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Nitric oxide-induced immune switching in experimental inflammatory autoimmune diseases

Hyun-Jeong Kwak[1], Hyung-Jin Kim[1], Jae-Sung Park[1], Chang-Duk Jun[1], Mun-Young Lee[2], Tae-Kyun Shin[3], Hun-Taeg Chung[1]*

[1]Department of Microbiology and Immunology, [2]Department of Neurosurgery, Wonkwang University School of Medicine and Medicinal Resources Research Center of Wonkwang University, Iksan, Chonbuk, Korea, [3]Department of Veterinary Medicine, Cheju University, Cheju, Korea

= Abstract =

Background: Nitric oxide (NO) production has been described as a double-edged sword eliciting both pro- and anti-inflammatory effects in different immune reactions. This work was undertaken to investigate the immunoregulatory role of NO in experimental allergic encephalomyelitis (EAE) and experimental allergic uveitis (EAU). **Method:** We examined whether molsidomine (MSDM), a NO donor, administration to the myelin basic protein (MBP)- or interphotoreceptor retinoid binding protein (IRBP)-immunized rats could suppress EAE development by shifting toward the Th2 cytokine response. In the EAE experiments, the rats were treated orally with MSDM (10 mg/kg/day) at the early stage (−1 4 days) or throughout the experimental period (−1 15 days). **Results:** This resulted in significant amelioration of the disease and mild clinical symptoms, while MBP-immunization without MSDM administration showed severe EAE development. A marked reduction in inflammation was also observed in the spinal cord, indicating the crucial role of NO in the pathogenesis of EAE in *in vivo*. In the EAU experiments, a 24 h pre-treatment with MSDM prior to IRBP immunization resulted in significant inhibition of the disease. Furthermore, MSDM administration for 21 days completely reduced the incidence and severity of EAU. To investigate whether MSDM could modulate cytokine switching from Th1 to Th2, culture supernatants of MBP- or IRBP-stimulated inguinal lymphocytes were analyzed. MSDM treatment enhanced IL-10 secretion but decreased IFN-γ. IL-4 was undetectable in all groups. In contrast, the MBP-or IRBP-immunized rats without MSDM secreted high concentrations of IFN-γ, but low concentrations of IL-10. **Conclusion:** In conclusion, NO administation suppresses EAE and EAU by modulating the Th1/Th2 balance during inflammatory immune responses. This work further suggests that NO may be useful in the therapeutic control of autoimmune disease.

Key Words: nitric oxide, inducible nitric oxide synthase, autoimmune disease, aminoguanidine, molsidomine, interferon-γ, interleukin-10

Correspondence : Hun-Taeg Chung, M.D., Ph.D.
Department of Microbiology and Immunology Wonkwang University School of Medicine 344-2, Shin-Yong Dong, Iksan, Chonbuk 570-749, Korea
Tel: 063-850-6762, Fax: 063-851-5066
E-mail: htchung@wonkwang.ac.kr

INTRODUCTION

Nitric oxide (NO) has been known to mediate vasodilation (1, 2), neurotransmission (3), and immune-

mediated cytotoxicity. In addition, NO has been implicated in the pathogenesis of experimental allergic encephalomyelitis (EAE), a chronic inflammatory demyelinating disease of the central nervous system (CNS), and experimental allergic uveitis (EAU), a neural retinal inflammatory disease (4).

Both EAE and EAU have been described as being a Th1 cell-mediated disease (5-7). Thus it has been suggested that they play a pivotal role in the pathology of Th1 proinflammatory cytokines such as interferon-γ (IFN-γ) and interleukin-2 (IL-2). In animals with EAE, increased levels of NO and iNOS mRNA have been detected in the CNS (8-10), and the administration of iNOS inhibitors or NO scavengers inhibited the onset of disease (11, 12). This has been considered as one of the therapeutic approachs in autoimmune disease. In contrast, some studies have demonstrated that iNOS inhibition does not ameliorate EAE and diabetes (13). Furthermore, recent studies using knockout mouse strains with a disrupted iNOS gene have shown at best only minor suppression of autoimmune disease. In some case, both pharmacological inhibition and genetic inactivation of iNOS have led to an increase in pathogenesis.

High NO doses have been reported to induce apoptosis of thymocytes and splenic T cells. On the other hand, low NO doses protect cells from anti-CD3-induced apoptosis in the thymocytes. Interestingly, Th1 cells rather than Th2 cells are more susceptible to apoptosis. This implies that NO may regulate the Th1/Th2 balance by promoting or suppressing apoptosis at high or low concentrations. In murine lymphocytes, exposure to NO suppresses IL-2 gene expression at the level of transcription. Thus, it is most likely that NO may modulate the Th1/Th2 balance by favoring the Th2 response (14).

In this study, a possible role of NO in autoimmune disease, such as EAE and EAU was examined. The results suggest that NO may be responsible for driving Th1-cell switching to a Th2-cell dependent immune response. To our knowledge, this is the first attempt to inhibit the autoimmune disease by exogenous NO.

MATERIALS AND METHODS

1. Animals

Male Lewis rats (8- to 12-wk old) were obtained from the Jackson Laboratory (Japan) and housed in an air-conditioned and pathogen-free room with a moderate temperature and humidity. Throughout the experiment, food and water were provided *ad libitum*, and they were kept under 12 h light and dark cycle.

2. Reagents

The guinea pig myelin basic protein (GPMBP) and complete Freund's adjuvant (CFA) were purchased from Sigma (St. Louis, MO., USA). The interphotoreceptor retinoid binding protein (IRBP)-derived peptides were synthesized and purified by Peptron, Co. Ltd. (Taejon, Korea). The peptide sequence was derived from bovine IRBP, as determined and reported by Borst et al15). A series of truncated forms of the peptide 1169-1191 (PTARSVGAADGSSWEGVGVVPDV) were synthesized and used. Rat IFN-γ and IL-4 were purchased from Endogen (Cambridge, MA., USA) and rat IL-2 and IL-10 were purchased from Pharmingen (San Diego, CA., USA).

3. Induction of EAE and EAU

The rats were immunized with either 200 μg of GPMBP or 100 μg of IRBP in 100 μl of an emulsion with CFA (1:1, v/v) that had been supplemented with *M. tuberculosis* to a final concentration of 2.5 mg/ml, with the pertussis toxin (PTX) by subcutaneous injection into two hind footpads.

4. Administration of MSDM

Rats were treated orally with (10 mg/kg/day) molsidomine (MSDM; Sigma, St. Louis, MO., USA), dissolved in their drinking water, in a volume of 0.1 ml, beginning 24 h prior to being immunized with GPMBP and IRBP to induce EAE and EAU, respectively. The MSDM treated GPMBP immunized rats were divided

into three groups. One group received MSDM daily (from day −1 to day 15) and the other two received MSDM at either the early stage (from day −1 to day 4) or at a later stage (from day 7 to day 10) from the beginning of immunization with GPMBP. In the IRBP immunized rats, MSDM treatment was continued through to day 21 after immunization. The control groups included animals that received an equal amount of MSDM without the antigens.

5. Clinical assessment of EAE and EAU

All animals were observed daily for clinical symptoms of disease from day 5 through to day 20 after immunization. The clinical disease severity of EAE and EAU was assessed and scored as described previously (16, 17): in EAE, grade 0, asymptomatic; 1, flaccid distal half of tail; 2, entire tail flaccid; 3, ataxia, hind limb weakness; 4, hind limb paralysis, forelimb weakness; and 5, morbidity or death. The incidence and severity of EAU were examined with a biomicroscope in the pre-clinical stages of disease on day 4 and daily thereafter to assess the disease development. In EAU, a grade 0 represented no disease; 1, enlargement of the iris vessel; 2, cellular infiltrates in the anterior chamber; 3, fibrin deposition around the pupil and less hypopyon; 4, fibrin plunging out of the pupil and the development of severe inflammatory hypopyon.

6. Histological examination of EAE and EAU

Experimental rats in each group were sacrificed on day 13 under ether anesthesia. Spinal cord sections were embedded in paraffin after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, and examined under an optical microscope. Immunostaining was performed using a Histoplus Immunostaining kit (Vector Laboratories, Burlingame, CA., USA) as outlined in the manufacturer's instructions. In brief, deparaffinized sections in a citrate buffer were washed and incubated with non-immune goat serum to block the non-specific binding of the secondary antibodies. Subsequently, the sections were incubated in optimally diluted rabbit anti-iNOS (Transduction lab, CA., USA) for 1 hr. After three washes in PBS, the sections were incubated with biotinylated anti-rabbit or anti-mouse antibodies, and then with the avidin-biotin complex reagent (Vector Laboratories, Burlingame, CA., USA) and diaminobenzidine as chromogens (Sigma, St. Louis, MO., USA). All incubations were carried out at room temperature. The slides counter-stained with hematoxylin were dehydrated and mounted in balsam (Sigma, St. Louis, MO., USA).

7. Cytokine assay by ELISA

The lymph nodes, the inguinal and iliacs, were drained and the liquid was collected and pooled within each group at the peak of the disease (12 day after immunization). Duplicated cultures of 1×10^6 cells/0.2ml/well were stimulated with 30 μg/ml GPMBP and 50 μg/ml IRBP. The supernatants were collected for cytokine analysis after 48 h. IFN-γ and IL-4 were measured by ELISA using the respective antibodies from Endogen (Cambridge, MA., USA). The ELISA detection kits, purchased from Pharmingen (San Diego, CA., USA), were used to measure the IL-2 and IL-10.

8. Statistical analysis

Experiments were repeated at least three times. The response patterns were highly reproducible. The results are expressed as a mean±SD and were analyzed statistically using the students *t*-test or Mann-Whitney *U* test (clinical grade of EAE). Probability values < 0.05 were considered significant.

RESULTS

1. Effect of MSDM on Clinical Sign of EAE

To examine whether NO could be involved in inhibiting EAE and EAU, the clinical signs of disease in the MBP- and IRBP-immunized rats with or without the NO donor, molsidomine (MSDM), were evaluated throughout the experimental period. In the EAE groups, a total of 70 rats were used. These included 45 treated with MSDM, 15 with PBS in the presence of MBP immunization, 5 with MSDM with CFA immunization

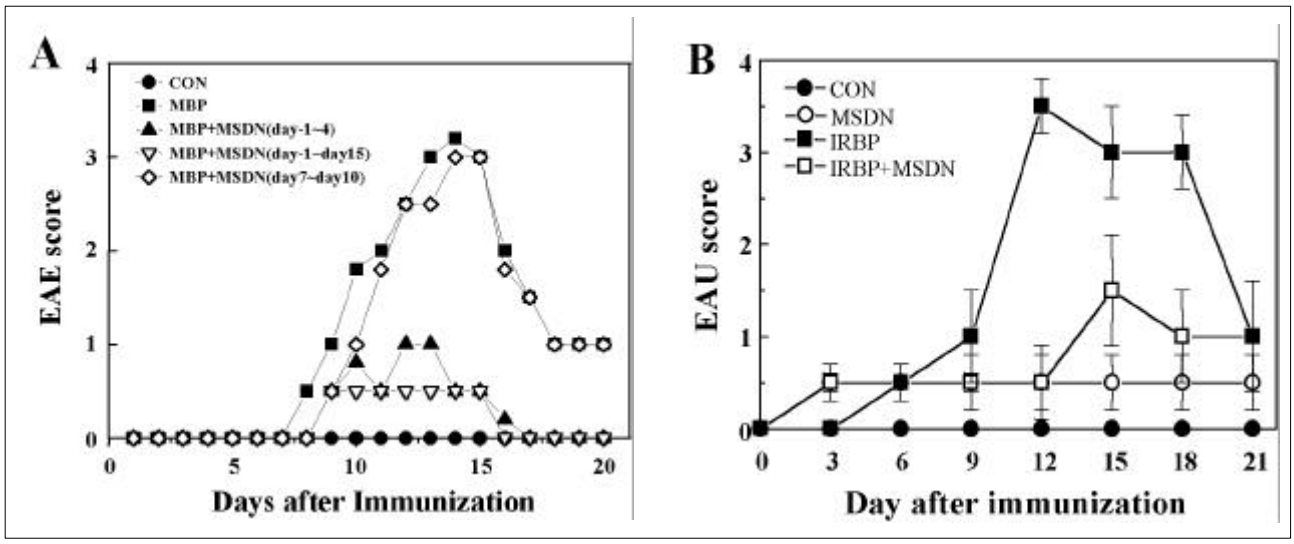

Fig. 1. Effect of MSDM treatment on the severity of EAE and EAU. (A) Lewis rats were immunized with 200 μg MBP in CFA and administered orally with MSDM (10 mg/kg). The MBP-immunized rats were treated with MSDM at the early stage (from day -1 to day 4), the later stage (from day 1 to day 10) and over the whole experimental period (from day -1 to day 15), respectively. (B) Lewis rats were immunized with 100 μg IRBP in CFA and administered orally with (□) or without (■) MSDM. The control group was administered orally with (○) or without (●) MSDM in the absence of IRBP immunization, respectively. Each curve represents the average daily disease score of four to six rats from more than three experiments. Each datum point represents the average daily disease score of four to six rats from at least three experiments. The disease severity was documented as the daily mean clinical score in each experimental group. The observed differences between each group are statistically significant ($p<0.05$, three distinct experiments).

and 5 control rats (untreated and 3 different sets of experiments). In the MBP-immunized rats, sets of 15 rats were treated with MSDM at either the early stage (from day −1 to day 4), at a later stage (from day 1 to day 10) or during the whole experimental period (from day −1 to day 15). The rats injected with MBP developed EAE on day 9 after immunization. By day 13, most rats (9 of 10, 90%) underwent sever EAE, with a clinical score of between 3 and 4. MSDM treatment for the whole experimental period or in the early stage clearly inhibited the clinical symptoms of EAE by 46.6% and 33.3%, respectively. In addition, a slight delay in disease onset was observed in a minority of MSDM-treated rats. However, significant EAE disease severity in the rats treated with MSDM at the later stage was observed, with clinical score between 3 and 4. The majority of animals in all groups began a normal recovery and had improved by at least one clinical score by day 18 (Fig. 1A).

In the EAU groups, a total of 46 rats, 17 treated with MSDM, 17 with IRBP-immunization, 7 with MSDM without IRBP immunization, and 5 untreated rats, were used in this study. Clinical observations revealed a marked and significant reduction in EAU by MSDM in all three experiments. As shown in Fig. 1B, severe EAU was induced in the rats that were immunized with IRBP without MSDM at day 12. These symptoms had gone by day 21. A peak clinical grade of 3 or 4 was observed at day 12. In contrast MSDM treatment in the IRBP-immunized rats showed not only a reduced the severity but also delayed the onset of clinical EAU. In the IRBP-immunized rats without MSDM, EAU had developed in 12.5 of the 17 treated rats (grade 3). However, MSDM (10 mg/rat/day, orally) completely suppressed the clinical expression of EAU in 13 out of the 17 treated rats (grade 1.5). In the control rats only injected with MSDM, EAU was not induced, only iris vessel dillatation was noted (Fig. 1B).

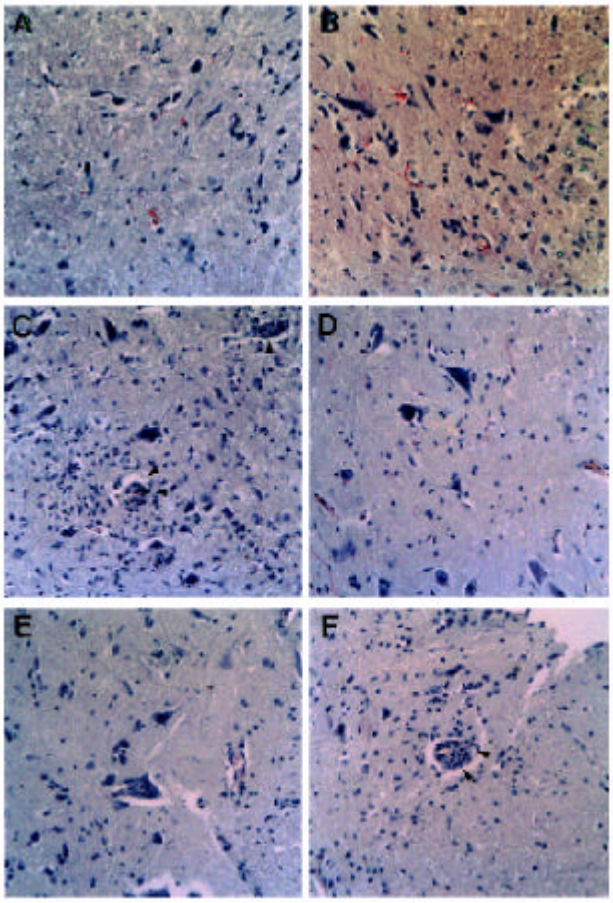

Fig. 2. Histologcal finding in the spinal cords of rats with EAE. Serial section from the rat spinal cords at day 13 were stained with H & E. Inflammation was not observed in the naive and CFA-immunized rats (A and B). MBP-immunized rats (C) and MSDM treated rats at late stage (F) strongly detected infiltrating inflammatory cells, while no inflammatory cells were noted in MSDM treated rats at the early stage (D) and for those treated over the whole experimental periods (E). (Hematoxylin-Eosin satin, Magnification: ×150).

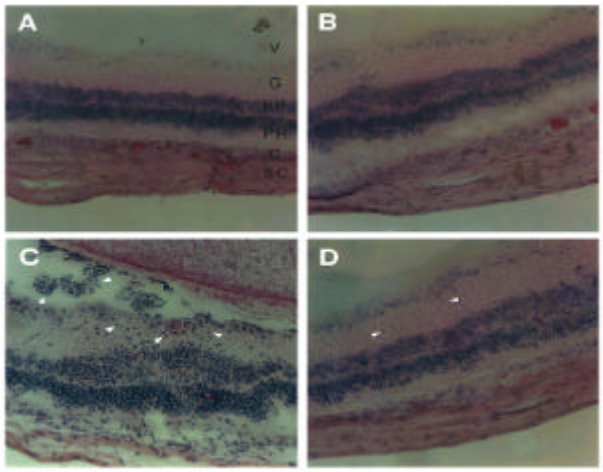

Fig. 3. Showing the effect of MSDM treatment on the histopathologic aspect of EAU in anterior and posterior segments of the eye. 12 days after footpads IRBP injection with (C) or without (D) MSDM, the eyes were harvested for histopathology. (A) Posterior segment of an eye of a normal rat shows the typical stratiform morphology of the segment (disease grade 0). (B) In the MSDM administered group, it shows as slightly edematous in the rods & cones (disease grade 0.5). (C) In the posterior segment of the eye at the peak of EAU, the retina is detached, edematous and infiltrated with inflammatory cell (arrowhead) in retina and vitreous. The photoreceptor layer is severely damaged and each layer shows an irregular form. A significant amount of inflammatory cells are observed in the entire retina (disease grade 3.5). (D) In contrast, only a few inflammatory cells are present in the retina (arrowhead) of the MSDM administered rat with IRBP immunization. The layer is less damaged and slightly irregular (disease grade 1.5). The rats were administrated with 10 mg MSDM. (V, vitreous; G, ganglion cell layer; BP, bipolar cell layer; PR, photoreceptor layer; C, choroids; S, sclera). (magnification ×200)

2. Effects of MSDM on the Histological Finding of EAE and EAU

Rats from each groups were sacrificed on day 13 after immunization, and their spinal cords and eyes examined for histopathological evidence as described in *Materials and Methods*. As expected, both of the MBP-immunized rats without MSDM (Fig. 2C) and with MSDM treatment at the later stage (from day 7 to day 10) (Fig. 2F) showed a large number of inflammatory cells infiltrating the perivascular lesions and parenchyma of the spinal cords of rats with EAE. No infiltrating cells were detected in the parenchyma of the MBP-immunzied rats with MSDM treatment at the early stage (from day 1 to day 4) (Fig. 2E) or throughout the whole all experimental period (from day 1 to day 15) (Fig. 2D). In addition, no infiltrating cells were observed in the CFA-immunized control spinal cords (Fig. 2B). In the EAU groups, the posterior segment of an eye from a

normal rat showed the typical stratiform morphology (Fig. 3A). The MSDM treated rats showed slight edematous signs in the rods and cones cells. However, the entire structure of the retina was similar to that of the normal rats (Fig. 3B). In the IRBP-treated groups without MSDM, severe inflammations was observed. The retina was detached, edematous, and was infiltrated with inflammatory cells. The photoreceptor layer was severely damaged (Fig. 3C).

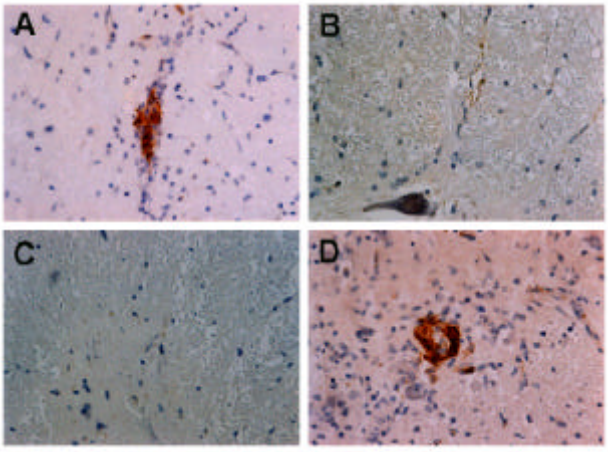

Fig. 4. Immunohistochemical detection of iNOS in the spinal cord of rats with EAE. Serial section from the rat's spinal cord at day 13 were immunostained with anti-iNOS antibodies. iNOS was detected in the MBP-immunized rats (A) and the MSDM treated rats treated at the later stage (D). In contrast, only a few iNOS was detected in the MSDM treated rats over the whole experimental period or at the early stage (C). (Magnification: ×240).

In parallel with the histological severity, the iNOS+ cells were easily identified in the perivascular lesions of the EAE in the MBP-immunized (Fig. 4A) and the MSDM-treated rats at the later stage (Fig. 4D). The spinal cords of the animals treated with MSDM for all experimental periods and the early stages did not develop EAE, and no iNOS+ cells were detected (Fig. 4B and C).

3. MSDM Administration Reduced Th1 Cytokine but Elevated Th2 Cytokine Secretion

The cytokine secretion pattern was analyzed in each group to examine the impact of exogenous NO as an immunoregulatory role to shift Th1 response toward Th2 response. The cultured lymphocytes derived from each groups at day 12 after the start of the experiment were cultured for 48 h in the presence of MBP or IRBP sensitization, respectively. Subsequently, the Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokine secretions were determined. There was also a significant increase in IFN-

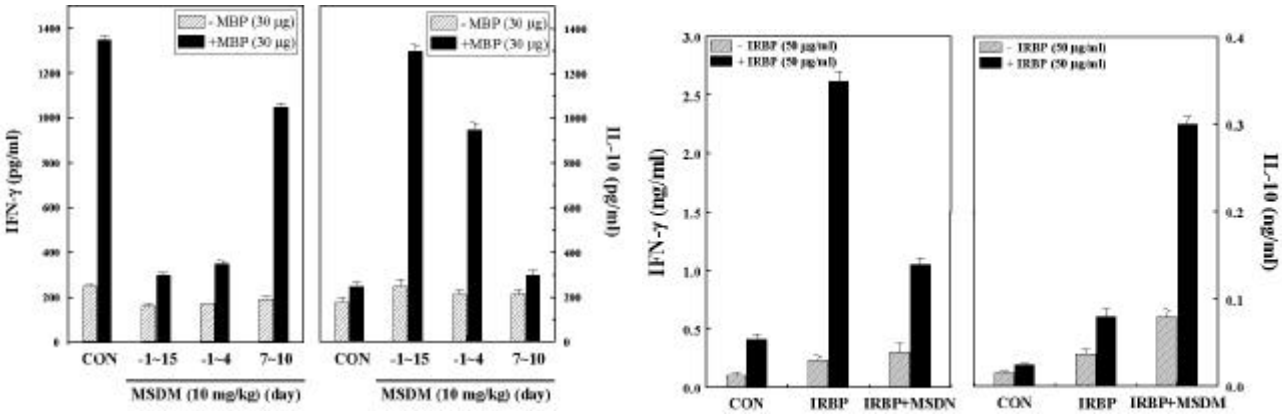

Fig. 5. Effect of MSDM on the secretion of Th1 or Th2 cytokine. Lewis rats were immunized with MBP (A) or IRBP (B) in CFA and administered orally with or without MSDM (10 mg/kg). 12 days after immunization, the draining lymph node cells were collected from each group, and cultured with or without MBP (30 μg/ml) or IRBP (50 μg/ml). The supernatants were collected at 48 h and assayed by ELISA. The cell density of all groups was adjusted to 1 x 10^6 cell/ml. Th1 (IFN-γ) and Th2 (IL-10) cytokine were measured and values are expressed as means SEM of three distinct experiments ($p < 0.05$).

γ secretion in the MBP-or IRBP-immunized group, while IL-10 secretion was not increased. In the groups orally treated with MSDM at the early stage and over the whole experimental period, IL-10 secretion had increased 4- to 4.5-fold compared to the MBP-immunized rats. Furthermore, IFN-γ production was not higher, indicating a shift toward the Th2-type response. The group treated with MSDM at the later stage did not show an up-regulation of IL-10, but showed a significant up-regulation of IFN-γ after MBP-immunization (Fig. 5A). This result correlated with the EAE development showed in Fig. 1A, indicating that NO administration at the later stage does not prevent EAE.

As shown in Fig. 5B, IRBP-elicited IFN-γ production correlated with EAU induction in the IRBP-immunized rats (Fig. 1B). In contrast, the same lymph nodes during this period did not show any Th2 cytokine, or IL-10, production (Fig. 5B). The next series was to determine whether MSDM treatment could switch the Th1 response to Th2. IFN-γ production was not increased in the MSDM-treated rats when compared with the non-treated animals. However, IL-10 production was markedly up-regulated (Fig. 5B). Overall, the results showed that exogenous NO treatment could regulate the immune response toward the Th2-type and prevent EAE and EAU induction.

DISCUSSION

Many reports have previously demonstrated that iNOS-derived NO conveys protection against many intracelluar bacteria and parasites. It assists in fighting several viral infections, and is implicated in the control of malignancies (18-21). On the other hand, NO might also promote tumor angiogenesis and metastasis (22, 23), and iNOS-dependent tissue destruction and/or disease has been observed in several rodent autoimmune models, such as experimental allergic encephalomyelitis (EAE) and uveitis (EAU), arthritis and glomerulonephritis (11, 24-28).

A frequently proposed cascade for the development of organ-specific autoimmune disease invokes the induction and expansion of Th1 in response to microbial antigens and IL-12, which produces IFN-γ and thereby activates macrophages and other effector cells for producing tissue damaging molecules such as reactive oxygen intermediates or NO. Several areas of this concept have been repeatedly challenged, particularly in the EAE mouse model, which shares similarities with human multiple sclerosis (MS). In mice where EAE was induced via immunization with the myelin basic protein (MBP) combined with microbial adjuvants, it has been shown that IFN-γ and NO are not only dispensible for developing EAE, but also clearly protects against disease progression or a relapse in susceptible mice and contributes to the resistance of strains where EAE cannot be elicited (16, 29). In IFN-γ +/+ PL/J mice, the pharmacologic inhibition or genetic deletion of iNOS was associated with an increased incidence and enhanced severity of EAE induced by immunization with MBP (30). This strongly suggests that NO has a protective, anti-inflammatory role. Indeed, MSDM administration, a known NO donor, can virtually block clinical disease and delay disease onset in the MBP- or interphotoreceptor retinoid binding protein (IRBP)-immunized rats. NO has been reported to be involved in the pathogenesis of EAE and EAU. This suggests that the area containing inflammation might exhibit iNOS enzyme. There are a number of possible mechanisms describing how NO down-regulates the immune response. NO inhibits lymphocytes proliferation by preventing Janus kinase activation, reduces leukocyte adhesion and infiltration, and inhibits other tissue-damaging pathways (e.g., NADPH oxidase). Furthermore, NO can, depending on the concentration, be either an inducer or suppressor of apoptosis and/or necrosis in a number of different systems (14). Several studies have indicated that elimination of inflammatory T cell and macrophages from the CNS during EAE might be the result of apoptosis (31-33). Recent evidence also indicates that Th1 cells are more prone apoptosis than Th2 cells (14), which would again be beneficial in regulating EAE.

Based on these results we propose that the immunoregulatory action of NO primarily targets the

Th1/Th2 balance in the immune response. NO administration can induce Th2 cytokine expression such as IL-10, but suppresses Th1 cytokine expression such as IFN-γ. Unexpectedly, another Th2 cytokine, IL-4, was not induced (data not shown). Previous reports demonstrated that EAU was ameliorated by IL-10. However, although IL-4 alone had no effect on its own, IL-4 in combination with IL-10 synergized EAU. This result also supports the results of this study. A similar regulatory effect might be exerted by NO at the level of antigen presentation, as it has been shown that NO induces IL-12 p40 gene transcription in macrophages but not the p35 gene. Because the p40 homodimer acts as an antagonist for IL-12 (34), it is likely that NO acts during the antigen presentation period to prevent EAE and EAU in this system. NO administration was observed to block EAE and EAU when NO was administered at the early stage. Because EAE and EAU inhibition by NO was effective at the early stage, we determined whether the shift in the Th1 response to Th2 occurred by the same mechanism. These results demonstrated that administering NO at the early stage induces IL-10 but suppresses IFN-γ.

In conclusion, we have shown that exogenously administered NO prevents EAE and EAU and controls Th1 reactivity, indicating systemic modulation of the Th1/Th2 balance. These findings demonstrate a modulatory function of NO in autoimmune disease. On the basis of our findings, NO may be beneficial for treating autoimmune disease.

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FOOTNOTES

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