A Reversible \(S\)-Adenosyl-\(L\)-Homocysteine Hydrolase Inhibitor Ameliorates Experimental Autoimmune Encephalomyelitis by Inhibiting T Cell Activation

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ABSTRACT

The reversible \(S\)-adenosyl-\(L\)-homocysteine hydrolase inhibitor DZ2002 [methyl 4-(adenin-9-yl)-2-hydroxybutanoate] suppresses antigen-induced-specific immune responses, particularly type 1 helper T cell (Th1)-type responses. Experimental autoimmune encephalomyelitis (EAE) is thought to be a Th1 cell-mediated inflammatory demyelinating autoimmune disease model of human multiple sclerosis (MS). In this study, we examined the effects of DZ2002 on active EAE induced by myelin oligodendrocyte glycoprotein (MOG) 35-55 in female C57BL/6 mice. Administration of DZ2002 (50 mg/kg/day i.p.) significantly reduced the incidence and severity of EAE, which was associated with the inhibition of MOG35-55-specific T cell proliferation and Th1-type cytokine production. In vitro studies also demonstrated that DZ2002 inhibited anti-CD3/28-induced naive T cell activation concomitant with the down-regulation of cyclin-dependent kinase (CDK) 4, CDK6, cyclin D3, and the up-regulation or protection of the CDK inhibitor p27. These findings highlight the fact that DZ2002 likely prevents EAE by suppressing T cell activation and suggest its utility in the treatment of MS and other Th1-mediated inflammatory diseases.

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the central nervous system (CNS). EAE is typically characterized by lymphocytic and mononuclear cell inflammatory infiltrates and demyelination throughout the CNS (Zamvil and Steinman, 1990). EAE can be induced in many susceptible strains of rodents and primates by immunizing with whole-brain homogenate or purified neural Ags such as myelin basic protein, proteolipid protein, and myelin oligodendrocyte glycoprotein (MOG) (Sospedra and Martin, 2005). Many clinical and pathological features of EAE show close similarity to human multiple sclerosis (MS); therefore, EAE has been commonly used as a model system to study the mechanism of MS pathogenesis and to test the efficacy of potential therapeutic agents for the treatment of MS.

\(S\)-Adenosyl-\(L\)-homocysteine (AdoHcy) hydrolase (EC 3.3.1.1) is a highly conserved 432-amino acid ubiquitous enzyme that catalyzes the hydrolysis of AdoHcy to adenosine and homocysteine in eukaryotic cells (Yuan et al., 1999). Conversely, inhibition of the AdoHcy hydrolase leads to the accumulation of intracellular levels of AdoHcy, a potent feedback inhibitor of all \(S\)-adenosyl-\(L\)-methionine-dependent cellular transmethylations, including methylations of proteins, lipids, and nucleic acids (Saso et al., 2001; Wu et al., 2005). Because lymphocytes seem more dependent upon transmethylation reactions than most other cell types for their activation/function (German et al., 1983), AdoHcy hydrolase is thought to be an attractive target for the design of immunosuppressive and anti-inflammatory agents. In recent years,
several AdoHcy hydrolase inhibitors have been characterized. These compounds block T cell proliferation and IL-2 production (Wolos et al., 1993b; Wu et al., 2005) and exhibit both prophylactic and therapeutic effects in T cell-mediated immune models, including 2,4-dinitrofluorobenzene (DNFB)-induced delayed-type hypersensitivity reactions (Saso et al., 2001; Wu et al., 2005), allogeneic skin graft rejection, and collagen- (Wolos et al., 1993a,c) and peptidoglycan polysaccharide-induced arthritis (Saso et al., 2001). To date, three types of AdoHcy hydrolase inhibitors have been described: the irreversible type I and type II inhibitors and the reversible type III inhibitors (Yuan et al., 1999). These inhibitors either take advantage of AdoHcy hydrolase’s 3’-oxidative activity (type I), thus becoming irreversibly trapped in the active site of the enzyme, its 5’-hydrolytic activity, which permanently alters the active site (type II), or by reversibly binding to the open form of the enzyme (type III). Due to the relatively long turnover rate of AdoHcy hydrolase (t_{1/2} ~ 24 h), the irreversible inhibitors manifest significant toxicity, whereas type III inhibitors show greatly reduced toxicity yet still retain a similar ability to block the enzyme’s activity (Yuan et al., 1999).

Recently, we reported that a potent (K_i = 17.9 nM) type III inhibitor of AdoHcy hydrolase, DZ2002, obstructs both T cell and macrophage activation/function through down-regulation of cytokine production and costimulatory molecule expression (Wu et al., 2005). Moreover, DZ2002 treatment reduced DNFB-induced delayed-type hypersensitivity responses (Wu et al., 2005), ovalbumin-induced lymphocyte proliferation, and Th1-type cytokine (IL-2 and IFN-γ) production (Fu et al., 2006).

Here, we determined whether DZ2002 could inhibit EAE development. Our results demonstrate that DZ2002 significantly reduced the incidence and severity of the disease, which was associated with direct inhibition of T cell proliferation and activation.

Materials and Methods

Reagents. AdoHcy hydrolase inhibitor DZ2002 was synthesized at Diazyme Laboratories (San Diego, CA). The peptide MOG35-55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Sangon Biological Engineering Technology and Service Co. (Shanghai, People’s Republic of China). Amino acid sequences were confirmed by amino acid analysis and mass spectroscopy. The purity of the peptide was greater than 95%. Complete Freund’s adjuvant (CFA) and Mycobacterium tuberculosis H37Ra were purchased from Difco (Detroit, MI). Bordetella pertussis toxin, dimethylsulfoxide, and 3,3’,5,5’-tetramethylbenzidine were supplied by Sigma-Aldrich (St. Louis, MO). RPMI 1640 was bought from GibCO/Life Technologies Inc. (Gaithersburg, MD), and fetal calf serum was obtained from Hyclone Laboratories (Logan, UT). [3H]Thymidine was provided by Shanghai Institute of Applied Physics, Chinese Academy of Science (Shanghai, People’s Republic of China). The ELISA kits for IL-2 and IFN-γ were purchased from PharMingen (San Diego, CA).

Induction, Treatment, and Clinical Evaluation of EAE. Female C57BL/6 mice, aged 6 to 8 weeks, were purchased from the Shanghai Experimental Animal Center, the Chinese Academy of Sciences. The animals were housed in specific pathogen-free conditions. Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica. We used active the EAE model as previously described (Suen et al., 1997; Youssef et al., 2002). Briefly, female C57BL/6 mice were immunized on day 0 by s.c. injections with 100 μl of an emulsion of MOG35-55 peptide in CFA, distributed over three sites: one along the midline of the back between the shoulders and two on either side of the midline on the lower back. The final concentrations of MOG35-55 and M. tuberculosis H37Ra were 200 and 200 μg per mouse, respectively. Each mouse additionally received 400 ng of Bordetella pertussis toxin by i.p. injection in 400 μl of PBS on day 0 and 72 h postimmunization. DZ2002 (50 mg/kg/day) was dissolved in PBS containing 0.4% dimethylsulfoxide and administered i.p. following MOG35-55 immunization and continued throughout the study (n = 10 mice). The dose of DZ2002 was chosen on the basis of previous in vivo data (Fu et al., 2006; Wu et al., 2005) and our own preliminary experiments. As a control, an equal volume of PBS containing 0.4% dimethylsulfoxide was injected daily into control mice i.p. (n = 10 mice). Clinical assessment of EAE was performed daily, and mice were scored for disease according to the following criteria: 0, no overt signs of disease; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; and 5, moribund state or dead (Sakurai et al., 2002).

Histopathology Analysis. To assess the degree of CNS inflammation and demyelination, mice in the DZ2002-treated and untreated groups were anesthetized with an i.p. injection of sodium pentobarbital (30 mg/kg) on day 18 (at the peak of the disease) and perfused with 20 ml of cold PBS. Spinal cords were removed and fixed in 10% formalin. Five-micrometer-thick transverse sections were taken from cervical, upper thoracic, lower thoracic, and lumbar regions of the spinal cord (four sections per mouse). The sections were stained with Luxol Fast Blue (E.M. Science, Cherry Hill, NJ) to assess demyelination and with H&E to assess leukocyte infiltration and inflammation. The signs of inflammation and demyelination in the anterior, posterior, and two lateral columns (four quadrants) of the spinal cord sections were scored microscopically. Each quadrant displaying infiltration of mononuclear cells was assigned a score of one inflammation, and the quadrants that showed perivascular lesion and loss of myelin staining were assigned a score of one demyelination. Thus, each animal had a potential maximum score of 16 inflammation and/or 16 demyelination, and this study represents the analysis of 10 representative mice from two different groups. The pathologic score (inflammation or demyelination) for each group was evaluated by an examiner blinded to the treatment status of the animal, and was expressed as the percentage positive over the total number of quadrants examined (Bright et al., 1999; Natarajan and Bright, 2002).

Proliferation Assay and Cytokine Measurement. Spleens or draining lymph nodes (LNs) (axillary and inguinal) (n = 10 mice/group) were removed, and cell suspensions were prepared as previously described with some modifications (Wu et al., 2005; Fu et al., 2006). Briefly, erythrocytes were lysed with Tri-NH4Cl. Remaining cells (5 × 10^5 cells/well) were then cultured in triplicate in RPMI 1640 supplemented with 10% fetal calf serum, 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2-mercaptoethanol in 96-well flat-bottom plates (Corning Incorporated, Corning, NY) in the presence of 10 μg/ml MOG35-55. After 48 h, culture supernatants were harvested and stored at −20°C. Concentrations of IL-2 and IFN-γ were determined by ELISA as described by the manufacturer. After 60 h, 0.5 μCi/well [3H]thymidine was added to each well for 12 h, and cells were harvested onto glass fiber filters. [3H]Thymidine incorporation was measured by a Microbeta Trilux liquid scintillation counter (PerkinElmer Life and Analytical Science, Boston, MA).

Preparation of Purified T Cells and Enriched Antigen-Presenting Cells. Primary T cells were purified by using immunomagnetic negative selection to delete B cells and I-A^d antigen-presenting cells (APCs) as previously described (Yang et al., 2002; Zhu et al., 2006). Lymph node cells were allowed to react with anti-I-A^d mAb and then incubated with magnetic particles bound to goat anti-
mouse Ig (QIAGEN GmbH, Hilden, Germany). A T cell population depleted of anti-I-A\(^b\)-labeled and surface Ig\(^+\) cells was obtained by removing cell-bound magnetic particles with a rare earth magnet. Purity of the resulting T cell populations was examined by flow cytometry and found to be consistently \(>95\%\).

Splenic APC-enriched populations were separated using immunomagnetic negative selection to delete the surface Ig\(^+\) cells (B cells) and T cells as previously described (Yang et al., 2002; Zhu et al., 2006). Splenocytes were allowed to react with a mixture of rat anti-mouse CD4 (GK1.5) and rat anti-mouse CD8 (2.43) mAb and then incubated with a mixture of magnetic particles bound to goat anti-rat and goat anti-mouse Ig (QIAGEN GmbH). An APC-enriched population was obtained by removing cell-bound magnetic particles. Purity of the resulting APC-enriched populations was examined by flow cytometry and found to be consistently \(>95\%\).

**Antigen Presentation Assay.** To examine anti-MOG35-55 T cell responses (Yang et al., 2002; Zhu et al., 2006), purified T cells (4 \(\times\) 10\(^5\) cells/well) from normal C57BL/6 mice or from MOG35-55-immunized mice with or without DZ2002 treatment were cultured with APC-enriched cells (1 \(\times\) 10\(^5\) well) from normal C57BL/6 mice in triplicate in 96-well flat-bottom plates coated with MOG35-55 (5 \(\mu\)g/ml). IL-2 and IFN-\(\gamma\) levels by ELISA. After 60 h, cells were pulsed with 0.5 \(\mu\)Ci/well \[^{3}H\]thymidine for 12 h before harvest and assessed for \[^{3}H\]thymidine incorporation.

To examine APC antigen presentation (Youssef et al., 2002; Nath et al., 2004), purified T cells (4 \(\times\) 10\(^5\) cells/well) from normal C57BL/6 mice or from MOG35-55-immunized mice were cultured with APC-enriched cells from normal C57BL/6 mice or from MOG35-55-immunized mice with or without DZ2002 treatment (1 \(\times\) 10\(^5\) cells/well) in triplicate in 96-well flat-bottom plates in the presence or absence of 10 \(\mu\)g/ml MOG35-55. IL-2 and IFN-\(\gamma\) levels and T cell proliferation were assayed, respectively.

**Primary T Cells Stimulated by CD3 and CD28 Cross-Linking.** Purified primary T cells (2 \(\times\) 10\(^5\) cells/well) were cultured with anti-CD28 mAb (1 \(\mu\)g/ml) in 96-well flat-bottom plates coated with anti-CD3 mAb (5 \(\mu\)g/ml). IL-2 and IFN-\(\gamma\) levels and T cell proliferation were assayed, respectively.

For CD25 and CD69 expression level determination, T cells were stimulated with anti-CD3/28 mAb for 12 h and analyzed for surface staining of CD25 and CD69 by flow cytometry as previously described (Zhu et al., 2006). In brief, cells were suspended at 5 \(\times\) 10\(^6\) cells/ml and stained with fluorescein isothiocyanate-anti-CD25 (BD4) and fluorescein isothiocyanate-anti-CD69 (PharMingen) for 30 min at 4\(^\circ\) C in PBS with 1% fetal calf serum. Cells were then washed twice in PBS. The percentage of cells expressing early activation markers CD25 and CD69 was determined by flow cytometry.

**Western Blotting.** T cells were activated with anti-CD3/28 mAb and harvested at 36 h postactivation for Western blotting. Whole-cell extracts were obtained from T cells as previously described (Zhu et al., 2006). One hundred micrograms of total cell protein was resolved by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Arlington Heights, IL). After saturation of nonselective binding sites with 5% bovine serum albumin in Tris-buffered saline-Tween 20, the membranes were probed overnight with the appropriate Ab against \(\beta\)-actin (Sigma-Aldrich), CDK4, CDK6, cyclin D3, and p27\(^{kip1}\) (Santa Cruz Biotechnology, Santa Cruz, CA), and subsequently incubated with a secondary horseradish peroxidase-labeled anti-IgG Ab. Finally, the membranes were treated with chemiluminescent detection reagents (Amersham Bioscience, Buckinghamshire, United Kingdom) and processed for autoradiography.

**Statistical Analysis.** Results are expressed as mean \(\pm\) S.E.M. One-way analysis of variance followed by Dunnett’s post test was used to determine differences between groups where appropriate. Comparisons between two groups were made by Student’s test for unpaired observations. \(P < 0.05\) was considered significant.

**Results**

**DZ2002 Ameliorates EAE in C57BL/6 Mice.** To test the protective efficacy of DZ2002 on actively induced EAE, MOG35-55-immunized C57BL/6 mice were treated with 50 mg/kg/day DZ2002 i.p. from the day of EAE induction. All mice (100%) in the vehicle-treated group developed severe EAE starting on day 10.7 \(\pm\) 0.8 and reached a maximum mean clinical score of 2.0 \(\pm\) 0.3 on day 14 (Table 1). In contrast, a small proportion (30%) of mice treated with DZ2002 showed mild signs of disease with a delay of disease onset (day 14.7 \(\pm\) 0.7, \(P < 0.05\) compared with vehicle-treated mice) and a maximum mean clinical score of 0.8 (60% reduction), which was significantly less than that in the vehicle-treated mice (Table 1; Fig. 1A). In addition to reducing the clinical score, DZ2002 also prevented loss of body weight (data not shown). These results suggest that DZ2002 significantly reduced the incidence and severity of clinical paralysis in EAE.

We further examined the effect of DZ2002 on the pathogenesis of inflammation and demyelination in the CNS of mice with EAE. Spinal cord sections from mice treated with DZ2002 following induction of active EAE were analyzed for the infiltration of mononuclear cells (inflammation) and myelin loss (demyelination). As shown in Fig. 1B, vehicle-treated mice showed profound inflammation and demyelination in the CNS that decreased following treatment with DZ2002. In vehicle-treated mice, 55 and 58% of spinal cord quadrants were positive for inflammation and demyelination, respectively (Table 1; Fig. 1B). Conversely, in DZ2002-treated mice, 18 and 20% of spinal cord quadrants were positive for inflammation (67% reduction) and demyelination.

**TABLE 1**

DZ2002 suppresses clinical and pathological symptoms of EAE

<table>
<thead>
<tr>
<th>Group</th>
<th>Clinical Symptoms</th>
<th>Pathological Index</th>
</tr>
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<tr>
<td></td>
<td>Incidence</td>
<td>Average Day of Onset</td>
</tr>
<tr>
<td>Vehicle</td>
<td>10/10 (100%)</td>
<td>10.7 (\pm) 0.8</td>
</tr>
<tr>
<td>DZ2002</td>
<td>3/10 (30%)(^*)</td>
<td>14.7 (\pm) 0.7(^*)</td>
</tr>
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\(^*\) \(P < 0.01\) compared with vehicle control; three independent experiments were performed with similar results.
DZ2002 inhibits CNS inflammation and demyelination in EAE. These results suggest that DZ2002 inhibits CNS inflammation and demyelination in EAE.

DZ2002 Reduces MOG35-55-Induced Lymphocyte Proliferation and Cytokine Production. Because EAE is mediated by Th1-type T cells, we examined whether T cell activation and Th1 cytokine production was altered in DZ2002-treated mice. Compared with vehicle-treated mice, systemic administration of DZ2002 suppressed splenic and LN (axillary and inguinal) cells' ability to respond to 10 μg/ml MOG35-55 (38% reduction; LN cells, 53% reduction, compared with vehicle-treated mice, respectively) (Fig. 2, A and B). MOG35-55-loaded antigen-presenting cells in lymph nodes were sufficient to activate T cell proliferation. DZ2002 also inhibited LN cell's proliferative responses in the absence of MOG35-55 (69% reduction compared with vehicle-treated mice) (Fig. 2B). Culture supernatants were examined for Th1 cytokines (IL-2 and IFN-γ). As shown in Fig. 3, DZ2002 treatment reduced MOG35-55-induced production of IL-2 and IFN-γ in splenocytes and LN cells. At the same time, DZ2002 also inhibited IL-2 and IFN-γ production in the absence of MOG35-55 (Fig. 3).

DZ2002 Directly Inhibited Anti-MOG35-55 T Cell Responses but Did Not Affect Antigen Presentation by APCs. It was possible that the inhibitory effect on T cell activation of DZ2002 reflected an alteration in T cells and/or APC function. To distinguish between these possibilities, we examined MOG35-55-specific T cell activation when only T cells or APCs were exposed to DZ2002. First, to determine anti-MOG35-55 T cell responses, T cells from normal C57BL/6 mice or MOG35-55-immunized mice with or without DZ2002 treatment were stimulated with MOG35-55 in the presence of enriched APCs prepared from normal mice.

Fig. 1. Administration of DZ2002 inhibits the clinical and pathological symptoms of EAE. Active EAE was induced in female C57BL/6 mice by immunization with MOG35-55 peptide in CFA. The mice (n = 10) were treated with DZ2002 at 50 mg/kg/day by i.p. injection. Clinical symptoms (A) were scored every day. Spinal cord sections from cervical, upper thoracic, lower thoracic, and lumbar regions were prepared (four sections per mouse), and the four quadrants of every spinal cord section were scored for the presence of inflammation or demyelination. The pathological score for each treatment group was expressed as a percentage over total number of quadrants examined (B). The average number of quadrants examined per mouse was 16; therefore, this study included the analysis of 160 spinal cord quadrants in each group (n = 10 mice). Results are expressed as mean ± S.E.M. of 10 mice. *, P < 0.05; **, P < 0.01 compared with vehicle control (unpaired Student’s t test). Three independent experiments were performed with similar results.

Fig. 2. Administration of DZ2002 reduces MOG35-55-induced splenocyte (A) and LN cell (B) proliferation. Active EAE was induced in female C57BL/6 mice by immunization with MOG35-55 peptide in CFA. The mice (n = 10) were treated with DZ2002 at 50 mg/kg/day by i.p. injection. Splenocytes and draining LN cells were recovered, and 5 × 10⁶ cells were incubated with 10 μg/ml MOG35-55 for 72 h. Cultures were pulsed with 0.5 μCi/well [3H]thymidine for the final 12 h. Results are expressed as mean ± S.E.M. of 10 mice. **, P < 0.01 compared with vehicle control (unpaired Student’s t test). Three independent experiments were performed with similar results.
The results of Fig. 4A demonstrate that in the presence of normal APCs, T cells from vehicle-treated EAE mice showed high proliferative responses following MOG35-55 stimulation, but T cells from DZ2002-treated mice exhibited significantly lower responses. We also examined the capacity of T cells from immunized mice to produce IL-2 and IFN-γ in response to MOG35-55 stimulation. T cells from mice treated with DZ2002 produced significantly lower amounts of IL-2 and IFN-γ upon MOG35-55 stimulation (Fig. 4A). Second, to examine APC antigen presentation, purified T cells from MOG35-55-immunized mice were cultured with APC-enriched cells from normal C57BL/6 mice or MOG35-55-immunized mice with or without DZ2002 treatment in the presence or absence of 10 μg/ml MOG35-55. Although T cells cocultured with APCs enriched from MOG35-55-immunized mice showed high proliferative responses and produced greater amounts of IL-2 and IFN-γ upon MOG35-55 stimulation (Fig. 4B), there was no substantial difference in proliferation, IL-2 and IFN-γ production from T cells activated by APC-enriched cells from MOG35-55-immunized mice or DZ2002-treated mice (Fig. 4B). These results indicated that administration of DZ2002 had a direct suppression on anti-MOG35-55 T cell responses but had no visible effect on antigen presentation by APCs.

DZ2002 Suppressed Anti-CD3/28 mAb-Induced Primary T Cell Activation. Because the main targets for DZ2002 are considered to be T cells in EAE, we further examined whether DZ2002 has any effect on anti-CD3/28 mAb-induced T cell proliferation from naive cells. In vitro stimulation of T cells with anti-CD3/28 mAb served to mimic the physiologic cross-linking of the T cell antigen receptor, which is required for antigen-induced stimulation of T cells (Alberola-Ila et al., 1997). Primary T cells were stimulated with anti-CD3/28 mAb, and then DZ2002 (6.25–100 μM) was added to cultures starting at 0, 12, 24, and 36 h, then cultured for a total of 48 h. Previous in vivo pharmacologic studies showed that the maximal blood levels of DZ2002 after i.p. injection of 50 mg/kg in mice is ~100 μg/ml, corresponding to a concentration of ~400 μM. Therefore, the in vitro concentrations of DZ2002 were chosen specifically to be significantly less than 400 μM. As shown in Fig. 5A, DZ2002 (6.25–100 μM) concentration-dependently inhibited anti-CD3/28 mAb-induced T cell proliferation at all time points. Since DZ2002 did not show cytotoxicity up to 100 μM, we believe that the immunosuppressive activities of DZ2002 are not due to any cytotoxicity.

IL-2 and IFN-γ are produced and released upon T cell activation. DZ2002 (6.25–100 μM) exerted a profound, con-
centration-dependent inhibitory effect on IL-2 production from anti-CD3/28-activated T cells at all time points (Fig. 5B). DZ2002 also significantly inhibited the production of IFN-γ (Fig. 5C). However, decreases in the production of IFN-γ were more significant than that of IL-2.

Anti-CD3 cross-linking induces IL-2R α-chain (CD25) assembly with β and γ subunits to form the high-affinity IL-2R. IL-2 regulates proliferation of T cells in concert with induced expression of high-affinity cytokine receptors. A reduction in CD25 expression would certainly impair the ability of T cells to respond to IL-2, an event that is required for T cell proliferation (Zella et al., 2000). Flow cytometric analysis revealed a marked delay of the appearance of CD25⁺ cells in DZ2002-treated T cells, as opposed
to the untreated controls (Fig. 6A). This delay was observed as early as 12 h after anti-CD3/28 stimulation and persisted until 24 h after stimulation (data not shown). Another early activation marker, CD69, was also analyzed on anti-CD3/28-stimulated T cells. Likewise, DZ2002 concentration-dependently decreased the expression of CD69 as early as 12 h after anti-CD3/28 stimulation (Fig. 6B).

**DZ2002 Affected Cell Cycle Regulation in Anti-CD3/CD28-Activated T Cells.** Although DZ2002 blocked the expression of the early activation markers CD25 and CD69 in anti-CD3/28-stimulated T cells as described above, we also observed that DZ2002 could inhibit T cell proliferation and Th1 cytokines production even when added 36 h after initial stimulation. These results indicated that DZ2002 might also affect some late events of T cell activation. The expression of G₁ regulatory proteins, including CDK4, CDK6, cyclin D3, and the CDK inhibitor p27, all play important roles during the late stages of the cell cycle progression. Thus, the effects of DZ2002 on G₁ regulatory proteins were examined. Consis-
ent with published results, anti-CD3/CD28 stimulation efficiently induced expression of CDK4, CDK6, and cyclin D3, and a significantly degraded p27 in activated T cells (Fig. 7). Treatment of T cells with DZ2002 at 0 h after initial stimulation, however, down-regulated the expression of CDK4, CDK6, and cyclin D3 and protected p27 from degradation (Fig. 7). Similar results could be observed when DZ2002 was added 24 h poststimulation (data not shown).

Discussion

The present study demonstrated that systemic administration of DZ2002, a potent type III inhibitor of AdoHcy hydrolase, prevented clinical manifestations of active EAE, an animal model of MS. The inhibition of EAE by DZ2002 was associated with a decrease in MOG35-55-specific T cell proliferation and Th1 cytokine (IL-2 and IFN-γ) production. In anti-CD3/28 mAb-induced T cell activation, DZ2002 inhibited T cell proliferation, IL-2 and IFN-γ production, CD25 and CD69 expression, down-regulated CDK4, CDK6, and cyclin D3, and up-regulated p27, suggesting that direct inhibition of T cell activation is the likely reason for the attenuation of EAE by DZ2002.

MS is an inflammatory demyelinating disease of the CNS that affects more than 1 million people worldwide (Dean, 1994). A substantial percentage of MS patients develop clinical paralysis and become wheelchair bound or generally disabled during their lives. The pathogenesis of MS is a complex process involving the differentiation of neural antigen-specific Th1 cells and activation of macrophages (Craner et al., 2005; Sospedra and Martin, 2005). Recently, we showed that DZ2002 effectively suppressed immune responses to ovalbumin in mice (Fu et al., 2006). These results indicated that AdoHcy hydrolase blockers possess inhibitory properties for antigen-specific immune responses that may play crucial roles in the pathogenesis of autoimmune diseases, such as MS. In this study, we tested the potential therapeutic efficacy of DZ2002 on MOG35-55-induced active EAE in C57BL/6 mice. DZ2002 reduced the incidence, onset and severity of EAE, and prevented weight loss in DZ2002-treated EAE mice. The dosages of DZ2002 used for the in vivo experiments were significantly below its LD50 (500 mg/kg i.p.) (Y.-F. Fu, Y.-N. Zhu, J. Ni, X.-G. Zhong, W. Tang, Y.-D. Re, L.-P. Shi, J. Wan, Y.-F. Yang, C. Yuan, F.-J. Nan, B.R. Lawson, and J.-P. Zu, unpublished data), and we did not observe any negative side effects in DZ2002-treated mice during these studies. Furthermore, we and others recently demonstrated that AdoHcy hydrolase inhibitors are effective in the treatment of several immune-mediated disease animal models, including DNFBI-induced delayed-type hypersensitivity reactions (Saso et al., 2001; Wu et al., 2005), allogeneic skin graft rejection, and collagen- (Wolos et al., 1993a,c) and peptidoglycan polysaccharide-induced arthritis (Saso et al., 2001). Taken together, the results suggested that AdoHcy hydrolase inhibitors should be effective therapeutic agents for autoimmune diseases.

EAE is mediated by myelin-specific CD4+ T cells (Craner et al., 2005). Activated CD4+ T cells that recognize one of the candidate CNS myelin antigens cause paralysis and CNS demyelination (Craner et al., 2005). At the same time, CD4+ Th1 cells secrete proinflammatory cytokines, including IL-2 and IFN-γ, which are up-regulated during acute diseases (Youssef et al., 2002; Aktas et al., 2003; Nath et al., 2004). In particular, IFN-γ is thought to play a key role in the pathogenesis of EAE (Panitch et al., 1987; Lovett-Racke et al., 2004). Previous studies demonstrated that suppressing IFN-γ production in the encephalitogenic Th1 cells might provide therapeutic benefit (Youssef et al., 2002; Aktas et al., 2003; Nath et al., 2004). In the present study, the inhibition of clinical symptoms by DZ2002 was associated with a decrease in both antigen-specific T cell proliferation and the production of Th1-type cytokines, such as IL-2 and IFN-γ. To further demonstrate direct inhibition of T cells involved in the attenuation of EAE by DZ2002, we examined MOG35-55-specific T cell activation when only T cells were exposed to DZ2002. In vivo administration of DZ2002 resulted in a significant decrease in anti-MOG35-55 T cell proliferation and Th1 cytokines (IL-2 and IFN-γ) production. In vitro, DZ2002 concentration-dependently inhibited anti-CD3/CD28-induced T cell proliferation and Th1 cytokine (IL-2 and IFN-γ) production.

IL-2 and the IL-2 receptor (CD25) play critical roles in the induction of the cell cycle progression in activated T cells and, in particular, Th1 cells (Nelson and Willerford, 1998). On the one hand, the inhibition of T cell proliferation was partially attributed to a decrease in IL-2 production. On the other hand, the appearance of CD25+ T cells was delayed by DZ2002 treatment during the first 12 to 24 h following activation. In addition, DZ2002 concentration-dependently decreased the expression of another early activation marker CD69 in anti-CD3/28-stimulated T cells from 12 h. These results demonstrate that modulation of cell surface early activation markers may be a mechanism involved in the inhibition of T cell proliferation by DZ2002.

Entry of quiescent (G0) T cells into the G1 phase of the cell cycle involves induction of a number of cell cycle activators, including CDK4, CDK6 (Nagasaki et al., 1997; Appelman et

Fig. 7. DZ2002 down-regulates the expression of CDK4, CDK6, and cyclin D3 and up-regulates the expression of p27 in primary T cells stimulated by anti-CD3/28. Lymph nodes were harvested from normal C57BL/6 mice. T cells were prepared from lymph node cells by eliminating B cells and APCs. Purified T cells (2 × 10⁶/well) were cultured with anti-CD3/28 mAb (1 µg/ml) in 96-well culture plates coated with anti-CD3 (5 µg/ml). DZ2002 (6.25–100 µM) was added at 0 h. T cells were cultured for 36 h to determine expression of β-actin, CDK4, CDK6, cyclin D3, and p27 by Western blotting. Three experiments were performed with similar results.
al., 2002), and D-type cyclins (Nagasawa et al., 1997). Cyclin D3 has been reported to be the major inducible D-type cyclin in T cells (Nagasawa et al., 1997). Mammalian CKDs are also negatively regulated by CKD inhibitors. They consist of two distinct multigene families, the CIP/KIP family (p21CIP1, p27KIP1, and p57KIP2) and the INK4 family (p16INK4a, p15INK4b, p16INK4c, and p19INK4a) (Sherr and Roberts, 1999).

Among the CKD inhibitors, p21, p57, p16, and p15 are not significantly expressed in lymphoid organs (Sherr and Roberts, 1999). Although p27, p18, and p19 are highly expressed in lymphoid organs and cells (Kwon et al., 1997; Sherr and Roberts, 1999), and T cells with a mutant p19 showed normal proliferation (Zindy et al., 2000). Thus, a potential role for p27 in the negative regulation of T cell proliferation has been proposed (Aktas et al., 2003). We found that treatment of T cells with DZ2002 down-regulated the expression of p24K, p24K6, and cyclin D3 and up-regulated or prevented the degradation of p27. This finding, together with the persistent immunosuppressive effect of DZ2002, even when added late in culture, suggests an inhibition of cell cycle progression at the late part of the G1 phase.

In addition to T cells, macrophages also play a pivotal role in the pathogenesis of EAE (Craner et al., 2005). In EAE, deletion of macrophages immediately before the onset of clinical signs has been shown to significantly reduce disease expression (Huitinga et al., 1990). First, APCs, such as macrophages, present myelin basic protein to encephalitogenic T cells (Matsumoto et al., 1992). Second, macrophage activation is thought to be mediated by IFN-γ, resulting in the release of a range of proinflammatory cytokines (IL-6, IL-12, and TNF-α) and other factors such as the free radical nitric oxide, which contributes significantly to the expansion of inflammatory responses and associated tissue damage (Liu et al., 2001). Our recent studies have shown that DZ2002 down-regulates the expression of the costimulatory molecules CD80 and CD86 on differentiated human monocytic THP-1 cells in vitro (Wu et al., 2005). However, in this study, in vivo treatment of DZ2002 had no effect on antigen presentation by APCs. Maybe, a modest decrease in CD80 and CD86 expression was not enough to affect macrophage antigen presentation function. Otherwise, the differences between the results could be due to differing experimental systems and protocols. Furthermore, in vitro DZ2002 suppressed the production of TNF-α and IL-12 (Wu et al., 2005). In the present study, DZ2002 reduced TNF-α and IL-12 production in splenocytes (supplemental Data Fig. 1). Taken together, these studies suggest that inhibition of APC-produced proinflammatory cytokines might contribute, at least in part, to the therapeutic effects of DZ2002 in EAE.

In conclusion, the present study demonstrated that DZ2002, a novel potent type III inhibitor of AdoHcy hydrolase, prevents experimental autoimmune encephalomyelitis and that the inhibitory effect of DZ2002 is likely related to direct inhibition of T cell proliferation and activation. Therefore, we believe that DZ2002 warrants further analysis as a potential therapeutic agent for immune-mediated diseases with a Th1-type bias, such as multiple sclerosis.

References
Sasso Y, Conner EM, Teegarden BR, and Yuan CS (2001) T-cell migration responses to cytokines (IL-6, IL-12, and TNF-α) and other factors such as the free radical nitric oxide, which contributes significantly to the expansion of inflammatory responses and associated tissue damage (Liu et al., 2001). Our recent studies have shown that DZ2002 down-regulates the expression of the costimulatory molecules CD80 and CD86 on differentiated human monocytic THP-1 cells in vitro (Wu et al., 2005). However, in this study, in vivo treatment of DZ2002 had no effect on antigen presentation by APCs. Maybe, a modest decrease in CD80 and CD86 expression was not enough to affect macrophage antigen presentation function. Otherwise, the differences between the results could be due to differing experimental systems and protocols. Furthermore, in vitro DZ2002 suppressed the production of TNF-α and IL-12 (Wu et al., 2005). In the present study, DZ2002 reduced TNF-α and IL-12 production in splenocytes (supplemental Data Fig. 1). Taken together, these studies suggest that inhibition of APC-produced proinflammatory cytokines might contribute, at least in part, to the therapeutic effects of DZ2002 in EAE.

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