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HISTOCHEMICAL LOCALIZATION OF ALKALINE AND ACID PHOSPHATASE AND THEIR PROBABLE ROLE IN GUINEA PIGS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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ABSTRACT
Experimental allergic encephalomyelitis has attracted wide attention as a meaningful model for probing the basis of autoimmune reactivity and defining its role in neurologic disorders characterized by inflammation and demyelination as in multiple sclerosis in man. Activated inflammatory cells and microglia can secrete a variety of enzymes and factors responsible for the breakdown of myelin in the autoimmune diseases like multiple sclerosis and experimental allergic encephalomyelitis. Experimental allergic encephalomyelitis was induced in the adult healthy guinea pigs by weekly intradermal injections 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 alkaline phosphatase was carried out by Gomori's Calcium-Cobalt method and acid phosphatase by Gomori's Lead Acetate method. The increased activity of alkaline phosphatase indicates its involvement in active transport needed for the phagocytic action at the lesion site. The increased activity of acid phosphatase might be due to increased activity in the microgliocytes or phagocytes or hypertrophied astrocytes. Probably the inflammatory cells such as lymphocytes and monocytes may be the cause of the increased acid phosphatase activity in the central nervous system of animals with experimental allergic encephalomyelitis, and that enzymes from these cells possess the capability of digesting myelin basic protein.

KEYWORDS: EAE, MS, Inflammation, Demyelination, Alkaline phosphatase, Acid phosphatase.

INTRODUCTION
Activated circulating monocytes, macrophages, and microglia in the central nervous system can secrete a variety of enzymes and factors that result in breakdown of myelin proteins, presentation of antigens to T and B lymphocytes, and generation of high oxidative stress, all of which may contribute to myelin and axonal damage.[1–6] Several enzymes have been histochemically localized and demonstrated in multiple sclerosis (MS) by various investigators. Elevated lysosomal enzyme activity has been reported in MS and experimental allergic encephalomyelitis (EAE); however it is not clear whether the elevated lysosomal enzyme concentration causes myelin breakdown or it simply reflects a secondary phenomenon due to inflammatory reaction or digestion of myelin debris.[7, 8, 9] Contradictory reports have been obtained regarding the alkaline phosphatase (ALP) activity in MS and EAE. The functional significance of these alterations and their role in demyelination is not yet clear, although the role of ALP in active transport process have been demonstrated suggesting the possible role in removal of myelin debris. Thus the present study is an attempt to localize histochemically and demonstrate ALP and acid phosphatase (ACP) activity in EAE in guinea pigs and their probable role in demyelination.

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

Experimental animals
Adult healthy animals of both sexes weighing 400 to 500 grams were used for the study. 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 pig 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 emulsion (homogenate) and Freund's complete adjuvant (Diffco Laboratories USA) were (at laboratory temperature) thoroughly mixed 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[22] into the foot pads of the animal.

Clinical observations or assessment
The weight and rectal temperature of the animals was recorded daily prior to feed the animals every day. The clinical assessment of the animals was made daily according to standard method of Keith and McDermott (1980)[10] 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 and urinary retention and ataxia,
- Severe paraperesis or paraplegia (prominent dragging of hind limbs and pronounced ataxia),
- Moribund state and
- Death

For the histochemical examination of Alkaline and Acid phosphatase:
Experimental animals were grouped into three groups of five 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 an over dose of ether anaesthesia. Immediately thorax of the animal was opened and perfused with 15 ml of cold (2 – 4^{o} C) neutral formalin intracardially, introduced through left ventricle and a vent in the right atrium allowing outlet. The spinal cord, brainstem and cerebellum were dissected out immediately and cut into small pieces of 3 – 5 mm and fixed in cold neutral formalin maintained at 2 – 4^{o}C in refrigerator for 12 – 24 hours.

Fresh frozen sections of 18 – 21 µm were cut in cryostat maintained at – 20^{o}C and mounted on clean glass slides without any adhesive. Alternate sections of the same tissue were used for the histochemical examination of alkaline and acid phosphatase. The slides were air dried for half an hour and incubated in the respective incubation media at 37^{o}C and processed with.
Calcium Cobalt method (after Gomori – 1951) for alkaline phosphatase[11]
Lead Acetate method (after Gomori 1952 –1956) for Acid phosphatase [12]

The incubated sections were compared with the control sections and examined under light microscope.

RESULT
Block deposits indicated alkaline phosphatase activity.
Brown to dark brown deposits indicate acid phosphatase 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. The control sections were incubated along with the treated sections for the histochemical examination of alkaline and acid phosphatase.

RESULTS AND OBSERVATIONS
The observation of the alkaline and acid phosphatase was demonstrated with respect to the colour intensity and thus graded as follows.

Grade	Alkaline Phosphatase	Acid Phosphatase
No activity –	No colour	No colour
Negligible ±	Grey	Faint brown
Mild +	Greyish black	Light brown
Moderate ++	Black	Brown
Strong +++	Jet black	Dark brown

OBSERVATION
ALP in control animals
Spinal cord:
Histochemical localization of ALP in the spinal cord is shown in Fig. 1 and 2. Activity of ALP is mainly localized in the blood vessels.
All the neurones in grey matter ±
Substantia gelatinosa externa ++
White matter ±

Brainstem: ALP activity could be demonstrated in the blood vessels, lepto meninges and the neuropil of brainstem nuclei.

Cerebellum:

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

ALP in experimental animals

Moderate ALP activity could be demonstrated in patches in the posterior funiculus towards the periphery and bordering the grey matter (Fig. 3 and 4) and white matter in the periphery of lateral and ventral funiculi. Increased (moderate) activity of ALP in a patch could be demonstrated at the junction of anterior and lateral funiculi which also showed demyelination when stained with Luxol fast blue and neutral red (Fig. 5). White matter bordering the lateral funiculus and anterior median fissure showed increased activity (Fig. 5). ALP was seen in and around the perivascular cuffing in the brainstem (Fig. 6 and 7).

ALP in the cerebellar cortex showed moderate to strong activity in granule cell layer and Purkinje cell layer and mild activity in the molecular layer (Fig. 8 and 9). The white matter did not show any activity (Fig. 9).

Acid phosphatase in control animals.

Spinal cord:

Histochemical localization of acid phosphatase in the spinal cord is shown in (Fig. 10 and 11).

Ventral grey horn cells	+++
Dorsal grey horn cells	+++
Lateral grey horn cells	+++
Internuncial neurones	++ to +++
Ependyma lining central canal	++
Substantia gelatinosa externa	++
White matter	+

Cerebellum:

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

Acid phosphatase in experimental animals

Acid phosphatase activity could be demonstrated to a fairly heightened degree in patches in the lateral funiculus which also showed demyelination when stained with Luxol fast blue and neutral red (Fig.12 and 13). Moderate acid phosphatase activity could also be demonstrated in the anterior funiculus (Fig. 12 and 13), and acid phosphatase could not be demonstrated in the dorsal funiculus. The cerebellar cortex showed moderate to strong activity in the granule cell layer, Purkinje cell layer, and mild activity in the white matter also (Fig. 14 and 15).

ILLUSTRATIONS

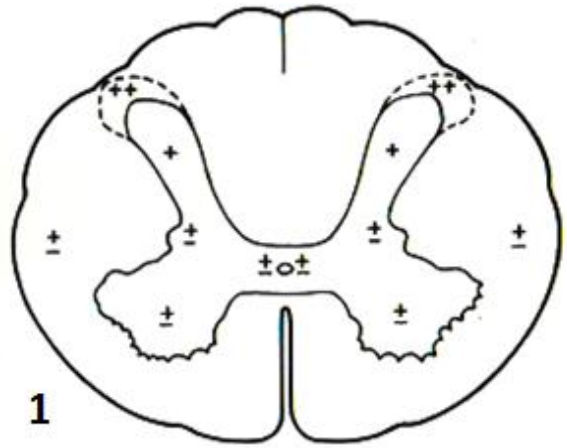

Fig. 1: Diagrammatic representation of the distribution of alkaline phosphatase in the control animal.

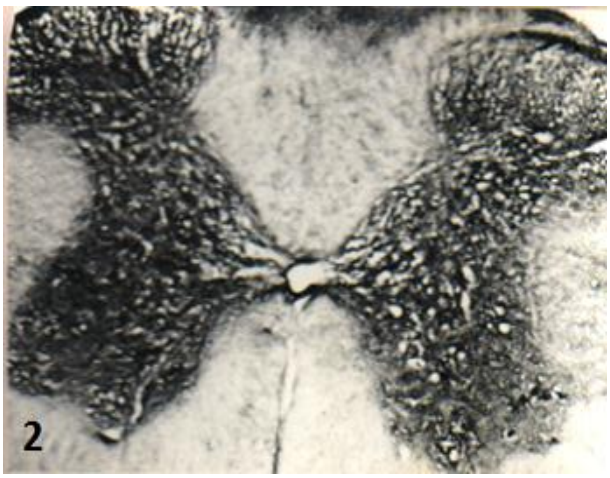

Fig. 2: Section of control spinal cord showing alkaline phosphatase Alkaline phosphatase (Gomori's method) X 50.3.

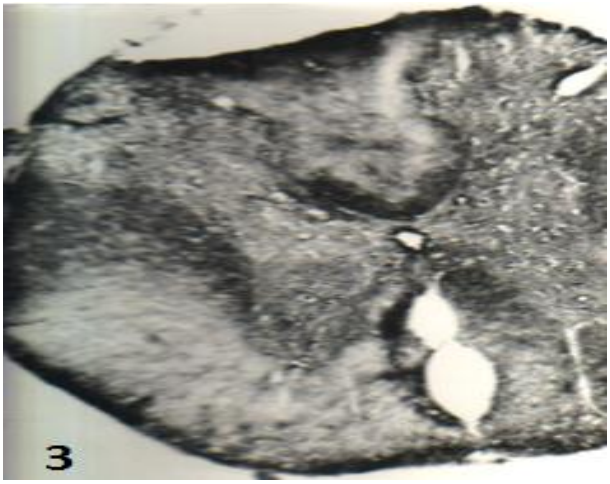

Fig. 3 Section of spinal cord with EAE showing increased alkaline phosphatase activity in patches in dorsal funiculus, towards the periphery and bordering the grey matter.
Alkaline phosphatase (Gomori's method) X 42.3

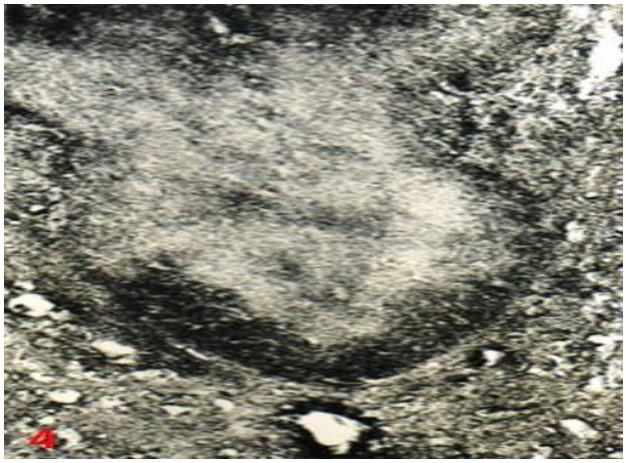

Fig. 4 A portion of Fig. 3 is magnified, showing increased activity in dorsal funiculus.
Alkaline phosphatase (Gomori's method) X 107.5

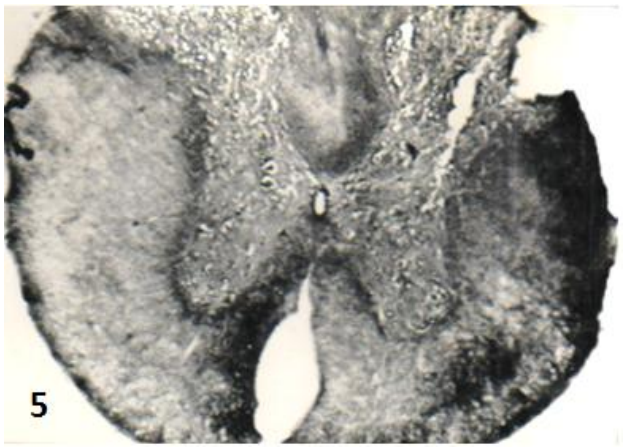

Fig. 5 Section of the spinal cord with EAE showing increased activity in a patch at the lateral funiculus, junction of lateral and anterior funiculi and bordering the anterior median fissure
Alkaline phosphatase (Gomori's method) X 42.3

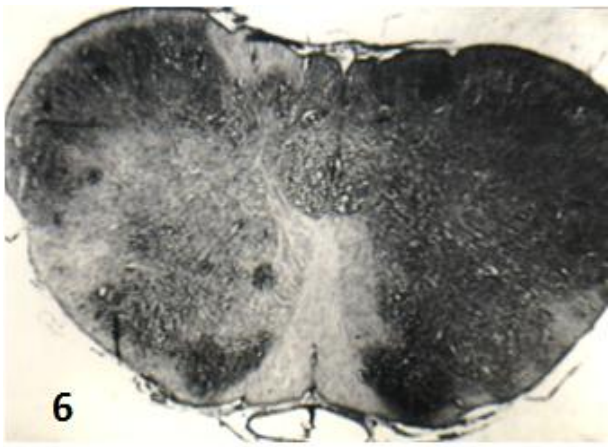

Fig. 6 Section of brainstem with EAE showing increased activity of alkaline phosphatase in and around the perivascular cuffing.
Alkaline phosphatase (Gomori's method) X 20.3

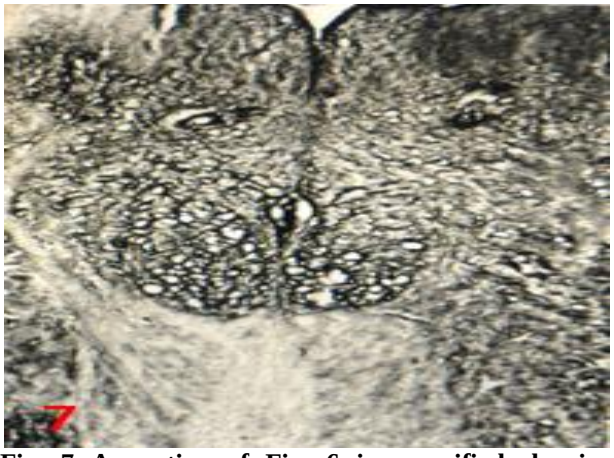

Fig. 7 A portion of Fig. 6 is magnified showing increased activity in the perivascular cuffings.
Alkaline phosphatase (Gomori's method) X 42.3

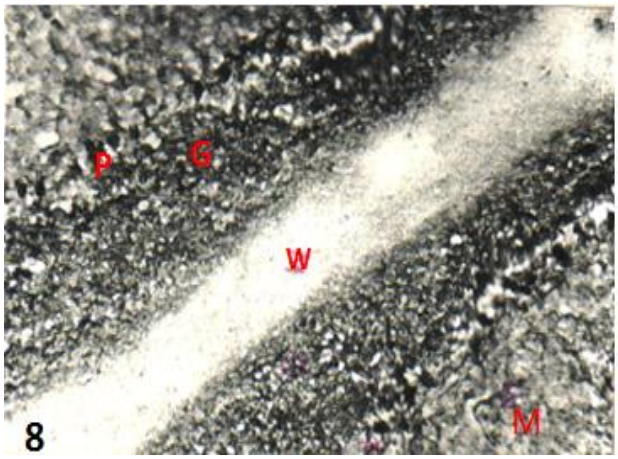

Fig. 8 Section of control cerebellum showing alkaline phosphatase activity
Alkaline phosphatase (Gomori's method) X 70.5
W – White matter G – Granule cell layer
M – Molecular layer P – Purkinje cell layer

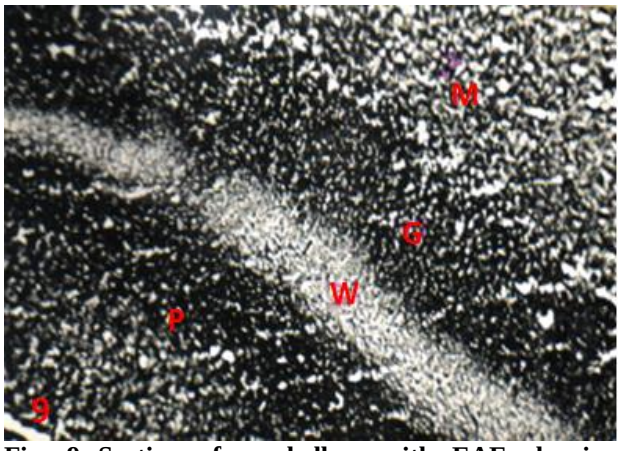

Fig. 9 Section of cerebellum with EAE showing moderate to strong activity in granule cell layer and Purkinje cell layer and mild activity in the molecular layer, no activity in white matter
Alkaline phosphatase (Gomori's method) X 70.5
W – White matter G – Granule cell layer
M – Molecular layer P – Purkinje cell layer

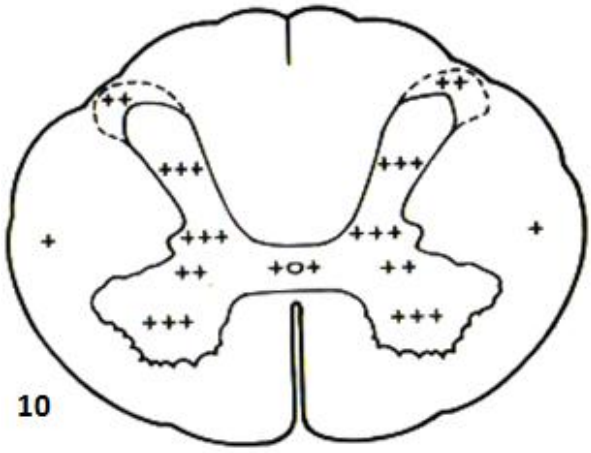

Fig. 10 Diagrammatic representation of the distribution of acid phosphatase in the spinal cord of control animal.

Fig. 11: Section of control spinal cord showing distribution of acid phosphatase activity. (Gomori's method) X 42.3.

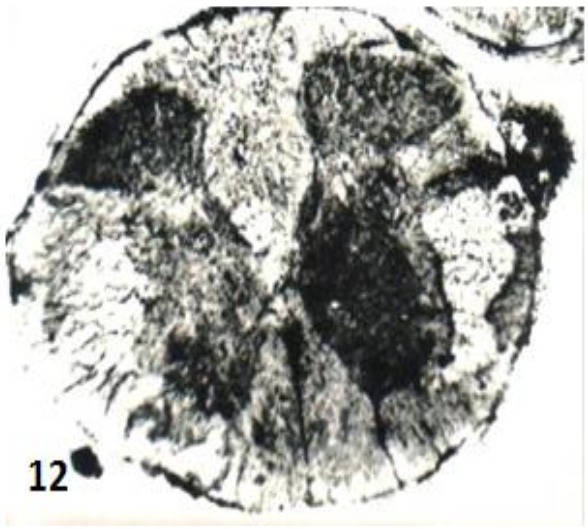

Fig. 12 Section of spinal cord with EAE showing moderate to strong acid phosphatase activity in patches in lateral funiculus
Acid phosphatase (Gomori's method) X 20.3

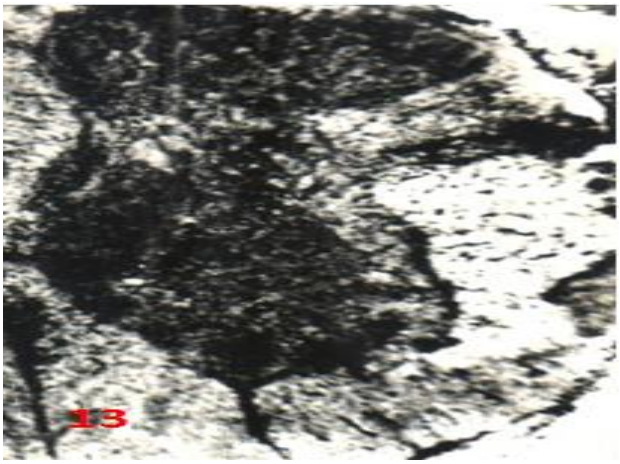

Fig. 13 Right portion of Fig. 12 is magnified
Acid phosphatase (Gomori's method) X 42.3

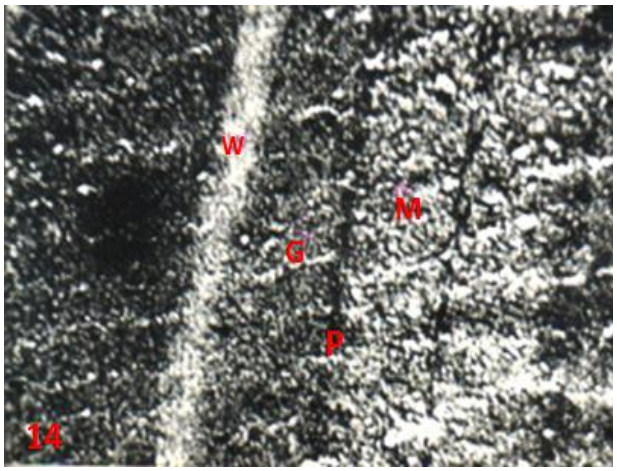

Fig. 14 Section of control cerebellum showing distribution of acid phosphatase activity
Acid phosphatase (Gomori's method) X 42.3
W – White matter G – Granule cell layer
P – Purkinje cell M – Molecular layer

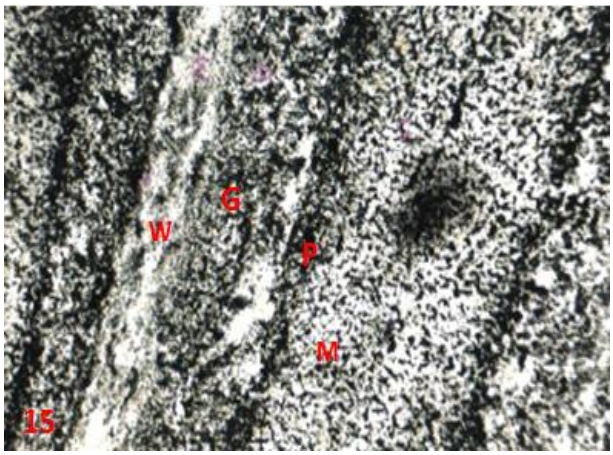

Fig. 15 Section of cerebellum with EAE showing moderate activity in molecular layer, strong activity in Purkinje cell layer and granule cell layer and mild activity in nerve fibre layer (white matter)
Acid phosphatase (Gomori's method) X 42.3
W – White matter G – Granule cell layer
P – Purkinje cell M – Molecular layer

DISCUSSION

ALP is a ubiquitously expressed enzyme which can neutralize endotoxins, an endogenous danger signal released during brain injury.[13] The localization of alkaline phosphatase activity in the early stages (before the onset of clinical symptoms) of EAE was similar to that in control animals. Inactive lesions with marked perivascular fibrosis showed no alkaline phosphatase reaction products. ALK activity in unaffected areas showed the same localization as that in control animals throughout the various clinical stages of EAE.[14] Alkaline Phosphatase is found primarily in cell membrane where active transport process takes place. The initial inflammatory and actively demyelinating stage with perivascular cuffs of mononuclear cells showed positive activity in correlation to the findings of Kato S and H. Nakamura (1987) who demonstrated high activity of alkaline phosphatase on the luminal surface in many endothelial cells.[14] This increased activity at the inflammatory and demyelinating lesions and in relation to endothelial cells might be related to the functioning of endothelial cells and the blood brain barrier. The functions of the endothelia, assessed by ouabain-sensitive K+-dependent p-nitrophenylphosphatase activity were impaired as EAE progressed, suggests the endothelial impairment of active transport of metabolites. With the development of inflammatory demyelination in EAE, the BBB in affected areas became more and more altered and gradual morphological and functional impairment of the BBB developed (15). An increase in blood-CSF barrier permeability; an important role of the blood-brain barrier for the central nervous system immunoglobulin homeostasis during EAE was suggested by Zlokovic BV et al (1989).[16]

Interaction between various inflammatory cells and endothelial cells lining selective blood vessels of the blood-brain barrier (BBB) is an initial, important event during inflammatory conditions of the central nervous system (CNS).[17] In disorders such as MS, the barrier becomes compromised with the result that there is an intense infiltration of the CNS by T lymphocytes whose subsequent activity appears to underlie the onset and progression of disease. Lymphocytes bind to cerebral endothelial cells, migrate across the blood vessel walls and enter the CNS parenchyma.[18] EAE, a rodent model for multiple sclerosis, have enabled us to understand some of the molecular mechanisms involved in immune cell entry into the CNS; in particular, the realization that alpha4-integrins play a predominant role in leukocyte trafficking to the CNS has led to the development of a novel drug for the treatment of relapsing-remitting multiple sclerosis, which targets alpha4-integrin mediated immune cell migration to the CNS. Immune cell entry into the CNS critically depends on the unique characteristics of the brain barriers maintaining CNS homeostasis.[19]

The total serum ALP activity is significantly higher in patients with auto- immune diseases when compared with the appropriate control groups. This could be due to leakage of ALP from cells that were killed or injured by the autoimmune processes and/or by abnormal cell activation. In either case, the ALP activity in blood was increased. It closely reflects the abnormal activation of T lymphocytes.[20] The bulk of the enzymatic activity is mostly membrane bound with the highest specific activity and total activity contained in a lysosomal-mitochondrial fraction.[21] Microglia (brain-resident) and macrophages, (mostly haematogenous), represent two related cell types involved in the brain pathology in MS and its autoimmune animal model, the EAE.[22] Together, they perform a variety of different functions: they are the primary sensors of brain pathology, they are rapidly recruited to sites of infection, trauma or autoimmune inflammation in EAE and MS and they are competent presenters of antigen and interact with T cells recruited to the inflamed CNS.[22]

In view of the infiltration of lymphocytes into the brain substance in acute EAE, it is suggested that these cells may contribute to the destruction of myelin which is usually attributed to the monocyte or macrophage.[21] In the present study an increased activity of alkaline phosphatase in the site of lesion and surrounding the perivascular cuffing, support the findings of Friede (1961)[23] who reported increased activity of the enzyme in and around the plaques of MS and EAE. The increased activity of the alkaline phosphatase at the lesion site may be due to the active transport needed for the phagocytic action of the microgliocytes to clear the myelin debris. Although microglial cells are known as the professional phagocytes and executer of innate immunity in the central nervous system (CNS), it is believed that microglia are rather neurotoxic in MS and EAE.[24] The ALP activity in the endothelium of the vessels decreases with the impairment of the function of the endothelial cells.[14]

Histochemical changes of lysosomal and myelinic hydrolases (proteases and acid phosphatase) have been investigated by many workers during the evolution of EAE. Lysosomes are known to participate in the early breakdown of myelin in EAE.[25, 26, 27 & 28] Increased activity of acid phosphatase and other hydrolases was demonstrated in plaques of MS[29], in the inflammatory lesions[30] and in the lymphocytes of EAE.[31] The increased lysosomal activities of peripheral blood lymphocytes were found prior to the manifestation of clinical symptoms – day eight after immunization.[31] Brain lysosomal enzyme activities were elevated markedly on the 1st day of EAE induction. Serum lysosomal enzyme activity was not altered significantly, but in the experimental allergy, lysosomal enzyme activity was altered in Type IV, but not in Type I.[32] In view of the infiltration of lymphocytes into the brain substance in acute EAE, Smith ME (1979) suggested that these cells may contribute to the destruction of myelin which is usually attributed to the monocyte or macrophage.[21] Since lipid is the major constituent of

myelin sheath, the possible role of lipases in myelin disintegration was first considered. Early perturbation, perhaps enzymatic may initiate the degradative course. Significant increase in levels of several lipases has been measured in the demyelinating studies. The myelin debris becomes incorporated within phagocytic cells where further digestion takes place.[33] In experimental allergic neuritis (EAN) break-down of myelin is attributed to macrophages, which among other factors contain and secrete proteases. In vitro studies have shown that cathepsin D, an acidic aspartyl endopeptidase, and plasmin can degrade myelin proteins.[34]

In the present investigation an increased acid phosphatase could be demonstrated in and around the lesion sites suggesting that the inflammatory cells such as lymphocytes and mononuclear leukocytes which surround the lesion are responsible for the increased activity and the probable role of their enzymes in the degradation or digestion of myelin basic protein. Similar results were recorded earlier, related to the proteolytic enzyme activity and suggested the probability of inflammatory cells such as lymphocytes may be the cause of the increased proteolytic activity in the CNS of animals with EAE, and that enzymes from these cells possess the capability of digesting myelin basic protein.[35] Many cytokines must be considered as effector and immunoregulatory molecules in neuroinflammatory diseases such as MS and EAE. The potential role of interferon-gamma (IFN-gamma) in the pathogenesis of demyelinating diseases, since this cytokine has a number of important effects such as macrophage activation, induction of MHC class I. class I antigens, and T cell homing. In EAE, increased numbers of IFN-gamma-secreting cells (IFN-gamma-sc) appear in the CNS shortly before onset of clinical signs. Myelin autoreactive T cells have the functional ability to produce this cytokine.[36]

The source of increased activity may be due to the increased activity in the lipid- laden gitter cells (microglia), probably within lysosomes around the plaques.[23, 37 & 38] This increased activity may reside in the astrocytes also.[39] The biochemical assay showed a significant increase of the enzyme activities during the disease and the increase was significantly correlated with the intensity of the disease.[40]

The distribution in the nervous tissue of the increase in acid phosphatase activity observed in animals with EAE, suggests that, the endogenous nervous cells may contribute to the lysosomal enzyme increase in EAE.[40] Probably the inflammatory cells such as lymphocytes may be the cause of the increased acid phosphatase activity in the central nervous system of animals with EAE, and that enzymes from these cells possess the capability of digesting myelin basic protein.

CONCLUSION

The localization of alkaline phosphatase activity in the early stages (before the onset of clinical symptoms) of EAE was similar to that in control animals. Increased activity of alkaline and acid phosphatase was seen in and around the lesions. This increased activity of acid phosphatase might be due to increased activity in the microgliocytes or phagocytes or hypertrophied astrocytes. Probably the inflammatory cells such as lymphocytes and monocytes may be the cause of the increased acid phosphatase activity in the central nervous system of animals with experimental allergic encephalomyelitis, and that enzymes from these cells possess the capability of digesting myelin basic protein. The increased activity of alkaline phosphatase at the lesion site might be accounted for the active transport needed for the phagocytic action of microgliocytes and macrophages to clear the myelin debris.

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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