adjuvant injected controls or controls. There was no appearance of oligoclonal bands (OCBs) in the CSF of the EAE rats (Fig. 5.2) as compared with the adjuvant injected control rats as revealed by the one dimensional SDS PAGE analysis using 7.5% light running gel. These results suggest absence of the oligoclonal bands which are synthesized intrathecaly and have a pathogenic role in the development of the autoimmunity. Moreover, these results point towards the role of the systemic antibodies in the establishment of the neuroinflammation and demyelination after MOG stimulated CNS autoimmunity.
**Characterization of Selected Model**

**Figure 5.1** Total protein in CSF of MOG induced EAE rats after 20 days and 31 days post induction. **P < 0.01, CNTRL vs. EAE (20), q = 23.29, ***P < 0.001 CNTRL vs. EAE (31), q = 34.19 and ** P < 0.01, EAE (20) vs. EAE (31), q = 10.89

**Figure 5.2** SDS PAGE of CSF for the determination oligoclonal bands. Unconcentrated CSF was resolved on light running 7.5% PAGE with Commassie blue stain. Note the absence of oligoclonal bands at both phases of the disease.
5.4.2 EAE elevated serum MOG and MBP specific antibodies.

After the confirmation that EAE does not induce the intrathecal synthesis of the autoantibodies against the myelin antigens, we performed a standard ELISA to confirm their presence in the sera from the first acute and first relapse phases of EAE in Wistar rats. EAE significantly (P < 0.05) elevated the anti-MOG antibodies in the serum after the first acute phase suggesting that MOG domains are recognized by the antigenic stimulation. However, there was no further increase in the antibody titer in the first relapse of the disease as no significant (P > 0.05) increment could be noticed. On the contrary, MOG sensitization led to the increase in the antibody titer of MBP specific antibodies. There was a significant increase the anti-MBP antibody responses in the first acute (P < 0.01) as well as the first relapse (P < 0.001) of the disease. Moreover, there was a significant (P < 0.01) increase in MBP antibodies when first acute phase was compared to the first relapse (Fig. 5.3). These results point towards the role of systemic antibody response in the pathology of MOG induced EAE in Wistar rats and at the same time highlight the cross antigen activation by MOG in this model where the neuroinflammatory phase is maintained by the anti-MBP antibodies.

5.4.3 EAE disrupted the blood brain barrier (BBB)

After immunization with 50 μg of MOG subcutaneously, Wistar rats were injected a macromolecular albumin binding dye “Evan's blue”. Since albumin is absent in CNS tissue, any albumin extravasation due to the breakdown of blood brain barrier can be quantified. BBB disruption was studied after the first acute phase and first relapse viz. 31 days, p.i. There was a significant (P < 0.01) (Fig. 5.4A) extravasation of Evans blue in the CNS parenchyma after the first acute phase as well as the first relapse of the disease suggesting breakdown of the barrier. However, there was no significant increment (P > 0.05) in the extravasation of dye when the first acute phase was compared with first relapse phase. These findings were confirmed by the recording of fluorescence intensity of the dye complex (Fig. 5.4B, C & D). The above mentioned changes were also seen as fluorescence intensity increased in the first acute phase and first relapse while no change was observed within the two phases. It seems like the BBB is disrupted by the acute inflammatory penetration of cells.
20 days p.i, but the remission phase may recover the incurred damage and the next bout of inflammation may lead to the opening of the barrier again.

**Figure 5.3**- ELISA for MOG and MBP specific antibodies in serum after EAE induced by MOG in rats at two different phases viz. after first acute episode and at the start of the first relapse. Concentration of antibodies was plotted as absorbance at 450nm. * P < 0.05, CNTRL vs. MOG (20) q = 9.6; * P < 0.05, CNTRL vs. MOG (31) q = 8.95; NS (non-significant), MOG (20) vs. MOG (31) q = 0.64; P < 0.01, CNTRL vs. MBP (20) q = 12.86; *** P < 0.001, CNTRL vs. MBP (31) q = 32.82; ** P < 0.01, MBP (20) vs. MBP (31) q= 19.95

**Figure 5.4**- Disruption of vascular endothelial barrier (blood brain barrier) after MOG induced EAE in Wistar rats. A, colorimetric estimation (absorbance at 595nm) of the extravasation of Evan's blue–albumin dye complex in brain. B, C & D fluorescence intensity of extravasated dye in adjuvant control, first acute phase of EAE and first relapse respectively (Original magnification-10x). **P < 0.01, CNTRL vs. MOG (20) q = 9.26, ** P < 0.01, CNTRL vs. MOG (31) q = 9.15; NS (non-significant) MOG (20) vs. MOG (31) q = 0.10.
5.4.4 Study of inflammation and demyelination.

5.4.4.1 Recruitment of inflammatory cells

Autoimmunity to MOG leads to the infiltration of several immune cells including lymphocytes, B-cells and plasma cells etc. There was a marked cellular infiltration in first acute phase of the EAE rats. The immune cells recruitment was more evident in the white matter as compared to grey matter (Fig. 5.5 B&D). Dense cellular deposition was noticed in the areas of extensive perivascular cuffing in white matter as well as grey matter but the white matter showed these changes to a greater extent (Fig. 5.5 B). The tissues in the areas of cellular penetration appeared to be irregular and vacuolated. The immune cell recruitment is indeed, the driving force for demyelination. These results confirmed the disruption of blood brain barrier in the cellular phase of the EAE which eventually leads to the massive cellular flux into the CNS parenchyma.

Figure 5.5- H & E histology of the rat brain following EAE. A, normal white matter tracts of an adjuvant injected control rat. B, vacuolated tissue, cellular infiltration and vascular cuffing present in the white matter of EAE rat. C, normal grey matter of control rat in contrast to a irregular vacuolated and inflammatory cell penetrated grey matter in EAE rat (D).Original magnification 20x.
5.4.4.2 Demyelination following EAE

5.4.4.2.1 Toulidine blue histology

Toulidine blue histology revealed that demyelination was more prominent in the white matter tracts and to a lesser extent in the grey matter (Fig. 5.6). The demyelinating episode was characterized by the presence of actively demyelinating plaques along with some plaques undergoing remyelination (shadow plaques) (Fig. 5.6 C). Typically several denuded axons were noticed with irregular myelin and vacuolated appearance was prominent. However, grey matter presented with moderate to severe axonal loss along with numerous demyelinated neurons (Fig. 5.6 F).

Figure 5.6- Toulidine blue histology of the adjuvant injected and EAE rats. Demyelinated white matter tracts with relative preservation of the axonal elements along with irregular myelin in EAE rats (B) in comparison to the compact and well arranged myelin with prominent myelinated axons in the white matter of the adjuvant control rats (A) - white arrows point towards the myelinated axons present in the normal white matter. Several actively demyelinating plaques in the EAE white matter were accompanied with shadow plaques undergoing remyelination with relative thin myelin (C). Appearance of normal grey matter with thick myelin sheaths and prominent nuclei (D) - white arrows point towards the thick myelin sheaths around axons. E, demyelination represented by thin myelin sheaths in the grey matter of the EAE rats (E)- black arrows point towards apparently degenerating axons. F represents the undergoing degeneration in grey matter with numerous fading axons (black arrow heads) and demyelinated white matter with vacuolated and irregular myelin. Original magnification of A, B, D & E-10x; C-4x; F-20x.
5.4.4.2.2 EAE decreases myelin basic protein expression in rats

Myelin basic protein expression was checked in order to confirm the findings presented by toulidine blue using monoclonal antibody against rat myelin basic protein. Expression of MBP decreased in the periventricular white matter (PVWM) with relative sparing of the axonal elements as revealed by the numerous denuded axons with vacuolated myelin (Fig. 5.7). The grey matter presented with moderate demyelination and severe loss of the axonal elements evidenced by fading cell bodies. These findings establish that MOG induced EAE leads to substantial demyelination in the white matter and severe axonal loss in the grey matter.

![Figure 5.7](image)

**Figure 5.7** - MBP expression in the EAE and adjuvant injected rats. MOG immunization decreased the MBP expression in the periventricular white matter (B, white arrows) and grey matter (D, white arrows) in comparison to the controls represented by A and C respectively. E, gross view of a coronal section of EAE rat brain depicting loss of MBP expression in white matter (black arrow) and demyelinated and degenerating neurons (white arrows). Original magnification- 20x.
5.4.4.2.3 EAE limits the activity of Oligodendrocyte specific CNPase.

CNP is mostly expressed by oligodendrocytes in their different stages of development. This protein is the marker of choice for detection of oligodendrocytes and myelin elements in tissue along with cell cultures. The first acute as well as the first relapse phases of MOG induced EAE marked steep loss ($P < 0.001$) of CNP (Fig. 5.8). However there was no significant change within the two phases and the CNPase decreased to about 43% and was maintained until the start of the first relapse.

![Figure 5.8](image)

Figure 5.8- CNPase activity in EAE Wistar rats after the first acute and first relapse phases. CNTRL vs. MOG (20) *** $P < 0.001$, $q = 15.09$; CNTRL vs. MOG (31) *** $P < 0.001$, $q = 19.23$; MOG (20) vs. MOG (31) NS-Non significant.

Discussion

Development of targeted therapies for neuro-inflammatory disorders especially M.S is currently a challenge for mankind because of the heterogeneity with the clinical disease and the models representing it. Here we have tried to fulfill the basic requirement of the model for human M.S that can be used for extensive studies for molecular pathology.

Several types of myelin antigens are used for development of autoimmune neuro-inflammatory models in a wide variety of laboratory animals (Mannie et al., 2009) etc. Apart from myelin antigens several gangliosides are also antigenic (Menge et al., 2005). The wide variety of CNS antigens which can elicit autoimmune response makes it very difficult to understand the disease because of the different types of strategies used by these antigens in
eliciting the autoimmunity (Kuerten and Angelov, 2008). The most important myelin antigens studied with respect to the M.S are MBP and MOG. Myelin basic protein is expressed by mature as well as developing oligodendrocytes in brain and spinal chord (Yang et al., 2011). MBP is present on the intracellular surface of the cells making them relatively inaccessible to the circulating antibodies but the T-cell mediated responses are well pronounced (Wekerle, 1993; Baxevanis et al., 1989). MOG on the other side is expressed by wide variety of myelinating cells and its domains are present on the extracellular side of the cells making both autoantibody and T-cell mediated mechanisms a possibility.

As discussed previously, that MOG immunization was able to elicit a remitting relapsing type disease much similar to remitting relapsing type human M.S in Wistar rats (Chapter 4). There are several changes which occur during the course of the human M.S. Human M.S has been demonstrated to elevate the total CSF protein (Awad et al., 2010) along with presence of oligoclonal bands (von Büdingen et al., 2010) in the CSF. While the identity of the individual proteins elevated following M.S is unknown; the oligoclonal bands present in CSF of majority of patients are IgM molecules. Elevated total protein in CSF was measured in the first acute as well as the first relapse of the EAE in wistar rats, however no oligoclonal bands could be detected at any of these phases. Elevation of total protein in CSF suggests intrathecal synthesis of these proteins. The absence of the oligoclonal bands (OBs) in CSF highlights the absence of the intrathecal synthesis of the auto-antibodies against myelin. These findings are in line with the earlier studies demonstrating the absence of OBs in response to MOG immunized DA rats (Rostrom et al., 2004).

For the establishment of the chronic relapsing EAE, antibodies specific to myelin antigens is a basic requirement (Fukaura et al., 2010) and in their absence from CSF suggests systemic synthesis. EAE rats showed presence of anti-MOG and anti-MBP antibodies (Bischof et al., 2004) in the sera of acute and relapsing phases of EAE. While the MOG specific antibodies showed a stable increase which did not surge after the acute phase, the MBP specific antibodies showed an exponential increase from acute phase to the first relapse phase. These results suggest that MOG can elicit antigen cross-reactivity to MBP also which may be
secondary to the generation of anti-MOG antibodies since there was a significant increase in the anti-MBP antibodies on the first relapse i.e. 31 days p.i.

This is an established fact that all the CNS antigens including MOG lead to the trafficking of several immune cells like macrophages, plasma cells and others which pass through the blood brain barrier in order to penetrate into the CNS (Alvarez et al., 2011). In the course of penetration, these cells lead to the damage of the BBB (Alvarez et al., 2011) which may be permanent if done by several toxic mediators or temporary, if caused by the passage of these cells. MOG caused persistent BBB damage which was evident through the first acute phase to the first relapse i.e. 31 days, p.i. Also the damage on the 31st day viz., first relapse was not significant when compared to the 20 days, p.i and can be ascribed to the intermittent remission phase (Hawkins et al., 1990). The disruption of BBB allows cellular infiltration along with the trafficking of several types of inflammatory mediators which can be instrumental in CNS tissue damage.

The combined effects of all aforesaid events are the driving force for the loss of myelin and tissue damage in CNS following EAE. In rats, the most affected region is periventricular white matter (PVWM) including corpus callosum, extreme end white matter and cortex (Zeis et al., 2008). Toluidine blue staining showed extensive demyelination in periventricular white matter. The plaques were somewhat diffuse with distinct edges surrounded with normal appearing myelin. The cortex presented with feebler demyelination but extensive axonal loss. Another striking feature of human M.S was evident in this model where some the plaques presented with some level of remyelination characterized by thin myelin sheaths surrounded by either normal myelin or denuded axons. The loss of the myelin was characterized loss of MBP expression in both PVWM and cortex. CNP amounts to about 4% of the brain protein. In rats, CNP expression peaks around 10 days of age at a time when oligodendrocyte precursors enter their terminal differentiation stage preceding myelination (Amur-Umarjee et al., 1990). The loss of the MBP expression was ascertained to the steep decline in the brain CNP activity. CNP activity was reduced to almost 43% in the acute phase and was maintained up to the first relapse. All these evidences suggest that loss of myelin is evident in acute phase and does not recover because of the recurrent bouts of
inflammation which can lead to progressive deficits as described in human RR type multiple sclerosis.

Present investigation is an attempt to study the mechanisms of the CNS autoimmunity described in humans and correlate them in an animal model for better understanding of human neuro-inflammation. Moreover, this study will lead to the decrease in the diversification of methodology and approach for the development of the effective therapies for this disabling disease.