

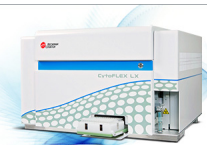


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J Immunol 2007; 179:7415-7423; ;
doi: 10.4049/jimmunol.179.11.7415
<http://www.jimmunol.org/content/179/11/7415>

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Therapeutic Effect of Anthracene-Based Anticancer Agent Ethonafide in an Animal Model of Multiple Sclerosis¹

Wen-Hua Piao,* Rudy Wong,* Xue-Feng Bai,[†] Jianhua Huang,[¶] Denise I. Campagnolo,* Robert T. Dorr,^{‡§} Timothy L. Vollmer,* and Fu-Dong Shi^{2*}

The side effects of cancer chemotherapeutic agents such as mitoxantrone (MIT) in multiple sclerosis (MS) patients justify the search for less toxic drugs. Ethonafide is an anthracene-based antineoplastic drug similar to MIT. With reference to MIT, we examined the effect of ethonafide on experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice, an animal model of human MS. We demonstrated that ethonafide is effective in preventing development of EAE as well as in ameliorating the severity of EAE when disease is ongoing. In relatively higher dosages, the effects of ethonafide and MIT on EAE were identical, whereas in lower dosages, MIT seemed more effective. Therapeutic effects of ethonafide were associated with the initial reduction in cellular counts of CD3⁺, CD4⁺, CD8⁺, B220⁺, CD11b⁺, NK cells, and NKT cells, followed by recovery of these cells from the bone marrow. Interestingly, the recovered autoreactive T cells in ethonafide-treated animals have reduced capacity to expand and produce cytokines in response to myelin Ag stimulation. Furthermore, CD4⁺CD25⁺ regulatory T cells were relatively resistant to depletion and/or recovered faster than T effector cells. The ability of regulatory T cells to resist depletion and replenish quickly during cell ablation therapy may provide an opportunity to reprogram the immune system. Moreover, we provided evidences that ethonafide has less cardiac toxicity compared with MIT. The effectiveness and the low cardiotoxicity of ethonafide might make it a promising immunosuppressive agent for clinical use in treating MS patients. *The Journal of Immunology*, 2007, 179: 7415–7423.

Multiple sclerosis (MS)³ is an important cause of progressive neurological disability, typically commencing in early adulthood (1). There is a need for safe and effective therapy to prevent progressive CNS damage and resultant disability that characterize the disease course. Mitoxantrone (MIT), now approved for the treatment of acute leukemia and several forms of MS in the United States and Europe (2, 3), is an anthracene-based anticancer agent whose efficacy in treating MS is believed to be due to inhibition of proliferation of T cells, B cells, and macrophages (4–7). However, MIT is only moderately effective in reducing the disease progression and the frequency of relapses in patients affected by relapsing remitting, primary progressive and secondary progressive MS in the short-term follow-up (3 years) (8). Thus, longer follow-

up studies are highly warranted to better explore the efficacy and safety of the drug, mainly with regard to the long-term risk of therapy-related leukemias and cardiotoxicity (9, 10). Due to its partial efficacy and unclear long-term safety profile, MIT is only used to treat patients with worsening relapsing-remitting and secondary progressive MS with evidence of worsening disability (11, 12). The dose-limiting toxicities of MIT in humans are acute myelosuppression and chronic cardiotoxicity (9). The latter condition is a dose-limiting side effect depending on the total cumulative dose (10, 13, 14). Therefore, there is a need to develop effective, less toxic drugs with particular reference to cardiotoxicity.

Azonafides are a chemically related class of anthracene-based anticancer agents. This group includes >160 analogs that have structural similarities to both anthracenes and the naphthylene-based agent amonafide (15, 16). Design features in the azonafides sought to prevent variable acetylation metabolism, which occurs in amonafides by avoiding primary amine side groups, and reduce cardiotoxicity by removing hydroquinones on the anthracene rings that are present in MIT. Ethonafide is the 6-ethoxy analog on the azonafide series, exhibiting high potency against solid tumor cell lines in vitro and in vivo (17). Ethonafide also inhibits topoisomerase II (18). Thus, ethonafide shares similar chemical structures and molecular targets with MIT. Nevertheless, the primary effect of ethonafide on MS has yet to be investigated.

In this study, we investigated the clinical and immunological effects of ethonafide in experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice, an animal model of human MS. We have determined the effective dosage of ethonafide for prevention as well as treatment of EAE. Additionally, we have explored several immunological parameters that may account for therapeutic effects of this compound. Our results showed that ethonafide is effective in prevention and treatment of EAE in this model. Further

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Received for publication April 18, 2007. Accepted for publication September 11, 2007.

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¹ This work was supported in part by AmpliMed Corporation. F.-D. Shi's Laboratory is supported by grants from the National Multiple Sclerosis Society, Muscular Dystrophy Association, and Barrow Neurological Foundation.

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³ Abbreviations used in this paper: MS, multiple sclerosis; MIT, mitoxantrone; EAE, experimental autoimmune encephalomyelitis; Foxp3, Forkhead box p3; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; p.i., postinjection; Treg, regulatory T.

investigation into the immunological mechanisms and side effects may reveal that this compound is a potential therapy for MS.

Materials and Methods

Mice

C57BL/6 (B6, H-2^b) mice were purchased from Taconic Farms. Mice were housed in animal facilities of the Barrow Neurological Institute (Phoenix, AZ). Female mice, 7–8 wk of age at the initiation of the experiments, were used. Experiments were conducted in accordance with institutional guidelines.

Antigens

The murine myelin oligodendrocyte glycoprotein (MOG)_{35–55} peptide (M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K) and proteolipid protein (PLP)_{139–151} peptide (H-S-L-G-K-W-L-G-H-P-D-K-F) were synthesized (Biosynth International).

Induction of acute EAE

To induce acute EAE, B6 mice were injected s.c. in the hind flank with 200 μ g of MOG_{35–55} peptide in CFA (Difco) containing 500 μ g of *Mycobacterium tuberculosis*, supplemented by a tail base i.v. injection of 200 ng of pertussis toxin (List Biological Laboratories) on days 0 and 2. The mice were observed daily for clinical signs of disease and scored on an arbitrary scale of 0–5, with gradations of 0.5 for intermediate scores (19): 0, no clinical signs; 1, flaccid tail; 2, hind limb weakness or abnormal gait; 3, complete hind limb paralysis; 4, complete hind limb paralysis with forelimb weakness or paralysis; and 5, moribund or deceased.

Ethonafide treatment

Ethonafide and MIT were obtained from AmpliMed. To detect the preventive efficacy of etthonafide, starting on the day of immunization for acute EAE, mice were treated with etthonafide at a dose of 2, 1, 0.5, 0.25, 0.125, and 0.05 mg/kg or PBS i.p. daily for a total of seven doses. To evaluate the treatment efficacy of etthonafide, on day 11 after inducing EAE, mice were treated with etthonafide at a dose of 1 and 0.5 mg/kg or PBS i.p. daily for a total of seven doses. These dosages were chosen based on the previous publications in cancer models (20–22), as well as the myelosuppressive activity (R. T. Dorr et al., unpublished data). MIT was used as a reference drug for effectiveness and toxicity. Control mice were injected with PBS.

Preparation of tissues and histological staining

Mice were anesthetized with pentobarbital and perfused by intracardiac puncture with 50 ml of cold PBS. Murine brains were removed, frozen immediately, and then stored at -80°C . For evaluation of inflammatory cell infiltrates, 10- to 15- μm serial cryosections were prepared. One section was stained with H&E and three other serial sections (15 μm) were stained for CD4, CD8, and CD11b Abs as follows. Briefly, cryosections of brain were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 3% BSA in PBS. The sections were incubated with rat anti-mouse CD4 (1/800; DakoCytomation), CD8 (1/800; DakoCytomation), and CD11b (1/800; Serotec) Ab for 1 h, washed with PBS, and then incubated with donkey anti-rat IgG secondary Ab conjugated to FITC (1/200; Fitzgerald). All images were captured with a fluorescent microscope.

Histological findings were graded into five categories: 0, no inflammation; 1, few infiltrating cells; 2, leptomeningeal infiltration; 3, mild perivascular cuffing; 4, extensive perivascular cuffing; and 5, extensive perivascular cuffing and severe parenchymal cell infiltration.

For heart tissue, mice were perfused by intracardiac puncture with 50 ml of 4% buffered formalin and paraffin embedded. Four-micrometer sections were used for H&E and Masson's trichrome staining with standard methods.

Echocardiographic assessment of heart function

We performed echocardiography using a 15-MHz high-frequency transducer (Sonos 5500 Agilent). All measurements were performed on trained, unsedated mice to avoid cardiodepressant effects of anesthesia. Measurements are averaged over three consecutive beats from the left ventricle (LV) end-diastolic dimension (EDD), LV end-systolic dimension (ESD), LV posterior wall (LVPW), the intraventricular septum (IVS), and the LV internal diameter (LVID). The LV volume was calculated using this equation: LV volume = $1.055[(\text{LVID} + \text{IVS} + \text{LVPW})^3 - (\text{LVID})^3]$. The LV shortening fraction (SF), a measure of LV systolic function, was calculated from the M-mode LV dimensions using the equation: SF (%) =

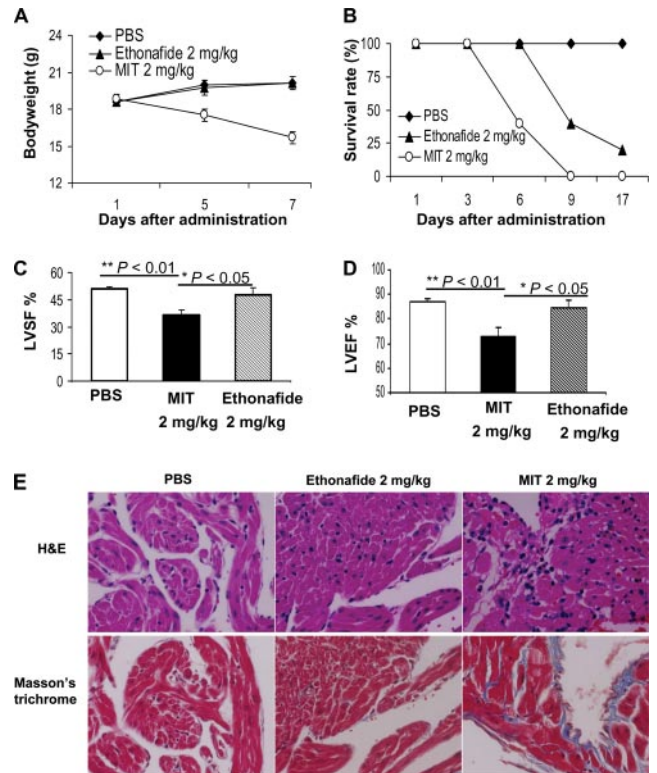


FIGURE 1. High-dose toxicity of etthonafide and MIT in B6 mice. EAE was induced in mice as described in *Materials and Methods*. Starting on the day of immunization for acute EAE, mice were treated with etthonafide (or MIT) at a dose of 2.0 mg/kg or PBS i.p. daily for a total of seven doses. The actual body weight of all mice was recorded until the mice died. Data represent mean \pm SD of total mice in each group. **A**, The plot of body weights among three different treatment groups. This is representative of two experiments ($n = 5$ for etthonafide or MIT group; $n = 10$ for PBS group, $p < 0.05$ compared with the PBS- and etthonafide-treated groups). **B**, The plot of survival rate after etthonafide and MIT treatment in EAE mice. This is representative of two experiments ($n = 5$ for etthonafide or MIT; $n = 10$ for PBS, $p < 0.05$ compared with PBS-treated group). **C** and **D**, Effects of etthonafide and MIT treatment on cardiac function as measured by 2-D M-mode echocardiography. All measurements were performed on trained, unsedated mice on day 6 after treatment. The LVSF and LVEF were calculated as in *Materials and Methods*. Results are the mean \pm SD ($n = 6$ for each group). All analyses were performed by a single examiner, who was blinded with respect to the experimental group to which each sample belonged. **, $p < 0.01$ compared with the PBS-treated group; *, $p < 0.05$ compared with etthonafide-treated group. **E**, Histological analysis of the hearts after etthonafide and MIT treatment. Mice were sacrificed on day 6 after treatment at the dose of 2 mg/kg and paraffin sections were prepared. The sections were stained with H&E (*top panel*, $\times 400$ original magnification) and Masson's trichrome methods (*bottom panel*, $\times 400$ original magnification). Data shown are representative of two experiments ($n = 3$).

$[(\text{LVEDD} - \text{LVESD})/\text{LVEDD}] \times 100$. The LV ejection fraction (EF) was calculated from the LV cross-sectional area (2-D short-axis view) using the equation: EF (%) = $[(\text{LVEDV} - \text{LVESV})/\text{LVEDV}] \times 100$, where LVEDV is LV diastolic volume and LVESV is LV systolic volume.

Cell preparation and T cell proliferation

Spleen mononuclear cells were suspended in culture medium containing DMEM (Invitrogen Life Technologies) supplemented with 1% (v/v) MEM (Invitrogen Life Technologies), 2 mM glutamine (Flow Laboratory), 50 IU/ml penicillin, 50 mg/ml streptomycin, and 10% (v/v) FCS (both from Invitrogen Life Technologies). Spleen mononuclear cells (4×10^5 cells) in 200 μl of culture medium were placed in 96-well round-bottom microtiter plates (Nunc). Ten microliters of MOG_{35–55}

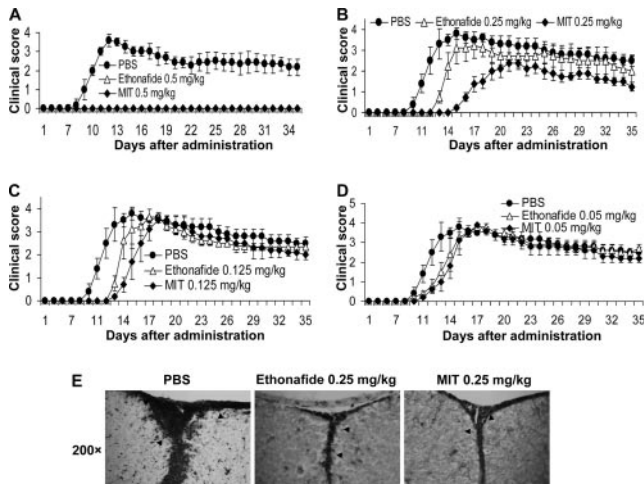


FIGURE 2. Ethonafide prevents EAE in B6 mice. EAE was induced in mice as described in *Materials and Methods*. The mice were observed daily for clinical signs of disease and scored. Starting on the day of immunization for acute EAE, mice were treated with ethonafide (or MIT) at five different doses of 1, 0.5, 0.25, 0.125, and 0.05 mg/kg or PBS i.p. daily for a total of seven doses. After immunization with MOG, the mice were observed until termination of the experiment. Means of clinical scores were calculated for the mice. Data represent mean \pm SD of total mice in each group. Statistical evaluation was performed to compare experimental groups and corresponding control groups. The experiment was repeated two times with similar results. The comparison of the mean clinical score is shown at different doses. A, 0.5-mg/kg dose (1-mg/kg dose group not shown because results are identical to those of the 0.5-mg/kg dose group). B, 0.25-mg/kg dose; C, 0.125-mg/kg dose; and D, 0.05-mg/kg dose. E, Ethonafide treatment leads to weak infiltration of inflammatory mononuclear cells in the CNS. On day 20 postimmunization, the mice were sacrificed and cryosections of brain tissue were prepared and stained with H&E. PBS-treated mice showed severe inflammation characterized by leptomeningeal region cell infiltration and extensive perivascular cuffing of the brain than those of the brain of ethonafide- or MIT-treated mice ($\times 200$ original magnification). Data shown are representative of two experiments ($n = 3$).

peptide (10 $\mu\text{g/ml}$), PLP_{139–151} peptide (10 $\mu\text{g/ml}$), or Con A (5 $\mu\text{g/ml}$; Sigma-Aldrich) were then added in triplicate into the wells. After 3 days of incubation, the cells were pulsed for 18 h with 10- μl aliquots containing 1 μCi of [*methyl*-³H]thymidine (sp. act., 42 Ci/mmol; Amersham Biosciences). Cells were harvested onto glass fiber filters, and the thymidine incorporation was then measured. The results are expressed as cpm.

Spleen mononuclear cell phenotypes

Spleen mononuclear cell suspensions were collected from PBS-, ethonafide-, and MIT-treated mice on days 7, 20, 32, and 44. Single-cell suspensions were prepared and stained with fluorescently labeled Abs to mouse Ags. Abs were directly labeled with one of the following fluorescent tags: FITC, PE, allophycocyanin, PE-Cy5, PE-Cy-7; CD25 (7D4), CD3 (145-2C11), CD4 (GK1.4), CD8 (53-6.7), NK1.1 (PK136), CD45RA (OX-33), and CD11b (OX-42). Appropriate isotype controls were always included. All samples were analyzed on a FACSaria using Diva (BD Biosciences).

Intracellular cytokine staining and FACS analysis

Single-cell suspensions were prepared and stained with fluorescent-labeled Abs to mouse Ags. Abs were directly labeled with one of the following fluorescent tags: FITC, PE, allophycocyanin, PE-Cy5, PE-Cy-7; CD25 (7D4), CD3 (145-2C11), CD4 (GK1.4), CD8 (53-6.7), IFN- γ (XMG1.2), IL-10 (JESS-16E3) (BD Biosciences), and Foxp3 (FJK-16s; eBioscience). Intracellular flow cytometry for cytokines and Foxp3 was performed using Cell Fix/Perm (BD Biosciences) according to the manufacturer’s instructions. Appropriate isotype controls were always included. All samples were analyzed on a FACSaria using Diva (BD Biosciences).

Statistical analysis

Differences between groups were evaluated by ANOVA. The Fisher exact test and Mann-Whitney *U* test analyzed disease incidence and severity, respectively.

Results

Effects of ethonafide on acute EAE and acute toxicity

To determine the effect of ethonafide on EAE in comparison with MIT, the B6 mice were treated with PBS, MIT, or ethonafide at high doses of 2, 1, and 0.5 mg/kg by i.p. for 7 days. EAE was induced in B6 mice and clinical scores were monitored subsequently. Since weight loss is a well-known side effect of most antineoplastic and immunosuppressant drugs in mice models, as well as an indicator of EAE course, body weight was recorded for all mice on days 1, 5, 7, 15, and 32 to make an estimate of the tolerability of the treatment. In ethonafide-treated mice, the weight loss was not significant compared with control EAE mice. In contrast, at the highest dose (2 mg/kg), as shown in Fig. 1A, the mean lost weight in MIT-treated mice was significantly higher than in PBS mice ($p < 0.05$). In terms of mortality, the mean time of death in the MIT-treated group was earlier than in the ethonafide-treated group during 15 days after immunization ($p < 0.05$). Ethonafide caused 80% death by the 15th day; MIT caused 100% death of mice by day 7 (Fig. 1B). However, no death occurred at this time point (at 15th day) in the PBS-treated group.

Table I. Clinical evaluation of EAE after ethonafide or MIT treatment^a

	% Incidence	% Mortality	Day of Onset	Maximum Clinical Score	Mean Clinical Score
PBS	90 (9/10)	0 (0/10)	11.4 \pm 0.83	4.0 \pm 0.83	3.15 \pm 0.92
Ethonafide (mg/kg)					
0.05	100 (5/5)	0 (0/5)	12.6 \pm 1.28	4.0 \pm 0.0	2.93 \pm 0.55
0.125	100 (5/5)	0 (0/5)	13.4 \pm 0.48**	3.7 \pm 0.24*	2.89 \pm 0.73
0.25	100 (5/5)	0 (0/5)	13.4 \pm 0.64**	3.3 \pm 0.44*	2.67 \pm 0.52*
MIT (mg/kg)					
0.05	100 (5/5)	0 (0/5)	13.2 \pm 0.96*	4.0 \pm 0.0	2.80 \pm 1.13
0.125	100 (5/5)	0 (0/5)	14.4 \pm 0.88**	3.6 \pm 0.16*	2.39 \pm 1.01*
0.25	60 (3/5)	0 (0/5)	16.0 \pm 0.67**	2.2 \pm 0.78**	1.47 \pm 0.56**

^a Mice were induced to develop EAE and treated with PBS, ethonafide, or MIT as described in *Materials and Methods*. The mice were observed until termination of the experiment after immunization with MOG, and the incidence, mortality, day of onset, and maximal disease grade were determined during the observation period. Means of maximum scores are calculated for the mice and had evidence of disease. Data represent mean \pm SD of total mice in each group. Statistical evaluation was performed to compare experimental groups and corresponding control groups, respectively (*, $p < 0.05$ vs PBS; **, $p < 0.01$ vs PBS). The experiment was repeated two times with similar results. The 0.5- and 1-mg/kg treatment groups did not develop EAE and therefore are not shown.

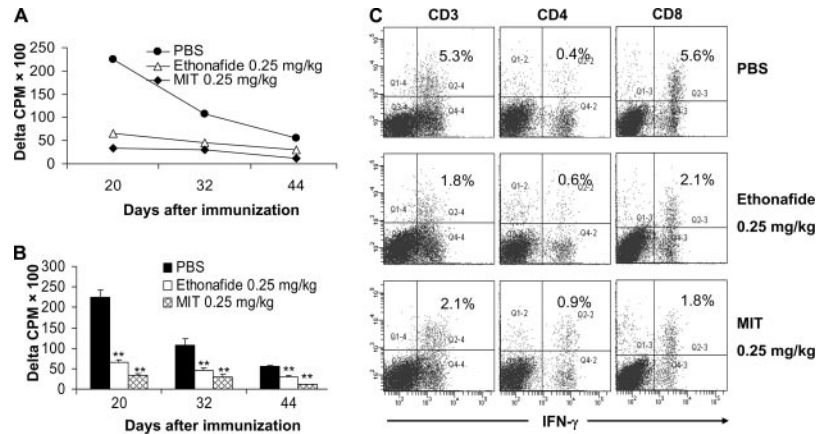


FIGURE 3. Effects of ethonafide on autoreactive T cell response in EAE mice. *A* and *B*, Proliferative responses of splenic T cells in ethonafide- and MIT-treated mice. On the day of immunization for acute EAE, mice were treated with ethonafide (or MIT) at dose of 0.25 mg/kg or PBS i.p daily for a total of seven doses. Mice were killed on days 20, 32, and 44 after immunization, and mononuclear cells were isolated from their spleens. Proliferative responses to the Ag (MOG₃₅₋₅₅ peptide) shown were assessed in triplicate wells for each experiment. Results are expressed as Δ cpm (mean cpm stimulated cultures – mean cpm unstimulated cultures). The ANOVA analysis showed a significant variation with time (**, $p < 0.001$) or treatments (PLP, MOG). *C*, Comparison of IFN- γ secretion stimulated by MOG in ethonafide- and MIT- treated mice (same treatment as in *A* and *B*). The dot plots generated after gating on lymphocytes (by forward vs side scatter) are shown. The *top row* is PBS, *middle row* is ethonafide, and *bottom row* is MIT; *left column* is CD3, *middle column* is CD4, and *right column* is CD8. Single-cell suspensions of spleen were prepared from mice treated with ethonafide and MIT or PBS on day 20 after immunization. The expression of CD3, CD4, and CD8 vs intracellular IFN- γ by splenocytes was compared with an appropriate isotype control within this gate.

At the lower dose (1 mg/kg), the mean lost weight was similar in three different treatment groups (data not shown), but the health of mice treated by MIT was worse than that treated by ethonafide. In the 0.5-mg/kg dose group, there was no difference in the three different treated groups (data not shown).

In an attempt to assess whether our study's dosages induce cardiotoxicity, we first performed echocardiography in EAE mice treated by PBS, ethonafide, and MIT. At the lower dose (1 mg/kg), the mean percentage of LVEF and LVSF in the MIT-treated group was slightly lower than in the PBS- and ethonafide-treated groups (data not shown). However, at the high dose (2 mg/kg), as shown in Fig. 1C, the mean percentage of LVSF in MIT-treated mice (36.5 ± 2.55) was significantly decreased than in the PBS- and ethonafide-treated mice (50.8 ± 1.30 and 47.5 ± 3.96 , respectively) on day 6 after treatment ($p < 0.01$ vs PBS control mice, $p < 0.05$ vs ethonafide-treated mice, respectively). In terms of LVEF, as shown in Fig. 1D, the mean percentage in the MIT-treated group (73.5 ± 3.48) was significantly decreased than in the PBS- and ethonafide-treated

groups (87.3 ± 0.83 and 84.7 ± 3.18 , respectively) on day 6 after treatment ($p < 0.01$ vs PBS control mice, $p < 0.05$ vs ethonafide-treated mice, respectively). We next performed histological analysis of heart tissue in EAE mice treated by ethonafide and MIT. No noticeable histopathological changes were observed in the hearts of ethonafide- and MIT-treated mice at doses of 1 and 0.5 mg/kg (data not shown). In contrast, at the high dose (2 mg/kg), in an H&E staining (Fig. 1E, *top panel*), obvious focal inflammatory cells were observed in MIT-treated mice. In a Masson's trichrome staining (Fig. 1E, *bottom panel*), obvious fibrosis was observed in MIT-treated groups.

To examine the efficacy of ethonafide to prevent the development of EAE, the mice were treated by ethonafide and MIT with five different doses (1, 0.5, 0.25, 0.125, and 0.05 mg/kg per mouse per injection) for seven consecutive days. A single s.c. immunization of C57BL/6 mice with MOG₃₅₋₅₅ peptide along with CFA and pertussis toxin induced moderate to severe encephalomyelitis (mean maximum clinical score, 4.0 ± 0.62 ; mean clinical score, 3.15 ± 0.92) in the majority of animals. The average date of disease

Table II. Effects of ethonafide and MIT on the T cell proliferative response in EAE mice^a

	Day 20	Day 32	Day 44
	Mean DCPM \pm SD	Mean DCPM \pm SD	Mean DCPM \pm SD
PBS			
PLP	1,550 \pm 129.5	1,420 \pm 72.2	1,018 \pm 34.0
MOG	22,478 \pm 1,685.3	10,793 \pm 1,574.7	5,568 \pm 208.2
Ethonafide			
PLP	1,323 \pm 111.9	1,435 \pm 335.3	926 \pm 277.7
MOG	6,492 \pm 747.8**	4,573 \pm 642.0**	2,964 \pm 479.1**
MIT			
PLP	1,549 \pm 195.0	1,354 \pm 359.3	829 \pm 24.9
MOG	3,414 \pm 277.7**	3,024 \pm 528.0**	1,096 \pm 96.2**

^a Single-cell suspensions were prepared as in *Materials and Methods* using three mice from each group on different days (days 20, 32, and 44). The data are reported as Δ cpm (DCPM) (mean cpm stimulated cultures – mean cpm unstimulated cultures). Statistical evaluation was performed to compare experimental groups and corresponding control groups (**, $p < 0.01$ vs PBS). The experiment was repeated two times with similar results.

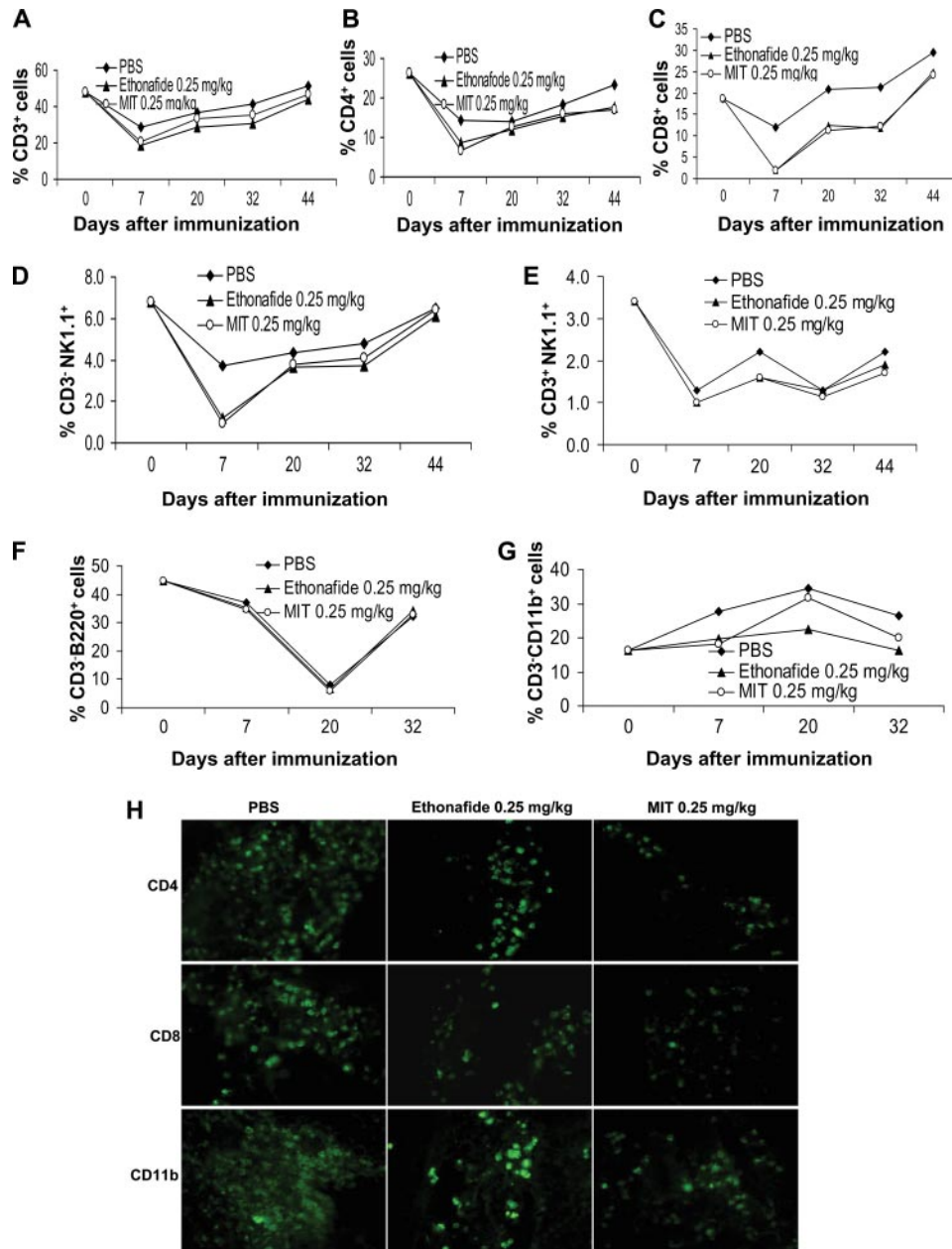


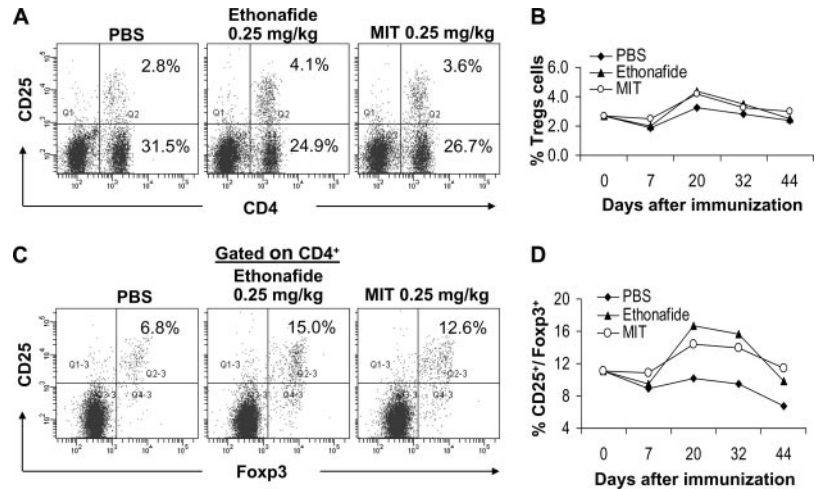
FIGURE 4. Phenotype assay of splenic T cells, B cells, and macrophages in ethonafide- and MIT-treated mice. On the day of immunization for acute EAE, mice were treated with ethonafide (or MIT) at a dose of 0.25 mg/kg or PBS i.p. daily for a total of seven doses. Mice were killed on days 7, 20, 32, and 44 after immunization, and mononuclear cells were isolated from their spleens. The single cells were stained with PE-Cy7-labeled CD4, FITC-labeled CD8, PE-Cy5-labeled CD3, PE-labeled NK1.1, PerCP-labeled B220, and PE-labeled CD11b. Samples were analyzed on a FACSARIA using Diva software. The dot plots generated after gating on lymphocytes (by forward vs side scatter) are shown for T cells and B cells (for macrophages, gated on lymphocytes and monocytes). The results of the average percentage values in CD3⁺, CD4⁺, CD8⁺ T cells, NK, NKT cells, B cells, and macrophage are shown at the different time points. *A*, CD3⁺ T cells; *B*, CD4⁺ T cells; *C*, CD8⁺ T cells; *D*, NK cells; *E*, NKT cells; *F*, CD3⁺ B220⁺ cells; and *G*, CD3⁺ CD11b⁺ cells. *H*, Ethonafide treatment reduces accumulation of inflammatory cells in the CNS during EAE. Representative fluorescent images of the brain sections were taken from PBS-, ethonafide-, and MIT-treated mice on day 20 after immunization and stained for CD4, CD8, and CD11b Ab. Sections from PBS-treated mice showed both perimeningeal/perivascular and parenchymal infiltrates. In contrast, sections from ethonafide- and MIT-treated mice lacked lymphocytes and macrophage/microglia infiltrates on day 20. Data shown are representative of two experiments ($n = 3$). The *top row* is CD4, *middle row* is CD8, and *bottom row* is CD11b; *left column* is PBS, *middle column* is ethonafide, and *right column* is MIT.

onset was 11.4 ± 0.83 . The disease was associated with rapidly progressing ascending paralysis appearing around days 11–15. Strikingly, none of the mice treated with 1 or 0.5 mg/kg ethonafide or MIT developed EAE (Fig. 2, *A–D*).

The effects of ethonafide and MIT in EAE are also shown in Table I. At the dose of 0.25 mg/kg, ethonafide- and MIT-treated groups developed EAE, with a mean maximum clinical score of 3.3 ± 0.44 and 2.2 ± 0.78 , respectively. The disease severity was

significantly less than that of PBS control mice and the mean day of disease onset was clearly later than that of the PBS control mice ($p < 0.01$); At the dose of 0.125 mg/kg, the ethonafide-treated group and MIT-treated group showed similar maximum clinical scores and delayed the onset of EAE ($p < 0.05$ and $p < 0.01$, respectively). At the lowest dose of 0.05 mg/kg, the ethonafide-treated group showed similar clinical scores and mean days of EAE onset to those of the PBS control group. However, in the

FIGURE 5. Ethonafide or MIT treatment increases expression of Foxp3. The cells were analyzed by flow cytometry for expression of Foxp3 from spleens isolated on days 7, 20, 32, and 44 after immunization of mice treated with ethonafide, MIT, or PBS. The average percentage of CD4⁺CD25⁺ cells was gated on lymphocytes from individual mice. The expression of Foxp3 in relation to CD25⁺ cells was gated on CD4⁺ cells. The dot plots are representative of two experiments ($n = 3$). **A**, A representative dot plot result on day 20 after immunization in CD4 and CD25. **B**, A plot of the average percentage in CD4⁺CD25⁺ cells at the different time points. **C**, The expression of Foxp3 by CD4⁺ cells in relation to CD25⁺ cells gated on CD4⁺ cells on day 20 after immunization. **D**, The plot of the average percentage in Foxp3⁺CD25⁺ cells gated on CD4⁺ cells at the different time points.



MIT-treated group, the median day of EAE onset was delayed compared with control mice ($p < 0.05$).

Histological analysis revealed that the inflammatory infiltrates in control mice were more extensive than those in the ethonafide- or MIT-treated mice (Fig. 2E). The ethonafide- and MIT-treated mice showed moderate inflammation with mean histological scores of 2.17 ± 0.44 and 1.74 ± 0.44 , respectively ($p < 0.05$ vs PBS control), compared with the PBS-treated control EAE mice (histological score, 3.50 ± 0.41). Therefore, ethonafide-treated mice showed significantly reduced EAE severity and less CNS inflammation. Collectively, our results indicated that ethonafide was effective in preventing the development of EAE. In relatively higher dosages, the effects of ethonafide and MIT on EAE were identical, whereas in lower dosages, MIT seemed more effective. The 0.25-mg/kg dose was chosen for subsequent studies because both ethonafide and MIT demonstrated clinical benefit at this dosage and, yet, no significant mortality was observed in either the ethonafide- or MIT-treated animals.

Effects of ethonafide on autoreactive T cell response in EAE mice

To address whether ethonafide affected expansion of MOG₃₅₋₅₅-specific T cell responses in EAE mice, mononuclear cells were isolated from the spleens of immunized animals treated with PBS, ethonafide, or MIT (0.25 mg/kg i.p.). Experiments were conducted after day 20 postinjection (p.i.) when significant cells were replenished. As shown in Fig. 3, A and B, and Table II, splenocytes from PBS control mice and both ethonafide and MIT groups mounted significant proliferative responses to MOG₃₅₋₅₅ peptide. However, the proliferation of T cells in response to the MOG₃₅₋₅₅ peptide was significantly decreased in the ethonafide-treated mice and MIT-treated mice in comparison to the PBS control mice on days 20, 32, and 44 after immunization. We also determined the production of the Th1 cytokine IFN- γ and Th2 cytokine IL-10 in splenocytes from EAE PBS-, ethonafide-, and MIT-treated mice. When stimulated with MOG₃₅₋₅₅ peptide, splenocytes from the EAE group produced significant amounts of IFN- γ , detectable on days 20 and 32 after immunization (day 44 was undetectable). The frequency of IFN- γ was strongly reduced in the ethonafide-treated group and the MIT-treated group compared with that of the PBS-treated EAE control mice on day 20 (Fig. 3C). Strikingly, both the ethonafide-treated and the MIT-treated mice had a 2- and 3-fold decrease in IFN- γ production by CD3⁺ and CD8⁺ T cells, respectively. However, both the ethonafide-treated and the MIT-treated mice had no difference in IFN- γ production by CD4⁺ T cells.

There was no significant difference in IL-10 production among the three groups (data not shown). These results demonstrated that ethonafide is able to down-modulate T cell cytokine effector functions, particularly the “inflammatory” Th1 responses.

Thus, our data suggest the recovered autoreactive T cells after cell ablation therapy might partially lose their autoaggressiveness as reflected by reduction in autoantigen-induced cell expansion and production of Th1 cytokines. These effects may explain, at least in part, the therapeutic effects of ethonafide in EAE.

Effects of ethonafide on the lymphocyte subpopulation and macrophages during EAE

To address how ethonafide might alter the encephalitogenic potential of autoreactive T cells, we first examined the kinetics of various lymphocyte subpopulations during EAE. For this purpose, we quantified the CD3, CD4, CD8, NK, and NKT cells by flow cytometry. Compared with that of control EAE mice, the percentage of CD3⁺ T cells significantly reduced in the spleens of ethonafide- and MIT-treated mice on days 7, 20, 32, and 44 (Fig. 4A; $p < 0.05$); the percentage of CD4⁺ T cells significantly decreased in the spleens of ethonafide- and MIT-treated mice on days 7, 20, 32, and 44 (Fig. 4B; $p < 0.05$); and the percentage of CD8⁺ T cells

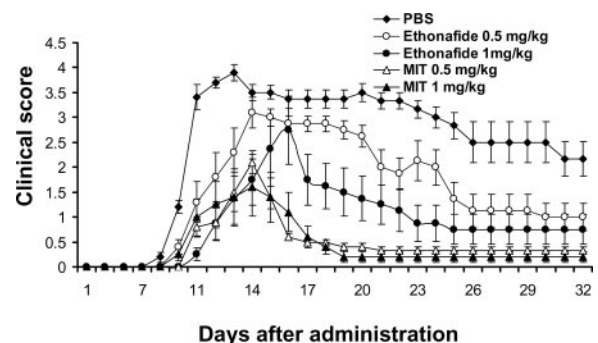


FIGURE 6. Therapeutic effect of ethonafide on acute EAE. Mice were induced to develop EAE and treated with PBS (control group), ethonafide, or MIT as described in *Materials and Methods*. On day 11 after developing EAE, ethonafide or MIT was injected at the dose of 0.5 and 1 mg/kg for 7 consecutive days. After immunization with MOG, the mice were observed until termination of the experiment. Means of clinical scores are calculated for mice. Data represent mean \pm SD of total mice in each group. Statistical evaluation was performed to compare experimental groups and corresponding control groups. The experiment was repeated two times with similar results.

Table III. Clinical evaluation of EAE after ethonafide or MIT-treatment^a

	% Incidence	% Mortality	Day of Onset	Maximum Clinical Score	Mean Clinical Score
PBS	100 (6/6)	17 (1/6)	10.4 ± 0.17	4.2 ± 0.16	2.87 ± 0.32
Ethonafide (mg/kg)					
0.5	100 (5/5)	20 (1/5)	11.8 ± 0.72*	3.9 ± 0.26	1.86 ± 0.35**
1	60 (3/5)	0 (0/5)	12.3 ± 0.55**	3.7 ± 0.22*	1.17 ± 0.24**
MIT (mg/kg)					
0.5	100 (5/5)	0 (0/5)	12.2 ± 0.68*	2.4 ± 0.36**	0.59 ± 1.67**
1	60 (3/5)	0 (0/5)	13.3 ± 0.55**	2.8 ± 0.36**	0.51 ± 0.21**

^a Mice were induced to develop EAE and treated with PBS, ethonafide, or MIT as described in *Materials and Methods*. The mice were observed until termination of the experiment after immunization with MOG, and the incidence, mortality, day of onset, and maximal disease grade were determined during the observation period. Means of maximum scores are calculated for the mice and had evidence of disease. Data represent mean ± SD of total mice in each group. Statistical evaluation was performed to compare experimental groups and corresponding control groups (*, $p < 0.05$ vs PBS; **, $p < 0.001$ vs PBS). The experiment was repeated two times with similar results.

significantly decreased in the spleens of ethonafide- and MIT-treated mice on days 7, 20, 32, and 44 (Fig. 4C; $p < 0.05$). Additionally, the percentage of NK (CD3⁻NK1.1⁺ cells) cells significantly reduced in the spleens of ethonafide- and MIT-treated mice on days 7, 20, and 32 (Fig. 4D; $p < 0.05$) and the percentage of NKT cells significantly reduced in the spleens of ethonafide- and MIT-treated mice on days 7, 20, and 44 (Fig. 4E; $p < 0.05$).

We also examined whether ethonafide or MIT affects B cells or macrophages. Compared with that of control EAE mice, the percentage of B cells (B220⁺CD3⁻ cells) reduced on day 7 but did not markedly change during EAE (days 20 and 32; Fig. 4F). The percentage of macrophages (CD11b⁺CD3⁻ cells), however, decreased in the spleens of ethonafide- and MIT-treated mice on days 7, 20, and 32 (Fig. 4G).

Given the similarity in peripheral immune response in ethonafide- and MIT-treated mice, we asked whether there were altered immune responses within the CNS. We analyzed the migration of inflammatory cells into the CNS by staining brain sections at various time points for CD4⁺ T cell, CD8⁺ T cell, and macrophage/microglia (CD11b⁺ cells) markers. We found that abundant CD4⁺ T cell, CD8⁺ T cell, and macrophage/microglia (CD11b⁺ cells) were present in the CNS sections of PBS-treated mice on day 20 after immunization. However, these cells appeared dramatically reduced in both ethonafide- and MIT-treated mice (Fig. 4H).

Collectively, ethonafide and MIT have a similar impact on CD3, CD4, CD8, NK, NKT, macrophages, and B cells: dramatic reduction 1 wk after EAE induction, followed by partial recovery starting the second week. By day 44, these cells were nearly completely recovered.

Distinctive influence of ethonafide on CD4⁺CD25⁺ T cells

We quantified the frequency of regulatory T (Treg) cells in ethonafide- and MIT-treated mice. Compared with control EAE mice, the percentage of Tregs increased 1.5- and 2-fold in the spleens of ethonafide- and MIT-treated mice on day 20 (Fig. 5A). Additionally, the percentage of Tregs increased in MIT-treated mice during EAE (days 7, 20, 32, and 44 p.i.; $p < 0.05$). Treg cells were relatively resistant to the cell depletion effect of ethonafide (Figs. 5, A and B). Furthermore, notably, such differences between ethonafide-treated mice and PBS-treated mice on day 7 and day 44 became less pronounced (Fig. 5B). Overall, analysis of ethonafide- and MIT-treated mice showed significant differences on days 20 and 32 ($p < 0.05$) in the number of Tregs compared those of with PBS-treated mice.

Although CD25 expression is not a definitive marker for Tregs, the transcription factor Foxp3 does seem to determine differentiation of the regulatory phenotype and distinguishes

Tregs from activated T cells (23, 24). We next asked whether Tregs from ethonafide- and MIT-treated mice show distinguishable features from Tregs of EAE controls. We first determined the expression of Foxp3 by CD4⁺ cells. Compared with EAE control mice, ethonafide- and MIT-treated mice had a 1.5-fold increase in the spleen on day 20 after immunization (data not shown). The percentage of Foxp3 by CD4⁺ cells in MIT-treated mice increased during EAE (days 7, 20, and 32 p.i.; $p < 0.05$; data not shown), but we did not find a significant difference in ethonafide-treated mice on day 7. We next determined the expression of Foxp3 in relation to CD25 expression by CD4⁺ cells. Compared with EAE control mice, ethonafide- and MIT-treated mice had a 1.5-fold increased percentage of CD25⁺Foxp3⁺ cells in the spleen on day 20 (data not shown). The percentage of Foxp3 by CD25⁺ cells increased in MIT-treated mice during EAE (days 7, 20, 32, and 44 p.i.; $p < 0.05$; data not shown), but we did not find a significant difference in ethonafide-treated mice on day 7. The level of expression of Foxp3 in relation to CD25⁺ cells gated on CD4⁺ cells in spleens of ethonafide- and MIT-treated mice was also higher than that of EAE control mice on day 20 (Fig. 5C) and during EAE (days 20, 32, and 44 p.i.; $p < 0.05$; Fig. 5D). Collectively, our results indicated that Treg cells from ethonafide- and MIT-treated mice were relatively resistant to cell depletion or recovered more quickly than other cell populations during EAE.

Therapeutic effect of ethonafide on acute EAE

To examine the efficacy of ethonafide to treat existing EAE, a group of mice was first immunized with MOG. When signs of EAE began to manifest, these mice were randomly divided into three groups that received ethonafide, MIT, and PBS, respectively, of two different doses (1 and 0.5 mg/kg per mouse per injection) for 7 consecutive days from day 11 after developing EAE. As demonstrated in Fig. 6, both 1 and 0.5 mg/kg showed various degrees of reduction in EAE severity. Compared with PBS-treated mice, the mean disease clinical scores of mice treated by ethonafide and MIT were significantly reduced at both doses ($p < 0.001$; Table III). Additionally, the therapeutic effects on EAE were more pronounced in MIT-treated mice.

Discussion

One of the strategies to treat autoimmune diseases such as MS is the removal of autoaggressive T cell populations. This strategy has led to the introduction of cytotoxic agents initially used for cancer patients into MS therapy. MIT is an anthracene-based anticancer agent that is now approved by the Food and Drug Administration

in several forms for MS patients. MIT is recommended as a first-line drug for patients with malignant forms of MS and as a second-line drug in those with relapsing-remitting MS and the secondary progressive MS (8). Although data on long-term efficacy and safety of MIT in MS patients are lacking, recent studies suggested that MIT was tolerated in a 3-year follow-up study and that disease remission is well maintained with MIT in the treated patients (8, 25). Nonspecific cytotoxicity effects on lymphocytes is believed to be a major mechanism responsible for the clinical effects of MIT in MS patients (26), other immunological mechanisms such as effects on Treg cells have not been investigated.

The effects of the MIT analog pixantrone (BBR2778) is also explored in EAE models (27–29). However, the available data are not sufficient to draw a conclusion regarding efficacy and cardiotoxicity of this compound. Ethonafide shares similar chemical structures and molecular targets with MIT. Design features in the azonafides sought to prevent variable acetylation metabolism which occurs in amonafides by avoiding primary amine side groups, and reduce cardiotoxicity by removing hydroquinones on the anthracene rings, which are present in MIT (17). The current study has identified that ethonafide is effective in preventing the development of EAE as well as in ameliorating the severity of EAE when the disease is ongoing. This study also showed that ethonafide has less cardiotoxic effect compared with MIT. Thus, these results provide basis for further investigation of this drug as a potential therapy for MS.

Previous studies showed that ethonafide has a breadth of anti-tumor activity which is similar to the spectrum of activity of doxorubicin and exhibits greater inhibitory potency than MIT against several human tumors growing in the SCID mouse model (17). Prior in vitro studies have shown markedly less cardiotoxicity in vitro for the azonafides compared with other classes of DNA intercalators (15, 16, 30). In our study, we also provided evidences that ethonafide has less cardiotoxicity compared with MIT. These features suggest that ethonafide might have important preclinical advantages over existing classes of DNA intercalators. It is conceivable that ethonafide has cytotoxic effects on lymphoid cells as well as on somatic cells. Our current results have demonstrated that the therapeutic effects of ethonafide in EAE were associated with drastic reduction of a number of lymphocyte populations such as CD3, CD4, CD8, CD11b, NK cells, and NKT cells, followed by replenishing of these populations. The kinetics of these cellular changes in mice receiving ethonafide and MIT is similar. It is of note that at some medium dosages, MIT appears to be more effective in the prevention and treatment of EAE.

Cell ablation triggers a reconstitution program that leads to the comprehensive renewal of the T cell repertoire. This process may provide opportunities to eliminate the self-reactive T cells and to boost the naturally occurring immunoregulatory events that come into play to suppress the neo-generated autoimmune repertoire. Indeed, our results demonstrated that after treatment with ethonafide or MIT, the remaining or regenerated autoreactive T cells become blunted in myelin-Ag-induced proliferation. Furthermore, the production of IFN- γ by these T cells is reduced. Therefore, autoreactive T cells may lose their pathogenicity. Interestingly, we found that CD4⁺CD25⁺ Tregs either were relatively resistant to deplete or recovered more quickly than effector T cells after ethonafide or MIT treatment at a number of time points studied. These recovered Treg cells expressed higher levels of Foxp3. Although further functional characterization of these Treg cells is needed, the preferential replenishing of Treg cells may contribute to the blunted response of autoreactive T cells.

While this manuscript was being prepared, the study by Condomines et al. (31) has reported that high-dose cyclophosphamide

treatment in patients induced a severe T cell count decrease without depleting Treg cells. Our findings, along with the report by Condomines et al. (31), support the concept of reprogramming the immune system after cell ablation (32). Furthermore, we envisage such reprogramming would include the natural restoration of regulatory events that curb the autoreactive T cell clones and introduction of other reagents to induce regulatory elements in the context of a renewed host immune system.

Collectively, our study has justified the search for a potentially less toxic agent such as ethonafide and further investigation of the sequential events renewing our immune system after cell ablation. Once the mechanisms governing the restoration of the immune system are uncovered, our capacity to gain control and reeducate the immune system will increase as well as our ability to treat autoimmune disease.

Acknowledgments

We thank Susan Rhodes for various assistances. We also thank Linda Swanson for reading, critiquing, and editing this manuscript.

Disclosures

The authors have no financial conflict of interest.

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