

Appendix A

The Basis for Antioxidant Therapies and Analysis of Oxidative Stress and Peroxynitrite in the Pathogenesis of Multiple Sclerosis

The following includes a review demonstrating the role of oxidative stress by the action of peroxynitrite (PN) as a cause of MS demyelination.

Enzyme produced nitric oxide (NO) has various biological functions and is important as a toxic defense molecule against infectious organisms (1) (2). Super oxide (O₂⁻) and NO act to kill invading microbes in phagocytes and (PN) (ONOO⁻) is formed by the reaction of NO and O₂⁻ (3). Inducible type-2 isoform of nitric oxide synthase (iNOS, NOS-2) produces a high-level sustained NO synthesis (2). iNOS mediated O₂⁻ and ONOO⁻ increased the antibacterial activity of macrophages ; both O₂⁻ and NO are important mediators of cellular immune response (3). In L-arginine depleted macrophages iNOS generates both O₂⁻ and NO that interact to form the potent oxidant ONOO⁻ which results in nitrotyrosine (NT) formation and NT serves as a biochemical marker of PN induced damage (3) (4). This iNOS derived ONOO⁻ was inhibited by iNOS blockade (3). Since L arginine is depleted in inflammatory sites during macrophage infiltration, iNOS mediated O₂⁻ and ONOO⁻ could be particularly important in the cytotoxic actions of macrophages and this pathway of O₂⁻ and ONOO⁻ formation could cause subsequent autotoxicity (3) (5).

NT was detected in CNS tissue from mice with acute experimental allergic encephalomyelitis (EAE), the animal model of MS. (4). PN is formed very early in EAE development and correlates with disease activity and NT positive cells display a widespread distribution in brain and spinal cord during severe disease and are associated with parenchymal sites(6). Incubation of myelin suspension with a donor of PN results in the formation of the lipid peroxidation product(7). In an animal study PN was found to induce primary axonal damage with severe myelin alteration, demyelination, and NT formation (8). When oligodendrocytes cells (OL) which produce myelin are exposed to PN in vitro, PN dose dependently reduced the viability of the OL and induced cell death (10). PN is generated at sites of

inflammation and NT is abundant in lesions of acute EAE and in active MS plaques (6) (10).

The cerebrospinal fluid (CSF) level of a reliable marker of oxidative stress in vivo, was three times higher in subjects with definite MS than in a benchmark group of subjects with other neurological diseases (11). iNOS is upregulated in the CNS of animals with EAE and patients with MS; raised levels of nitrate and nitrite the oxidation end products of NO are found in the CSF and serum of MS patients; nitrite+nitrate and PN were increased by 81% and 61% respectively in the CSF of MS patients compared to controls (12) (13). In MS patients in extreme exacerbation, NO oxidation products in CSF were remarkably elevated compared to both those MS patients after methylprednisolone administration and those in remission (14). There is a significant increase in DNA oxidation within plaques compared to normal appearing white matter in MS cerebellar specimens (15). Levels of NO oxidation products were four times higher in the CSF of MS patients compared to controls and a correlation was found between CSF nitrite/nitrate ratio and relative brain atrophy (16). NT immunostaining of CSF proteins was greatly increased in MS samples compared to controls (13). The CSF nitrite levels of MS patients correlated with clinical disease activity (17).

Interferon-gamma (IFN-gamma), a potent inducer of iNOS, is produced in immune cells before MS exacerbations; an exogenous IFN-gamma treatment causes MS attacks (17)(18). Observations in an animal study strongly indicate that denervation is because of interferon-gamma induced-elicited NO production (19).

Monocytes/macrophages expressing iNOS mRNA were prevalent in the plaque areas with extensive damage from postmortem MS patients brain tissue and in said plaque areas there was an accumulation of cell debris positive for NT (20). From biopsies of acute MS lesions immunohistochemical staining revealed codistribution of iNOS and NT positive cells; NT was more widespread in the monocyte/macrophage lineage (21). In postmortem MS brain tissue, strong immunoreactivity (IR) for iNOS mRNA and was detected in the active lesions and for all lesions iNOS IR was detected within lesion areas of the brain with levels that correlated with lesion activity; in the acute lesions intense NT staining was detected within the parenchyma with a distribution pattern suggestive of (IR) on cell membranes and/or myelin membrane (22). iNOS mRNA was detected in all

samples of postmortem brain tissue from MS patients but none in the control brains and iNOS protein was also exhibited in the vast majority of cells that exhibited iNOS mRNA and iNOS positive cells were much more frequent in, but not limited to the areas of the plaque and were more concentrated around and within proximal areas of demyelination; only MS positive brains exhibited significant concentration of NT; specific staining for NT was significantly denser in areas in and around MS plaques and appeared more intense within areas of demyelination (23). In postmortem brain tissue from MS patients there was strong IR for iNOS in all the active lesions with iNOS positive cells diffusely distributed in the parenchyma within regions of the active MS lesions and at the outer edge of the demyelinated region, and in control brain and spinal cord no or minimal iNOS IR could be detected (24) (25). Macrophages isolated from these active MS lesions showed IR for iNOS and these isolated macrophages spontaneously produced NO in vitro (25). In postmortem brain tissue, from MS patients NT was detected in tissue adjacent to regions of intense iNOS staining in and around chronic active plaques, iNOS intense regions included cells with ingested NT positive material associated with lipid (26).

There is a high prevalence of vitamin D deficiency and insufficiency in MS patients (27). The vitamin D metabolite (1,25-D₃) in activated human T lymphocytes blocked IFN- γ production in a concentration-dependent fashion (28). 1,25-D₃ dose dependently inhibited iNOS mRNA expression in activated mouse macrophages and reduced NO release and PN production (29). Treatment of mice with a 1,25-D₃ completely inhibited EAE induction and progression (30). Treatment of rats with 1,25-D₃ resulted in the reduction of EAE disease activity and significant clinical improvement and a marked decrease in the number of macrophages (31)(32)(33). Hypercalcemia often prevents further administration of 1,25-D₃ (34). 1,25-(OH)₂-24OXO-16eneD₃ is a metabolite from the vitamin D analog 1,25(OH)₂-16eneD₃ (34). Suppression of EAE in mice was observed with 1,25(OH)₂-24OXO-16eneD₃, comparable to the suppression induced with the parent compound and more potent than 1,25-D₃ and no hypercalcemia was seen with 1,25-(OH)₂-24OXO-16eneD₃ (34). MC1288 a nontoxic 1,25-D₃ analog strongly inhibited EAE symptoms in rats and resulted in a reduced incidence of a second paralytic attack and a improved clinical score compared to 1,25-D₃ treatment (35). Ro 63-2023 an analog of 1,25D₃ inhibits IFN- γ production in vivo without producing hypercalcemia

and in mice Ro 63-2023 treatment provides long term protection from EAE when administered after the first peak of disease(36).

Remyelination to an extent does occur in MS (37) (38), and complete remyelination is claimed to be possible (39). The premyelinating OL are present in some but not all chronic lesions of MS (40). Where OL are absent there replacement is needed.

The plasma and serum retinol level of MS patients were lower compared to control patients with studies suggesting an association between retinol levels and the clinical disease activity in MS patients (41) (42). Retinol plays a role in the regulation of OL differentiation and retinol has been suggested to play a role in their migration (43). Retinol treated P19 embryonal carcinoma cells differentiate into OL and when these cells were transplanted into the brains of neonatal pups, the cells migrated up several millimeters from the site of the graft and appeared capable to myelinate the axons from the host neurons (44).

An in vitro study with blood cell samples from MS and control patients found combination treatment with retinol and interferon beta-1b an agent used in the general treatment of MS, lead to a significant increase in suppressor cell function over that achieved with interferon beta-1b use alone and that in the control samples which were examined for the number of interferon gamma-secreting cells, retinol administration decreased the number of said cells (45).

Retinol administration to rats prevented the development of EAE (46) (47). In another study after on set of EAE, retinol administration to rats resulted in an improved clinical course (48).

Strategies for the treatment of MS would include arresting the overproduction of PN through inhibiting iNOS induction. In macrophage cells activated to produce NO, retinol depresses the levels of NO in a dose depended manner (49). In another study, results demonstrated retinol inhibited the production of NO from activated macrophages in a dose and time depended manner (50).

When MS patients were administered retinol and vitamin D metabolites through a cod liver oil supplementation with other proposed therapeutic

agents for a period of one to two years the number of exacerbation was less than one half the number expected (51).

When MS patients were administered retinol and vitamin D metabolites through a cod liver oil supplementation with other proposed therapeutic agents of the sixteen patients in the study one deteriorated, one withdrew, four remained stable and eleven improved (52).

When MS patients were administered retinol and vitamin D metabolites through a cod liver oil supplementation with other proposed therapeutic agents, nine of ten patients who completed the study showed improvement; two of the patients were bedridden for about five years and not once during this time could the patients even as much as feed themselves and with the treatment one of the patients was able to get out of bed and take a few steps with support, and the other patient was able to walk with little support such as holding on to one finger of an assistant; seven of the patients lost practically all of their paralytic symptoms(53).

Antioxidants are necessary for optimal in vitro myelination(54).In a study of antioxidant therapy MS patients were administered various antioxidants during one month, two times a year, which resulted in a significant reduction(2-3) of relapse frequency(55).Inosine a precursor of the antioxidant uric acid was administered to MS patients for a year or more, and 3 of the 11 patients showed some evidence of clinical improvement and there was no sign of disease progression in the remaining remaining patients (56).Another study reports that employment of antioxidant therapy in MS patients resulted in an improvement of their condition that correlates with positive changes in the process of lipid peroxidation (57).

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