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*J Immunol* 2001; 167:5904-5912; doi: 10.4049/jimmunol.167.10.5904

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Inhibition of Nitric Oxide Synthase Initiates Relapsing Remitting Experimental Autoimmune Encephalomyelitis in Rats, Yet Nitric Oxide Appears to be Essential for Clinical Expression of Disease

Nikki C. O’Brien,†† Brett Charlton, † William B. Cowden, ‡ and David O. Willenborg

Myelin basic protein-CFA-induced experimental autoimmune encephalomyelitis (EAE) in Lewis rats is an acute monophasic disease from which animals recover. In this model, spontaneous relapses do not occur and rats develop a resistance to further active reinduction of disease. Previously, we reported that oral administration of the NO synthase inhibitor N-methyl-L-arginine acetate (L-NMA) to recovered rats precipitated a second episode of disease in 100% of animals. Further studies now show that this second clinical episode is actually a chronic relapsing disease that persists for months. This occurs only in rats that have recovered from actively induced EAE and not in rats recovered from passively induced EAE, suggesting the need for a peripheral Ag depot to induce secondary disease. We have also determined that clinical signs of EAE in L-NMA-treated recovered rats do not appear until L-NMA treatment has stopped. This is despite the fact that, at the same time point, CNS inflammatory lesions in symptomless animals receiving L-NMA are qualitatively and quantitatively similar to those with severe disease symptoms from whom L-NMA treatment has been withdrawn. The latter animals have significantly higher levels of reactive nitrogen intermediates in the cerebrospinal fluid than the former group. This study examines the mechanism of reinduction of disease by L-NMA treatment, and the findings suggest a dual role for NO in regulation of pathology in EAE that is dependent on site and timing of NO production. The Journal of Immunology, 2001, 167: 5904–5912.

Nitric oxide is a free radical produced from the oxidation of the terminal guanidino nitrogen of arginine, and this reaction is catalyzed by a NADPH-dependent enzyme, NO synthase (NOS). Three isoforms of this synthase have been described (1–3). NO produced from neuronal NOS (nNOS) acts as a modulator of neurotransmission (4), while NO from endothelial NOS (eNOS) regulates vascular tone and adhesion of circulating blood cells (5). Both nNOS and eNOS are constitutive enzymes, with a dependence on increased intracellular levels of calcium for enzymatic activity (6); however, both isoforms also have the ability to be induced under certain physiologic conditions such as nerve injury or sheer stress (7). These two enzymes regulate a number of physiologic functions in normal healthy individuals. In contrast, inducible NOS (iNOS) is calcium independent and up-regulated during inflammation and infection, and this up-regulation can be sustained over a prolonged period, culminating in the production of large quantities of NO (2). High levels of NO have been implicated in the pathogenesis and tissue damage found in some disease states (8), such as inflammatory autoimmune diseases. There have been, for example, numerous studies in the last few years that have focused on the role that NO plays in autoimmune experimental encephalomyelitis (EAE) (9).

EAE is an autoimmune CD4 T cell-mediated disease of the CNS that is often used as a model of the human demyelinating disease multiple sclerosis (10). Pathologically, EAE is characterized by infiltration of the CNS by lymphocytic and mononuclear cells, a breakdown in blood-brain barrier permeability, astrocytic hypertrophy (11), and demyelination (12), which cumulatively contribute to clinical expression of disease. Clinically, the disease often manifests as an ascending paralytic disease of the hindlimbs with occasional forelimb involvement. The clinical course of the disease varies depending on the animal species and strain and type of Ag used for immunization (13).

Myelin basic protein in CFA (MBP-CFA)-induced EAE in the Lewis rat is an acute monophasic disease from which affected animals fully recover. This recovery is associated with an absence of spontaneous relapses and a long-term resistance to active reinduction of disease (14–16). Previously, we have reported (17, 18) increased serum levels of reactive nitrogen intermediates (RNIs), indicators of increased NO production, during the recovery phase of MBP-CFA-induced EAE in the Lewis rat. These levels remained elevated after the recovery period and increased even further early after a rechallenge with MBP-CFA. All rechallenged animals were totally refractory to a second episode of disease (18). Oral treatment of rats with a NOS inhibitor, N-methyl-L-arginine acetate (L-NMA), beginning at peak disease on day 11 postprimary immunization, resulted in significant prolongation of disease and an alteration in the presentation of clinical symptoms from that of...
solely hindlimb paresis/paralysis to severe forelimb involvement as well. It was found that these animals recovered only after the cessation of L-NMA treatment and the consequent rise in systemic NO production. Treatment of fully recovered rats with L-NMA beginning 24 h before a rechallenge with MBP-CFA led to decreased serum RNI levels and resulted in a second episode of EAE in 100% of otherwise totally resistant animals. Intriguingly, L-NMA treatment of fully recovered rats, even in the absence of a rechallenge immunization, led to a second clinical episode of disease.

Further studies on this model have now shown that animals recovered from actively induced EAE and treated with L-NMA, whether or not they are reimmunized with MBP-CFA, develop not only a second episode of disease, but this disease is a chronic relapsing one observed over many months. Unlike these animals, however, rats recovered from passively induced disease do not relapse following treatment, suggesting the need for a peripheral Ag depot to induce a second episode of disease. The mechanism of reinduction of disease by NOS inhibitors has been examined, and evidence suggests that a T lymphocyte escape from proliferation inhibition by NO may play a role.

We also demonstrate in this study that clinical signs of EAE in L-NMA-treated recovered rats do not appear during treatment with the inhibitor, but only after treatment is stopped. While receiving L-NMA, rats that show no clinical signs of disease nevertheless have quantitatively and qualitatively the same CNS inflammatory infiltrates as rats whose treatment was terminated 3 days earlier and who have severe clinical signs. The former animals also have a similar proportion of iNOS-producing cells in the CNS as do the latter, but significantly less RNIs in their cerebrospinal fluid (CSF). These findings suggest a dual role for NO in regulation of pathology in EAE that is dependent on site and timing of production.

Materials and Methods

Animals

Lewis rats (8–12 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 either the Animal Holding Facility at The Canberra Hospital or at the John Curtin School of Medical Research. Throughout the experiment, food and water were provided ad libitum, and they were housed under 12 hourly light and dark cycles.

Induction of active EAE

MBP was purified from frozen guinea pig spinal cord, according to the method of Eylar et al. (19). Guinea pig MBP in saline was emulsified in an equal volume of incomplete Freund’s adjuvant containing 4 mg/ml heat-killed Mycobacterium butyricum. Rats were anesthetized before immunization with 100 μl of emulsion into each hind footpad for the initial induction of EAE. For rechallenge, rats were immunized with 50 μl of emulsion into each front footpad and 100 μl intradermally in the nuchal region. Total dose received for each immunization per rat was 25 μg of guinea pig MBP and 400 μg of M. butyricum. Both routes of immunization produce clinical EAE of equal severity inclusive with the day of onset, incidence, and development of resistance.

Passive EAE

Donor rats were immunized with MBP-CFA, and spleens were removed 10 days postimmunization. Single-cell suspensions were prepared and cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% heat-inactivated FCS, 2.5 × 10^{-3} 2-ME, 1 μg/ml penicillin-streptomycin-neomycin solution, and 2 μg/ml Con A at 37°C, 5% CO₂ for 72 h. Cells were collected into HBSS and washed twice before counting and injecting the reseptated cells at a concentration of 40 × 10⁶ viable cells in 500 μl of HBSS.

Evaluation of clinical signs of EAE

Rats were examined on a daily basis, and clinical scores were recorded from day 7 postimmunization to day 22. Scores were then recorded every other day until rechallenge at day 35, in which scoring was resumed on a daily basis. Clinical disease severity was assessed and scored as previously described (15) using a scale from 1 to 5 as follows: 0, asymptomatic; 1, flaccid distal half of tail; 2, entire tail flaccid; 3, ataxia, difficulty in righting; 4, hindlimb weakness; 5, hindlimb paralysis. Where necessary, the score was divided to indicate the disease score falling between two categories, e.g., 3.5.

Inhibition of NO production with L-NMA

L-NMA was synthesized using the method outlined by Pathy et al. and described in Ref. 17. Lewis rats were housed individually and given L-NMA via their drinking water, as outlined in Results. The concentration of L-NMA needed to reduce NO production in MBP-CFA-immunized animals to that of naive animals has previously been established as 15 mM/day with the volume of fluid consumed between 15 and 25 ml/rat (14). As the initial anesthesia and immunization procedure causes the animals to temporarily reduce their fluid consumption by half, it is necessary to double the concentration of L-NMA in the drinking water for 24 h following immunization.

The L-NMA solution was prepared daily and filter sterilized, and 25 ml was decanted into sterile 50-ml tubes. The daily volume consumed per rat was recorded at the same time each day and expressed as milligrams of L-NMA per rat per day.

Measurement of NO production

The level of nitrate and nitrite in plasma samples was determined as an indirect measurement of NO production in vivo, as outlined by Rockett et al. (20), and modified and described in detail by Cowden et al. (17). Briefly, 30-μl aliquots of serum were added in duplicate to a V-bottom microplate (Nunc, Roskilde, Denmark). Standard curves were generated using normal rat plasma to which sodium nitrite or potassium nitrate had been added at concentrations of 1 mM to 1 μM. To measure nitrate, the addition of nitrate reductase and NADPH (20 μl; Boehringer Mannheim, Mannheim, Germany) for 30 min was 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 proteins. The plate was centrifuged, and 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 appropriate standard curves. The results were expressed as micromolar concentrations of RNI, i.e., the sum of both nitrate and nitrite concentrations.

RNI levels determined in CSF and spinal cord tissue

Samples of CSF were obtained from rats following anesthesia. The rats were shaved over the back of the skull and neck, and a 25-gauge needle (1 inch minus the hub) attached to 40 cm of 0.5-mm cannula tubing (Dural Plastics and Engineering, Sydney, Australia) connected to a 1-ml syringe was inserted ~6 mm behind the occipital prominence. The dura was penetrated, and the needle passed the spine of the atlas before entering the cisterna magna. CSF flows into the tubing, and suction is gently applied using the syringe. The suction must be released before removing the needle so that blood does not contaminate the samples. Aliquots of between 20 and 100 μl/ml were routinely obtained and transferred in duplicate to a V-bottom immunoassay plate (Nunc) for RNI determination. To correlate RNI data between the CSF and serum taken from the same animal, the standard curve for these analyses was made with distilled water.

MBP-specific lymphocyte proliferation assay

Draining lymph nodes and spleens were harvested on day 10 following rechallenge and made into a single-cell suspension gently teasing the node or spleen through a metal sieve (400 mesh) into mixed lymphocyte culture medium supplemented with 1 μl/ml antibiotics. The cells were washed twice and counted. The cell suspension for each sample was diluted in complete mixed lymphocyte culture medium (containing 10% FCS; 1% sodium pyruvate, L-glutamine, and nonessential amino acids; 1 μl/ml antibiotics; and 0.5 μl/ml 2-ME) to give a final cell concentration of 5 × 10⁶/ml. A total of 200 μl cell suspension was added in triplicate to a 96-well round-bottom plate (Nunc). Each sample was incubated with Ag at 5, 10, and 20 μg/ml guinea pig MBP or with no Ag to determine nonspecific proliferation. The cells were incubated for 24 h and 3 and 7 days at 37°C and 5% CO₂, then labeled with 5 μCi of [3H]thymidine and incubated overnight. Cell cultures were harvested and radioactivity was determined on a Packard liquid scintillation instrument (Packard Instrument, Downers Grove, IL). The stimulation index was calculated as the degree of proliferation of Ag-stimulated cells divided by unstimulated cells from the same sample.
Histological analysis of H&E-stained paraffin sections from rat spinal cord

Spinal cords were removed from rats following perfusion with 30 ml of saline, followed by 60 ml of 10% neutral buffered Formalin, and placed in 10% neutral buffered Formalin for 7-day fixation before paraffin embedding. Cross-sections from the spinal cord were placed side by side in paraffin blocks, and 5-μm serial sections were cut and stained with H&E. To quantify the number of inflammatory lesions between animals, eight sections were cut at four different levels, with 100 μm between levels, through each the lumbar-sacral, thoracic, and cervical areas of the spinal cord. A minimum, therefore, of 96 sections per rat, at 12 different levels of the spinal cord, was assessed in a blinded fashion. A lesion was considered as containing not <10 inflammatory cells.

Immunohistochemistry

Immunohistochemical procedures were performed using an Innogenex IHC kit (San Ramon, CA) following the manufacturer’s instructions. Briefly, paraffin sections on silanized slides were taken to water before immersing slides in citrate buffer (pH 6) at 95°C for 20 min for Ag retrieval. Slides were cooled for 20 min at room temperature, and endogenous peroxidase activity was blocked using 0.3% H₂O₂ in methanol. Primary Ab, either biotinylated mouse monoclonal anti-rat ED-1 1:100, polyclonal anti-iNOS 1:500, or polyclonal anti-nNOS 1:1000 (Sapphire Biosciences, Sydney, Australia) in TBS (pH 7.6) was added to cover appropriate sections and incubated at room temperature for 1 h, followed by secondary biotinylated anti-rabbit Ab for 1 h at room temperature and HRP-streptavidin conjugate for 30 min. The reaction product was visualized using 3-amino-9-ethyl-carbazole, and each section was counterstained with Mayer’s hematoxylin. ED-1-positive staining was assessed and expressed as the number of positive cells/inflammatory lesion.

Results

Chronic relapsing EAE results from l-NMA treatment of rats recovered from actively induced disease

Rats were immunized with MBP-CFA and allowed to develop disease and recover. Thirty-four days after immunization (15 days after recovery), they were placed on oral l-NMA (15 mM) treatment in the drinking water and reimmunized with MBP-CFA 24 h later. Treatment was continued for either 8 or 12 days and then discontinued. As reported previously, such treatment results in reduced levels of serum RNI and a secondary clinical disease episode in the majority of animals within 2–5 days of removal of treatment (18). All animals were then observed long-term, up to 100 days postrechallenge. As shown in Fig. 1, not only did 15 of 16 animals have a second episode of disease, but they all, with one exception, developed multiple relapses and remissions, e.g., eight episodes in animal 4, group B. A similar pattern of relapsing remitting disease was seen in animals treated identically with oral l-NMA, but not rechallenged with Ag (data not shown). Rechallenged animals not treated with l-NMA do not develop disease.

The observation of relapsing disease following l-NMA treatment in the absence of antigenic rechallenge raised the question of...
ory cells capable of being reactivated, as shown by the early onset of disease following active challenge in a cohort of rats receiving the same initial donor cell population. One interpretation of this result is that there is the need for a peripheral Ag depot to develop a secondary disease following l-NMA treatment, and suggested the effect of the treatment may be at the level of proliferation of new effector cells.

l-NMA in vivo allows enhanced proliferation of MBP-specific lymph node cells ex vivo

Rats that had recovered from actively induced EAE were placed on oral l-NMA treatment at day 34 and reimmunized with MBP-CFA 24 h later. Another group of recovered rats received a rechallenge, but no l-NMA treatment, and a group of naive rats received a primary immunization only. Ten days after immunization, draining lymph nodes were taken from each group for proliferation assays, as described in Materials and Methods. Stimulation indices for individual animals responding to three different doses of MBP in vitro are presented in Table I. These data are representative of repeated proliferation assays. The proliferation in the l-NMA-treated group was significantly greater than the untreated group at both 10 and 20 μg MBP. The untreated rechallenged group did not differ significantly from animals receiving only the primary immunization 10 days previously.

l-NMA treatment allows secondary clinical episodes of EAE only after discontinuation of the treatment

In the course of our original work (18), recovered rats were treated with l-NMA for two different lengths of time following active reimmunization, and it was noted that the animals showed clinical signs of disease apparently only after withdrawal of treatment. To formally test whether this is the case, rats were immunized with MBP-CFA and allowed to develop disease and recover as usual. On day 34 after disease induction, all animals were put on 15 mM l-NMA in the drinking water for 24 h and then rechallenged with MBP-CFA. Rats were allocated to one of four groups in which l-NMA treatment was continued for 8, 12, 17, or 21 days and then removed. Another group of naive rats was immunized with the same inoculum of MBP-CFA to show the challenge inoculum was encephalitogenic. As shown in Fig. 3, in all cases animals remained clinically well while receiving l-NMA and only developed

Table I. Proliferation of lymph node cells from rechallenged rats untreated or treated with l-NMA

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<th>Group</th>
<th>Animal</th>
<th>Stimulation Index (S.I.) at Dose of MBP</th>
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*Draining lymph node cells taken 10 days after rechallenge and incubated with or without Ag for 72 h, at which time [3H]thymidine was added for 6 h prior to harvest of cells. These data are from one of five experiments all with similar results.

The differences between group B and group A at the 5-, 10-, and 20-μg doses are p < 0.2, p < 0.02, and p < 0.02, respectively (Wilcoxon ANOVA).

FIGURE 2. Rats (n = 8) recovered from passive EAE and rechallenged with MBP-CFA exhibit early onset and recovery; in fact, the clinical course of EAE in these animals was finished before the controls (n = 8) became symptomatic.

whether relapses could also be induced in animals that had recovered from passively induced disease using a similar treatment.

l-NMA treatment of rats recovered from passive EAE does not induce a second episode of disease

Recipients of 40 × 10⁶ Con A-activated spleen cells from MBP-CFA-immunized donors developed typical adoptive EAE with clinical disease onset at days 4 and 5 posttransfer and recovery by days 10 and 11 (data not shown). Such recovered animals do not spontaneously relapse. However, when actively challenged with MBP-CFA, the rats develop an early (days 5–8) onset of disease, which has been interpreted as the persistence and reactivation of some of the originally transferred cells that had reverted to memory cells (14, 16). When the recipient rats that had recovered from passive EAE were placed on oral l-NMA treatment for 12 days and subsequently followed for 8 wk, there were no second episodes of disease in any of the animals (0 of 9). The same result was obtained in repeated experiments using both l-NMA treatment and a more specific iNOS inhibitor, aminoguanidine. There was no evidence of subclinical disease when four animals were examined histologically 5 days after removal of l-NMA treatment (data not shown). Fig. 2 illustrates that the animals in this group had mem-

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*Draining lymph node cells taken 10 days after rechallenge and incubated with or without Ag for 72 h, at which time [3H]thymidine was added for 6 h prior to harvest of cells. These data are from one of five experiments all with similar results.

The differences between group B and group A at the 5-, 10-, and 20-μg doses are p < 0.2, p < 0.02, and p < 0.02, respectively (Wilcoxon ANOVA).
signs of disease 2–4 days after the drug was removed. Again, these L-NMA-treated animals all developed relapsing remitting disease.

Lesion burden and distribution in rechallenged rats treated with L-NMA and presenting either clinically well or with overt disease

We examined the histology of groups of L-NMA-treated rechallenged animals that were either still on treatment and not clinically ill or taken off treatment 2–3 days previously and were clinically ill. Forty sections from three levels of spinal cord, lumbar-sacral, thoracic, and cervical, were examined and scored from each animal in a blinded fashion. Fig. 4 shows that the animals still being treated with L-NMA and showing no clinical signs (group C) had just as many lesions as clinically ill animals that had treatment withdrawn 2 days before taking tissue for histology (group A). This was true whether histology was taken day 8 after rechallenge (L-NMA treatment for 6 days) or day 15 (L-NMA treatment for 13 days) after rechallenge. There was also no difference in the distribution of the lesions between the two groups with respect to the level of the spinal cord involved. Group B in Fig. 4 shows the lesion burden in naive animals, 8 and 15 days after primary immunization with MBP-CFA.

Macrophage infiltration in the CNS of rechallenged rats treated with L-NMA and presenting either clinically well or with overt disease

Quantitatively, there was no apparent difference with respect to inflammatory lesions between clinically well, rechallenged rats still on L-NMA and those taken off treatment that were clinically ill. This did not, however, preclude a qualitative difference with respect to cell types involved. Since the macrophage is essential for clinical expression of disease (21), we examined the localization of ED-1 cells in the inflammatory infiltrates between rechallenged, L-NMA-treated animals with and without clinical signs. Twelve sections per rat from different levels of the lower spinal cord were stained for ED-1 cells, and positive cells were enumerated in a blinded fashion. Note was also taken of the overall extent of lesions between the two groups and again found to be similar. There was no difference in the extent of involvement of ED-1 cells within lesions between the two groups of animals.
(Fig. 5), nor was there a difference in distribution; cells were both perivascular and migrated into the parenchyma. Six sections from both the cervical and thoracic cord were also examined, and Fig. 6 shows the pattern of expression and distribution of ED-1⁺ cells. ED-1⁺ cells were equally represented in both clinically affected and unaffected animals at all levels of the spinal cord.

**iNOS expression in CNS tissue**

ED-1⁺ macrophages were equally abundant in clinically well, rechallenged rats still on L-NMA and those taken off treatment that were clinically ill. We therefore stained immunohistochemically for the presence of iNOS in sections of lower spinal cord from both groups of animals. Six to eight sections, serial to those used for the ED-1⁺ cells, were examined, and iNOS⁺ cells were counted. Note was also taken of the extent of inflammation, which again was equally intense in both groups. Both groups of animals had comparable numbers of iNOS⁺ cells, which were quite minimal, representing no >1–2% of the ED-1⁺ cells (data not shown).

**RNI levels in serum and CSF of rechallenged rats treated with L-NMA and presenting either clinically well or with overt disease**

The presence of iNOS does not necessarily translate to the production of NO. We therefore measured RNI levels, as an indicator of NO production by NOS, in CSF and serum 8 days after rechallenge (Fig. 7). RNI levels in both the CSF and serum of rats (n = 4) still on L-NMA treatment and with no clinical signs of EAE (group C) were ~20 μM on day 8 after rechallenge. Rats (n = 4) that had the L-NMA treatment terminated on day 6 and had clinical signs of disease on day 8 (group A) had 3–4 times this amount.

![FIGURE 6. Immunohistochemical staining for ED-1⁺ cells/inflammatory infiltrate in the spinal cord of rats rechallenged with MBP-CFA and treated with L-NMA. Treatment was removed at day 13 with subsequent clinical symptoms (A, C, and E). Histology was taken at day 15. A, Cervical spine; C, thoracic spine; and E, lumbar-sacral region. ED-1 positivity does not differ when compared with cervical spine (B), thoracic spine (D), and lumbar-sacral region (F) from rats still on L-NMA treatment with no clinical symptoms.](http://www.jimmunol.org/)
untreated controls, L-NMA treatment did not inhibit RNI production of disease by treatment with L-NMA, an inhibitor of NOS, reinduction of another episode of disease (15). We demonstrated lapses, and in fact develop a solid long-term resistance to active which animals recover fully. They do not show spontaneous re-

EAE in Lewis rats is normally an acute monophasic disease from Discussion

(70–80 μM) of RNI in both CSF and serum. As has been previously reported (17, 18), RNI levels in animals receiving a primary immunization (group B) have not yet significantly increased at the time of sampling.

In contrast to the lowered RNI levels in CSF of NMA-treated rechallenged animals, rats undergoing a primary course of EAE did not show a reduction in RNI levels in the CSF when treated with NMA throughout the disease course. Fig. 8 presents results of an experiment in which RNI levels in the CSF of Lewis rats were measured during a primary course of EAE with and without treatment with L-NMA (n = 4). Despite lowered serum RNI levels vs untreated controls, L-NMA treatment did not inhibit RNI production in the CSF.

Discussion

EAE in Lewis rats is normally an acute monophasic disease from which animals recover fully. They do not show spontaneous relapses, and in fact develop a solid long-term resistance to active reinduction of another episode of disease (15). We demonstrated previously (18) that rats can be induced to develop a second episode of disease by treatment with L-NMA, an inhibitor of NOS, either with or without subsequent rechallenge with MBP-CFA. The fact that such treatment allows a second episode of EAE to occur in rats previously believed to be resistant to a further course of disease is consistent with known roles of NO in inhibition of lymphocyte proliferation, leukocyte adhesion, and migration, and with the effects of NOS inhibitors on these parameters (5, 20).

In the present study, we have found that such treated rats not only develop a second episode of disease, but also subsequently develop relapsing remitting disease with up to eight episodes of clinical disease occurring over a period of 3–4 mo (the longest time period examined to date). Two other models of chronic relapsing EAE in the rat have been previously described. Polman et al. (22) reported that low dose cyclosporin A treatment of Lewis rats immunized with spinal cord homogenate plus CFA resulted in a chronic relapsing form of EAE. Understanding the mechanism of relapses in this model is somewhat complicated by the myriad of functions of cyclosporin A ranging from alteration of expression of class II MHC transcriptional activator genes on one hand (23) to osteopenia (24) and hypertrichosis (25) on the other. The other recently reported model is that of protracted, relapsing, and demyelinating disease in dark august rats immunized with syngeneic spinal cord and incomplete Freund’s adjuvant (26). The latter disease results in demyelinating inflammatory lesions mainly in the spinal cord and is associated with Abs to myelin ologodendrocyte glycoprotein and cellular reactivity to the rat MBP peptides 69–88 and 87–101. It is of special interest that the disease should in fact become relapsing since the lack of relapse following the first episo-
dose appears due to the production of high levels of NO. One might anticipate that following rechallenge there would also be increased NO levels, and if so, why do relapses occur in this circumstance? An obvious answer is that the primary and relapsing diseases are fundamentally different. In a primary disease, there are two factors limiting it to an acute monophasic disease: specific immune regulation and nonspecific NO production. Inhibiting NO in this case is enough to induce another episode. The second episode of disease can be thought of as a boost to the primary immune response. These animals would possibly have a different specific immune regulatory response that may now allow relapses even in the face of increased (above background) NO levels. We are currently examining the relapsing remitting model with respect to NO levels at various times, pathologic parameters such as demyelination, remyelination, glial reactivity, as well as immunologic parameters such as humoral and cellular reactivity and epitope spreading.

To address the mechanism by which L-NMA treatment may promote relapses, we examined whether animals that had recovered from passive EAE could also be induced to relapse by L-NMA treatment. When rats recover from passive EAE, they show no spontaneous relapses, but when actively immunized with MBP-CFA, they develop clinical disease with onset occurring significantly earlier than in naive rats (14, 16). This has been interpreted as a memory response in which a proportion of the originally transferred encephalitogenic cells persist long-term (27) in the recipient as memory cells. Active immunization then stimulates the expan-
sion of both primary effector cells and resident memory cells, resulting in the more rapid attainment of the threshold number of cells needed to elicit clinically apparent disease. To determine whether animals recovered from passively induced EAE could be made relapsing remitting, they were treated with L-NMA for 12 days and then observed for 2 mo. No relapses or evidence of sec-
ondary lesions were observed. Memory cells persisted in these animals, as evidenced by an early onset in actively challenged animals, and these results suggest that there is a need for an Ag/ adjuvant depot to drive relapses.

NO has been shown to inhibit macrophage class II expression (28) as well as to have a direct effect on T cell proliferation (29, 30), possibly by preventing activation of Janus kinase (31). RNI levels in serum increase as animals recover from EAE and remain elevated for long periods. Continuing elevated production of NO may prevent renewed expansion of effector cells in response to persistent Ag presence (the depot) through the ability of NO to

FIGURE 8. RNI levels determined in CSF from rats during primary active EAE (MBP-CFA). At day 7 postimmunization, L-NMA treatment inhibited RNI levels, but during peak disease (day 14) this inhibition was not maintained.
prevent both Ag presentation and proliferation. Inhibiting NO reverses this effect with subsequent renewed expansion of a new wave of effector cells. We have in fact shown in this study that there is a significant increase in Ag-specific lymphocyte proliferation in l-NMA-treated animals rechallenged with MBP-CFA when compared with untreated animals.

Gold et al. (32) described the enhancement of EAE in Lewis rats treated with another NOS inhibitor, l-N-iminoethyl lysine (l-NIL). However, they were unable to induce disease recurrence using this agent. The difference between their findings and ours could of course be due to the fact that l-NIL is more specific for iNOS than is l-NMA; the latter also inhibits the endothelial (eNOS) and neuronal (nNOS) enzymes. Dose and timing of treatment could also account for the different results, and most importantly, based on the findings presented in this work, we would now suggest that unless Gold et al. (32) had ceased l-NIL treatment before termination of the experiment, clinical disease would not have been seen. We have shown in this work the necessity for cessation of inhibitor treatment in order for clinical disease to occur (Fig. 3).

Histological sections were taken at the same time point following rechallenge from l-NMA-treated animals that were either still receiving l-NMA (and not clinically ill), or that had been taken off treatment 2–3 days previously (and showing clinical signs). We found no difference in the inflammatory lesion burden or in the distribution of lesions within the CNS between these groups. These findings are consistent with the known role of NO in leukocyte adhesion and migration, and with the effects of NOS inhibitors on these parameters (5). The clinically well animals had the same extent of macrophage (ED-1+) involvement in their lesions as did clinically ill animals, and both groups showed the same level of iNOS expression immunohistochemically. We therefore measured RNI levels in the CSF of these two groups and found that despite similar lesion severity, macrophage numbers, and iNOS expression, the clinically well animals had 3–4 times less RNI in their CSF. This suggested that l-NMA was able to cross the blood-brain barrier in rechallenged animals, where it then inhibited in situ NO generation. The fact that clinical disease was observed only after cessation of l-NMA treatment and a corresponding elevation in RNI levels in the CSF further suggested that NO production in the CNS was required for disease expression.

The clinical findings are inconsistent with our original observations in which l-NMA treatment administered throughout the course of primary EAE caused a worsening of clinical disease (17). In a primary course of EAE, continuous l-NMA treatment caused an enhanced clinical disease, whereas continuous l-NMA treatment in rechallenged animals allows cellular infiltration of the CNS, but prevents the onset of clinical signs until treatment is ceased. One clear difference in these two cases is in the levels of RNI found in the CSF of l-NMA-treated animals. Thus, l-NMA treatment in rechallenged animals kept RNI levels in the CSF low, while treatment with the same dose during primary disease did not prevent an EAE-induced rise in RNI levels in the CNS. The reason for this is not understood, but it could be that the first episode of EAE compromised the blood-brain barrier, rendering it more permeable to l-NMA at the time of rechallenge. Whatever the reason, clinical signs of disease in primary and second episode experiments correlated with CNS RNI levels ≥70 μM.

These findings support the notion that NO may have a dual function in the regulation of EAE. Its inhibition leads to a more severe disease in a primary course of EAE (17, 18, 32, 33), and as we have now shown, its inhibition can initiate a second episode of inflammatory cell accumulation in the CNS. In contrast, it appears as if the production of NO in the CNS, in excess of normal levels, is required for clinical expression of disease. The mechanism for the latter is not clear, but potential rationales can be envisaged. For example, NO may act as a tissue-damaging free radical directly or combine with the superoxide anion (O2−) to generate peroxynitrite (ONOO−⋅). Peroxynitrite is an oxidant as well as a nitrating agent, and can promote lipid peroxidation as well as nitration of tyrosine residues on proteins (34). Peroxynitrite also degrades to form the highly tissue-damaging hydroxyl radical, and any or all of these downstream molecules of NO could possibly play a role in clinical expression of disease. Recently, others have shown that peroxynitrite is formed early in development of EAE and correlated with clinical disease expression in hyperacute EAE in mice (35). Others have shown that destruction of peroxynitrite by a decomposition catalyst resulted in less severe clinical EAE and demyelination in mice (36). We have been unable to confirm these findings in our model. This could perhaps be due to differences between the mouse and rat models of EAE. We have examined the extent of nitrotyrosine formation in our model and have demonstrated only minimal positive staining throughout the CNS for the presence of nitrotyrosine at peak disease. Therefore, despite the fact that peroxynitrite has been strongly implicated as the pathogenic downstream molecule in mouse EAE (34–36), there are little comparative data to support the same hypothesis in the rat model.

In summary, our previous findings (17, 18) and those of others (32, 33) suggest that NO has a down-regulatory effect in primary EAE. This effect is probably mediated in the periphery through inhibition of T cell proliferation (29), as well as inhibition of cell adhesion and migration (5). In contrast, the present study has shown that NO may have a role to play in expression of clinical disease. A challenge, therefore, exists in exploiting NO as a potential therapeutic target. A better understanding of the mechanisms involved in the pathogenesis of CNS inflammatory diseases may lead to appropriate therapeutic approaches to the use of NO or inhibitors of its synthesis in treatment of such diseases.

References


