

## Chapter B8

### NITRIC OXIDE IN TMEV

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**Abstract:** We and others have previously investigated the role of inducible nitric oxide synthase (iNOS) on early acute and late chronic demyelinating disease induced by Theiler's Murine Encephalomyelitis Virus (TMEV). Infection of susceptible SJL mice with this virus serves as an excellent model of virus-induced demyelinating disease, such as multiple sclerosis (MS). iNOS transcripts and protein were detected in brains and spinal cords of TMEV-infected SJL mice during early acute disease, which resembles polioencephalomyelitis. Similar level of expression of iNOS has been found in resistant B6 mice, which develop only early acute disease. Weak iNOS staining was detected in reactive astrocytes and in leptomeningeal infiltrates in TMEV-infected SJL mice at 42 days post infection (p.i.), corresponding to early phase of chronic demyelinating disease, but not at 66 and 180 days p.i. corresponding to advanced and terminal stages of the disease, respectively. Results from other laboratories demonstrated that, blocking of NO by treatment of TMEV-infected SJL mice with amino guanidine (AG), a specific inhibitor of NO resulted in delay of late chronic demyelinating disease. However this protective effect of NO inhibitor depended on the temporal phase of the disease, type of cells expressing iNOS and the time of administration of AG. The results from our laboratory suggests that NO expressed during early acute disease is beneficial to the host through induction of apoptosis of infiltrating T cells and resolution of encephalitis, but its role in myelin/oligodendrocytes damage during late chronic demyelinating disease is not clear and it may depend on availability of superoxide and formation of peroxynitrite.

**Key words:** nitric oxide, TMEV, multiple sclerosis

## 1. INTRODUCTION

Theiler's virus (TMEV) induces a biphasic disease in susceptible strains of mice (such as SJL). Early acute disease (polioencephalomyelitis) is characterized by replication of the virus in gray matter(1-4). This phase of disease is associated with neuronophagia and inflammatory infiltrates in the cerebral gray matter and anterior horn cells of spinal cord(4, 5). Within two to three weeks the virus is partially cleared and approximately 35 days p.i. susceptible mice develop late chronic demyelinating disease. The virus persists at very low level in microglia/macrophages, astrocytes and oligodendrocytes(1, 5-9). Heavy inflammatory infiltrates comprised of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, macrophages, and few B cells are present, exclusively in the spinal cord(7, 9, 10). Demyelinating lesions are associated with perivascular and parenchymal inflammatory infiltrates. TMEV-induced late chronic demyelinating disease is an excellent animal model for human MS(1, 2, 7, 9, 11). Resistant strains of mice (such as C57BL/6) develop only early acute disease, clear the virus completely and do not develop demyelinating disease. MS is the most common inflammatory demyelinating disease in humans. It has been suggested that T cells specific for a myelin component such as MBP (Myelin Basic protein, PLP (proteolipid) and/or MOG (myelin oligodendrocytes protein), and activated microglia/macrophages participate in myelin damage(12-15). Epidemiological evidence suggests that MS could be triggered by virus (es) acquired before puberty.

The mechanism(s) of the pathogenesis of inflammatory demyelinating diseases is not well understood. Although oligodendrocytes/myelin may be damaged by a direct attack of cytotoxic T cells, other cells, including CD4<sup>+</sup> T cells, activated macrophages, and microglia may contribute to myelin destruction by the production of cytokines, such as IL-1, IFN- $\gamma$ , and TNF- $\alpha$ , and reactive oxygen and reactive nitrogen species(14, 16). Nitric Oxide (NO) is a short lived, highly reactive molecule with free radical properties. It is synthesized by nitric oxide synthase (NOS) by converting of L-arginine to L-citrulline, reviewed in(17, 18). There are three isoforms of NOS; NOS-type I and NOS-type III are calcium dependent and are constitutively expressed. This results in the production of physiological levels of NO which acts as second messenger in the NO signaling pathway in the neuronal and cardiovascular system. The NOS-type II (iNOS, inducible NOS) is calcium independent and is produced in large amounts during innate and adaptive immune responses(19-24). NO produced during an innate immune response clearly contributes to the killing of intracellular microorganisms and parasites(25-27). Less is known about a role of NO in the generation and regulation of an adaptive immune response, including inflammation.

It has been suggested that NO may exhibit cytotoxic, regulatory, and immunosuppressive properties, including induction of apoptosis and autoimmune disease, reviewed in (28-30). Such a wide array of biological actions of NO suggests that iNOS may have different functions in different cells. The immunoregulatory action of NO is likely mediated through its effect on cytokines and other inflammatory molecules by regulation of the transcription factor, NF-activating protein-1 (AP-1) by targeting the Th1/Th2 balance(29, 31-33). On the other hand, NO produced by microglia/macrophages could be a potent neurotoxin and mediates TNF- $\alpha$  neurotoxicity toward oligodendrocytes(16, 34-36). The toxic effect of NO is likely attributed to the nitrosylation of target iron-sulfur proteins, including enzymes involved in DNA replication and repair and in blocking mitochondrial respiration(37, 38). The uncoupling of the electron transfer chain in mitochondria will result in production of oxygen free radicals. Reaction of NO with a superoxide will lead to formation of extremely toxic peroxynitrite (ONOO<sup>-</sup>)(39-41). Peroxidations of membranes and swollen oligodendrocyte cell bodies have been described in the CNS of patients with MS(42). Nitrosylation of tyrosine in proteins has been demonstrated in a number of neurodegenerative and inflammatory conditions of the CNS(43-45).

In the inflammatory conditions of CNS, such as viral encephalitis and EAE (experimental allergic encephalomyelitis), iNOS is expressed in macrophages, microglia and/or astrocytes, and possibly neurons but not oligodendrocytes(41, 43, 45-51). The role of iNOS has been examined in a number of encephalitis's induced by viruses, such as corona, flavi, rhabdo, Borna, rabies, herpes, Japanese encephalitis, Venezuelan encephalitis and several others, revealing that NO may contribute to the pathogenesis of the disease(52-60). Alternatively, NO may have a protective role in a viral infection.

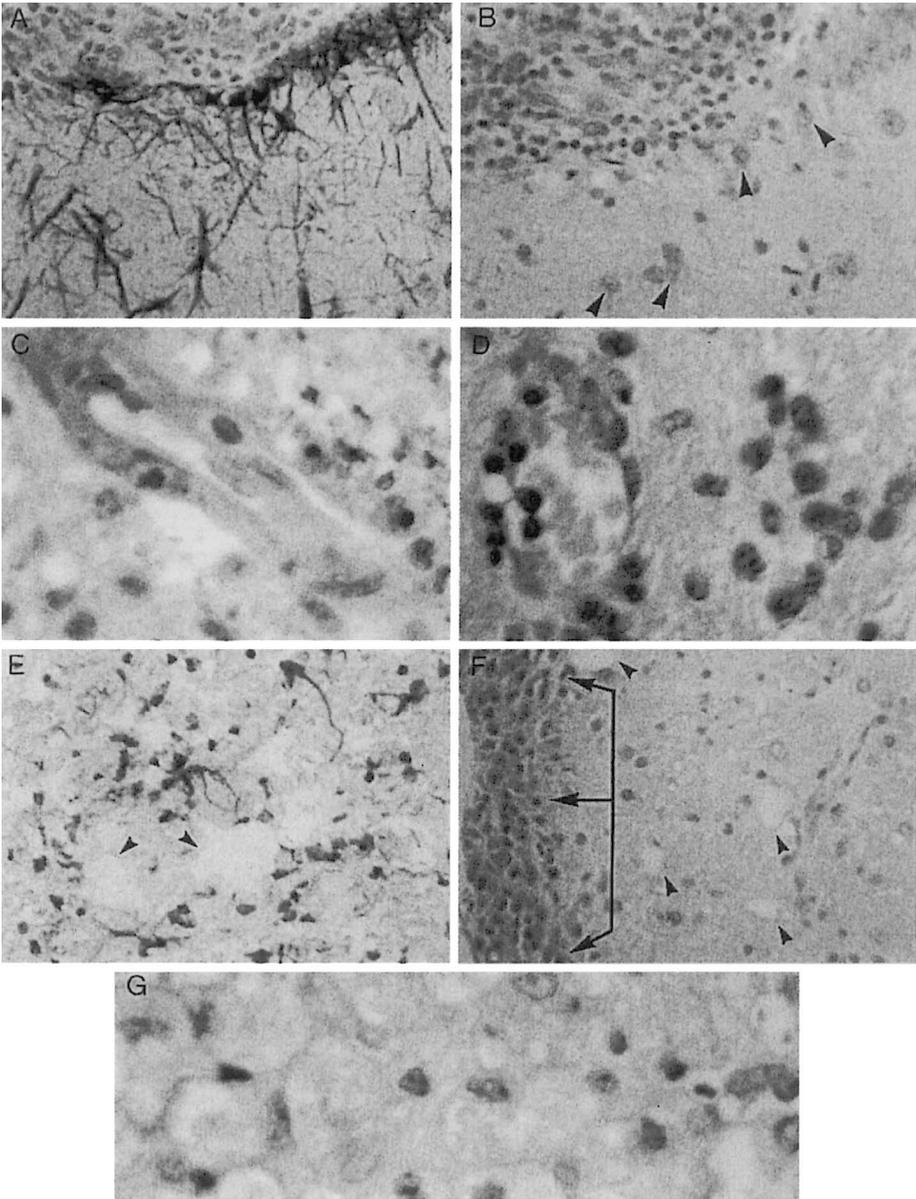


Figure B8-1.

IHC localizations of GFAP (A) and iNOS protein in the CNS of SJL mice at 10 (A-C), 6 (D), 42 (E and F), and 66 (G) days p.i. (A) Encephalitis and poliomyelitis phase: an area of intense leptomeningeal inflammation accompanied by reactive astrogliosis in the subjacent brain parenchyma, which is highlighted by GFAP staining. (B) Same field from an immediately adjacent section exhibiting iNOS-like staining in reactive subpial astrocytes (arrowheads) as well as in scattered monocytes/macrophages in the overlying leptomeningeal infiltrate (top

portion of photomicrograph). (C) Encephalitis and poliomyelitis phase: iNOS-like localization in hypertrophic endothelial cells of a blood vessel amidst an area of necrotizing encephalitis. (D) Encephalitis and poliomyelitis phase. iNOS-like staining in cells of the monocyte/macrophage lineage and in vascular endothelial cells from an area of spinal grey matter inflammation. (E) Early stage of spinal cord demyelination exhibiting a number of scattered iNOS-positive astrocytes in a background of incipient vacuolar changes (arrowheads) suggestive of early demyelination involving the lateral column. (F) Spinal cord demyelination phase: Focal, weak, and ill-defined iNOS-like immunoreactivity is detected among leptomeningeal infiltrates (the three arrows) which are encroaching upon the lateral funiculus of the spinal cord. Note vacuolar change in the lateral column (arrowheads). (G) Spinal cord demyelination phase: Lack of iNOS-like staining in large foamy (myelin-laden) macrophages in full-blown demyelinating lesions in the posterior columns of the thoracic cord. (Original magnifications: A, B, E, F x400; C, D, G x1000)

## 1.1 iNOS in TMEV infection.

We have examined the expression of iNOS transcripts and protein in the CNS of TMEV-infected mice during early acute and late chronic demyelinating disease(61). Both susceptible (SJL) and resistant (B6) strain of mice were infected i.c. with the DA strain of TMEV and infected mice were sacrificed at 0, 3, 6, 10 days p.i. (SJL and B6), corresponding to early acute disease and at 39, 42, 66–67 and 180 days p.i. corresponding to late chronic demyelinating disease (SJL only). Mock-infected mice were injected i.c. with medium alone and they were sacrificed at 6 and 39 days p.i. iNOS transcripts were determined by RT-PCR, followed by Southern blotting, as described(61); the presence of iNOS protein was determined by immunohistochemical staining using affinity-purified polyclonal rabbit anti-iNOS antibody (61). These antibodies are iNOS specific and do not react with NOS type I and NOS type II.

Although both strains of TMEV-infected mice develop polioencephalomyelitis (gray matter inflammatory disease), confirmed by histological analysis (Fig.1), none of the animals showed overt neurological signs. We did not detect any transcripts for iNOS in brain and spinal cord of mock-infected animals examined at day 6 and 39 p.i. and very little iNOS transcripts were present at day 3 p.i. In contrast, high levels of expression of iNOS transcripts were detected in the CNS of TMEV-infected SJL and B6 mice at 6 and 10 days p.i. In general, higher levels of iNOS expression were detected in brains of SJL and B6 mice than in the spinal cords. Immunohistochemical staining of CNS tissue enabled the identification of cells which expressed iNOS during early acute disease. At 6 and 10 days p.i. iNOS was expressed in strains of TMEV-infected mice in areas of intense inflammation in reactive astrocytes (Figs. 1A & B), monocytes/macrophages and hypertrophic endothelial cells (Figs. 1C, 1D). Few perivascular

monocytes-like cells in leptomeninges were also iNOS positive, while lymphocytes were negative(61). Normal endothelial cells and smooth muscles cells were consistently negative for iNOS. To confirm that reactive astrocytes were the major cell type expressing iNOS, we stained adjacent sections with antibodies to GFAP (glial fibrillary acidic protein) or with antibodies to iNOS. Cells which were positive for iNOS were also positive for GFAP. The profile of iNOS staining in the spinal cord was similar to that described for encephalitis. We have not detected any iNOS-like immunoreactivity in normal interfascicular fibrous astrocytes, protoplasmic astrocytes, Bergmann's glia or oligodendrocytes. Rod-shaped microglia-like cells were also consistently negative. iNOS protein was essentially undetectable in the brain and spinal cord of naive mice.

We have examined expression of iNOS in late chronic demyelinating disease(61). iNOS transcripts were detected in spinal cords of TMEV-infected SJL mice at 39 days p.i., which corresponds to the beginning of inflammatory demyelinating disease (61). Less iNOS transcripts were detected in brains of these mice. Immunohistochemical staining confirmed expression of iNOS protein in fibrillary astrocytes in areas of inflammation (Fig.1E) and in leptomeningeal infiltrate (albeit weak) at 42 days p.i. (Fig.1F). iNOS protein was not detected in the CNS of TMEV-infected B6 mice at 42 and 67 days p.i. and, as expected, there were no overt pathological changes in the brains and spinal cords of these mice. In contrast, extensive inflammatory disease, demyelination and gliosis were demonstrated in TMEV-infected SJL mice at day 39, 67 and 180 p.i. However, we did not detect any iNOS transcripts nor iNOS protein at day 67 p.i. (advanced phase of demyelinating disease) or at 180 days p.i. (terminal phase of disease) associated with hind leg paralysis and incontinence). Foamy-myelin-laden macrophages were always negative (Fig.1G).

### 1.1.1 DISCUSSION

We have demonstrated that during early acute disease induced in TMEV-infected SJL and B6 mice iNOS is expressed in the brain and spinal cord of infected animals, predominantly in hypertrophic astrocytes, monocytes/macrophages and endothelial cells in the area of inflammation (61). During late chronic demyelinating disease, iNOS has been detected in fibrous astrocytes and meningeal infiltrates of spinal cord but only during the early stages (39 days p.i.) of late chronic demyelinating disease (SJL mice) and not at 66days p.i. or at 180 days p.i. (terminal stage of the disease). We have also demonstrated that during early acute disease there is high level of apoptosis of T cells undergoing activation induced cell death, while there is paucity of apoptosis of T cells infiltrating the CNS of TMEV-infected SJL

mice during late chronic demyelinating disease (unpublished observations). We suggest that NO produced by reactive astrocytes and macrophages/microglia during early acute disease triggers apoptosis of infiltrating T cells. Conversely, lack of expression of iNOS in the CNS during late chronic demyelinating disease (days 66 and 180 p.i.) may relate to the very low level of apoptosis of these T cells. Induction of apoptosis of MBP-specific T cells by NO producing astrocytes (functioning as antigen presenting cells) has been described(62). Molina-Holgado(63) recently reported that in brain astrocytes infected in vitro with TMEV there is high expression of iNOS and that IL-4 and IL-10 down regulate expression of iNOS by modulation of NF- $\kappa$ B activity. We have demonstrated high level of expression of proinflammatory cytokines in the CNS of TMEV-infected mice during early acute and late chronic demyelinating disease (64). However, IL-4 transcripts have been detected primarily during late chronic demyelinating disease. It is therefore possible to suggest that IL-4 down regulates expression of NO in astrocytes in SJL mice during late chronic demyelinating disease, which may result in inhibition of apoptosis. According to this postulate, expression of iNOS during early acute disease would be beneficial to the host, because NO could be responsible for eliminating infiltrating T cells through down regulation of Bcl-2 and induction of AICD, leading to restoration of homeostasis in the CNS. Thus, NO may function as an anti-inflammatory or immunosuppressive molecule during early acute disease. Immunosuppressive effect of macrophage-derived NO on T cells has also been reported (25, 28, 29). However, lack of iNOS expression (down regulated perhaps by IL-4/NF- $\kappa$ B) during late chronic demyelinating disease in TMEV-infected mice may be detrimental to the host by blocking apoptosis of infiltrating T cells. On the other hand, down regulation of NO production in the CNS of TMEV-infected mice may represent an effort of the host to curb formation of peroxynitrite, an extremely toxic molecule, which may lead to peroxidation of membranes and formation of nitrotyrosine. Whether nitrotyrosine is present in the CNS of TMEV-infected mice is not known. NO is a weak oxidant and acts rather like an antioxidant and only in reaction with superoxide it forms peroxynitrite, very toxic molecule. We and others demonstrated the expression of iNOS and nitrotyrosine in reactive astrocytes and monocytes/macrophages in Multiple Sclerosis lesions(43, 45). In general the expression of iNOS and nitrotyrosine seems to depend on the extent of lesion activity (acute vs. chronic).

The unexpected protective or destructive role of NO in induction and progression of EAE is also well known, reviewed in(65).

The effect of specific iNOS inhibitor, amino guanidine (AG) on mice infected with DA strain of TMEV has been examined(66). Treatment of

TMEV-infected mice starting at day 28 p.i. till day 49 p.i. significantly decreased inflammation, demyelination and axonal necrosis. This treatment was associated with decreased apoptosis of CNS infiltrating cells, presumably monocytes or lymphocytes. Early treatment (starting at day 7 p.i.) was not effective(66). When TMEV-infected mice were treated starting at day 14 p.i., only demyelination and axonal necrosis were decreased while the level of inflammation was not changed(66). These results suggest that inflammation and demyelination and axonal damage may be differentially down regulated by treatment of TMEV-infected mice with NO inhibitors at different times of infection. The outcome of treatment may depend on the level of activation of macrophages/microglia and astrocytes, which produce NO but also on availability of superoxide. The role of iNOS in TMEV induced demyelinating disease has also been examined using BeAn strain of the virus (instead of DA strain routinely employed in our laboratory). The BeAn strain of TMEV replicates with different kinetics in the CNS of SJL mice(67). For example, mice infected with BeAn show clinical signs of disease such as extensor spasm and hind leg of paralysis at 50 days p.i., whereas, mice infected with DA strain of TMEV do not show these signs earlier than 180 days p.i. Treatment of SJL mice infected with BeAn strain of TMEV with AG during the effector phase of the disease (initiating treatment at 15 days p.i.) resulted in significant delay of inflammatory demyelinating disease (67). Interestingly, iNOS was detected at low levels in monocytes/macrophages (MOMA-positive) in leptomeninges and perivascular spaces, but not in astrocytes, in TMEV infected mice between 0 and 15 days p.i.(67). The maximum expression of iNOS was observed at day 60 p.i. In contrast, iNOS expression was high in the CNS of SJL mice infected with DA strain of TMEV between 6 and 10 days p.i., but was not detectable at 66 and 180 days p.i. Treatment of TMEV (BeAn)-infected mice with AG in the induction phase (starting at day 1 p.i.) did not alter the course of the disease(67). The authors suggested that protective effect of AG on inflammatory demyelinating disease observed in BeAn infected mice when treatment had begun 15 days p.i. was associated with suppression of activation and proliferation of inflammatory macrophages and lymphocytes(67). The results of these experiments suggest that the effect of NO on inflammatory demyelinating disease induced by these two strains of TMEV may depend on the kinetics of disease, time of appearance of inflammatory infiltrates, type of cells which express iNOS and time of administration of the inhibitor.

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