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Inhibition of nitric oxide synthase-2 reduces the severity of mouse hepatitis virus-induced demyelination: implications for NOS2/NO regulation of chemokine expression and inflammation

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Infection of C57BL/6 mice with mouse hepatitis virus strain V5A13.1 (MHV-V5A13.1) results in an acute encephalitis followed by a chronic, progressive demyelinating disease with clinical and histological similarities to the human demyelinating disease Multiple Sclerosis (MS). Studies were undertaken to evaluate the contribution of NOS2 generated NO in demyelination in MHV-infected mice. MHV-infected animals were treated daily with either 8 mg of aminoguanidine (AG), a selective inhibitor of NOS2 activity, or PBS by intraperitoneal (i.p.) injection. MHV-infection of mice resulted in 20% mortality in both groups with surviving mice clearing virus below levels of detection, as measured by plaque assay, by day 12 postinfection (p.i.). A significant decrease in the severity of clinical disease was observed in AG-treated animals as compared to mice receiving PBS at days 7 and 12 p.i. ($P \leqslant 0.001$ and 0.003, respectively) however, by day 21 p.i. AG-treated mice exhibited the same severity of clinical disease as control animals. Examination of brain and spinal cords from infected mice revealed a pronounced reduction in the severity of inflammation at day 7 p.i. in mice treated with AG as compared to control mice. By day 12 p.i. there was a significant decrease ($P \leqslant 0.02$) in the severity of demyelination in AG-treated mice as compared to control animals yet both PBS and AG treated mice had a similar degree of demyelination by day 21 p.i. Analysis of chemokine mRNA transcripts by RNase protection assay revealed that AG-treated mice had significantly lower levels ($P \leqslant 0.007$) of transcripts for the C-C chemokine monocyte chemoattractant protein-1 (MCP-1) at day 7 p.i. as compared to control animals. By day 12 p.i., AG-treated mice and control mice had similar levels of chemokine transcripts. Together, these data suggest that inhibition of NOS2/NO slows the progression of MHV-induced demyelination. One potential mechanism by which this may occur is through controlling inflammation through modulation of chemokine expression in the CNS.

Keywords: MHV; nitric oxide synthase; nitric oxide; chemokines; inflammation; demyelination

Introduction

Infection of susceptible strains of mice with neuradapted strains of mouse hepatitis virus (MHV), a member of the coronaviridae family of viruses, results in a chronic demyelinating disease characterized clinically by progressive hindlimb paralysis and histologically by mononuclear cell infiltration and demyelination of the CNS (Houtman and Fleming, 1996a; Compton *et al*, 1993; Kyuwa and Stohlman, 1990; Lavi and Weiss, 1989). MHV-induced demyelination is considered a relevant animal model of the human autoimmune demyelinating disease Multiple Sclerosis (MS) because the two diseases display a similar histological and clinical disease profile (Houtman and Fleming, 1996a; Lane and Buchmeier, 1997).

Although the mechanism(s) by which demyelination occurs in MHV-infected animals is not clear, it is thought that components of the immune

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response play a role in demyelination (Wang *et al*, 1990; Houtman and Fleming, 1996b). We have recently demonstrated that MHV-V5A13.1 infection of the CNS of mice results in an orchestrated expression of both C-C and C-X-C chemokines (Lane *et al*, 1998). The subfamilies of chemokines have been shown to selectively attract distinct leukocyte populations during periods of inflammation (Taub and Oppenheim, 1994). Therefore, chemokine expression may be important in contributing to demyelination in MHV-V5A13.1-infected mice through attracting inflammatory cells e.g. T lymphocytes and macrophages into the CNS to sites of virus persistence (Lane *et al*, 1998). Once present, this population of cells may participate in demyelination by activating inflammatory cells as well as resident glia to produce cytotoxic products such as nitric oxide (NO), a gaseous, lipophilic free radical generated by nitric oxide synthase. NO has been postulated to contribute to the pathology of MS (Bo *et al*, 1994; De Groot *et al*, 1997; Giovannoni *et al*, 1997; Merrill *et al*, 1993). Furthermore, inhibition of nitric oxide synthase type 2 (NOS2 the enzyme responsible for high-level output of NO) through treatment with aminoguanidine (AG), a selective inhibitor of NOS2 activity, ameliorates the severity of clinical and histological EAE, suggesting a role for NO in contributing to pathology in this model of autoimmune demyelination (Cross *et al*, 1994; Zhao *et al*, 1996; Brenner *et al*, 1997).

In addition to the reduction in the severity of demyelination in EAE, treatment with AG results in a pronounced decrease in the severity of inflammation in the CNS (Cross *et al*, 1994; Zhao *et al*, 1996; Brenner *et al*, 1997). Brenner *et al* (1997) have shown that AG-treatment modulates cytokine expression in the CNS suggesting that NOS2-generated NO may influence inflammation by regulating proinflammatory factors. In support of this are studies demonstrating NO regulation of cytokine and chemokine expression (Marcinkiewicz *et al*, 1995; Remick and Villarete, 1996; Brenner *et al*, 1997; Hogaboam *et al*, 1997; Merril and Murphy, 1997). Therefore, NO may contribute to the pathology of demyelinating diseases such as MS and EAE not only by exerting cytotoxic effects on oligodendrocytes (Merrill *et al*, 1993) but also by regulating proinflammatory factors which are important in initiating and maintaining inflammatory conditions.

In order to evaluate the role of NOS2 generated NO in MHV-induced demyelination, we treated MHV-infected mice with AG, a selective inhibitor of NOS2, and evaluated the severity of clinical and histologic disease. In addition, we have analyzed the chemokine mRNA profile in AG-treated animals in an attempt to determine whether NOS2 generated NO modulates chemokine expression in the CNS of MHV-infected mice.

Results

NOS2 is expressed in MHV-V5A13.1-infected mice with demyelination

Infection of C57BL/6 mice with 10 pfu of MHV-V5A13.1 resulted in an acute viral encephalitis with 20% of mice dying between 8 and 10 days p.i. By day 12 p.i., the surviving mice developed the clinical characteristics of MHV-induced demyelination e.g. hind-limb paralysis and awkward gait. Examination of brains and spinal cords of mice at day 12 p.i. revealed the presence of demyelinating lesions. Immunohistochemical staining revealed NOS2 protein associated with areas of demyelination and tissue damage in both brain and spinal cord tissue sections of persistently infected mice at day 12 p.i. (Figure 1A). In addition, NOS2-positive cells were observed in areas with little to no tissue damage as well as surrounding vessels within the CNS (Figure 1B). NOS2-positive cells were not detected in the brains of sham-infected mice (Figure 1C). The majority of cells expressing NOS2 morphologically resembled astrocytes which is consistent to what we and others have reported (Sun *et al*, 1995; Lane *et al*, 1997).

Effects of AG on survival, viral clearance, and clinical disease

Having determined that NOS2-positive cells were present in the CNS in MHV-V5A13.1-infected mice, we next wished to determine if NOS2/NO was participating in clinical and/or histological disease. Mice were infected with virus and either treated with AG (8 mg/mouse/day, i.p.) or sterile PBS. A total of 52 mice, 29 treated with AG and 23 treated with PBS were used in these studies. Mortality in AG-treated mice did not differ from control mice as 20% of the mice in each group died between 8–10 days p.i. Furthermore, no difference in the kinetics of clearance of MHV was observed between AG-treated mice and control mice as both groups of mice cleared virus below limits of detection (<100 pfu/g) by day 12 p.i. as determined by viral plaque assay (Table 1). AG-treated animals exhibited significantly less severe clinical disease symp-

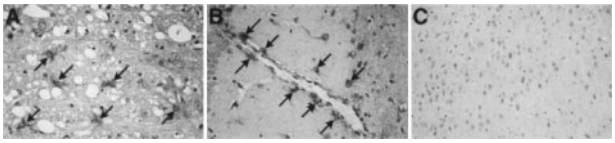

Figure 1 NOS2 expression in the CNS of MHV-V5A13.1-infected mice. (**A**) NOS2-positive cells (arrows) are detected at day 12 p.i. in the brains of mice within areas of demyelination. (**B**) Cells staining positive for NOS2 (arrows) surrounding a vessel within the brain (day 12 p.i.). (**C**) Sham-infected mouse brain stained for NOS2. No positive cells are detected. Original magnification, $\times 200$.

toms at days 7 ($P \leqslant 0.001$) and 12 ($P \leqslant 0.003$) as compared to PBS-treated mice (Table 1). However, by day 21 p.i., both AG and PBS-treated mice revealed similar clinical scores (Table 1).

Effect of AG on MHV-V5A13.1-induced histology

In order to correlate the decrease in clinical symptoms observed in AG-treated mice with a reduction in the severity of histologic disease, brains and spinal cords from mice treated daily with 8 mg AG and mice treated with PBS alone were obtained on days 7, 12, and 21 p.i. and stained with either hematoxylin-esoin or Luxol fast blue to assess the severity of inflammation and demyelination, respectively. AG-treatment resulted in a profound reduction in both inflammation and demyelination. Examination of brains from PBS-treated mice at day 7 p.i. revealed numerous inflammatory foci. Among the histologic features at this time were increased numbers of inflammatory cells in the meninges as well as perivascular cuffing (Figure 2). In contrast, brains from AG treated mice at day 7 revealed limited inflammation as compared to the PBS-control mice (Figure 2). By day 12, numerous inflammatory cells and white matter destruction were detected in spinal cords of infected mice treated with PBS (Figure 2). In contrast, AG-treated mice had only limited numbers of inflammatory cells present and this correlated with only mild demyelination (Figure 2). In order to provide a more quantitative interpretation of these data, slides containing brains and spinal cord sections from PBS-treated and AG-treated mice at days 12 and 21 p.i. were blinded and scored as described in Materials and methods. The results in Table 1 show that the demyelination in AG-treated mice at day 12 p.i. is significantly reduced ($P \leqslant 0.02$) in comparison to PBS-treated mice at the same time. By day 21 p.i., the demyelination in AG-treated mice remained qualitatively less severe than PBS-treated mice, however, this difference was not statistically significant.

Chemokine expression in the CNS of MHV-infected mice

In order to determine if the decrease in inflammation and demyelination in AG-treated mice was the result of a modification of proinflammatory signals, chemokine mRNA expression in the brains of infected mice treated with either PBS or AG was evaluated by RNase protection assay using a probe set designed to detect multiple chemokine transcripts. At day 7 p.i., there was a similar chemokine mRNA profile detected the brains of both PBS and AG-treated mice. Semi-quantitative analysis of the autorad (not shown) revealed a greater than 50% reduction in the levels of MCP-1 ($P \leqslant 0.007$) in AG-treated mice as compared to PBS-treated animals (Figure 3). In addition, there was a marked reduction in RANTES mRNA levels in AG-treated mice although this difference was not significant. IP-10 mRNA levels were similar between AG and PBS treated mice at day 7 p.i. By day 12 p.i., both AG and PBS groups of mice had similar levels of mRNA transcripts for MCP-1, IP-10, and RANTES (Figure 3).

Table 1

Treatment[a]	*Day*[b]	*Titer*[c]	*Demyelination*[d]	*Clnical*[e]
PBS	7	7100±141	ND	1.2±0.6
	12	<100	2.3±0.2	2.6±0.4
	21	ND	2.7±0.5	1.0±0.5
Aminoguanidine	7	6800±566	ND	0.4±0.4**
	12	<100	1.5±0.4*	1.4±0.4***
	21	ND	2.1±0.6	0.8±0.9

[a]C57B1/6 mice were infected i.c. with 10 pfu MHV-V5A13.1 and then received daily i.p. injections of either PBS or 8 mg AG.
[b]Days post-infection.
[c]Viral titer was determined by plaque assay as described in Materials and methods. In both groups of mice, virus was cleared below limits of detection (100 pfu/g tissue). PBS-treated mice at days 7 and 12 p.i., n=2 and 3, respectively. AG-treated mice at days 7 and 12, n=2 and 4, respectively. Data presented as mean±s.e.m.
[d]Severity of demyelination was scored using Luxol Fast Blue staining or paraffin-embedded brain and spinal cord sections from infected mice. Scores represent mean±s.e.m. *$P \leqslant 0.02$.
[e]Clinical disease was scored as the mean clinical score in each experimental group at the indicated time point. AG-treated mice had significantly reduced clinical signs at days 7 (**$P \leqslant 0.001$) and 12 p.i. (***$P \leqslant 0.003$) as compared to PBS treated mice. PBS-treated mice: day 7, n=15; day 12, n=7; day 21, n=5. AG-treated mice: day 7, n=15; day 12, n=9; day 21, n=3. Scores represent mean±s.e.m.

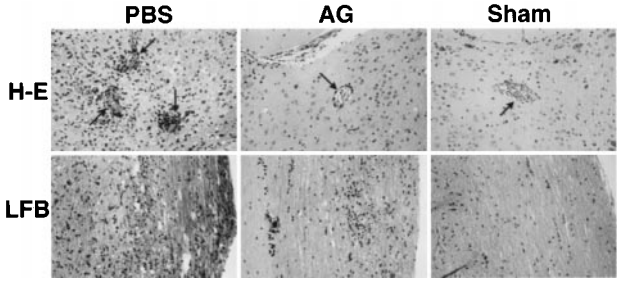

Figure 2 Inflammation and demyelination in MHV-V5A13.1-infected mice. AG-treatment resulted in a marked reduction in the severity of both inflammation and demyelination at days 7 and 12 p.i. Top row. Brains from either PBS-treated, AG-treated, or sham-infected mice at day 7 p.i. and stained with hematoxylin-eosin. Perivascular cuffing is indicated with arrows. Note the limited number of inflammatory cells present in the AG-treated brain as compared to PBS. Bottom row. Luxol fast blue staining of spinal cord sections from mice at day 12 p.i. Numerous cellular infiltration accompanied by myelin destruction is observed in PBS-treated animals while only limited inflammation and demyelination is detected in AG-treated mice. Original magnification, ×200.

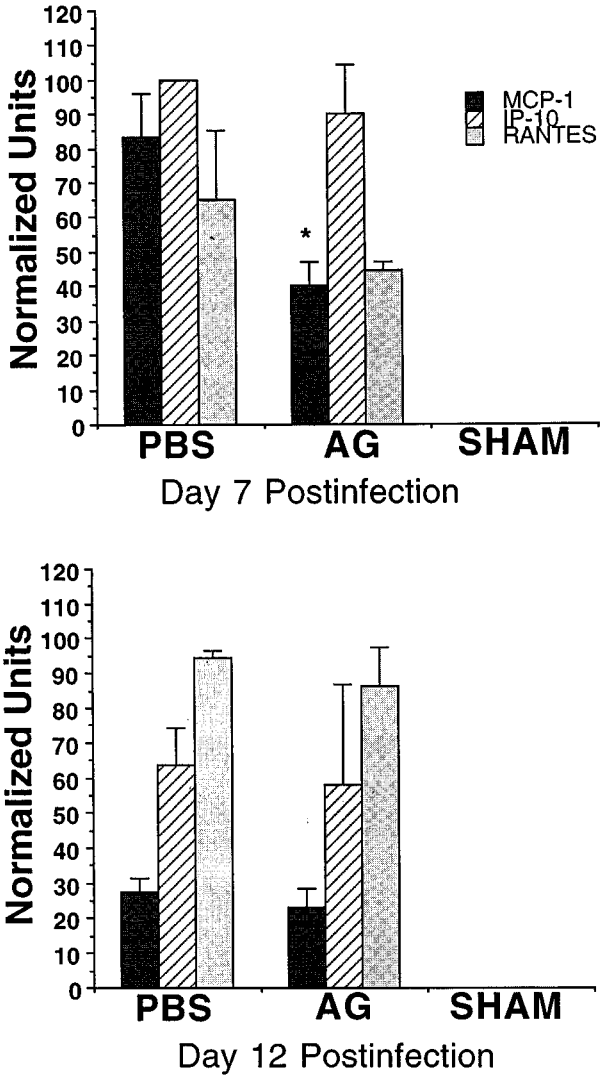

Figure 3 Effect of AG treatment on chemokine mRNA expression in the brain. Semiquantitative analysis of RPA autoradiography (not shown). Data are measured in normalized units and presented as average ± s.e.m. Sample sizes are as follows: day 7, n=3; PBS, n=3; AG and day 12, n=2; PBS, n=3; AG. (*) $P<0.007$.

Discussion

Mice infected with MHV-V5A13.1 undergo an acute encephalitis followed by the onset of demyelination which may last the lifetime of the animal. In this study we have demonstrated that NOS2 is upregulated, as determined by immunohistochemical staining, in mice undergoing demyelination. Expression of NOS2 was detected in brain and spinal cords of infected mice. NOS2 was predominantly detected in areas with pronounced neuropathology, however, NOS2 could be detected within regions of the CNS without apparent damage. These data suggested that NOS2/NO may be participating in demyelination in mice infected with MHV-V5A13.1. Astrocytes appeared to be the predominant cell type expressing NOS2 during the late stages of disease. This is consistent with a report by Sun *et al* (1995) which demonstrated that astrocytes were responsible for NOS2 expression during the chronic stage of MHV-induced demyelination. In order to evaluate the contributions of NOS2/NO in CNS disease following infection with MHV-V5A13.1, mice were treated AG, a selective inhibitor of NOS2, and examined for alterations in viral clearance from the CNS as well as changes in both clinical and histological disease.

Inhibition of NOS2 activity did not alter the kinetics of viral clearance from the CNS as compared to control mice treated with PBS. These data support an earlier study from this laboratory which demonstrated that NOS2/NO does not contribute to clearance of the neuroattenuated MHV strain OBLV60 from the CNS (Lane *et al*, 1997). We have also demonstrated that daily treatment of mice with AG resulted in a significant decrease in the severity of clinical disease as compared to PBS-treated animals. AG-treatment also resulted in a pronounced decrease in the severity of inflammation during the acute stage of disease. Correlating with the clinical scores, AG-treated mice exhibited a significant reduction in the severity of demyelination at day 12 p.i. as compared to the PBS-treated control mice. Together, these data argue for a role for NOS2/NO in contributing to the pathogenesis of MHV-V5A13.1-induced demyelination. In support of the observation that NO contributes to demyelination following viral infection of the CNS is a recent study by Rose *et al* (1998) which has shown that AG-treatment results in a decrease in clinical disease as well as inflammation and demyelination in mice infected with Theiler's virus.

The mechanism(s) by which NO may participate in demyelination are not clear. NO can directly exert a toxic effect on cells through the inhibition of Fe-S containing enzymes required for mitochondrial respiration and DNA replication (Stuehr and Nathan, 1989; Kwon *et al*, 1991). However, NO can also react with O_2^- to generate peroxynitrite which is a strong oxidant that can damage cell membranes and proteins via its interaction with proteins, lipids, and DNA as well as the nitration of tyrosine residues in proteins (Beckman, 1996; Beckman and Koppenol, 1996). NOS2-positive cells were present within areas of robust demyelination in the brains and spinal cords of chronically infected mice which argues that NO is exerting a toxic or damaging effect on oligodendrocytes. Whether NO blocks cellular respiration and/or if peroxynitrite is contributing to cellular damage in MHV-infected mice is not known as this time.

We have recently demonstrated that MHV-V5A13.1 infection of the CNS results in an orchestrated expression of both C-C and C-X-C

chemokines (Lane *et al*, 1998). Expression of proinflammatory chemokines may be important in contributing to the pathogenesis of MHV-induced demyelination by attracting effector cell e.g. activated T lymphocytes and macrophages to sites of viral persistence. The reduction in inflammation and demyelination observed in AG-treated animals suggested that the inhibiting NOS2 expression resulted in an alteration in expression of proinflammatory factors in the CNS. Interestingly, AG-treated mice had significantly lower levels of transcripts for the C-C chemokine MCP-1 at day 7 p.i., a time in which there was also a pronounced decrease in the inflammation in these animals as compared to the PBS-treated control mice. MCP-1 is thought to be a potent chemoattractant for monocyte/macrophage during periods of inflammation, thus the limited inflammation observed in AG-treated animals may be the result of reduction in expression of this chemokine (Berman *et al*, 1996; Taub *et al*, 1995). Supporting this are reports postulating that expression of MCP-1 contributes to the pathogenesis of EAE through recruitment of inflammatory cells into the CNS (Glabinski *et al*, 1995; Hayashi *et al*, 1995; Berman *et al*, 1996; Glabinski *et al*, 1997; Adamus *et al*, 1997).

How NOS2/NO may influence MCP-1 expression is not known at this time. MCP-1 transcripts are readily detected in the CNS of AG-treated mice indicating that MCP-1 is expressed in the absence of NOS2 generated NO. However, the fact that significantly higher levels of MCP-1 mRNA transcripts are present in the CNS in PBS-treated mice as compared to AG-treated mice indicates that NO exerts some regulatory role in MCP-1 expression. NO is known to regulate the expression and activation of transcription factors such as NF-κB and AP-1. Therefore, NO may modulate transcription of MCP-1 through interactions with factor(s) which enhance expression of this gene. Recent studies have indicated an interaction in expression between NOS2 and MCP-1 (Gryzbicki *et al*, 1998; Hogaboam *et al*, 1997). Gryzbicki *et al* (1998) have shown that following induction of cerebral trauma in mice there is a coordinate expression of MCP-1 and NOS2 suggesting these factors are regulators of cellular events following trauma.

We have recently examined the contributions of NOS2/NO in clearance of MHV-OBLV60 from the CNS (Lane *et al*, 1997). In contrast, the present study has examined the role of NOS2/NO in contributing to MHV-V5A13.1-induced demyelination in persistently infected mice. It is important to recognize a fundamental difference between the two MHV strains used in these studies. MHV-OBLV60 is a neuronotropic virus which does not normally persist in the brains of immunocompetent mice and remains primarily associated with neuronal tracts. MHV-OBLV60 is cleared from the CNS and mice do not develop demyelination at any point following clearance. In contrast, MHV-V5A13.1 infects both neurons and glia during acute infection. Although the bulk of virus is eliminated from the CNS, virus will persist in astrocytes as well as oligodendrocytes and the majority of surviving mice develop demyelination. Therefore, while NOS2/NO does not appear to contribute to clearance of either MHV strain, there is a potential role for NOS2/NO in contributing to the pathology of CNS disease in mice persistently infected with MHV-V5A13.1.

It should be emphasized that AG-treatment did not completely block demyelination in NHV-V5A13.1-infected mice but slowed its progression. By day 21 p.i., AG-treated animals had a similar level of demyelination as PBS-treated control mice. The mechanism(s) by which demyelination occurs in MHV-infected mice are very complex and most likely no one single effector molecule or cell population will be found to be the sole mediator of this disease process. Rather, MHV-induced demyelination may occur via overlapping events which occur during the course of infection. The data presented in this paper support that possibility that NOS2 generated NO may accelerate demyelination in MHV-infected animals by upregulating expression of chemotactic signals, such as MCP-1, which attract inflammatory cells into the CNS. Although further studies are required to fully define the role of NOS2/NO and chemokines in CNS disease, regulation of expression of these factors may have eventual therapeutic value for human CNS diseases.

Materials and methods

Virus and mice

The MHV strain V5A13.1 was derived from the wild type MHV-4 as previously described (Dalziel *et al*, 1986). Infection of susceptible strains of mice with the neuroattenuated MHV-V5A13.1 results in a chronic demyelinating disease in susceptible animals (Dalziel *et al*, 1986; Lane *et al*, 1998). Age-matched (5–7 weeks) male C57BL/6 mice (H-2^b background) were used for studies described. Following anesthetization by inhalation of methoxyflurane (Pitman-Moore Inc., Washington Crossing, NJ, USA), mice were injected i.c. with 10 pfu of MHV-V5A13.1 suspended in 30 μl of sterile saline. Control animals were injected with sterile saline alone. Animals were sacrificed by methoxyflurane anesthesia at days 7, 12, and 21 p.i. and brains and spinal cords removed. One-half of each brain of all sacrificed animals was used for plaque assay on the DBT astrocytoma cell line to determine viral burden (Lane *et al*, 1998). The remaining half brain as well as spinal cords were either fixed in 10% normal buffered formalin for paraffin-embedding or stored at -70°C for RNA isolation.

AG treatment of mice
AG is a selective inhibitor of NOS2 activity which readily crosses the blood brain barrier (Cross *et al*, 1993; Zhao *et al*, 1996; Brenner *et al*, 1997). A total of 52 mice were used for experiments described in these studies: 29 treated with AG hemisulfate salt (Sigma Immunochemicals, St. Louis, MO, USA) and 23 treated with sterile PBS. Following infection with MHV-V5A13.1, mice were immediately injected intraperitoneally (i.p.) with either 8 mg AG (dissolved in 0.5 ml sterile PBS) or PBS alone (0.5 ml). Animals were treated daily for a total of 21 days. Such treatment of mice results in a decrease in NOS2 activity as measured by nitrite/nitrate anions in the plasma (Lane *et al*, 1997).

Clinical disease
Following infection with MHV-V5A13.1, mice were evaluated daily for signs of clinical disease and scored according to a recently described scale (Houtman and Flemming, 1996b). Scoring was based as follows: 0, no abnormality; 1, limp tail; 2, limp tail and partial hindlimb weakness; 3, complete hindlimb paralysis; 4, death.

Histology
Brains and spinal cords were removed from mice at scheduled time points and fixed overnight in 10% normal buffered formalin. Tissues were then embedded in paraffin. Sections were stained with both hematoxylin and eosin to detect inflammation and luxol fast blue to detect areas of demyelination. Slides were blinded and independently read by three investigators. Scoring was based on a recently described scale (Houtman and Fleming, 1996b). A score of 0 indicated normal tissue; 1 indicated mild inflammation with limited white matter destruction; 2 represented a moderate increase in both inflammation and demyelination; 3, represented a pronounced increase in both inflammatory lesions and demyelination; and 4 indicated numerous inflammatory/demyelinating lesions. Scores were averaged between the three investigators and presented as the mean $\pm$ standard error of the mean (s.e.m.).

Immunohistochemistry
Anti-mouse NOS2 antibody (rabbit polyclonal anti-NOS2; Transduction Laboratories, Lexington, KY, USA) at a 1/200 dilution (2% normal goat serum in PBS) was used to detect NOS2 in the brains of MHV-infected mice. Staining was performed on brain and spinal cord tissue fixed in 10% normal buffered formalin and embedded in paraffin according to previously described protocols (Lane *et al*, 1997). The ABC Elite (Vector Laboratories) staining system was used according to manufacturer's instructions and diaminobenzidine was used as a chromagen. All sections were counterstained with hematoxylin.

RNase protection assay
Total RNA was extracted from brain and spinal cords using the TRIzol reagent as previously described (Lane *et al*, 1998). For RPA analysis designed to detect chemokines, a multi-probe set designed to detect lymphotactin, regulated on activation, normal T cell expressed and secreted (RANTES), eotaxin, macrophage inflammatory protein-1 alpha, beta, and 2 (MIP-1α, MIP-1β, MIP-2), interferon inducible protein-10 (IP-10), macrophage chemoattractant protein-1 (MCP-1), and T-cell activation-3 (TCA-3) (mCK-5; Pharmingen, San Diego, CA, USA). Probes for L32 and GAPDH were included to verify consistency in RNA loading and assay performance. RPA analysis was performed with 10 μg total RNA using a previously described protocol (Lane *et al*, 1998). For quantification, autoradiographs were scanned (Scanjet 4C/T, Hewlett Packard, San Jose, CA, USA) and band density was assessed with NIH image 1.57 software (Lane *et al*, 1998).

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