CHAPTER 6
6.1 Focus of the Present Study

Neuronal as well as glial cell loss is central to the pathology of Multiple Sclerosis and its experimental counterpart, the experimental autoimmune encephalomyelitis (EAE). This study encompasses the bystander mechanism of cellular apoptosis in EAE. The study was aimed at

- Study of the generation of inflammatory mediators in the CNS following EAE in rats.
- Evaluation of reactive oxygen and nitrogen stress in EAE rat CNS after first acute episode.
- Induction of cellular apoptosis in EAE.
- Effect of the ongoing cellular death on the cholinergic neurons.

6.2 Experimentation

- Induction of Experimental Autoimmune Encephalomyelitis in Wistar Rats using MOG (35-55) in Complete Freund's Adjuvant and sacrifice of animals after the first acute phase of the disease i.e. 20 days p.i.
- Study of the secretion of Th-1 type cytokines by several inflammatory cells in periventricular tissue.
- Study of the depletion of the non enzymatic as well as enzymatic antioxidants along with the peroxidation of the biological membranes in the brains of EAE rats as a consequence of the generation of oxygen free radicals.
- Determination of nitrite content in the brain cell lysates and serum following EAE using Griess reaction.
- Estimation of MPO and peroxynitrite in the brain tissue to study the peroxidase catalyzed oxidation of nitrite.
- Immunohistochemical detection of the cytosolic shuttling of the Bax protein leading to the mitochondrial dysfunction.
- Induction of apoptosis by study of Caspase-3 activity and PARP activity in cell lysates.
Mechanism of Cell Death

- Immunohistochemical localization of Choline-o-acetyl transferase, the most important enzyme in the acetyl choline synthesis to mark the cholinergic cellular death after EAE.

6.3 Introduction

The axonal as well as glial cellular loss has been described earlier in experimental autoimmune encephalomyelitis (EAE) (Herz et al., 2010). The patterns of cell death differ with the mechanism involved. The most rapid type of cell death occurs through the cytotoxic action of T-lymphocytes of CD8+ phenotype (Sobottka et al., 2009) which lead to the release of granzymes on the surface of cells. However, this type of cell death occurs mostly during the peak (13-15 days, p.i) of the disease when the T-lymphocyte infiltration is at its maximum.

Apart from the aforementioned mechanism, the cell death continues in the normal appearing tissues in CNS at a much slower but progressive rate leading to the accumulation of the neurological deficits. Enhanced cerebrovascular permeability succeeds organ specific target autoimmunity towards myelin antigens (Gay and Esiri, 1991). The challenged cerebrovascular protection leads to the marked cellular infiltration of the circulating immune cells especially monocyte derived macrophages and T-lymphocytes (Benveniste, 1997; Jerome et al., 2005). This polarized immune environment leads to the activation of the microglia and cause onset of acute multiple sclerosis lesions (Castano et al., 1998).

The development of the lesions in EAE is not solely dependent on the demyelinating events. Macrophages and T-lymphocytes release many inflammatory mediators including cytokines (Jerome et al., 2005) and reactive oxygen and nitrogen intermediates (ROS & RNI) that are contributing factors of the cell death in neurodegenerative disorders (Jerome et al., 2005). It has been already described that ROS can lead to the fragmentation of the nuclear DNA. RNI can react with the superoxide to form peroxynitrite. Moreover, the presence of the peroxidase secreting polymorphonuclear cells in the vicinity of the RNI can have serious implications on the cellular survival. The inflammatory reaction in conjunction with oxidative environment produced in can lead to the potentiation of the already challenged...
neuronal as well as glial cells paving way to the neurological deficits that are dependent upon the type and anatomy of the cells being affected.

One of the particular neurological deficits that affect about half of the individuals suffering from M.S is the cognitive dysfunction (Peyser et al., 1980) and difficulties in learning and remembering new information represent the other most common cognitive deficits (Rao et al., 1991; Bobholz and Rao, 2003). Verbal memory deficits are observed in the progressive form of the disease and visuospatial memory deficits are observed in the relapsing–remitting form (Gaudino et al., 2001). Hippocampus is archicortical cholinergic rich structure and acts as a memory device (Manns et al., 2003) especially sensitive to various insults including inflammation (Larisa et al., 2005). Cellular death in this region can lead to depleted acetylcholine synthesis and can trigger memory deficits. Choline acetyl transferase is the most important enzyme in the acetylcholine synthesis as has been documented to be decreased in the progressive course of M.S which is an indicator of cholinergic cell death (Giulia et al., 2005). Apart from this, recently, hippocampal atrophy has also been documented in clinical investigations (Sicotte et al., 2008) suggesting delayed cell death.

This study is an attempt to investigate the potential interactions of the immune environment with the cellular survival in order to understand the mechanisms of the cellular death in the EAE.

6.4 Results

6.4.1 EAE upregulates Th-1 type cytokines in brain.

Antigen stimulated organ specific autoimmunity generated by MOG towards CNS recruits several cellular components including stimulated T-lymphocytes which are mainly MHC class I restricted CD8+ and MHC class II restricted CD4+ T-lymphocytes. The measurement of Th-1 type cytokines viz. TNF-α and IFN-γ was done to confirm the infiltration of the CD4+ T-lymphocytes due to autoimmune stimulation of T-lymphocytic populations in EAE rats, 20 days post induction. There was a significant increment in the TNF-α (P < 0.001) and IFN-γ (P < 0.05) levels following MOG immunization after first acute episode (Fig. 6.1). The elevation in the cytokines didn’t yield a clear cut pattern as
TNF-α increased more in comparison to the IFN-γ suggesting its persistent role in the progression of the disease. However, comparatively lower expression of IFN-γ may be responsible for the induction of remission 20 days post immunization (p.i).

Grossly, the sustenance of pro-inflammatory cytokines in this phase suggests that inflammation doesn’t stop in the tissues even at the start of the remission phase which extends from 21-30 days post induction.

6.4.2 EAE generates reactive oxygen species overload.

Proinflammatory cytokines lead to the activation of macrophages and microglia. These in turn lead to the direct generation of reactive oxygen species which is persistent in the event of cellular flux in almost all the phases of the disease. There was a significantly higher (P<0.01) amount of the lipid peroxides estimated as MDA formation in the EAE rats when compared to the adjuvant injected controls. Moreover, both the enzymatic and non enzymatic cellular antioxidants suffered a significant decrease in the EAE rats. There was a generalized decrease in non protein thiols (GSH) (P < 0.01). The enzymatic antioxidants viz, catalase (P < 0.001) and superoxide dismutase (P < 0.01) also recorded a parallel decrease (Table 6.1). Therefore, MOG induced EAE leads to an oxidized environment following after the first acute phase in rats.

![Figure 6.1](image.png)

**Figure 6.1**- Th-1 type cytokines (TNF-α & IFN-γ) in the brain of EAE rats immunized with MOG (35-55) 20 days post induction. TNF-α *** P < 0.001, CNTRL vs. EAE (t=6.76); IFN-γ * P < 0.05, CNTRL vs. EAE (t=2.90)
Table 6.1 - Increase in lipid peroxidation and depletion of non-enzymatic as well as enzymatic antioxidants in brain 20 days post immunization. Lipid peroxidation, **P < 0.05 (t=3.74); GSH, ***P < 0.001(t=8.04), Catalases, ***P < 0.001(t=54.55), SODs, **P < 0.01(t=3.30).

6.4.3 EAE elevates nitrite in serum and brain.

Neuroinflammation after EAE leads to the formation of reactive nitrogen species apart from reactive oxygen species through several mechanisms which include the direct secretion of NO from inflammatory cells and upregulation of iNOS and increased expression of NADPH Diaphorase+ cells. Primary metabolite of NO, the nitrite in the brain and serum of the EAE rats was estimated 20 days, p.i. There was a significant (P < 0.001) increase in the concentration of nitrite conforming its direct formation in the tissue after EAE. The similar estimation in serum also showed a significant increase (P < 0.01) in the nitrite content of the serum (Fig. 6.2). Higher expression of NO in CNS leads to its leakage into vasculature in event of the disrupted blood brain barrier. Increase in the NO can also lead to the formation of several potential cytotoxic reactive nitrogen intermediates and prime neurodegeneration.

Figure 6.2 - EAE significantly upregulates NO concentration in brain and blood serum 20 days post induction. NO concentration was determined using Griess reaction. Brain, ***P < 0.001 (t= 23.19); Serum **P < 0.01(t= 5.74).
6.4.3 EAE generates peroxynitrite (potential implication of the peroxidase catalyzed oxidation of nitrite).

Recent evidences have confirmed the polymorphonuclear (PMN) cell infiltration after EAE in various laboratory animals. These PMNs secrete peroxidases. A significant increase was estimated ($P < 0.001$) in myeloperoxidase (MPO) (Fig. 6.3A), a direct proof of the PMN infiltration and activation after MOG induced EAE. In event of the increased primary metabolite of NO, the nitrite can be converted into several other potentially toxic nitrogen intermediates such as nitrogen dioxide ($\text{NO}_2$) and peroxynitrite ($\text{ONOO}^-$). There was a significant increase ($P < 0.001$) in the peroxynitrite formation following the first acute episode (Fig. 6.3B). These results suggested peroxidase dependent oxidation of nitrite to generate highly cytotoxic molecule peroxynitrite after the first acute phase in EAE in Wistar rats.

---

**Figure 6.3** - Peroxidation catalyzed oxidation of nitrite. A, elevation of PMN secreted MPO in the periventricular tissue after EAE. B, Nitrite oxidizes to peroxynitrite in the EAE periventricular white matter. MPO, ***$P < 0.001$ (t=6.27), ONOO-, ***$P < 0.001$ (t=9.88)
6.4.4 EAE leads to the mitochondrial dysfunction by Bax shuttling.

Under the prevalent oxidizing conditions, the functioning of the mitochondria can be compromised. In order to evaluate if generation of reactive oxygen and nitrogen intermediates can modulate functions of proteins affecting cellular survival, the expression of Bax protein following EAE at the end of the first acute episode was studied. There was a generally, a remarkable increase in the Bax expression in the EAE brains. However, the frontal cortex (Fig. 6.4D&E) showed a higher expression as compared to the underlying white matter (Fig. 6.4B&E). But the Bax localization was higher in the white matter when compared to the adjuvant injected control (Fig. 6.4A). These changes were associated with the elevation of the toxic metabolite peroxynitrite generation which is potent in upgrading the cellular death mechanisms by cytosol to mitochondrial shuttling of the Bax and thereby changing the permeability of mitochondrial membranes and leading to the leakage of several proapoptotic factors.

![Figure 6.4](image)

Figure 6.4- Expression of Bcl-2 associated X protein (Bax) in the EAE and adjuvant control brains. A&B, control and EAE white matter respectively representing enhanced expression of Bax. C & D are cerebral cortex of adjuvant injected control and EAE arts respectively showing very significantly elevated Bax expression. Note the Bax expression in cerebral cortex (black arrows) as compared with the white matter (White arrows). E, Coronal section of EAE rat brain depicting the selective higher expression of Bax in the cortex. (White arrows). WM (white matter); CTX (cortex). Original magnification A, B &E -10x; C&D- 20x.
6.4.5 EAE elevates activity of Caspase-3 and PARP.

Increase in the expression of Bax after EAE suggested permeabilisation of the mitochondrial membranes due to the generation of toxic intermediates like peroxynitrite (ONOO-). These results were further elaborated by the study of some downstream targets of cellular apoptosis including measurement of the activity of central executor Caspase-3 and DNA repair enzyme PARP in the cellular lysates. There was a significant increase ($P < 0.05$) in the activity of Caspase-3 in the EAE rats when compared with adjuvant controls (Fig. 6.5A). A similar trend was seen in the activity of DNA repair enzyme PARP, which showed higher increment ($P < 0.05$) when compared to the adjuvant injected rats (Fig. 6.5B). These results suggest higher rate of cellular apoptosis in EAE rats. The elevation of PARP can lead to the depletion of the cellular energy resources and together with ROS-mediated DNA damage can lead to the neuronal cell death.

![Figure 6.5](image)

**Figure 6.5** Activities of cellular Caspase-3 and PARP after EAE in Wistar rats. A, Caspase-3 activity in EAE rats in comparison to adjuvant controls. B, PARP activity in EAE rats in comparison to controls. Caspase-3, *P* < 0.05 ($t = 2.86$); PARP activity, *P* < 0.05 ($t = 2.32$).
6.4.6 EAE leads to the degeneration of cholinergic neurons.

In order to assess whether the ongoing cellular death can affect the cholinergic neurons in hippocampus, immunohistochemical studies were carried out on 5-8μm thick serial coronal sections from both the animal groups using a monoclonal antibody against Choline-O-Acetyl Transferase (ChAT). ChAT is an important but not rate limiting enzyme in the biosynthesis of neurotransmitter Acetylcholine. We noticed a remarkable decrease in the expression of the ChAT in the EAE rats suggesting cholinergic neuronal death after the first acute episode of EAE in Wistar rats. Moreover, there was a specific decrease in the ChAT expression in the CA2 of hippocampus (Fig. 6.6). Therefore it is quite possible that ongoing neuroinflammation may lead to the death of cholinergic neurons and trigger memory deficits.

![Figure 6.6](image)

**Figure 6.6**- ChAT-immunoreactive neurons (ChAT-ir) in hippocampus of adjuvant injected control and EAE rats 20 days p.i. Note the remarkable decrease in the ChAT-ir neurons in the hippocampus of the EAE rats. Loss of ChAT expression was especially noticeable in CA2. Original magnification 20x.

6.5 Discussion

The progression of the human multiple sclerosis is influenced by the axonal loss(Soulika et al., 2009) apart from the demyelination which is mediated by either phagocytosis of the intact myelin or by macrophages and the loss of glial cells. While phagocytosis is prominent in the peak period of the disease, the glial loss impairs the axon- myelin assembly to a significant extent (Pohl et al., 2011). The denuded axons after demyelination also experience transport dysfunction and die in due course of time (Raine and Cross, 1989). In this experiment analysis, the mechanism of cellular loss due to the mediators of inflammation has been elaborated as previously described as the “Bystander Mechanism” of cellular loss in multiple sclerosis or EAE.
Mechanism of Cell Death

Human MS as well as EAE has been described to be mediated by CD4+ T-cells apart from the macrophages, B-cells (Traugott et al., 1983) etc. as described in Chapter 2 already. Stimulated CD4+ T-cells release several proinflammatory cytokines including TNF-α and IFN-γ which lead to the activation of the recruited macrophages (monocyte derived) and the local microglia (Woodroofe and Cuzner, 1993). These in turn also secrete these proinflammatory cytokines leading to a strong inflammatory response. Stimulated macrophages and microglia secrete several reactive oxygen and nitrogen intermediates into the tissue creating an oxidative polarized condition which can mediate the damage to the genomic DNA (Zhang et al., 1994) and lead to the loss of function of several biomolecules including proteins due to the change in structural conformation. There was a significant increment in the Th-1 cytokine expression (TNF-α and IFN-γ) which may lead to the activation of macrophages and microglia and generation of ROS and RNI (Bernstein and Miller, 2010; Pasichna et al., 2007). Consistent with these inferences, a significantly depleted antioxidant defense in the form of both enzymatic as well as non enzymatic antioxidants (Table 6.1) which is due to the ROS was recorded. These findings were further strengthened by the significantly higher amount of the peroxidation of the lipids in the EAE rats. It is worth mentioning that brain is especially sensitive to the ROIs due to the high lipid content.

Apart from the ROS, RNI's have been extensively studied for their role in inflammatory as well as the tissue degradation in human MS and EAE. Nitric oxide is an important signaling molecule in central nervous system with diverse roles (Bredt et al., 1990). The elevated nitrite/nitrate ratio has been postulated to be a biological indicator of the neurodegenerative phase of MS (Zaffaroni, 2003). Nitric oxide has been implicated in blocking of the axonal conduction in sufficiently higher concentrations (Smith et al., 2001) and other studies have highlighted its role in priming the neurodegenerative events (Xiaoping et al., 2006). Nitrite estimation showed a significantly higher amount of nitrite in the brain and serum of EAE rats after first acute phase. While its increase in the CNS tissue can have local effects, the increment in the serum may mark the beginning of the neurodegenerative phase of EAE.

Recently the presence of the polymorphonuclear cells e.g. neutrophils has been highlighted in the course of EAE and multiple sclerosis (Gray et al., 2008). Neutrophils release myeloperoxidase (MPO) and has been previously used as a marker for their content and
activity. Under oxidative conditions, MPO can catalyze the formation of nitrogen dioxide and peroxynitrite (ONOO⁻) (Vliet and Eiserich, 1997) from nitrite, the primary metabolite of nitric oxide. Till date no cellular antioxidant has been reported against peroxynitrite meaning a higher risk of cellular death in these conditions. There was a significantly raised peroxynitrite content and MPO in the EAE rats. The presence of myeloperoxidase in the vicinity of microglia can lead to the devastating consequences to the neuronal as well as glial health (Lefkowitz and Lefkowitz, 2008). In other words, peroxidase dependent oxidation of nitrite leads to the formation of peroxynitrite along with the further activation of microglia. This cues towards an alternate mechanism of toxic metabolite generation in EAE.

RNIs individually and especially ONOO⁻ has been reported to alter the protein function or can lead to the loss of activity which can have deleterious effects on the cellular health (Brown, 2010). Apart from this, RNIs can lead to the translocation of Bax (Bcl2 associated X protein) from cytoplasm to the outer membrane of the mitochondria leading to their permeability transition (Snyder et al., 2009). This shift can lead to the mitochondrial depolarization and loss of cellular ATP and thereby priming cells for apoptosis (Brown, 2010). The increased permeability of mitochondrial membranes leads to the release of several apoptotic factors into the cytoplasm e.g. Cytochrome-c and trigger the activation of proteases (Caspases) (Snyder et al., 2009). A significant increase in the activity of the central executor Caspase-3 (Fig.6.5) was noticed following EAE after the first acute phase of the disease. Caspase-3 can lead to the fragmentation of DNA by activating Caspase activated DNase (CAD) and deregulation of the cytoskeleton by break down of laminins. In event of any genomic DNA damage, the cell cycle regulator protein P53 is upregulated (Sendoel et al., 2010) which in turn can also lead to the translocation of Bax (Kashyap et al., 2010). These changes stimulate the activation DNA repair enzyme poly ADP ribose polymerase (PARP) in an attempt to repair the damaged DNA. Elevated Caspase-3 raised the PARP activity significantly (12.5%) in EAE brains. It is a well known fact that PARP operates with NADPH consumption for repair of the damaged DNA. In an attempt of repair the DNA, excessive energy resources are depleted. The mitochondrial dysfunction along with the elevated PARP leads to severe energy crisis and cells undergo apoptosis (Fig. 6.7) which includes cells neuronal or glial origin.
As discussed earlier, the progression of disease is also dependent upon the cellular loss within the plaques. The signs of the disability depend upon the spatial arrangement of the plaques in the cerebrospinal axis (Confavreux et al., 2000). Almost 50% of the M.S patients experience cognitive impairment during the course of the disease. Hippocampus is a cholinergic rich region considered as the memory retrieval device of the brain (Dupret et al., 2010). Any disease that can lead to the cellular loss in hippocampus can accumulate significant amount of cognitive deficits. Choline o-acetyl transferase (ChAT) decreased significantly (Fig.6.6) in the EAE rat hippocampus which may be due the loss of the ChAT expressing neurons. It is worth mentioning that the maximum amount of the ChAT depression was noticed in the CA2. These findings are consistent with the other reports regarding hippocampal synaptic loss and atrophy during EAE (Giulia et al., 2005).

In conclusion, these findings establish the role of bystander mechanism of cell death in the progression of the disease and demonstrate the role of peroxidase dependent oxidation of nitrite as an alternate mechanism of cell death in rat model of human multiple sclerosis.
**Figure 6.7**- Graphical abstract of the findings of the mechanism of cellular death during EAE. Autoimmunity developed towards the CNS antigens by immunization recruits T-lymphocytes (CD4+ and CD8+). CD4+ T-lymphocytes secrete Th-1 type cytokines which can lead to the activation of macrophages and microglia. These cellular stimulations can lead to the generation of ROS and RNI. ROS can lead to the direct cellular DNA damage. RNI in presence of peroxidases and lead to the formation of peroxynitrite. Peroxynitrite can make several essential proteins non functional and mediate the shuttling of the Bax from cytoplasm to the outer mitochondrial membrane triggering apoptosis by activation of PARP.