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Nitric Oxide Plays a Critical Role in the Recovery of Lewis Rats from Experimental Autoimmune Encephalomyelitis and the Maintenance of Resistance to Reinduction

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Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the CNS and an animal model for the human demyelinating disease, multiple sclerosis. In the Lewis rat, myelin basic protein (MBP)-CFA-induced EAE is an acute monophasic disease from which animals recover fully, do not relapse, and develop a robust long-term resistance to further active reinduction of disease. In this paper, we report that rats recovering from MBP-CFA-induced EAE have significantly increased serum levels of reactive nitrogen intermediates indicative of increased NO production. These levels remain elevated after the recovery period and increase even further early after a rechallenge with MBP-CFA, and all animals are totally refractory to a second episode of disease. Oral treatment of rats with N-methyl-L-arginine (L-NMA), beginning at peak disease on day 11 postimmunization, results in significant prolongation of disease and an alteration in the presentation of clinical symptoms from that of solely hind limb paresis/paralysis to severe fore limb involvement as well. Treatment of fully recovered rats with L-NMA 24 h before a rechallenge with MBP-CFA leads to decreased serum reactive nitrogen intermediate levels and results in a second episode of EAE in 100% of animals. Furthermore, L-NMA treatment of fully recovered rats in the absence of a rechallenge immunization leads to spontaneous relapse of disease. The Journal of Immunology, 1999, 163: 6841–6847.

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3 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; iNOS, inducible NO synthase; NMA, N-methyl-L-arginine acetate; RNI, reactive nitrogen intermediates; eNOS, endothelial NOS; MBP, myelin basic protein; L-NIL, L-N(1-iminoethyl)lysine.

E xperimental autoimmune encephalomyelitis (EAE) is an organ-specific cell-mediated autoimmune demyelinating disease of the CNS. The pathology of EAE is characterized by lymphocytic and mononuclear cell infiltration of the CNS, an increase in blood-brain barrier permeability, astrocytic hypertrophy, and often demyelination; all of which contribute to the observed clinical expression of disease (1, 2). The cellular and molecular mediators of the disease are not yet wholly defined but there is considerable evidence that the initiating effector lymphocyte is a CD4+ T cell of the Th1 subset since the majority of cell lines transferring EAE are of this subset (3, 4). Th1 cells predominately produce IL-2, IFN-γ, and lymphotoxin, whereas their Th2 counterparts produce IL-4, IL-5, IL-6, IL-10, and IL-13 and are thought to possibly down-regulate inflammation (5). In addition to the initiating T cells, recruited macrophages, resident microglia (6), endothelial cells (7, 8), and astrocytes (9) all produce their own repertoire of cytokines, chemokines, and other molecules which may further contribute to the inflammatory response and to pathology.

One putative pathogenic molecule in EAE is NO, produced by the oxidation of arginine in a reaction catalyzed by the enzyme NO synthase (NOS). The inducible form of NOS (iNOS) is up-regulated during inflammation, and this up-regulation can be sustained over a prolonged period of time. An early study demonstrated the increased secretion of reactive nitrogen intermediates (RNI) by inflammatory leukocytes isolated from the CNS and the periphery of rats with hyperacute EAE (10). More recently, increased levels of NO and iNOS mRNA have been localized to the CNS of mice with EAE and correlated with disease severity (11–13). Furthermore, aminoguanidine, an iNOS inhibitor, has been shown to delay disease onset and decrease severity in murine EAE (14). Thus, NO is often considered a pathogenic molecule. Not all studies on the role of NO in EAE are in agreement however. We (15) and others (16) have shown that treatment of Lewis rats with iNOS inhibitors to prevent NO production enhances actively induced EAE. We further demonstrated that the relatively resistant PVG rat produces up to four times higher serum RNI levels within 48 h of myelin basic protein (MBP)-CFA immunization when compared with Lewis rats and that treating the PVG rats with N-methyl-L-arginine (L-NMA) reduced the NO levels to that of the Lewis rat and rendered them highly susceptible to disease induction. These data strongly suggest that NO can in fact act as a down-regulatory molecule in EAE.

The clinical course of EAE is greatly dependent on the type of Ag, type of immunization (active or passive), species, and strain of animal used to induce disease. In the Lewis rat, EAE actively induced with guinea pig MBP, is an acute monophasic illness from which the rats fully recover. Following recovery from this clinical episode, the rats do not show relapses and within 2 wk of recovery develop a total, long-term resistance to further active reinduction of disease (17–19). Despite numerous studies, the regulatory mechanisms that determine both the recovery process and the subsequent protection against reinduction of active EAE are not fully understood.
understood. Based on our studies with iNOS inhibitors described above (15) indicating a protective role for NO, we advanced the hypothesis that recovery from EAE in the Lewis rat and the subsequent resistance to active reinduction of disease may both be a function of increased production of NO. Here, we report a positive correlation between recovery from disease and the level of circulating RNI. Furthermore, RNI levels not only remain elevated at the time of development of resistance but following rechallenge with MBP-CFA, the levels increase another 2- to 4-fold and remain elevated for up to 2 wk. None of these rechallenged rats develop disease. If rats are treated with L-NMA beginning at peak clinical disease, there is both a prolongation of disease and a change in the presentation of clinical symptoms. Treatment of fully recovered rats with L-NMA 24 h before a rechallenge with MBP-CFA results in a decrease in RNI to below prechallenge levels, and 100% of animals develop a second episode of disease. Remarkably, when recovered rats are treated with L-NMA alone, i.e., in the absence of a rechallenge with Ag, 100% of animals develop a relapse of EAE. These results suggest a central role for NO in the immunoregulation of acute monophasic EAE in the Lewis rat.

Materials and Methods

Animals

Female Lewis rats (8- to 12-wk old) were obtained from the Animal Breeding Establishment at the Australian National University. They were bred under pathogen-free conditions and subsequently maintained in the Animal Holding Facility at The Canberra Hospital. Throughout the experiment, food and water were provided ad libitum, and they were housed under 12-h light and dark cycles.

Induction of EAE

MBP was purified from frozen guinea pig spinal cord according to the method of Eylar et al. (20). Guinea pig MBP in saline was emulsified in an equal volume of ICFA containing added heat-killed Mycobacterium butyricum (4 mg/ml). Rats were anesthetized before immunization with 100 μl of emulsion to each hind footpad for the initial induction of EAE. For rechallenge, rats were immunized with 50 μl of emulsion to each front footpad and 100 μl intradermal in the nuchal region. Total dose received for each immunization per rat was 25 μg of guinea pig MBP and 400 μg Mycobacterium butyricum. Preliminary studies indicated that both routes of immunization produced EAE with equal incidence, day of onset, severity, and development of resistance.

Evaluation of clinical signs

Rats were examined on a daily basis and clinical scores recorded from day 7 to day 22 postimunization. Scores were then recorded every other day from day 22 until the time of rechallenge. From rechallenge, they were again examined daily for clinical signs. Clinical disease severity was assessed and scored as described previously (17) using a scale from 1 to 5: 0, asymptomatic; 1, flaccid distal half of tail; 2, entire tail flaccid; 3, ataxia, difficulty in righting; 4, hind limb weakness; and 5, hind limb paralysis.

Histological examination

Rats for study were deeply anesthetized and perfused with 30 ml saline followed by 60 ml 10% neutral buffered formalin. Spinal cords were removed, fixed for 7 days in 10% formalin, and embedded for sectioning. The lumbar-sacral spinal cord was transected, and the halves were embedded side by side for longitudinal sectioning. Six 5-μm sections were cut at various levels through the cord with 50 μm between levels. For quantification, a minimum of 30 sections was counted at different levels.

Inhibition of NO production with L-NMA

L-NMA was prepared using the method outlined by Patthy et al. (21). Lewis rats were housed individually and given L-NMA via their drinking water at times as described in Results. The concentration of L-NMA needed to reduce RNI levels of MBP-CFA-immunized rats to that of unimmunized rats has previously been established as 15 mM/day with the volume of fluid consumed between 15 and 25 ml/rat (15). Because the immunization procedure causes the animals to temporarily reduce their fluid consumption by half, it was necessary to double the concentration of L-NMA in the drinking water for the first 24 h after immunization. The L-NMA solution was prepared daily, filter sterilized, and provided ad libitum to animals housed individually. The daily volume consumed per rat was measured and recorded at the same time each day to ensure delivery of the indicated minimal dose.

Measurement of NO production

The level of nitrate and nitrite in serum samples was determined as an indirect measurement of NO production in vivo as outlined by Rockett et al. (22) and modified and described in detail by Cowden et al. (15). Briefly, 30-μl aliquots of serum was added in duplicate to a V-bottom microplate (Nunc, Roskilde, Denmark). Standard curves were generated using normal dialyzed rat serum to which sodium nitrite or sodium nitrate had been added at concentrations ranging from 1 mM to 1 μM. To measure nitrate, the addition of nitrate reductase and NADPH (20 μl; Boehringer Mannheim, Mannheim, Germany) for 30 min is required for conversion to nitrite. Nitrite was measured by the addition of 100 μl of Greiss reagent to all wells. Trichloroacetic acid (100 μl) was added to precipitate protein, the plates were centrifuged, and the OD of each sample was read at 540 nm with a reference wavelength of 650 nm using a microplate reader (Molecular Devices, Menlo Park, CA). Nitrate and nitrite levels were quantified by reading against the appropriate standard curves. The results were expressed as micromolar (μM) concentrations of RNI, i.e., the sum of nitrate and nitrite concentrations.

Results

RNI levels in serum of rats during a primary episode of actively induced EAE

Rats were bled for determination of serum levels of RNI before immunization on day 0 with 25 μg MBP-CFA and again on days 1, 2, 7, 14, and 21 after immunization. Rats were also assessed for clinical signs of EAE over this period. Clinical signs first appeared on day 11, peaked on day 14, and all animals had recovered by day 21 (Fig. 1). Serum RNI levels increased slightly 24 h after immunization and then remained constant until day 7 when they again began to increase. By day 14 (peak disease), RNI levels had reached ~8 times background levels and remained elevated as the animals reached full recovery. These findings are similar to those made earlier (15).

RNI levels in serum of animals after a primary immunization and subsequent rechallenge

One group of rats was immunized with 25 μg MBP-CFA while another group received no immunization. Both groups were bled for RNI determination as above, and then on day 35 postimmunization, the MBP-CFA-treated group was given a second injection of 25 μg MBP-CFA, and the untreated group was given a primary immunization with the same inoculum. Rats were again bled on the day of immunization or rechallenge and on days 1, 2, 7, 14, and 21 postimmunization. There was an increase in NO production in the MBP-CFA-immunized group when compared with unimmunized controls (Fig. 2). As before, the increase in serum RNI remained significantly elevated (p < 0.05) from day 14 to day 21 after
primary immunization, and as shown here remained elevated out to the time of rechallenge at day 35. After rechallenge, RNI levels in the MBP-CFA-pretreated group decreased slightly in the first 24 h and then increased to four times prerechallenge levels by 48 h and remained increased for 2 wk before returning to prerechallenge levels. No animal in the MBP-CFA group displayed clinical signs of disease following the rechallenge, whereas all animals receiving the primary immunization had severe EAE (data not shown).

Treatment of rats with L-NMA at peak disease inhibits spontaneous recovery and alters the clinical presentation of disease

Rats were immunized with MBP-CFA and developed EAE beginning on day 9 postimmunization (Fig. 3). By day 11, most rats were severely affected, with clinical scores between 4 and 5. At this time, eight rats were put on oral L-NMA and the remaining six were maintained on normal drinking water. The majority of animals in both groups began normal recovery and improved by at least one clinical score by days 15–16 and remained so for the duration of the experiment. Of the treated animals, four of eight became symptom free by days 16–17, and remained so for the duration of the experiment. The untreated group showed less than one lesion/longitudinal section, which was confined mainly to the meninges. Previous studies from this laboratory (17) have shown that this level of lesion burden does not differ significantly from animals receiving only the primary immunization some 55 days earlier and therefore most likely represents residual lesions from that immunization rather than new lesions. In comparison, the rats treated with L-NMA that did not develop clinical signs of EAE (2 at 15 mM and 4 at 7.5 mM L-NMA) were killed on day 20 after rechallenge, and their spinal cords were examined for histological evidence of EAE as described in Materials and Methods. These were compared with three randomly chosen rats from the untreated rechallenged group and three from the primary immunized group taken at the same time. As expected, the primary immunized group showed an extremely heavy lesion burden, on average 15 lesions/section (Fig. 6). The untreated rechallenged animal developed disease (Fig. 5A), whereas all of the naive immunized rats developed EAE (Fig. 5D).

Histological analysis of L-NMA-treated and untreated rechallenged rats

The rats treated with L-NMA that did not develop clinical signs of EAE (2 at 15 mM and 4 at 7.5 mM L-NMA) were killed on day 20 after rechallenge, and their spinal cords were examined for histological evidence of EAE as described in Materials and Methods. These were compared with three randomly chosen rats from the untreated rechallenged group and three from the primary immunized group taken at the same time. As expected, the primary immunized group showed an extremely heavy lesion burden, on average ~15 lesions/section (Fig. 6). The untreated rechallenged group showed less than one lesion/longitudinal section, which were confined mainly to the meninges. Previous studies from this laboratory (17) have shown that this level of lesion burden does not differ significantly from animals receiving only the primary immunization some 55 days earlier and therefore most likely represents residual lesions from that immunization rather than new lesions. In comparison, the rats treated with L-NMA that did not develop clinical EAE, nonetheless, had extensive lesions throughout the lower spinal cord, 8–10 lesions/section depending on the dose of L-NMA. These lesions were found throughout the parenchyma of the white matter as well as in the meninges. The lesions

Treatment of recovered rats with L-NMA at the time of rechallenge inhibits the rapid increase in NO production and results in re-induction of EAE

Rats were immunized with MBP-CFA and allowed to develop disease and recover as normal. On day 35, they were divided into three groups. One group received normal drinking water and the other two received L-NMA in the water at either 7.5 or 15 mM beginning 24 h before rechallenge with MBP-CFA. Another group of naive rats was included to demonstrate the encephalitogenicity of the emulsion used for the rechallenge. L-NMA treatment was discontinued when this latter group had all developed clinical signs of EAE, i.e., on day 12. RNI levels were measured, and animals were observed for disease. Rats that had recovered from active EAE had elevated levels of RNI at the time of rechallenge as shown previously. Rechallenged nontreated rats showed a rapid increase in RNI, whereas both L-NMA-treated groups showed an initial decrease during the first 48 h followed by a slow increase to the level of the untreated rats by day 7 (Fig. 4; data shown only for 15 mM). Most important, 8 of 10 and 6 of 10 rats in the 15 and 7.5 mM L-NMA-treated groups, respectively, developed a pronounced second clinical episode of EAE (Fig. 5, B and C). No untreated rechallenged animal developed disease (Fig. 5A), whereas all of the naive immunized rats developed EAE (Fig. 5D).

FIGURE 2. RNI concentration in the serum of Lewis rats (n = 4) immunized with MBP-CFA on day 0 and rechallenged at day 35 with MBP-CFA. This is compared with control animals (n = 4) receiving only a primary MBP-CFA injection on day 35.

FIGURE 3. Prolonged disease in animals treated with L-NMA beginning at peak disease. Rats were put on 15 mM L-NMA in the drinking water from days 11 to 22 postimmunization (n = 8) or maintained on normal drinking water (n = 6). All L-NMA-treated rats showed a protracted or second episode of disease.

FIGURE 4. RNI concentration in the serum of Lewis rats (n = 4) immunized with MBP-CFA on day 0 and rechallenged at day 35 with MBP-CFA and treated or not treated with 15 mM oral NMA on days 34–46. Animals given only the MBP-CFA immunization on day 35.
were qualitatively the same as those seen in animals undergoing primary disease. The significant difference in the extent and distribution of these lesions and those seen in the untreated animals suggests that these must represent new lesions. These data along with the clinical scores in both of the l-NMA-treated groups indicates a 100% susceptibility to disease recurrence under such treatment.

**l-NMA treatment of recovered rats results in a second episode of disease in the absence of antigenic rechallenge**
We next investigated whether treatment of recovered rats with l-NMA alone without subsequent antigenic rechallenge might result in a spontaneous relapse of disease. Rats were immunized as before and allowed to recover from disease. On day 35 postimmunization, they were put on 15 mM l-NMA in the drinking water for 8 days. Another group was untreated and simply observed for spontaneous relapses. Remarkably, four of nine l-NMA-treated rats developed clinical signs of EAE beginning days 10–12 after initiation of treatment (data not shown). Histology of the lower spinal cord was examined from the five rats not developing clinical disease and compared with those from five nontreated rats. All five animals had extensive lesions when compared with untreated rats (Fig. 7), and the distribution of lesions in the parenchyma as well as the meninges again indicated new inflammatory episodes. Thus, 100% of l-NMA-treated rats showed spontaneous relapses following treatment in the absence of antigenic rechallenge, whereas none of the untreated rats relapsed.

**Discussion**
Actively induced acute EAE in the Lewis rat provides an important model for the study of multiple sclerosis. Rats develop severe disease from which they recover fully. The animals do not show spontaneous relapses and in fact with time develop a long-term...
resistance to active reinduction of disease (17–19). Understanding the recovery and resistance mechanism(s) will provide important insights into how such control mechanisms might be absent or defective in the multiple sclerosis patient and result in the chronic relapsing and progressive nature of that disease. Evidence to date clearly points to both nonspecific and specific (Ag) mechanisms being involved. Thus, CFA alone has been reported by some investigators (23, 24) to render Lewis rats resistant to subsequent challenge with MBP-CFA, whereas other investigators have reported no such protection (17, 18, 25, 26). The results obtained in such experiments may depend on the timing between pretreatment and challenge as described by Waxman et al. (27). Steroids also appear to play a role in recovery from EAE as well as being important in the resistance of some strains of rats to disease induction (28–30). With respect to specific regulatory mechanisms, Abs (31, 32), suppressor cells (33–35), or other serum suppressor factors (36) have been postulated to play a role. It is most likely of course that a number of both specific and nonspecific mechanisms act in concert to down-regulate disease.

Here, we describe the apparent involvement of NO in this immunoregulation of EAE. Rats recovering from MBP-CFA-induced EAE have significantly increased levels of serum RNI. These levels increase further after rechallenge with MBP-CFA, and all animals are refractory to a second episode of disease. In the experiments reported here, this effect is non-neuroantigenic specific in that animals rechallenged with CFA alone show increased RNI levels after rechallenge to determine whether the observed difference is related to dose or is in fact due to inhibitor selectivity.

A more likely explanation for the different results may lie in the timing and dose of inhibitors given. We have demonstrated that not only does serum RNI increase during recovery from clinical EAE and remain elevated to day 35 but when these animals are rechallenged, the elevated RNI levels double again within the next 24–48 h (Fig. 2). In our experiments, treatment with the NOS inhibitor began 24 h before rechallenge, which resulted in a decrease from these elevated serum RNI levels back to almost back to baseline at the time of the secondary immunization may not be adequate to lower RNI levels from an already increased baseline at the time of the secondary immunization. It is possible therefore that the t-NIL treatment simply failed to decrease the RNI levels adequately. We are currently assessing t-NIL in our system to determine whether the observed difference is related to dose or is in fact due to inhibitor selectivity.

FIGURE 7. Rats were put on 15 mM oral l-NMA for 8 days beginning day 35 after immunization with MBP-CFA. No further immunization was given. Five rats not developing clinical signs of EAE were killed 20 days after the initiation of NMA treatment, and histologic lesions of EAE were counted. These were compared with lesions from five immunized but untreated rats killed at the same time. A minimum of 30 sections per rat was examined.
Treatment of recovered rats with 1-NMA alone, without Ag rechallenge, was sufficient to cause a spontaneous relapse of disease in 100% of animals. This effect may be operating at one or two levels. It is known that recovered rats have MBP-reactive T cells, presumably memory cells, which can proliferate in vitro in response to Ag (17, 19) and can also transfer disease to naive recipients (42). MBP-reactive cells have not therefore been deleted in recovered animals, and it is evident that something in the in vivo environment is down-regulating their state of activation. Recovered rats also have an Ag depot which could be the source of stimuli for driving the development of new MBP-reactive precursors; this also is apparently down-regulated since spontaneous relapses normally never occur. NOS inhibition by 1-NMA treatment may work by reversing the regulatory mechanism at either or both of these levels. Experiments with passively induced EAE and with removal of the Ag depot will address this question.

Two groups have recently used iNOS knockout mice (iNOS−/−) in the study of the role of NO in EAE, and both reported that average disease severity scores were higher in actively immunized iNOS−/− mice than in wild-type controls (43, 44). Fenyk-Melody et al. (43) also demonstrated that (129SvEv × PL/J)F1 iNOS−/− mice had a greatly decreased ability to recover from disease. Furthermore, aminoguanidine-treated wild-type PL/J mice had an increased incidence and severity of disease and also a decreased remission rate. These authors concluded that iNOS (and by implication NO) may in some instances play a protective role in autoimmune-mediated tissue destruction. The other study also reported an increase in incidence and severity of disease but no evidence for decreased recovery rates in the iNOS−/− animals could be observed because of the chronic nature of the MOG35–55 peptide-mentioned, has been shown to promote recovery from EAE (54), which could promote relapses of disease.

With respect to the mechanism by which down-regulation of NO production by 1-NMA treatment allows reinduction or spontaneous relapses of EAE, there are numerous possibilities. NO has a number of known effects on immune responses. It has been shown to inhibit macrophage lysis (45) expression, which would have the effect of preventing T cell expansion due to the lack of Ag presentation. NO is also known to have a direct effect on T cell proliferation (46, 47), probably by preventing activation of Janus kinase (48). This inhibition of T cell proliferation by NO appears in fact to be a specific impairment of Th1 CD4+ T cells while sparing Th2 cells (49). Because EAE is a function of Th1 cells, the increase in NO may selectively limit the proliferation of the encephalitogenic effector population. Also, expression of selectins, VCAM, and ICAM-1 have been found to be down-regulated by NO and hence can significantly alter lymphocyte migration (50, 51). Finally, NO can in some circumstances stimulate the cytokine-mediated release of corticotrophin-releasing factor (52–54) with resultant production of corticosterone. Corticosterone has been shown to be very effective in down-regulation of EAE (54). Thus, treating with 1-NMA and lowering NO levels in recovered animals and preventing an early increase following rechallenge could allow for re-expression of Ilα on macrophages and promote Ag presentation and T cell expansion; release both memory and precursor cells from the antiproliferative effect of NO; lead to the re-expression of adhesion molecules and promote migration of effectors into the CNS; and reduce the levels of corticosterone, all of which could promote relapses of disease.

The ability of NO to contribute to recovery from disease may involve nonimmunological as well as immunological mechanisms. In addition to stimulating corticosterone production which, as mentioned, has been shown to promote recovery from EAE (54), NO is known to induce apoptosis or necrosis in T effector cells (13) and to protect oligodendrocytes against destruction by lipid peroxidation (55). Thus, inhibiting these two functions at the level of the target tissue could lead to the lack of recovery and prolonged disease that we observed.

In summary, we have demonstrated that the absence of relapses and the resistance to reinduction of disease seen in Lewis rats immunized with MBP-CFA can be reversed by treatment of rats with oral 1-NMA, a specific inhibitor of NOS. It is apparent therefore that although NO can have detrimental effects and contribute to immune-mediated pathology, it can also act in a positive way to down-regulate the immune response. The data presented here sound a cautionary word about the possible use of NOS inhibitors in the therapy of autoimmune disease.

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In summary, we have demonstrated that the absence of relapses and the resistance to reinduction of disease seen in Lewis rats immunized with MBP-CFA can be reversed by treatment of rats with oral 1-NMA, a specific inhibitor of NOS. It is apparent therefore that although NO can have detrimental effects and contribute to immune-mediated pathology, it can also act in a positive way to down-regulate the immune response. The data presented here sound a cautionary word about the possible use of NOS inhibitors in the therapy of autoimmune disease.


