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Coronavirus-Induced Demyelination Occurs in the Absence of Inducible Nitric Oxide Synthase

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Demyelination induced by mouse hepatitis virus (MHV), strain JHM, is in large part immune mediated, but little is known about the mechanisms involved in this process. Previous results suggest that inducible nitric oxide synthase (NOS2) contributes transiently to MHV-induced demyelination. Herein, we show that equivalent amounts of demyelination were evident at day 12 after MHV infection in mice genetically deficient in NOS2 ($NOS2^{-/-}$) and in C57BL/6 mice. Furthermore, using an established adoptive transfer model and pharmacological inhibitors of NOS2 function, we could demonstrate no effect on MHV-induced demyelination. These results indicate that NOS2 function is not required for demyelination in mice infected with MHV.

Mouse hepatitis virus (MHV), strain JHM, causes both acute and chronic diseases of the central nervous system (CNS) (9, 25). Intranasal or intracranial inoculation of susceptible mice with MHV results in a uniformly fatal encephalitis. Encephalitis is prevented by adoptive transfer of antiviral antibodies or T cells at the time of infection with wild-type virus or if mice are infected with attenuated virus (9, 25). However, mice surviving the acute disease often develop chronic demyelination of the CNS, with clinical and pathological features resembling those of the human disease multiple sclerosis (9, 25). Although initial reports suggested that MHV-induced demyelination resulted from direct viral lysis of oligodendrocytes, more recent work suggests that the immune system has a critical role in the development of MHV-induced demyelination (8, 28, 31). Mice with severe combined immunodeficiency (SCID) or those unable to generate mature B and T lymphocytes due to a genetic deficiency in recombination activating gene 1 function ($RAG1^{-/-}$) were shown not to develop demyelination after infection with MHV. However, adoptive transfer of splenocytes from immunocompetent donors into these MHV-infected mice resulted in demyelination by day 7 posttransfer (p.t.) (8, 31).

No single factor has been shown to be critical for the effector stage of MHV-induced demyelination, although recent data suggest that CD4 T cells have an important role in this process (11). Demyelination is unaffected by neutralization of tumor necrosis factor alpha activity or by the genetic absence of gamma interferon, perforin, or interleukin-10 (13, 14, 19, 26). Nitric oxide (NO) is a pleiotropic molecule important in vascular regulation, neuronal function, and immunological processes (15). Of the three isoforms of nitric oxide synthase (NOS), NOS2 is recognized as an important inflammatory mediator, with both protective and immunopathological capabilities (17). Macrophage production of NO by NOS2 is part of the effector phase of the immune response to numerous pathogens, including viruses (15, 23). Because of its cytotoxicity, NO has been suggested as a mediator of myelin damage.

In mice acutely infected with MHV, expression of NOS2 by macrophages is upregulated, whereas NOS2 synthesis is confined to astrocytes during chronic demyelination (6, 27). This induction of NO during MHV-induced chronic disease may have a role during the early phases of demyelination, since aminoguanidine (AG) treatment delays the onset of inflammation and clinical disease (10). However, demyelination is not abrogated in AG-treated mice infected with MHV, and by day 21 postinfection (p.i.), equivalent amounts of demyelination are detected in control and drug-treated animals. As another approach to defining the role of NOS2 in MHV-induced demyelination, $NOS2^{-/-}$ mice were infected with MHV and compared with MHV-infected C57BL/6 (B6) mice. Furthermore, to address the potential effector role of NO during the early phases of the demyelinating disease, NOS2 activity was inhibited by treatment with L-N^6-(1-iminoethyl)lysine (L-NIL), a more selective inhibitor of NOS2 than AG (16), in MHV-infected $RAG1^{-/-}$ mice.

The neuroattenuated variant of MHV JHM, J2.2-v1 (kindly

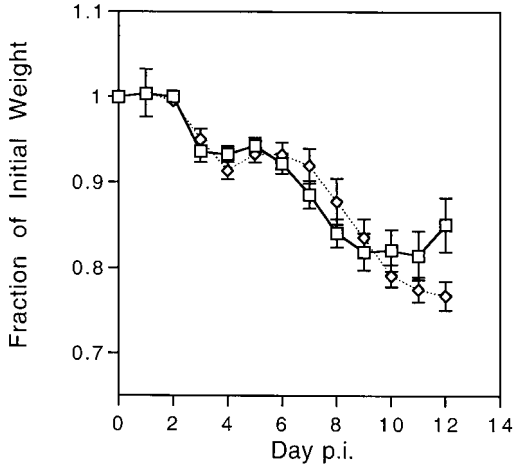

FIG. 1. Infection with MHV results in equivalent weight loss in B6 (◇) and $NOS2^{-/-}$ (□) mice. Eight B6 and nine $NOS2^{-/-}$ mice were inoculated with 10^3 PFU of MHV intracranially. Mice were monitored for mortality and weighed daily. One B6 mouse died on day 10 p.i. Values are expressed as a fraction of initial weight ± standard error. Although the mice were age matched, the average weight at the time of inoculation of B6 mice was 21.56 ± 0.57 g, while $NOS2^{-/-}$ mice weighed 17.82 ± 0.50 g. No statistical difference between groups was noted at any time point ($P > 0.05$).

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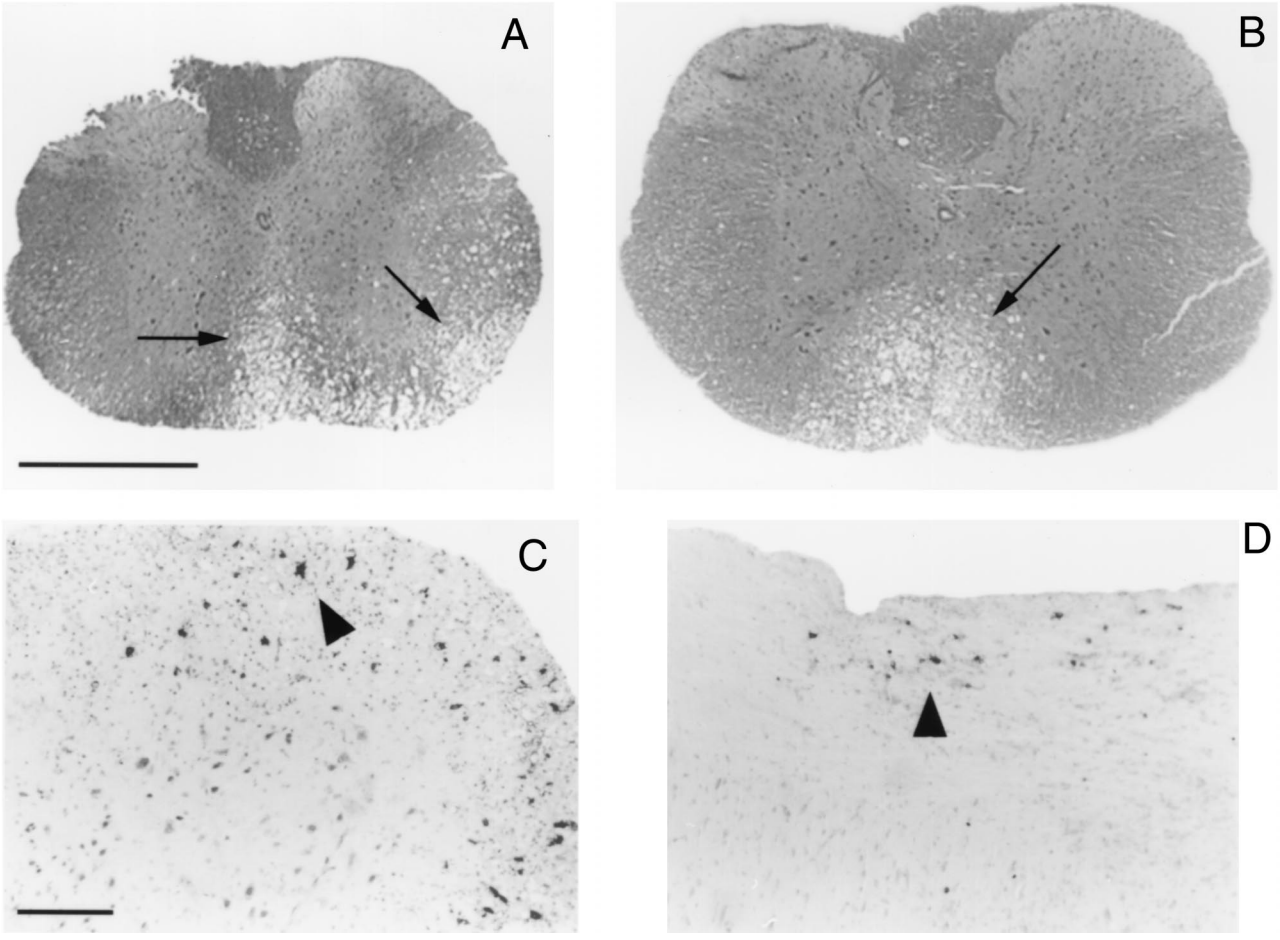

FIG. 2. MHV infection of B6 and NOS2$^{-/-}$ mice results in equivalent amounts of demyelination and levels of antigen in the CNS. (A and B) Spinal cords were harvested from mice infected with MHV J2.2-v1 at 12 days p.i. and were stained for myelin with luxol fast blue (31). Equivalent areas of inflammation and demyelination (arrows) are evident in coronal sections of spinal cord from B6 (A) and NOS2$^{-/-}$ (B) mice. The amount of demyelination detected in all of the B6 and NOS2$^{-/-}$ mice harvested at 12 days p.i. was quantified, and the results are summarized in Table 1. (C and D) Spinal cord sections were stained for viral antigen (arrowheads) by immunohistochemistry (31). Antigen is detected primarily in the white matter of B6 (C) (coronal section) and NOS2$^{-/-}$ (D) (longitudinal section) mice infected with MHV. Bars, 500 μm (A and B) and 200 μm (C and D).

provided by J. Fleming, University of Wisconsin, Madison), was used in all experiments and inoculated as previously described (31). *P* values were calculated using Student's *t* test. Intracranial inoculation of B6 mice with MHV J2.2-v1 resulted in mild acute disease developing around 7 days p.i., followed by hind-limb weakness and demyelination in all mice by 12 days p.i., as described previously (4). To determine if inducible NO was required for demyelination, NOS2$^{-/-}$ and wild-type B6 mice were infected in parallel with MHV J2.2-v1. A total of 19 B6 mice (National Cancer Institute, Bethesda, Md.) and 23 NOS2$^{-/-}$ mice (B6 background; provided by S. Murphy, University of Iowa) were infected with MHV. Some mice (11 B6 mice and 14 NOS2$^{-/-}$ mice) were analyzed for demyelination and virus titers, whereas others (8 B6 mice and 9 NOS2$^{-/-}$ mice) were monitored for morbidity and mortality. Nearly identical patterns of clinical disease were observed in both sets of mice. Several mice in each group developed symptoms of mild encephalitis (ruffled fur and slight hunching) at approximately 6 days p.i. All mice developed hind-limb weakness by 12 days p.i. Both B6 and NOS2$^{-/-}$ mice infected with MHV lost weight beginning at 3 days p.i., with more rapid weight loss observed at 7 to 8 days p.i. (Fig. 1), consistent with the progression to demyelinating disease. Although a slight difference in the fraction of the initial weight was observed at day 12 p.i., no statistically significant difference in weight was observed between groups at any time point. Both groups showed weight gain after 12 days p.i. (data not shown).

Histological examination of spinal cord white matter from both B6 and NOS2$^{-/-}$ mice infected with MHV revealed similar amounts of demyelination and inflammation (Fig. 2; Table 1). For purposes of quantification, 12 to 20 coronal sections throughout the length of the spinal cord were stained with luxol fast blue and digitized. Using VTrace software (Image Analysis Facility, University of Iowa), areas of demyelination were outlined and compared to outlines of the total area of white matter in each section. The fraction of demyelination was calculated by dividing the total area of demyelination by the total area of white matter per spinal cord.

NOS2$^{-/-}$ mice were not deficient in virus clearance. Virus was cleared from the brains of three out of eight B6 mice and of seven out of eight NOS2$^{-/-}$ mice by day 12 p.i. Virus was cleared inefficiently from the spinal cords of both groups, in agreement with previous results (29), and similar titers were observed in those mice with detectable levels of virus (Table 1). Additionally, immunohistochemical examination revealed similar quantities of virus antigen remaining within the spinal cord, almost exclusively in the white matter (Fig. 2).

One caveat to these experiments is that mice in which the

TABLE 1. Demyelination in B6 and NOS2$^{-/-}$ mice at 12 days p.i.

Mice	Mean % demyelination ± SE (no.[a])	No. of samples with detectable virus/ total tested[b]	Titer (mean $\log_{10}$ PFU/g of tissue ± SE)[c]
B6	20.8 ± 2.5 (8)	5/5	3.84 ± 0.25
NOS2$^{-/-}$	21.5 ± 2.0 (12)	4/5	3.88 ± 0.12

[a] Number of samples analyzed for demyelination.
[b] A subset of animals was analyzed for infectious virus. Spinal cords were harvested, and virus titers were determined. The limit of detection is 1.6×10^2 PFU/g of tissue.
[c] Titers of samples with detectable virus.

NOS2 gene was genetically disrupted might develop compensatory mechanisms that could mask the physiological contribution of NOS2 to demyelination. In addition, NO is well known to function in both the inductor and effector phases of the immune response (5, 18, 30). The results of Lane et al. (10) suggested that NOS2 was involved in the induction phase of the immune response and that AG treatment transiently inhibited lymphocyte entry into the CNS. To confirm the results obtained with the NOS2$^{-/-}$ mice and to assess the requirement for NOS2 function in the effector stage of the demyelinating process, we used the previously described adoptive transfer model (31).

MHV J2.2-v1-infected RAG1$^{-/-}$ mice (B6 background; Jackson Laboratories, Bar Harbor, Maine) develop delayed acute disease, initially exhibiting symptoms at approximately 12 to 15 days p.i., without evidence of demyelination. However, adoptive transfer of 5×10^6 immune splenocytes from one or two donor mice at 3 days p.i. resulted in the development of demyelination at 6 to 7 days p.t. Donor splenocytes were harvested from immunocompetent B6 mice 6 days after intraperitoneal (i.p.) infection with wild-type MHV (31). In order to determine whether inhibition of NOS2 can abrogate demyelination, mice were treated with pharmacological inhibitors of NOS2 at the time of transfer (3 days p.i.). The dosage of each drug and frequency of administration were based on previous reports showing inhibition of NOS2 activity in other animal studies involving the CNS (2, 5, 12). By not treating mice until 3 days p.i., any contributions that NOS2 makes to the initial immune response to the virus would not be inhibited. Although AG is a selective inhibitor of NOS2, it inhibits diamine oxidase and nonenzymatic glycosylation and has been shown to have weak antioxidant properties (20, 21). L-NIL is a more selective inhibitor of NOS2 (3, 16) and has been shown to inhibit the development of experimental allergic encephalomyelitis and other autoimmune diseases (1, 5).

Starting on the day of adoptive transfer, 375 μg of L-NIL (Sigma, St. Louis, Mo.) in 500 μl of saline was administered i.p. to 11 mice twice daily. Six control mice received saline alone. Treatment with L-NIL did not alter the clinical or pathological changes observed after infection with MHV J2.2-v1. Mice receiving L-NIL exhibited neurological symptoms similar to those of control mice receiving saline (data not shown). The amount of demyelination was not statistically different between the two groups (Fig. 3; Table 2), demonstrating that NOS2 function is not required for MHV-induced demyelination. L-NIL treatment did not affect virus clearance (Table 2). Although L-NIL-treated adoptive transfer recipients had slightly higher virus titers recovered from the spinal cords, this difference was not statistically significant. In other experiments, five mice received daily i.p. injections of 8 mg of AG (Sigma) in 500 μl of phosphate-buffered saline, starting on the day of adoptive transfer, while four received phosphate-buffered saline alone. Similar results were obtained when AG was substituted for L-NIL, with no effects on clinical disease or the extent of demyelination detected (data not shown).

Although NO may have a transient role in the early steps of demyelination (10), our results demonstrate conclusively that NOS2-generated NO is not necessary for disease to develop. These results are in general agreement with the results of Lane et al. (10), although we did not observe any transient effects of NOS2 inhibition either in MHV-infected NOS2$^{-/-}$ mice or in the adoptive transfer model. One difference between the two studies is that in the adoptive transfer model, MHV-specific effector T cells are activated before transfer, since donor mice were immunized with live MHV only 6 days prior to harvest of splenocytes. Inhibition of NOS2 under conditions in which activated T cells are delivered to infected RAG1$^{-/-}$ mice at 3 days p.i. targets the effector phase of demyelination. Lane et al. (10) concluded in their study that NO may have an important role in expediting the initial entry of lymphocytes into the infected CNS, a part of the pathogenic process not explicitly examined in our study.

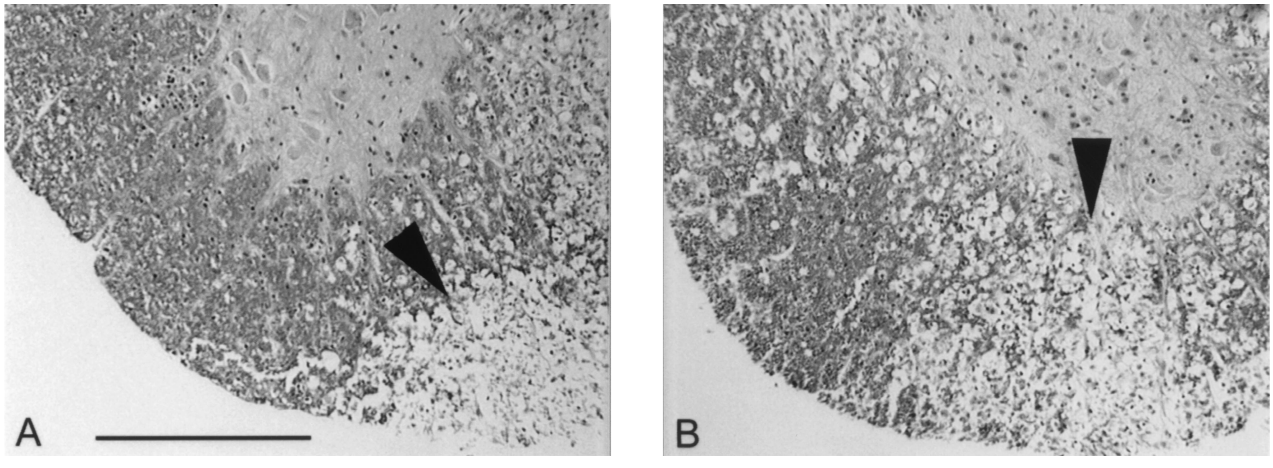

FIG. 3. Pharmacological inhibition of NOS2 activity does not change the pattern of demyelination observed in MHV-infected RAG1$^{-/-}$ mice following adoptive transfer. Spinal cord sections from saline-treated (A) and L-NIL-treated (B) mice were stained with luxol fast blue. Large areas of demyelination (arrowheads) are detected in both the presence and absence of drug. Normal myelin is immediately adjacent to lesions in both panels, with a small patch of demyelination beginning in the upper left of panel B. The amount of demyelination detected in all control and drug-treated animals is summarized in Table 2. Bar, 200 μm.

TABLE 2. L-NIL-treated RAG1$^{-/-}$ mice at 7 days p.t.

Group[a]	Mean % demyelination ± SE (no.[b])	No. of samples with detectable virus/ total tested[c]	Titer (mean $\log_{10}$ PFU/g of tissue ± SE)[d]
L-NIL	21.2 ± 2.5 (9)	4/5	3.61 ± 0.03
Saline	20.8 ± 2.4 (6)	4/4	3.16 ± 0.20

[a] Mice were treated with i.p. injections of L-NIL or saline.
[b] Number of samples analyzed for demyelination.
[c] A subset of animals was analyzed for infectious virus. Spinal cords were harvested, and virus titers were determined. The limit of detection is 1.6×10^2 PFU/g of tissue.
[d] Titers of samples with detectable virus.

The results presented here as well as several previous studies suggest that demyelination in MHV-infected animals is redundant and/or a result of several independent mechanisms. Overexpression of tumor necrosis factor alpha and gamma interferon within the CNS results in demyelination (7, 22), but neither is required for the demyelination detected after MHV infection (19, 26). Furthermore, no specific cell population is essential for MHV-induced demyelination. Neither CD4 T cells, CD8 T cells, nor hematogenous macrophages are required for MHV-induced demyelination (8, 32). Of note, MHV-induced demyelination differs from that observed in other model systems since inhibition of NO production by AG ameliorates demyelination in rodents with adoptive experimental allergic encephalomyelitis and those infected with Theiler's murine encephalomyelitis virus (2, 24).

This redundancy may be less apparent in the induction phase of the demyelinating process. Lane et al. suggested that the transient delay in demyelination observed after treatment with AG was a consequence of a reduction in chemokine production (10). In support of this conclusion, Lane et al. more recently showed that neutralization of RANTES (regulated on activation, normal-T-cell expressed and secreted), a chemokine that serves as a chemoattractant for both T cells and macrophages, resulted in a reduction in the amount of demyelination (11). However, even though these initial steps may be less redundant, all experimental interventions performed thus far have had only transient effects on the demyelinating process. Therefore, our results are consistent with a model in which no single factor is absolutely required for demyelination in MHV-infected mice.

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