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HISTOCHEMICAL LOCALIZATION OF MONOAMINE OXIDASE AND GLUCOSE 6 PHOSPHATE DEHYDROGENASE AND THEIR PROBABLE ROLE IN GUINEA PIGS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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ABSTRACT
The pathogenesis and etiology of the demyelinating diseases, in particular Multiple Sclerosis, has been a subject for discussion and speculation since a long time. Several experimental models have been worked out to evolve the pathogenesis and etiology of demyelination. Experimental Allergic Encephalomyelitis is one such model, extensively studied for probing the basis of autoimmune reactivity and defining its role in neurologic disorders characterized by inflammation, demyelination including neurodegeneration as in multiple sclerosis in man. The two oxidoreductases, Monoamine oxidase and Glucose 6 phosphate dehydrogenase play a significant role in the pathogenesis of Experimental Allergic Encephalomyelitis involving hypertrophied astrocytes and mitochondria. Experimental Allergic Encephalomyelitis was induced in the adult healthy guinea pigs by weekly intradermal injection of homologous whole brain and spinal cord antigen together with complete Freund's adjuvant into the foot pad of the animal. The animals were observed for clinical features of the disease after injection. Histochemical localization of Monoamine oxidase was examined by Tryptamine – tetrazolium method of Glenner and Glucose 6 phosphate dehydrogenase activity by the method of Cohan. The increased activity of Glucose 6 phosphate dehydrogenase and Monoamine oxidase at the margins and in the immediate vicinity of the plaques might be due to hypertrophied astrocytes leading to an increased activity of oxidoreductases in experimental allergic encephalomyelitis. The Purkinje neurones show hyper activity of both the enzymes.

KEYWORDS: Experimental allergic encephalomyelitis, Multiple sclerosis, Inflammation, Demyelination, Monoamine oxidase, Glucose 6 phosphate dehydrogenase.

INTRODUCTION
Monoamine oxidases (MAO) are a family of enzymes that catalyze the oxidation of monoamines.[1,2] They are found, bound to the outer membrane of mitochondria in most cell types in the body. They belong to the protein family of flavin-containing amine oxidoreductases. The presence of MAO in areas lacking a blood-brain barrier, within astrocytes and in tanycytes that have direct contact with the cerebrospinal fluid, has important functional implications. This enzyme may control in part the entrance of circulating biogenic amines into the brain. An additional role for MAO B may be related to the intimate association between astrocytic processes and synaptic terminals. Thus, the presence of MAO-B may facilitate degradation of monoamine neurotransmitters released from terminals.[3] Glucose-6-phosphate dehydrogenase (G6PD) is a cytosolic (cytoplasmic) enzyme responsible for catalyzing the first and rate-limiting step in the hexose monophosphate pathway. G6PD provides critical protection against reactive oxygen species (ROS) and oxidative stress.[4] Relatively little is known about which CNS cell types are affected by oxidative injury in Multiple Sclerosis (MS) lesions. Extensive oxidative damage to proteins, lipids, and nucleotides occur in active demyelinating MS lesions, predominantly in reactive astrocytes. Enhanced antioxidant enzyme production in inflammatory MS lesions may reflect an adaptive defense mechanism to reduce ROS-induced cellular damage.[5] Thus the present study is an attempt to localize histochemically and demonstrate MAO and G6PD activity in guinea pigs with Experimental Allergic Encephalomyelitis (EAE) and their probable role in demyelination.

MATERIAL AND METHODS
Experimental Allergic Encephalomyelitis (EAE) was induced in the adult healthy guinea pigs of both sexes by weekly intradermal injections of homologous whole brain and spinal cord antigen together with complete Freund's adjuvant (CFA) in the ratio of 1:1 into the foot pad of the animal.

Experimental animals
Adult healthy animals of both sexes weighing about 450 to 500 grams were used for the study. All animals were acclimatized to the laboratory conditions prior to the experimentation. During acclimatization and throughout the experimental period the animals were given free access to water and standard diet for the animals.

Preparation of brain antigen
Fresh homologous whole brain and spinal cord of guinea pigs were dissected out and washed in freshly prepared normal saline. Nine parts by weight of homologous whole brain and spinal cord tissue was mixed with ten parts by weight of freshly prepared normal saline. The normal saline and homologous CNS tissue was homogenized by a homogenizer to a fine emulsion (homogenate). The homogenate was collected in a sterilized sample tube and preserved in the freezer for further use.

Adjuvant
The homologous CNS tissue homogenate and Freund's complete adjuvant (Diffco Laboratories USA) were thoroughly mixed at laboratory temperature in the ratio of 1:1 just before injection.

Dose and route of injection
0.5 ml of homologous whole brain and spinal cord antigen (CNS emulsion + complete Freund's adjuvant) was injected intradermally with a fine syringe needle into the foot pad of the animal.

Clinical observation or assessment
The weight and rectal temperature of the animals was recorded daily prior to feeding the animals every day. The clinical assessment of the animals was made daily according to standard method of Keith and McDermott (1980)[6] and the following clinical symptoms were observed:

- Weight loss
- Mild paraperesis (weakness of one or both hind limbs),
- Moderate paraperesis (slight dragging of hind legs) with fecal impaction, urinary retention and ataxia,
- Severe paraperesis or paraplegia (prominent dragging of hind limbs and pronounced ataxia),
- Moribund state
- Death.

For the histochemical examination of MAO and G6PD the experimental animals were divided into three groups of seven animals each. Animals of first group received one injection, animals of second group received two injections and the animals of third group received three injections at weekly intervals. The animals were sacrificed at random whether they were clinically ill or not on the days 2, 4 and 6 after first, second and third injection from each group. All the animals were sacrificed by decapitation. Immediately the spinal cord, brainstem and cerebellum were dissected out carefully. The tissue was freezed rapidly at – 40°C with automatic freezer in the cryostat (Minotome, USA). Fresh frozen sections of 18 – 21 µm were cut in cryostat maintained at – 30°C and mounted on clean glass slides without any adhesive. Alternate sections of the same tissue were used for the histochemical examination of MAO and G6PD. The slides were air dried at room temperature and incubated in the respective incubation media at 37°C for 45 minutes and washed with distilled water and mounted with glycerin jelly. The slides for MAO activity was incubated and examined by Tryptamine – tetrazolium method of Glenner et al (1957) [7] and the slides for G6PD activity by the method of Cohan (1959)[8] subsequently modified by Bara (1965).[9] The control sections were incubated along with the treated sections in the same incubating media and similar conditions for the histochemical examination of MAO and G6PD. The incubated sections were compared with the control sections and examined under light microscope.

RESULT
Bluish purple diformazon deposits indicate sites of MAO activity.

Dark purple coloured deposits indicate sites of G6PD activity.

One set of control animals were injected with 0.5 ml of complete Freund's adjuvant alone and the other set were injected with 0.5 ml of freshly prepared normal saline to compare with CFA injected ones. The control animals were sacrificed with the experimental animals of each group.

RESULTS / OBSERVATIONS
The observation of MAO and G6PD was demonstrated with respect to the colour intensity and thus graded as follows:

Grade		MAO	G6PD
No activity	–	No colour	No colour
Negligible	±	Faint bluish purple	Faint purple
Mild	+	Light bluish purple	Light purple
Moderate	++	Bluish purple	Purple
Strong	+++	Dark bluish purple	Dark purple

Monoamine oxidase
Histochemical localization of Monoamine oxidase in the spinal cord is shown in Fig. 1.

Ventral grey horn cells	**– to ±**
Dorsal grey horn cells	±
Lateral grey horn cells	–
Internuncial neurons	–
Ependyma lining the central canal	±
Substantia gelatinosa externa	++
White matter	+

Cerebellum

Cortex	
Molecular layer	++

Purkinje layer	–
Granule cell layer	+
White matter	++ **to** +++

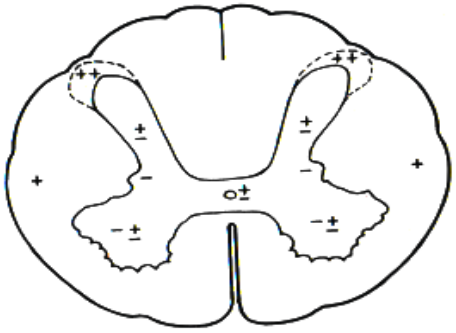

Fig. 1: Diagrammatic representation of the distribution of Monoamine oxidase in the spinal cord of control animal.

Glucose 6 phosphatase dehydrogenase

Histochemical localization of Glucose 6 phosphatase dehydrogenase in the spinal cord is shown in Fig. 2

Ventral grey horn cells	+++
Dorsal grey horn cells	+
Lateral grey horn cells	+
Internuncial neurons	++
Ependyma lining the central canal	++
Substantia gelatinosa externa	++ **to** +++
White matter	± **to** +

Cerebellum

Cortex	
Molecular layer	++
Purkinje layer	++ **to** +++
Granule cell layer	++
White matter	–

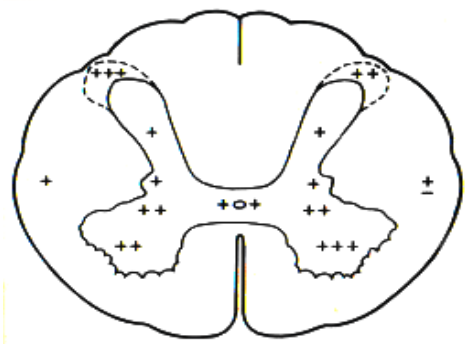

Fig. 2: Diagrammatic representation of the distribution of Glucose -6-phosphate dehydrogenase.

Observation in experimental animals: MAO

MAO activity was intensely present in the EAE plaques in the lateral funiculus of the spinal cord (Fig. 4), close to substantia gelatinosa which is the only region where MAO activity is demonstrable in the normal (control) spinal cord (Fig. 3). MAO activity was also found in moderate to intense degree dispersed throughout the white and grey matter in patches (Fig. 5). MAO activity is notably increased in the white matter of cerebellum (Fig. 7) compared to control cerebellum (Fig. 6). The Purkinje cell layer and molecular layer showed moderate degree of activity. Granule cell layer showed increased activity in patches (Fig. 7).

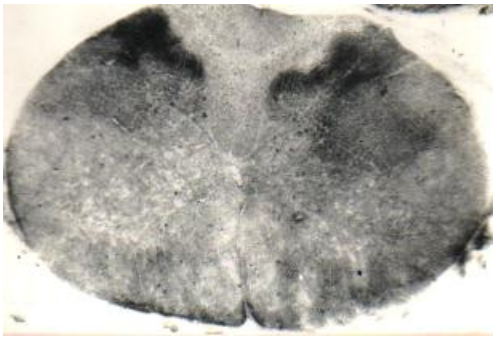

Fig. 3: Section of control spinal cord showing moderate MAO activity in substantia gelatinosa externa. MAO (Glenner) X20.3.

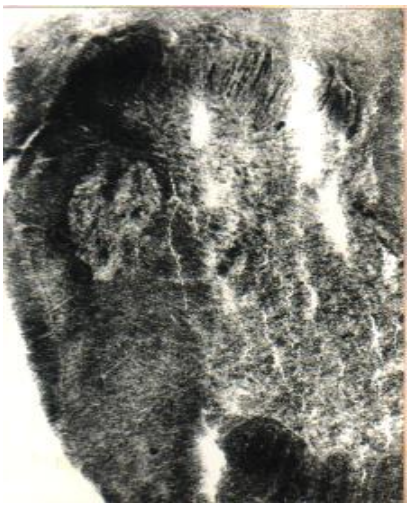

Fig. 4: Section of spinal cord with EAE showing moderate to strong activity in and around a lesion in the lateral funiculus close to substantia gelatinosa MAO (Glenner) X 42.3.

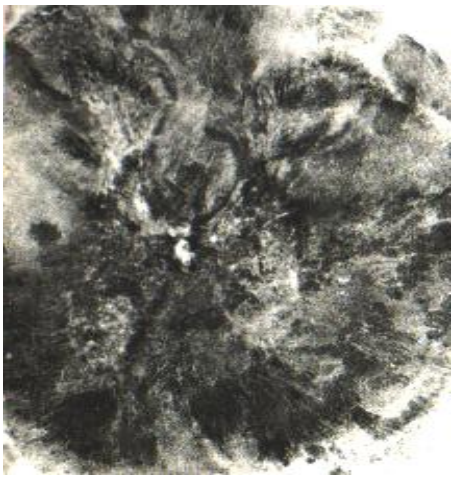

Fig. 5: Section of spinal cord with EAE showing moderate to intense degree MAO activity dispersed throughout the white and grey matter in patches MAO (Glenner) X 42.3.

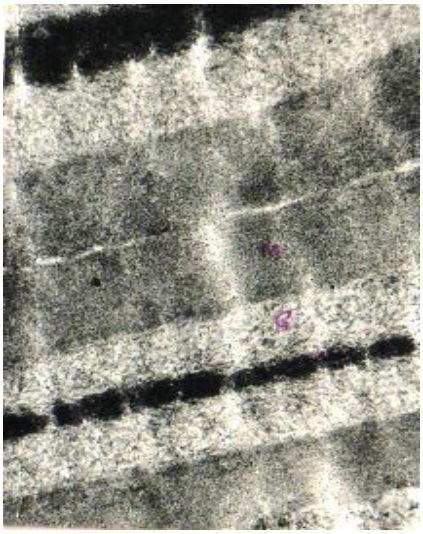

Fig. 6: Section of control cerebellum showing MAO activity, G – Granule cell layer, M – Molecular layer, P – Purkinje cell layer, W – White matter. MAO (Glenner) X 64.3

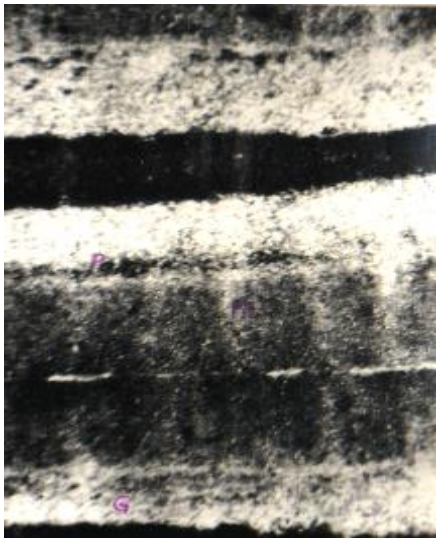

Fig. 7: Section of cerebellum with EAE showing increased activity in molecular layer, moderate activity in Purkinje cell layer, increased activity in granule cell layer in patches and very strong activity in white matter. MAO (Glenner) X 64.3.

Observation in experimental animals: G6PD

G6PD activity could be demonstrated intensely in patches in the ventral funiculus of the spinal cord (Fig. 9) compared to control spinal cord (Fig. 8). Neuroglia in the white matter and grey matter of the spinal cord showed strong activity (Fig. 10 & 11). Strong activity could also be demonstrated in the molecular and Purkinje cell layers of cerebellum (Fig. 13 & 15) compared to control (Fig. 12 & 14). Moderate to strong activity could be seen in granule cell layer (Fig. 13 & 15), whereas mild activity was seen in white matter of cerebellum (Fig. 13).

Fig. 8: Section of control spinal cord showing distribution of G6PD, G6PD (Cohen) X 20.3.

Fig. 9: Section of spinal cord with EAE showing strong activity in patches in ventral funiculus. G6PD (Cohen) X 20.3.

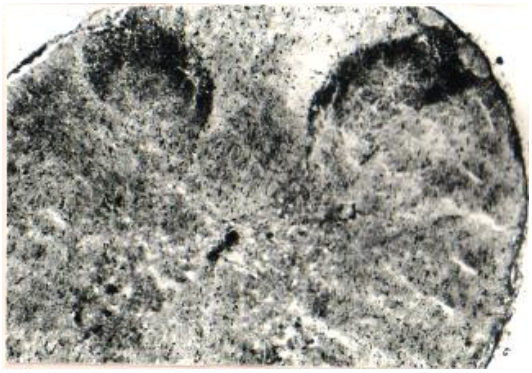

Fig. 10: Section of spinal cord with EAE showing strong activity in neuroglia in white and grey matter. G6PD (Cohen) X 42.3.

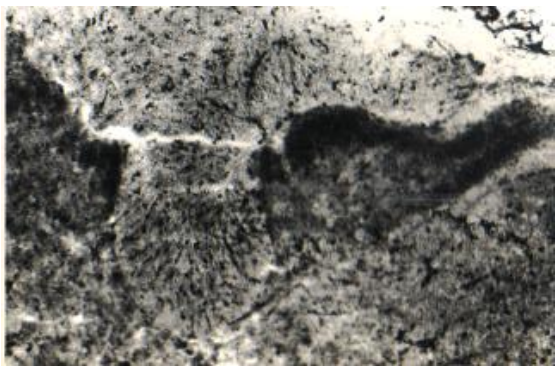

Fig. 11: Section of spinal cord with EAE showing strong activity in neuroglia in white and grey matter. G6PD (Cohen) X 50.3.

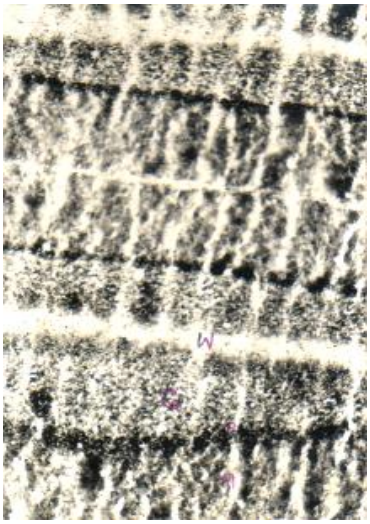

Fig. 12: Section of control cerebellum showing distribution of G6PD activity, G6PD (Cohen) X 64.

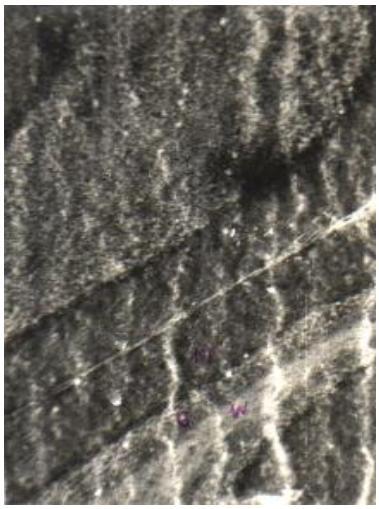

Fig. 13: Section of Cerebellum with EAE showing strong activity in molecular layer and Purkinje cell layer. Moderate activity in granule cell layer and mild activity in white matter G6PD (Cohen) X 64.

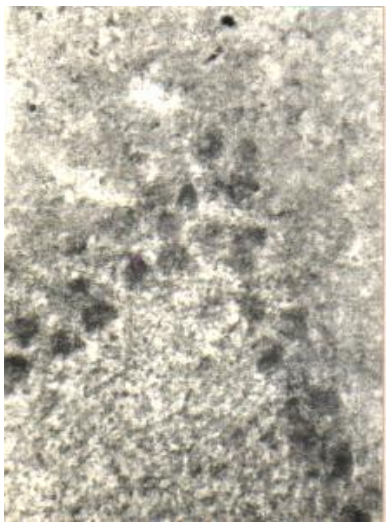

Fig. 14: Section of control cerebellum showing G6PD activity in Purkinje neurones, G6PD (Cohen) X 256.

Fig. 15: Section of cerebellum with EAE showing increased activity in Purkinje neurones. G6PD (Cohen) X 256 G – Granule cell layer M – Molecular layer, P – Purkinje cell layer W – White matter.

DISCUSSION

Marked activity of oxidative enzymes has been observed in a variety of biologically active or proliferating cells, such as osteoclasts[10,11] phagocytes,[12] foreign body cells[13] and macrophages.[14] An increase of enzyme activity in oligodendroglia occurs during the period of myelination, both in situ[15] and in vitro.[16] Roizin and Kolb (1957)[17] and Ibrahim and Adams (1965)[18] have reported, that the oxidases and peroxidases activities increased significantly around the demyelinating plaques of MS. In the present investigation, the two oxidoreductases studied i.e. MAO and G6PD showed increased activity in and around the margins of the lesions. The increased activity at the margins and in the immediate vicinity of the plaques might be due to hypertrophied astrocytes leading to an increased activity of oxidoreductases in EAE. Similar findings by Ibrahim and Adams (1963)[19] showed that the increased neuroglial population in the surroundings of the plaques in MS was mainly due to increased number of oligodendrocytes and hypertrophied astrocytes exhibiting oxidative activity. In our observation however, there was no increase in the population of oligodendroglia at or around the lesion sites, rather they were decreased in number or were on their way of disintegration.

During demyelination, involvement of oxidative stress was evident by disruption of mitochondrial structure and temporal decline in reduced glutathione (GSH) levels, later returning to normal.[20] Wender et al (1976)[21] investigated histoenzymic pattern of oxidative enzymes (G3PD, IDH, SD, G6PD, HBD, NADPH: dehydrogenase) in EAE produced in rats and the results obtained by him lead to following conclusions: (1) The neuroglia, including the white matter oligodendroglia of immunized rats, exhibits increased oxidoreductase activities; (2) The neuroallergic reaction induces some

stimulation of the oxidoreductive metabolism of oligodendroglia; (3) The enzymatic hyperactivity in EAE does not show any relation to the morphological signs of alterations of the myelin sheath.

G6PD is an oligodendrocyte enriched enzyme. Its activity in oligodendrocytes and myelin is more in younger rats but progressively lower in older rats.[22] The activity of Glycerol phosphate dehydrogenase (GPDH), G6PD, and lactate dehydrogenase (LDH) was significantly lower in both neurons and astrocytes than in oligodendrocytes isolated from the brains of developing rats.[23] MS is a chronic inflammatory disease of the CNS marked by infiltration of monocyte-derived macrophages in the brain parenchyma. Macrophages contribute to disease pathology by secretion of inflammatory mediators, such as reactive oxygen species (ROS), which are involved in various processes underlying MS pathology, including monocyte migration across the blood-brain barrier, phagocytosis and degradation of myelin, axonal degeneration, and oligodendrocyte damage.[24] High concentrations of ROS cause oxidative stress, which induces transcriptional activation of phase II detoxification enzymes, such as the antioxidant protein NAD (P) H: quinone oxidoreductase 1 (NQO1).[24] Oxidative stress may, in the form of O2 +, H2O2, OH+, peroxyl radicals and hydroperoxides, contribute to the development of endothelial injury in MS.[25,26] Cerebral endothelial cells (CEC) also express cholinesterases, monoamine oxidase, alkaline phosphatase and aromatic decarboxylases for catabolizing humoral transmitter substances; g-glutamyl transpeptidase (GGT) also appears to be another marker specific for CEC.[27] While ROS act as tertiary messengers, excess oxidants reduce barrier function, and involve reorganization of tight junction architecture.[28] ROS also activate kinase pathways and transcription factors.[29,30]

EAE and MS are no more primary inflammatory and demyelinating diseases as axonal damage and neurodegeneration occur in the acute and sub-acute phases of the disease.[31] MS disability results from neuronal and axonal loss – the hallmark of neurodegenerative diseases. Neurodegeneration is initiated by microglial activation and mediated by oxidative stress and excitotoxicity. The same sequence of events has been consistently observed in MS. In both pathological states, peroxynitrite is the common initiating factor of oxidative stress and excitotoxicity and is thus a potential interesting therapeutic target.[32] Oxidative stress leads to multiple lipid and protein damages via peroxidation and nitration processes. The pathomechanisms of excitotoxicity are complex; involving glutamate overload, ionic channel dysfunction, calcium overload, mitochondriopathy, proteolytic enzyme production and activation of apoptotic pathways. Inflammation not only causes axonal and neuronal loss but also initiates the degenerative cascade in the early stage of MS.[32]

Oxidative stress must be controlled by supplying known antioxidant nutrients and by minimizing effects of substances that stimulate ROS.[33] An imbalanced production of ROS plays a role in the pathogenesis of a number of human diseases such as ischemia/reperfusion injury, atherosclerosis, neurodegenerative diseases, cancer, and allergy.[34] Relatively little is known about which CNS cell types are affected by oxidative injury in MS lesions. The presence of extensive oxidative damage to proteins, lipids, and nucleotides occur in active demyelinating MS lesions, predominantly in reactive astrocytes and myelin-laden macrophages. Oxidative stress can be counteracted by endogenous antioxidant enzymes that confer protection against oxidative damage. Particularly, hypertrophic astrocytes and myelin-laden macrophages expressed an array of antioxidant enzymes. Enhanced antioxidant enzyme production in inflammatory MS lesions may reflect an adaptive defense mechanism to reduce ROS-induced cellular damage.[35] However, the increase in oxidative enzyme activity in astrocytes depend on the salt concentration of the environment, and this was considered a specific metabolic response which was seen only in astrocytes.[36] ROS contribute to the formation and persistence of MS lesions by acting on distinct pathological processes. In brain tissue of MS patients, enhanced expression of Nrf2/ARE-regulated antioxidants is suggestive of the occurrence of oxidative stress in these lesions.[37]

Mitochondria are intimately involved in pathways leading to the neuronal cell death characteristic for the areas of epileptogenesis.[38] However, mitochondrial injury, resulting in energy failure, is a key element of neurodegeneration in MS and is apparently driven by radical production in activated microglia.[39] Given the central role of the mitochondria in many important cellular functions including energy production, it is reasonable that its dysfunction is the key contributor to neurodegenerative process of MS.[40] Deficient mitochondrial metabolism may generate more ROS that can wreak havoc in the cell. Therefore, mitochondrial dysfunction is an attractive candidate for neuronal degeneration. Recently, several lines of evidence suggests that mitochondrial dysfunction is present in patients with MS. Mitochondrial DNA alterations, mitochondrial structural changes, defective mitochondrial DNA repair events, abnormal mitochondrial enzyme activities, mitochondrial gene expressions, increased free radical production and oxidative damage have been reported in patients with MS and EAE in mouse models.[40] Regardless of the exact mechanism responsible for mitochondrial dysfunction due to excess NO synthesis, an increasing body of evidence now suggests that the intracellular antioxidant content, especially glutathione (GSH) concentrations, may be a key factor in determining cellular vulnerability. A lowered neuronal GSH concentration has been considered to be a critical factor contributing to NO/ONOO2-mediated mitochondrial damage and neurotoxicity.[41,42,43,44] Unlike neurons, in

astrocytes a high GSH status is maintained despite endogenously formed NO[45] or exogenously added ONOO2.[40] In this context, high antioxidant concentrations, including those of GSH[46,47] vitamin E, and ascorbate, as well as the activities of glutathione metabolism enzymes,[48] might account for the specific resistance of astrocytes to oxidative stress; G6PD may contribute to the high resistance of astrocytes against NO/ONOO2-mediated cellular damage.[49] Oxygen species are key participants in damage caused by virus infections, progression to cancer, neurodegenerative processes (including cell death, motor neuron diseases and axonal injury), and both infarction and brain edema. Therefore, tissues must be protected from this oxidative injury by expression of stress-response genes and genes encoding antioxidant enzymes and activation of other related transcriptional regulatory proteins.[34]

G6PD is a cytosolic (cytoplasmic) enzyme responsible for catalyzing the first and rate-limiting step in the hexose monophosphate pathway and provides critical protection against ROS and oxidative stress.[50] Cell growth and proliferation are affected by G6PD.[51] G6PD activity is dispensable for pentose synthesis, but is essential to protect cells against even mild oxidative stress.[52] Wender et al. (1976)[12] reported strong G6PD activity of the cellular infiltrates and neuroglia in the vicinity of inflammatory foci in animals with EAE and also the neuroglia in the white matter which were quite remote from the inflammatory plaques. In the present investigation, the neuroglia in the white matter as well as grey matter exhibited strong activity of G6PD. This increased activity may be due to increased number of hypertrophied astrocytes. The mitochondrial population has been reported to increase in hypertrophied astrocytes.[14,15] Luse (1958)[53] and Nelson et al. (1963)[54] have reported that the normal astrocytes contain a few mitochondria, whereas activated astrocytes contained increased mitochondrial population. This suggests that the neuroglial enzyme hyperactivity may be indicative of an increased biological activity provoked by exogenous factors, such as the cerebral antigen or intoxication.

Saragea et al. (1965)[55] observed an increased activity of MAO in the brain of Guinea pigs with EAE, but could not explain the relationship of this hyperactivity and the intensity of the disease. Monoamine oxidases (MAO) are a family of enzymes that catalyze the oxidation of monoamines; plays a central role in the catabolism of monoaminergic neurotransmitters and partly in the conversion of a number of pharmacological agents from inactive to active states.[56] They are found, bound to the outer membrane of mitochondria in most cell types in the body.[1,2] Because of the vital role that MAOs play in the inactivation of neurotransmitters, MAO dysfunction (too much or too little MAO activity) is thought to be responsible for a number of psychiatric and neurological disorders like schizophrenia [57,58] depression [59] and migraines.[60,61]

MAO-B is located in astrocytes (both protoplasmic and fibrillary astrocytes throughout the brain) and serotonin-containing neurons, whereas oligodendrocytes do not contain this enzyme. MAO-A has been demonstrated in the endothelial cells lining cerebral blood vessels and the ependymal cells lining the ventricle. The presence of MAO-B in areas lacking a blood-brain barrier, within astrocytes and in tanycytes that have direct contact with the cerebrospinal fluid, has important functional implications.[3] This enzyme may control in part the entrance of circulating biogenic amines into the brain, may be related to the intimate association between astrocytic processes and synaptic terminals and facilitate degradation of monoamine neurotransmitters released from terminals.[3] MAO serves a protective function by regulating levels of exogenous, dietary amines; many of which would otherwise exert potent pressor effects and may constitute an important functional aspect of the blood-brain barrier, where it is thought to prevent the entry of potentially toxic false neurotransmitters. It has been suggested that increased MAO levels may represent a risk factor for a cell's potential to sustain oxidative injury.[61] Hence increased activity of MAO in Purkinje neurones in our studies suggests that there may be a possibility of Purkinje neurones to have potential for oxidative injury in EAE. Purkinje cells play a fundamental role in controlling motor movement. Most Purkinje cells release a neurotransmitter called GABA (gamma-aminobutyric acid), which exerts inhibitory actions on certain neurons and there by reduces the transmission of nerve impulses. These inhibitory functions enable Purkinje c ells to regulate and coordinate motor movements.[56] In the present investigation the Purkinje cells displayed high activity of G6PD. G6PD protects against endogenous ROS mediated neurodegeneration. With increased oxidative stress, G6PD expression and / or activity in vitro[62,63] and in vivo is increased.[64] Increased neuronal G6PD expression has been observed in the hippocampus of Alzheimer's disease patients.[65] Our results suggest that G6PD may be an essential protective enzyme preventing ROS initiated neurodegenerative oxidative damage to Purkinje neurones associated with EAE and this hyperactivity might have been stimulated by the brain antigen.

CONCLUSION

In the present investigation, the two oxidoreductases studied i.e. MAO and G6PD showed increased activity in and around the margins of the lesions. The increased activity at the margins and in the immediate vicinity of the plaques might be due to hypertrophied astrocytes leading to an increased activity of oxidoreductases (G6PD and MAO) in EAE. The oligodendroglias around the lesions and in immediate vicinity of the lesions were decreased in number or on their way of disintegration. The neuroglia in the white matter as well as grey matter exhibited strong activity of G6PD and MAO. The neuroglial enzyme hyperactivity may be indicative of an increased biological activity provoked by exogenous

factors, such as the cerebral antigen or intoxication. Increased activity of MAO in Purkinje neurones suggests that there may be a possibility of Purkinje neurones to have potential for oxidative injury in EAE. High activity of G6PD in Purkinje neurons of cerebellum suggests that the G6PD may be an essential protective enzyme preventing ROS initiated neurodegenerative oxidative damage to Purkinje neurones associated with EAE and this hyperactivity might have been stimulated by the brain antigen.

DEDICATION

This research paper is dedicated to my supervisor late Professor Dr. G. C. Sensharma, Member of the International Association of Neuroanatomists, Professor of Neuroanatomy and Head of the Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

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