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Sodium Phenylacetate Inhibits Adoptive Transfer of Experimental Allergic Encephalomyelitis in SJL/J Mice at Multiple Steps

Subhajit Dasgupta,* You Zhou,† Malabendu Jana,* Naren L. Banik,‡ and Kalipada Pahan2*

Experimental allergic encephalomyelitis (EAE) is the animal model for multiple sclerosis. The present study underlines the importance of sodium phenylacetate (NaPA), a drug approved for urea cycle disorders, in inhibiting the disease process of adoptively transferred EAE in female SJL/J mice at multiple steps. Myelin basic protein (MBP)-primed T cells alone induced the expression of NO synthase (iNOS) and the activation of NF-κB in mouse microglial cells through cell-cell contact. However, pretreatment of MBP-primed T cells with NaPA markedly inhibited its ability to induce microglial expression of iNOS and activation of NF-κB. Consistently, adoptive transfer of MBP-primed T cells, but not that of NaPA-pretreated MBP-primed T cells, induced the clinical symptoms of EAE in female SJL/J mice. Furthermore, MBP-primed T cells isolated from NaPA-treated donor mice were also less efficient than MBP-primed T cells isolated from normal donor mice in inducing iNOS in microglial cells and transferring EAE to recipient mice. Interestingly, clinical symptoms of EAE were much less in mice receiving NaPA through drinking water than those without NaPA. Similar to NaPA, sodium phenylbutyrate, a chemically synthesized precursor of NaPA, also inhibited the disease process of EAE. Histological and immunocytochemical analysis showed that NaPA inhibited EAE-induced spinal cord mononuclear cell invasion and normalized iNOS, nitrotyrosine, and p65 (the RelA subunit of NF-κB) expression within the spinal cord. Taken together, our results raise the possibility that NaPA or sodium phenylbutyrate taken through drinking water or milk may reduce the observed neuroinflammation and disease process in multiple sclerosis patients. The Journal of Immunology, 2003, 170: 3874–3882.

Materials and Methods
Reagents
FBS and DMEM/F-12 were from Invitrogen (Carlsbad, CA). Sodium phenylbutyrate (NaPB) and Abs against mouse macrophage iNOS were purchased from Calbiochem (La Jolla, CA). [α-32P]dCTP, and [γ-32P]ATP were purchased from NEN (Boston, MA).
Rabbit antinitritesyrosine, rabbit anti-NF-κB p65, and goat anti-gram fibrilary acidic protein (GFAP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat anti-mouse pan macrophage marker (moma-2) and rat anti-mouse CD11b were purchased from BioSource International (Camarillo, CA).

Preparation of NaPA

NaPA was prepared by titrating equivoluminal amounts of phenytoacetylene (Sigma-Aldrich, St. Louis, MO) and sodium hydroxide to pH 7.0.

Microglial cells

Mouse BV-2 microglial cells (kind gift from V. Bocchini of University of Perugia, Perugia, Italy) were maintained in DMEM/F-12 containing 10% FBS and stimulated with MBP-primed T cells under serum-free condition.

Isolation of MBP-primed T cells

Female SJL/J mice were immunized s.c. with 400 μg bovine MBP and 60 μg Mycobacterium tuberculosis (H37RA; Difco Labs, Detroit, MI) inIFA (Calbiochem). Lymph nodes were collected from these mice on day 10 postimmunization, and single cell suspension was prepared in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells cultured at a concentration of 4–5 × 10^6 cells/ml in six-well plates were incubated with 50 μg/ml MBP for 4 days. The nonadherent cells were collected and passed through the nylon wool column preincubated for a period of 30 min with RPMI 1640 supplemented with 10% FBS at 37°C, 5% CO2. The first 15–20 ml eluant was collected, centrifuged at 500 × g, and resupended in RPMI 1640-FBS. Viability of the cells was checked by trypan blue exclusion. By flow cytometry, using FITC-labeled anti-CD3 (BioSource International), >98% cells were found as CD3-positive T cells (9). These T cell populations were used to stimulate microglial cells.

T cell proliferation assay

T cells (2 × 10^5) cultured in 96-well U-bottom microtiter plates were stimulated with different concentrations of MBP in the presence or absence of NaPA. Unstimulated cells were kept as controls. After 72 h of stimulation, cells were pulsed with [3H]thymidine (0.5 μCi/well) for another 24 h. The level of [3H]thymidine incorporation in cells was assessed, as described earlier (9).

Assay for NO synthesis

Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen, using Griess reagent, as described earlier (8–12).

Immunoblot analysis for iNOS

Immunoblot analysis for iNOS protein was conducted, as described earlier, using Abs against mouse macrophage iNOS and 125I-labeled protein A (13, 14).

RNA isolation and Northern blot analysis

Cells were taken out of the culture dishes directly by adding Ultraspec-II RNA reagent (Biotexc Laboratories, Houston, TX), and total RNA was isolated, according to the manufacturer’s protocol. For Northern blot analysis, 20 μg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels, electrotransferred to Hybond nylon membrane, and hybridized with 32P-labeled iNOS cDNA probe, as described earlier (11–14).

Preparation of nuclear extracts from BV-2 microglial cells and EMSA

Nuclear extracts from BV-2 glial cells were prepared using the method of Dignam et al. (15), as described earlier by us (11–13). Nuclear extracts were used for EMSA utilizing the NF-κB DNA-binding protein detection system kit (Life Technologies), according to the manufacturer’s protocol.

Assay of transcriptional activities of NF-κB in BV-2 microglial cells

To assay the transcriptional activity of NF-κB, cells at 50–60% confluence in 12-well plates were transfected with 0.5 μg of pBIX-Luc, an NF-κB-dependent reporter construct, using the LipofectAMINE Plus method (Life Technologies) (16, 17). All transfections included 50 ng/μg total DNA of pRL-TK (a plasmid encoding Renilla luciferase, used as transfection efficiency control; Promega, Madison, WI). After 24 h of transfection, cells were treated with MBP-primed T cells (0.7:1 of T cell:glia) for 6 h. Firefly and Renilla luciferase activities were obtained by analyzing total cell extract according to standard instructions provided in the Dual Luciferase Kit (Promega) in a TD-20/20 Luminometer (Turner Designs, Palo Alto, CA).

Induction of EAE

Specific pathogen-free female SJL/J mice (3–4 wk old) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). EAE was elicited by passive transfer of MBP-reactive T cells. Donor mice were immunized s.c. with 400 μg bovine MBP and 60 μg M. tuberculosis in IFA. Animals were killed 11–12 days post immunization, and the draining lymph nodes were harvested. Single cell suspensions were treated with RBC lysis buffer (Sigma-Aldrich), washed, and cultured at a concentration of 4–5 × 10^6 cells/ml in six-well plates in RPMI 1640 supplemented with 10% FBS, 50 μg/ml MBP, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. On day 4, cells were harvested and resuspended in HBSS. A total of 2 × 10^4 viable cells in a volume of 200 μl was injected into the tail vein of naive mice. Pertussis toxin (150 μg/mouse; Sigma-Aldrich) was injected once via i.p. route on 0 day post transfer (dpt) of cells. Cells isolated from donor mice immunized with CFA or IFA alone were not viable after 4 days in culture with MBP, and therefore were not transferred. Animal maintenance and experimental protocols were approved by the University of Nebraska at Lincoln Animal Care Committee. Animals were observed daily for clinical symptoms. Experimental animals were scored by a masked investigator, as follows: 0, no clinical disease; 0.5, piloerection; 1, tail weakness; 1.5, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 3.5, forelimb weakness; 4, forelimb paralysis; 5, moribund or death.

Treatment with NaPA and NaPB

Groups of mice were treated with NaPA through drinking water in a blinded fashion at doses ranging from 2.5 to 15 mg/ml from 0 dpt. Mice were also allowed to drink water containing NaPA (10 mg/ml) at various phases of the disease (before acute phase, before chronic phase, and during the late chronic phase). Controls received normal drinking water without NaPA. During NaPB treatment, mice received i.p. injection of 400 mg of NaPB/kg body weight/day from 0 dpt. Control mice received the injections of saline as the vehicle for NaPB. Statistical analysis was determined by the RS/1 multicomparison procedure using a one-way ANOVA and Dunnett’s test for multiple comparisons with a common control group. Differences between means were considered significant when p values were less than 0.05.

Histological and immunofluorescence microscopy

On 15 dpt (acute phase), six mice from each of the following groups (control, vehicle-treated EAE, and EAE mice receiving NaPA from 8 dpt) were anesthetized with a mixture of ketamine (66.6 mg/kg) and xylazine (6.66 mg/kg) by i.p. injection. Whole spinal cord was dissected out from each mouse following perfusion with PBS (pH 7.4), and then with 4% (w/v) paraformaldehyde solution in PBS. The tissues were further fixed for at least 2 h in the same fixative at room temperature, followed by three PBS washes (15 min each). Samples were incubated in PBS containing 0.05% Tween 20 (PBST), 10% sucrose for 3 h, and then 30% sucrose overnight at 4°C. The spinal cord tissues were then embedded in OCT (TissueTek, Elkhart, IN) at −80°C, and processed for conventional cryosectioning. Frozen longitudinal sections (8 μm) were treated with cold methanol (−20°C), followed by two rinses in PBS. Samples were blocked with 3% BSA in PBST for 2 h and incubated in PBST containing 1% BSA and one of the following Abs for single labeling (rabbit anti-iNOS (1:100), rabbit anti-nitritesyrosine (1:100), rabbit anti-NF-κB p65 (1:50)). For double labeling, tissue sections were incubated with rabbit anti-iNOS (1:300) along with any one of the following Abs (rat anti-mouse moma-2 (1:25), rat anti-mouse CD11b (1:30), and goat anti-GFAP (1:100). After three washes in PBST (15 min each), sections were further incubated with Cy2 and Cy5 (Jackson Immunoresearch Laboratories, West Grove, PA). For negative controls, a set of sections was incubated under similar conditions without the primary Abs. The samples were mounted and observed under a Bio-Rad (Hercules, CA) MRC1024ES confocal laser-scanning microscope.

For histological analysis, routine histology was performed to obtain perivascular cuffing and morphological details of spinal cord tissues of EAE mice. Paraformaldehyde-fixed tissues were embedded in paraffin, and serial sections (4 μm) were cut. Sections were stained with conventional H&E staining method. Digital images were collected under bright field setting using a ×40 objective.
Results

MBP-primed T cells induce the expression of iNOS in BV-2 microglial cells: inhibition by NaPA

Because we are using MBP-primed T cells for the adoptive transfer of EAE in female SJL/J mice, at first we examined the effect of NaPA on the function of MBP-primed T cells. T cells isolated from lymph nodes of MBP-primed mice proliferated in response to MBP, and the maximum proliferation was observed at 50 or 100 μg/ml of MBP (Fig. 1A). However, pretreatment of MBP-primed T cells with NaPA dose dependently inhibited the proliferation in response to MBP (Fig. 1B). Recently, we have found that MBP-primed T cells induce the production of NO and the expression of iNOS in mouse BV-2 microglial cells and primary microglia through cell-cell contact (9). To examine the effect of NaPA on contact-mediated expression of iNOS in microglial cells by MBP-primed T cells, either NaPA-treated MBP-primed T cells were added to BV-2 microglial cells or MBP-primed T cells were added to NaPA-treated BV-2 microglial cells. It is evident from Fig. 2A that MBP-primed T cells markedly induced the production of NO in BV-2 microglial cells. Because the induction of NO production was maximum at the ratio of 0.7:1 of MBP-primed T cells and microglia (Fig. 2A), we used the same concentration of T cells for further studies. However, the NO production was reduced when microglial cells were cocultured with NaPA-treated MBP-primed T cells (Fig. 2B). About 98% inhibition of NO production was observed when MBP-primed T cells were treated with 5 mM NaPA (Fig. 2B). To understand the mechanism of NaPA-mediated inhibition of NO production, we analyzed the level of iNOS protein and mRNA. Western blot analysis for iNOS protein and Northern blot analysis for iNOS mRNA clearly showed that MBP-primed T cells alone induced the expression of iNOS protein (Fig. 3A) and iNOS mRNA (Fig. 3B) in microglial cells, and that the expression of iNOS was reduced when microglial cells were cocultured with NaPA-treated MBP-primed T cells. Similarly, addition of MBP-primed T cells to NaPA-treated BV-2 microglial cells also resulted in the inhibition of NO production (Fig. 2C). However, NaPA alone had no effect on the production of NO in BV-2 microglial cells (data not shown). In addition, we also show that MBP-primed T cells isolated from donor mice receiving NaPA-containing drinking water were also less efficient than that isolated from normal donor mice to induce the production of NO (Fig. 4) in BV-2 microglial cells.
MBP-primed T cells induce the activation of NF-κB in BV-2 microglial cells: inhibition by NaPA

Because the activation of NF-κB plays an important role in the expression of iNOS (11–14, 18), we examined the possibility as to whether NaPA inhibits the ability of MBP-primed T cells to induce the expression of iNOS in microglial cells by inhibiting the ability of the former cell type to induce the activation of NF-κB in the latter one. Activation of NF-κB was monitored by both DNA binding and transcriptional activity of NF-κB. Treatment of BV-2 glial cells with MBP-primed T cells resulted in the induction of DNA-binding activity of NF-κB (Fig. 5A). This gel shift assay detected a specific band in response to MBP-primed T cells that was competed off by an unlabeled probe. Consistent to the effect of NaPA on the induction of NO production, the DNA-binding activity of NF-κB in BV-2 glial cells was also inhibited when they were cocultured with NaPA-treated MBP-primed T cells (Fig. 5B). We then analyzed NF-κB-dependent transcription of luciferase in MBP-primed T cell-stimulated BV-2 glial cells. Consistent to the effect of NaPA on DNA-binding activity of NF-κB, the transcriptional activity of NF-κB was also reduced when they were cocultured with NaPA-treated MBP-primed T cells (Fig. 5C).

NaPA treatment inhibits the encephalitogenicity of MBP-primed T cells

Because NaPA treatment markedly inhibited the ability of MBP-primed T cells to induce the expression of iNOS and the activation of NF-κB in microglial cells, we examined whether NaPA-treated MBP-primed T cells can induce the disease process of EAE, a chronic relapsing model for MS, in female SJL/J mice. By adoptive transfer, MBP-primed T cells were capable of inducing the clinical symptoms of EAE (Fig. 6), displaying an acute phase of clinical signs peaking at 15 dpt and subsequently, a pattern of relapsing-remitting signs in the chronic phase. In contrast, NaPA-treated MBP-primed T cells were unable to induce the disease. Only piloerection was observed as the highest clinical symptom in few of the animals receiving NaPA-treated MBP-primed T cells in comparison with complete hind limb paralysis and/or partial front limb paralysis at the acute phase of the disease in most of the mice receiving MBP-primed T cells (Fig. 6).

NaPA treatment of donor mice inhibits the generation of encephalitogenic T cells

Because NaPA treatment of MBP-primed T cells in vitro blocked its ability to induce EAE in female SJL/J mice, we examined whether NaPA treatment of donor mice inhibits the generation of encephalitogenic T cells. SJL/J mice were immunized with MBP/IFA/M. tuberculosis, as described above, and from day 0 of immunization, donor mice received drinking water containing 10 mg/ml of NaPA. On day 10, the draining lymph nodes were removed and lymph node cells were activated in vitro with 50 μg/ml of MBP for 4 days. A total of 2 × 10^7 viable cells was adoptively transferred into naive SJL/J recipients, and on 0 dpt of cells, 150 ng of pertussis toxin was injected once via i.p. route. In contrast to the MBP-primed T cells isolated from mice drinking normal water, MBP-primed T cells isolated from mice drinking NaPA-containing water were much less efficient in transferring the disease to recipient mice, as judged by a reduction in disease severity (p < 0.001 on 16 dpt) (Fig. 6).
NaPA inhibits clinical symptoms and disease severity in adoptively transferred model of EAE

Next, we examined the ability of NaPA to prevent clinical manifestations of adoptively transferred EAE. By adoptive transfer, we have achieved 100% incidence of EAE in female SJL/J mice (Table I). Because NaPA is water soluble, we investigated the possibility as to whether animals can be treated with this drug through drinking water. First, we decided to select a proper dose of NaPA. During dose selection, only five mice were taken in each group. Mice received drinking water containing different concentrations of NaPA (2.5, 5, 10, and 15 mg/ml) from day 0 of transfer of MBP-primed T cells. Clinical symptoms were monitored everyday until 25 dpt. We found that NaPA was not very effective in inhibiting the clinical symptoms at a dose of 2.5 mg/ml (Fig. 7). However, it inhibited the clinical symptoms at a dose of 5 mg/ml, and the maximum inhibition was observed at the dose of 10 or 15 mg/ml (Fig. 7). To understand whether the inhibition of clinical symptoms by NaPA is specific, we examined the effect of sodium acetate, an inactive analog of NaPA, on the disease process of EAE. In contrast to the inhibitory effect of NaPA on the disease process of EAE, sodium acetate at a dose of 10 mg/ml did not inhibit the clinical symptoms of EAE (Fig. 7). Therefore, mice received drinking water containing 10 mg/ml of NaPA in additional experiments including at least six or more animals in each group. The results summarized in Table I demonstrate that NaPA at the dose of 10 mg/ml could reduce both the incidence and the clinical signs of EAE dramatically in comparison with mice receiving drinking water without NaPA. Because this adoptive transfer model of EAE exhibited acute, remission, and chronic phases, we studied the effect of NaPA on the disease process of EAE until 61 dpt. It is evident from Fig. 8 that NaPA markedly inhibited the clinical symptoms in acute (p < 0.001) as well as chronic (p < 0.001) phases of EAE. Considering an event as a relapse when it showed an increase of at least one full point of the clinical score after 22 dpt, NaPA-treated EAE group had no relapses compared with three relapses in the control EAE group (Fig. 8).

NaPB also inhibits clinical symptoms and disease severity in adoptively transferred model of EAE

NaPB, usually known as tributyrate in the pharmacy, is a synthetic precursor of NaPA. Under in vivo situation, NaPB undergoes one cycle of \(-\)oxidation to be converted into NaPA. Similar to NaPA, NaPB is also a prescribed drug for urea cycle disorders in children (19). Therefore, we investigated whether, similar to NaPA, NaPB is also capable of inhibiting the clinical symptoms of EAE. Because NaPB is costlier than NaPA, we treated mice through i.p. injection. Mice received 400 mg/kg body weight/day of NaPB from day 0 of transfer. Because NaPB was solubilized in sterile saline, control EAE animals also received sterile saline as the vehicle. It is evident from Fig. 8 and Table I that NaPB markedly reduced the incidence of EAE and inhibited the clinical symptoms in acute as well as chronic phases of EAE.

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Table I. Effect of NaPA and NaPB on clinical symptoms of EAE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Mean Peak Clinical Score</th>
<th>Suppression of EAE (%)</th>
</tr>
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<tbody>
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<td>Vehicle</td>
<td>9/9</td>
<td>3.3</td>
<td>Incidence Score</td>
</tr>
<tr>
<td>NaPA (drinking; 10 mg/ml) from 0 dpt</td>
<td>0/9</td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>NaPB (i.p. injection; 400 mg/kg/day) from 0 dpt</td>
<td>0/6</td>
<td>0.9</td>
<td>100</td>
</tr>
<tr>
<td>NaPA-treated MBP-primed T cells</td>
<td>0/6</td>
<td>0.25</td>
<td>100</td>
</tr>
</tbody>
</table>

EAE was induced in female SJL/J mice through adoptive transfer of MBP-primed T cells. Two groups of mice receiving MBP-primed T cells were treated with NaPA and NaPB, respectively, from 0 dpt. A clinical score of 2 was considered as the incidence of EAE in mice. Differences are significant (p < 0.001).
NaPA inhibits the disease progression in adoptively transfurred model of EAE

Next, we investigated whether NaPA could be used to prevent EAE progression. To achieve this goal, mice were treated with NaPA in three different groups. In the first group, mice were allowed to drink water containing 10 mg/ml of NaPA from the onset of acute phase (8 dpt). The results clearly show that the inhibitory effect of NaPA on the clinical symptoms started within 4 days of treatment (from 12 dpt), and the inhibition was significant \((p < 0.05)\) from 13 dpt onward until the duration of the observation (Fig. 9A). In the second group, mice received NaPA-containing drinking water from the onset of early chronic phase (22 dpt). Although NaPA did not inhibit the following relapse peaking at 25 dpt, the inhibitory effect of NaPA on clinical symptoms was apparent from 26 dpt (Fig. 9B). This inhibition was significant \((p < 0.05)\) from 28 dpt onward until the duration of the observation (Fig. 9B). In the third group, NaPA treatment began in the late chronic phase (45 dpt) and was continued until 75 dpt. Fig. 9C clearly shows that NaPA, in this instance, also halted disease progression. However, in contrast to the first or second instance, the inhibitory effect of NaPA started after 8 days of treatment (from 53 dpt), and the inhibition was significant \((p < 0.05)\) from 57 dpt onward. The EAE disease severity was always below or around stage 0.5 from 60 dpt until the duration of the study (75 dpt) (Fig. 9C). These results clearly suggest that drinking water containing NaPA can control the ongoing relapsing-remitting EAE whether given either early (at the onset of acute disease) or later when mice were in the chronic phase following the acute and several relapse incidents.

Is the effect of NaPA on the clinical symptoms of EAE reversible?

To test the reversibility of the NaPA block, mice were allowed to drink water containing 10 mg/ml of NaPA from 8 to 52 dpt and maintained for the next 34 days with normal drinking water without NaPA (Fig. 9D). Even after withdrawal of NaPA, clinical symptoms of EAE were much lower (maximum 0.5) for ~15 days in that group compared with that in the control EAE group that did not receive any NaPA. However, clinical symptoms started increasing after 67 dpt. Although there was an increase in clinical symptoms after withdrawal of NaPA, this group of mice always maintained the EAE disease severity lower than that of the control EAE group \((p < 0.05)\) for the duration of the observation (Fig. 9D). These observations suggest that the inhibitory effect of NaPA on the clinical symptoms of EAE is partly reversible.

NaPA inhibits the infiltration of inflammatory cells into the spinal cord of EAE mice

H&E staining of longitudinal sections of spinal cord was conducted to determine whether the diminished clinical disease in NaPA-treated mice correlated with reduced CNS infiltration of blood mononuclear cells. Blood vessels of the spinal cord of mock
control mice were completely devoid of inflammatory infiltrates (data not shown), while remarkable perivascular cuffing of mononuclear cells was observed in the spinal cord of EAE mice at the peak (15 dpt) of the disease (Fig. 10A). In contrast, very few immune cell infiltrates were found in the spinal cord of mice at 15 dpt receiving NaPA-containing drinking water from the onset (8 dpt) of the acute disease (Fig. 10B). Similarly, spinal cord of EAE mice at the late chronic phase (52 dpt) also contained infiltrated mononuclear cells (Fig. 10C). However, that infiltration was absent in the spinal cord of EAE mice at 52 dpt receiving NaPA-containing drinking water from the onset (22 dpt) of the chronic phase (Fig. 10D).

**Effect of NaPA on the level of inflammatory molecules (iNOS, nitrotyrosine, and p65) in the spinal cord of EAE mice**

Because iNOS and its surrogate marker nitrotyrosine participate in the pathophysiology of EAE and MS (3, 5, 20), we investigated the effect of NaPA on the in vivo expression of iNOS and nitrotyrosine in the spinal cord of EAE mice. On 15 dpt, EAE animals were sacrificed, and longitudinal sections of spinal cord were double immunolabeled with iNOS and either moma-2 (A, iNOS; B, moma-2; C, both) or CD11b (D, iNOS; E, CD11b; F, both) or GFAP (G, iNOS; H, GFAP; I, both) and observed under a confocal laser-scanning microscope. Scale bar = 10 μm.

In the spinal cord of EAE mice by immunofluorescence microscopy. The level of iNOS was almost undetectable in spinal cords isolated from control mice (data not shown). However, marked increase in expression of iNOS was found in areas surrounding the infiltration zone within the spinal cord of EAE mice at the peak (15 dpt) of the disease (Fig. 11). Double-immunolabeling experiment showed that iNOS was localized mainly to moma-2-positive infiltrated macrophages, CD11b-positive microglia, and GFAP-positive astrocytes (Fig. 11). Consistently remarkable expression of nitrotyrosine was observed in the spinal cord of EAE mice at the peak (15 dpt) of the disease (Fig. 12). However, the expression of nitrotyrosine was reduced markedly in spinal cords of EAE mice receiving NaPA-containing drinking water from the onset (8 dpt) of the acute phase (Figs. 11 and 12). Similarly, the expression of other proinflammatory markers such as TNF-α and calcium-activated neutral protease (calpain) also increased in the spinal cord of EAE mice compared with that of control mice. However, NaPA treatment inhibited the expression of these molecules in vivo in the spinal cord of EAE mice (data not shown). These results suggest that clinical treatment of NaPA is capable of inhibiting the level of proinflammatory molecules in the spinal cord of EAE mice.

Consistent to the necessity of activation of NF-κB in the expression of proinflammatory molecules (8, 11–14, 18) and to earlier observations by us (6) and others (21) that NF-κB is activated in the CNS of EAE animals, in this study we have demonstrated that the expression of p65 (the RelA subunit of NF-κB) is induced/stimulated markedly in the spinal cord of adoptively transferred EAE mice (Fig. 12) compared with that of control mice without EAE (data not shown). Next, we examined the level of p65 in the spinal cord of NaPA-treated EAE mice. Consistent to the inhibitory effect of NaPA on in vivo expression of iNOS, this drug also inhibited the expression of p65 in vivo in the spinal cord of EAE mice (Fig. 12).
Discussion
The relapsing and remitting model of EAE is particularly useful in testing new therapeutic intervention in MS. Several lines of evidence presented in this work clearly establish that NaPA, a fairly nontoxic Food and Drug Administration-approved drug for humans, inhibits the disease process of EAE at multiple steps in an adoptively transferred model in SJL/J mice. Our conclusion is based on the following. First, the proliferation of MBP-primed T cells in response to MBP was inhibited by NaPA. Second, MBP-primed T cells induced the expression of iNOS and the activation of NF-κB in microglial cells. However, pretreatment of these T cells with NaPA in vitro or in vivo inhibited their ability to induce the expression of iNOS and the activation of NF-κB in microglial cells. Third, MBP-primed T cells also cannot induce the production of NO in NaPA-treated microglial cells. Fourth, adoptive transfer of MBP-primed T cells, but not that of NaPA-treated MBP-primed T cells, induced the clinical symptoms of EAE in female SJL/J mice. In addition, NaPA treatment of donor mice also inhibited the generation of encephalitogenic MBP-primed T cells. Fifth, adoptive transfer of MBP-primed T cells was unable to induce the clinical symptoms of EAE in mice having drinking water containing NaPA. The disease progression of EAE was also inhibited in mice drinking NaPA-containing water. Finally, clinical treatment of EAE animals with drinking water containing NaPA was also capable of inhibiting the invasion of mononuclear cells into the spinal cord as well as the expression of inflammatory markers (iNOS, nitrotyrosine, and NF-κB p65) within the spinal cord.

Infiltration of leukocytes into the CNS, followed by activation of resident glial cells and concomitant production of wide varieties of inflammatory molecules are key neuroinflammatory events in EAE as well as MS. In EAE, chronological studies have demonstrated that myelin Ag-specific T cells home to the CNS early in the immune response, probably aided by chemotactic gradients provided by adhesion molecules and/or chemokines, and localize to the perivascular space. The entry of T cells into the parenchyma of the CNS places them in close proximity to microglia, a source of many inflammatory molecules, including NO. Recently, we have found that MBP-primed T cells induce the expression of iNOS and the activation of NF-κB in microglial cells through cell-cell contact (9). Interestingly, after NaPA treatment, MBP-primed T cells were unable to proliferate in response to MBP and to induce the expression of iNOS and the activation of NF-κB in microglial cells. Therefore, the inhibitory effect of NaPA on the disease process of EAE when mice received NaPA through drinking water from 0 dpt could be explained by its ability to deactivate MBP-primed T cells.

Interestingly, NaPA was also efficient in blocking the disease progression. Once neuroantigen-specific T lymphocytes enter into the CNS and initiate the inflammatory disease process, a large number of blood mononuclear cells, including nonantigen-specific T cells, are recruited to the BBB, which ultimately traverses through the leaky BBB into the CNS parenchyma to exacerbate the disease process. Interestingly, NaPA effectively blocks the observed invasion of mononuclear cells into the spinal cord. Leukocyte entry into the CNS began by an adhesion step at the BBB level. Although the mechanism of leukocyte invasion is not completely understood, this process most likely involves endothelial VCAM-1 expression (22), which is temporally correlated with the onset of clinical signs. Again, NO, a major mediator in immune and autoimmune functions, has been shown to increase permeability of the BBB, allowing substances to enter into the brain passively, leading to vasogenic edema and secondary brain damage (23). Although the precise molecular mechanisms for NO-induced breakdown of the BBB are not completely understood, in a cell culture model of the BBB, NO leads to a rapid breakdown in model barrier integrity and results in a reduction in endothelial cell ATP content and GAPDH activity (24). Mechanistically, if we consider the bigger scenario, expression of all these inflammatory adhesion molecules such as iNOS, ICAM-1, and VCAM-1 depends on the activation of NF-κB (25). Therefore, these inflammatory molecules being induced by activated NF-κB may guide inflammatory leukocytes into and through the CNS, thus contributing to their multiplication and finally to BBB disruption and broad-spectrum neuroinflammation. At present, we do not know whether NaPA crosses the BBB. However, we have found that NaPA effectively suppresses the activation of NF-κB in vitro in glial cells and in vivo in the spinal cord of EAE mice. Taken together, these results suggest that NaPA inhibits the established relapsing and remitting model of EAE probably by inhibiting the activation of NF-κB and thereby inhibiting the leukocyte invasion.

Apart from a role of NO in perivascular cutting, this molecule also has been implicated directly in oligodendrocyte loss and demyelination. Semi quantitative RT-PCR for iNOS mRNA in MS brains shows markedly higher expression of iNOS mRNA in MS brains than in normal brains (26). The reaction of NO with $O_2^{-}$ forms peroxynitrite, ONOO$^{-}$, a strong nitrosating agent capable of nitrosating tyrosine residues of a protein to nitrotyrosine. Consistently increased levels of nitrotyrosine have been found in demyelinating lesions of MS brains as well as in spinal cords of mice with EAE (20). Both NO and peroxynitrite may damage myelin-producing oligodendrocytes in demyelinating disorders such as MS (27) probably through the formation of iron-NO complexes of iron-containing enzyme systems, oxidation of protein sulfhydryl groups, nitration of proteins, nitrosylation of nucleic acids, increase in Ca$^{2+}$ overload, and DNA strand breaks (28). Consistently, intraventricular administration of 3-morpholinosydnonimine, a peroxynitrite donor, induces strong primary axonal damage with characteristics of primary acute axonopathy, together with severe myelin alteration, demyelination, and nitrotyrosine formation, as found in MS lesions (29).

Although all these observations suggested that NO produced from iNOS is involved in the pathogenesis of EAE and MS, the increase in clinical symptoms of EAE and mortality rate in the iNOS$^{-/-}$ mice compared with that of wild-type mice (30) suggests that the expression of iNOS may be beneficial for EAE. However, selective inhibition of iNOS in the CNS by intraventricular administration of antisense oligonucleotides blocks the disease process of EAE in mice (31). Therefore, at present, there are two distinct pictures regarding the involvement of iNOS in the disease process of EAE. First, chemical inhibitors or antisense oligonucleotides at the doses applied in vivo do not inhibit iNOS completely; therefore, partial inhibition of iNOS by chemical inhibitors of iNOS or antisense oligonucleotides against iNOS protects animals against EAE. Second, complete inhibition of iNOS in iNOS$^{-/-}$ mice worsens the clinical symptoms of EAE. These speculations suggest that NO produced up to the certain level from the activation of iNOS is beneficial for EAE. It is to be noted that NO inhibits the activation of T cells and thereby acts as an immunosuppressant (32). However, once iNOS is activated, it produces excessive amounts of NO for extended period of time (10, 17), and NO produced in excess is cytotoxic (5, 27, 29). Therefore, partial knockdown of iNOS by pharmacological compounds to inhibit the excessive production of NO, but not the complete knockout of the iNOS gene, is the feasible therapeutic approach for EAE and hence for MS. It is particularly useful to mention in this work that clinical application of NaPA is capable of attenuating the expression of iNOS and nitrotyrosine in vivo in the spinal cord of EAE mice.
Although there is no effective therapy against MS, different forms of IFN-β have been currently used to treat this disease. However, NaPA has several advantages over IFN-β. First, IFN-β has a number of side effects, including flulike symptoms, menstrual disturbances in women, decrease in neutrophil count and white blood cell count, increase in aspartate aminotransferase and alanine aminotransferase levels, and development of neutralizing Abs to IFN-β (33). However, NaPA is fairly nontoxic. It is used as a drug in urea cycle disorders in children. It is excreted through the urine, if excess. Second, MS patients are treated with IFN-β through painful injections that often lead to injection site reactions such as skin necrosis. However, NaPA can be taken through drinking water or milk, the least painful route. Third, IFN-β is costly, while NaPA is very cheap. Fourth, we have found that IFN-β alone can activate microglia to induce the production of NO and the activation of NF-κB (9), suggesting that apart from inhibiting the functions of activated T cells if IFN-β itself can enter into the CNS or if it is produced within the CNS by infiltrating macrophages, it may augment the inflammatory response. Consistent to our observation, it has been found that IFN-β is beneficial in relapsing/remitting MS, but not in acute MS attacks (33, 34), when the integrity of the BBB is questionable and the CNS is probably loaded with infiltrating macrophages. In contrast, NaPA alone cannot activate microglia; rather, it inhibits the activation of microglia.

In summary, we have demonstrated that NaPA, a commonly used drug for urea cycle disorders, inhibits neuroantigen-primed T cell-induced expression of iNOS and activation of NF-κB in vitro in glial cells and in vivo in the CNS of EAE mice and blocks the disease process of EAE, suggesting that this drug may be used for therapeutic intervention in MS.

Acknowledgments

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References