S-Adenosyl-L-homocysteine Hydrolase Inhibitor Mediates Immunosuppressive Effects in Vivo: Suppression of Delayed Type Hypersensitivity Ear Swelling and Peptidoglycan Polysaccharide-Induced Arthritis

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ABSTRACT
A specific and potent inhibitor of S-adenosyl-L-homocysteine (AdoHcy) hydrolase, 9-{[1'R,2'R,3'S'R]-2',3'-dihydroxycyclopentan-1'-yl}adenine (DHCaA), was evaluated for its immunosuppressive efficacy on murine T-cell proliferation in vitro and in several animal models, including delayed type hypersensitivity ear swelling and peptidoglycan polysaccharide-induced arthritis. The concanavalin A-induced [3H]thymidine incorporation into T cells was strongly inhibited by DHCaA with a 50% inhibition concentration (IC50) of 0.3 μM. In vivo, a dose-dependent reduction (39, 62, and 73%) of ear swelling was observed when 2,4-dinitrofluorobenzene-treated mice were orally administered with DHCaA at 1, 5, and 10 mg/kg, respectively. This inhibition in ear swelling dose dependently corresponded to the inhibition of AdoHcy hydrolase activity in the spleen. The more potent the AdoHcy hydrolase inhibitor, the stronger the immunosuppressive efficacy observed. In rat peptidoglycan polysaccharide-induced arthritis, orally dosed DHCaA significantly suppressed inflamed paw volumes with minimal effective dose of 0.1 mg/kg. At a dose of 1 mg/kg, DHCaA almost completely inhibited paw swelling. This inhibition of paw swelling was associated with an inhibition of interleukin-1β production in joint tissues. Histopathological evaluation of the joints in rats treated with 1 mg/kg showed a significant improvement in the reduction of the histopathological grading score from untreated scores of 10.44 to 4.78. Results from this study indicate that inhibitors of AdoHcy hydrolase could be effective anti-inflammatory agents.
pressive efficacy that has been observed with MDL28842 in different animal models, the direct linkage between AdoHcy hydrolase inhibition and immunosuppression has not been examined.

In this study, we used a more potent and specific inhibitor of AdoHcy hydrolase, DHCaA, to further test the hypothesis that inhibition of AdoHcy hydrolase leads to immunosuppression using both in vitro and in vivo experimental models, including DNFB-induced DTH ear swelling in mice and PG/PS-A in rats. The murine DTH response is a model of clinical allergic dermatitis and also a widely used model for investigating mechanisms of T-cell-mediated inflammation (Grabbe and Schwarz, 1998), whereas the rat PG/PS-A model exhibits chronic proliferative and erosive synovitis, resembling rheumatoid arthritis in humans (Cromartie et al., 1977). Our results from these two animal models demonstrated that the strong anti-inflammatory activity of DHCaA is at least partially resulted from its inhibitory activity against AdoHcy hydrolase.

Materials and Methods

Reagents. AdoHcy hydrolase inhibitors DHCeA, 9-[(1'R,2'S,3'R)-2',3'-dihydroxycyclopent-4'-en-1-yl]adenine (DHCeA), and (Z)-5'-fluoro-4',5'-didehydro-3'-deoxy-5'-fluoro-5'-deoxyadenosine (3'-deoxy-3'-fluoro-MDL28842) were synthesized at Tanabe Research Laboratories (San Diego, CA). Con A, acetone, olive oil, DNFB, adenosine (Ado), and bi-Hcy were purchased from Sigma (St. Louis, MO). RPMI-1640 and fetal calf serum were purchased from Life Technologies (Gaithersburg, MD). [3H]Thymidine was purchased from NEN (Boston, MA). PG/PS was purchased from Lee Labs (Grayson, GA). Methoxyfluorone (Metofan) was purchased from Schering-Plough (Union, NJ). Ado deaminase inhibitor erythro-9(2-hydroxy-3-nor)-adenine 160 HCl was purchased from Research Biochemicals (Rochester, NY). Ado deaminase inhibitor 9-(1'R,2'S,3'R)-2',3'-dihydroxycyclopent-4'-en-1-yl]adenine (DHCeA), and (Z)-5'-fluoro-4',5'-didehydro-3'-deoxy-5'-fluoro-5'-deoxyadenosine (3'-deoxy-3'-fluoro-MDL28842) were synthesized at Tanabe Research Laboratories (San Diego, CA). Con A, acetone, olive oil, DNFB, adenosine (Ado), and bi-Hcy were purchased from Sigma (St. Louis, MO). RPMI-1640 and fetal calf serum were purchased from Life Technologies (Gaithersburg, MD). [3H]Thymidine was purchased from NEN (Boston, MA). PG/PS was purchased from Lee Labs (Grayson, CA). Methoxyfluorone (Metofan) was purchased from Schering-Plough (Union, NJ). Ado deaminase inhibitor erythro-9(2-hydroxy-3-nor)-adenine 160 HCl was purchased from Research Biochemicals International (Natick, MA). Cocktail tablets of protease inhibitors (Complete) were obtained from Boehringer-Manheim (Mannheim, Germany). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β were from R&D Systems (Minneapolis, MN).

Animals. Female BALB/c mice aged 7 to 9 weeks and female Lewis rats weighing 85 to 110 g were obtained from Harlan Sprague-Dawley (San Diego, CA). The animals were housed in filter-top cages in an air conditioned room (23 ± 1°C, 55 ± 5% humidity). The light period in the room was 12 h (7:00 AM–7:00 PM). A standard diet (Purina, St. Louis, MO) and water were available ad libitum.

Preparation of Splenic Lymphocytes. The procedures for lymphocyte isolation were essentially according to the methods described previously (Johnson and Gordon, 1980). Briefly, mice were anesthetized with ketamine/xylazine (1:1) combination and killed by cervical dislocation. The spleen was removed and placed in 10 ml of 10 mM Tris-HCl, pH 7.4, containing protease inhibitor cocktail (1 tablet/25 ml of buffer). After homogenization, the supernatant was collected and used for the measurement of AdoHcy hydrolase activity. In addition, the supernatant was used for the measurement of AdoHcy hydrolase activities in spleens and joints were determined in this experiment. DHCaA was dissolved in water and daily administered orally to the mice 1 h before DNFB challenge. Ear thickness was measured 24 h after challenge using a digital micrometer (Mitutoyo, Japan) under light Metofane anesthesia. Results were expressed as the difference between the thickness of the left and the right ear. Spleens were taken from four mice in each group, which were randomly selected, after the measurement of the ear swelling and frozen until analysis.

PG/PS-Induced Arthritis. The PG/PS-A experiment was conducted essentially based on the methods described previously (Cromartie et al., 1977; Yocum et al., 1986; Widler et al., 1987). Forty-two female Lewis rats (95–120 g) were weighed and randomized into four groups using day 4 paw volume measurements as the criteria. The groups were as follows: group 1, PG/PS + vehicle (n = 9); group 2, PG/PS + 0.1 mg/kg DHCaA (n = 9); group 3, PG/PS + 0.3 mg/kg DHCaA (n = 9); and group 4, PG/PS + 1 mg/kg DHCaA (n = 9). A control group of nontreated animals (n = 6) was also included in this experiment. DHCaA was dissolved in water and daily administered by gavage from day 7 to day 27 of the experimental period. On day 0, animals were briefly anesthetized with Metofane and arthritis was induced via the intraperitoneal injection of a sterile aqeous solution of PG/PS at a dose of 25 μg of rhamnosel/g of body weight.

Measurement of Hind Paw Volume and Assessment of Arthritis. Arthritis was quantitatively determined daily (except non-treated control) by a measurement of the ankle volume using a plethysmometer (model 7140; Stoelting Co., Wood Dale, IL). For a consistent measurement, both hind limbs were shaved and a line was marked just above the ankle joint. Volume data were expressed as increase in milliliters (compared to day 0 reading). On day 28, the animals were sacrificed and the samples were obtained and data were recorded. The collected samples were as follows: 1) right hind limbs were fixed in 10% neutral-buffered formalin for histological processing, and 2) left hind limbs and spleens were frozen at −70°C for biochemical analysis.

Biochemical Examination of Spleen and Joint. The animals were killed by exanguination under Metofane anesthesia. The spleens from rats and mice, and left hind joints from rats were homogenized in hypotonic buffer (10 mM Na2HPO4, 10 mM NaCl, 1.5 mM magnesium acetate, pH 7.6) containing protease inhibitor cocktail (1 tablet/25 ml of buffer). After centrifugation, the supernatant was collected and the protein concentration was determined by the method of Bradford (1976). The supernatant was used for the measurement of AdoHcy hydrolase activity. In addition, the supernatant of joints was used for the assay of IL-1β concentration using an ELISA kit specific for rat IL-1β. IL-1β concentration in joints was expressed as amount (pg) per 1 mg of protein.

Determination of AdoHcy Hydrolase Activities in Tissue. AdoHcy hydrolase activities in spleens and joints were determined in the synthetic direction by measuring the rate of AdoHcy formation from Ado and Hcy according to the methods described previously (Yuan et al., 1994). The tissue supernatant (10 μl of spleen and 200 μl of joint) plus 20 μl of 10 mM Ado, 20 μl of 1 mM Ado deaminase inhibitor, and 50 μl of 62.5 mM Hcy were added to 400 μl of 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA and incubated at 37°C for 5 or 10 min. The reaction was terminated by the addition of 20 μl of 5 N HClO4. After centrifugation of the reaction mixture, the clear supernatant was collected and analyzed.

deoxy-3'-fluoro-MDL28842. After 48-h incubation, cells were pulsed with 1 μCi/well [3H]thymidine and cells were cultured for another 16 h. The cells were then harvested onto glass fiber filters and the incorporated radioactivity was counted using a liquid scintillation counter. Three spleens were used for each data point, and cells were cultured in the plate in triplicate.
for AdoHcy by HPLC (SPD-10AV; Shimadzu, Kyoto, Japan) using a C18 reversed phase column (Econosphere C18, 5 μm, 25 cm × 4.6 mm; Alltech, Deerfield, IL). The elution was carried out at a flow rate of 1 ml/min in two sequential linear gradients: 6 to 15% A over 0 to 9 min, 15 to 50% B over 9 to 15 min, where mobile phase A is acetonitrile and B is 50 mM sodium phosphate buffer, pH 3.2, containing 10 mM L-heptanesulfonic acid. The peak of AdoHcy was monitored at 258 nm. The concentration of AdoHcy was determined by the comparison of the peak area with that of a known quantity of authentic AdoHcy using a standard curve. One unit of enzyme activity was defined as the amount of enzyme that can synthesize 1 μmol of AdoHcy/min/mg of protein.

**Histopathological Analysis.** Right hind joints of rats stored in 10% neutral-buffered formalin were sent to Experimental Pathology Laboratories, Inc. (Research Triangle Park, NC) for processing and histopathological analysis. The histopathological scoring was assessed by Dr. Peter Mann according to the method of O’Byrne et al. (1991). Individual joints were assigned a score from 0 to 4 for each of the following four characteristics: 1) infiltration of cells, 2) pannus severity grade, 3) cartilage lesion severity grade, and 4) bone resorption severity grade.

**Statistics.** Results are expressed as mean and standard error. The time course data were analyzed using one-way ANOVA for repeated measurements. One-way ANOVA followed by Dunnett’s or Bonferroni’s post test was used to test for variances between groups for data collected at the experimental endpoint. For pathological scoring data, Wilcoxon matched pairs test was used. Significance was accepted at p < 0.05.

**Results**

**Inhibition of [3H]Thymidine Incorporation into T Cells by AdoHcy Hydrolase Inhibitors.** Figure 1 shows the inhibitory effects of three compounds on [3H]thymidine incorporation into Con A-stimulated murine T cells. The [3H]thymidine incorporation into T cells was strongly inhibited by DHCaA with an IC50 of 0.3 μM and mildly suppressed by DHCeA (a less potent hydrolase inhibitor) with an IC50 of 8 μM. On the other hand, 3'-deoxy-3'-fluoro-MDL28842, which has no inhibitory activity in enzyme-based assay, showed no inhibitory activity up to 10 μM in [3H]thymidine incorporation assay. These data indicated that inhibition of T-cell proliferation by AdoHcy hydrolase inhibitors is dependent on the potency of inhibitors against AdoHcy hydrolase.

**Effect of AdoHcy Hydrolase Inhibitors on Ear Swelling and Spleen AdoHcy Hydrolase Activity in Mouse DTH Model.** Figure 2 shows the correlation of inhibitory efficacy of DHCaA against ear swelling and the spleen AdoHcy hydrolase activity in the DTH model. When mice were administered orally with a single dose of DHCaA 1 h before DNFB challenge, a dramatic reduction of DNFB-induced ear swelling was observed. Inhibition of ear swelling by DHCaA was dose-dependent, ear swelling was inhibited by 39, 62, 73, and 72%, respectively at doses of 1, 5, 10, and 30 mg/kg. AdoHcy hydrolase activity in spleens collected from mice in the same DTH experiment was also dose dependently inhibited. Figure 3 shows the comparison of efficacy of DHCaA and DHCeA in the DTH model. Similar to the observations on T-cell proliferation, inhibition of the ear swelling was also dependent on the potency of AdoHcy hydrolase inhibitors against the enzyme activity, with more potent inhibitor (DHCaA) giving greater inhibition of ear swelling.

**Effect of DHCaA on PG/PS-Induced Joint Swelling.** Intraperitoneal injection of PG/PS resulted in a biphasic and chronic erosive polyarthritis typically observed in this model as shown in Fig. 4. Joints became swollen within 24 h after PG/PS injection, and reached to the first flare at day 4. This acute phase of arthritis was followed by a period of remission that lasted until day 14. The second flare then started and progressed into a chronic, erosive polyarthritis. Treatment of PG/PS-induced rats with DHCaA (0.1, 0.3, and 1 mg/kg from day 7 to day 27) significantly and dose dependently reduced the paw volumes (Fig. 4). DHCaA at 1 mg/kg near completely suppressed joint swelling throughout the second phase, a T-cell-dependent inflammation phase (Yocum et al., 1986; Wilder et al., 1987; Wahl et al., 1994). The increase in paw volume (ml) in each group on day 28 was as follows: for the left hind paws, 0.13 ± 0.02 (nontreated control), 0.75 ± 0.14 (≤0.05 compared with control (Dunnett’s method).
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**Effect of DHCaA on Histopathological Changes Induced by PG/PS.** The joint swelling data from day 28 correlated well with the histopathological scores. Typical control joints exhibit a smooth articular surface and a clearly defined synovial membrane (histology score of 0.50), whereas joints from animals injected with PG/PS exhibit marked pannus formation in addition to the minimal thickening and cellular infiltration. In contrast, joints from animals treated with DHCaA (1 mg/kg) did not exhibit any signs of inflammation or damage, indicating effective immunosuppressive activity.

**Fig. 3.** Effects of DHCaA and DHCeA on DTH ear swelling in BALB/c mice. Compounds were orally administered to the mice 1 h before initiation of ear inflammation. Twenty-four hours later, ear thickness was measured. Data are means ± S.E.M. **p < 0.01 and *p < 0.05 compared with vehicle control (n = 9, Dunnett’s method).**

(vehicle), 0.45 ± 0.11 (0.1 mg/kg), 0.25 ± 0.07 (0.3 mg/kg), and 0.19 ± 0.02 (1 mg/kg); and for the right hind paws, 0.13 ± 0.02 (nontreated control), 0.64 ± 0.17 (vehicle), 0.52 ± 0.14 (0.1 mg/kg), 0.25 ± 0.06 (0.3 mg/kg), and 0.17 ± 0.02 (1 mg/kg).

**Discussion**

T cells have been reported to play an important role in the development and the pathogenesis of both rheumatoid arthritis and experimental models of disease (Holmdahl et al., 1985a,b). A number of immunosuppressive agents are currently in use for the treatment of autoimmune diseases such as rheumatoid arthritis. Recently, a mechanism-based AdoHcy hydrolase inhibitor, MDL28842, has been reported to have an inhibitory effect on T-cell proliferation and activation (Wolos et al., 1993a). However, the precise biochemical mechanism by which AdoHcy hydrolase inhibitor mediates immunosuppressive effects is still unclear. We examined the efficacy of a more potent and specific inhibitor of AdoHcy hydrolase, DHCaA, in T-cell proliferation and two types of animal models of inflammation such as DTH and PG/PS-A, and compared the potency of enzyme inhibition with that of T-cell proliferation and immunosuppression.

The T-cell proliferation study measured by [3H]thymidine incorporation demonstrated that AdoHcy hydrolase inhibitor DHCaA (K_i = 90 nM) strongly inhibited T-cell proliferation with an IC50 of 0.3 μM, which was much stronger than the IC50 of 8 μM by DHCeA (K_i = 0.6 μM), a weaker inhibitor of AdoHcy hydrolase. The inhibitory activity of T-cell proliferation by these compounds correlated well with their inhibitory potency against AdoHcy hydrolase. Both DHCaA and DHCeA were also tested for their inhibitory activity on cell growth of several cell lines such as L929, HeLa, Vero, and CV-1. The IC50 values of cell proliferation inhibition by DHCaA and DHCeA using these cell lines were estimated to be around 130 μM (data not shown). Because DHCaA did not show a serious toxicity up to 100 μM, the inhibition of [3H]thymidine incorporation by DHCaA was assumed not to be due to any compound toxicity. In contrast, 3'-deoxy-3'-fluoro-MDL28842, which has no inhibitory activity against AdoHcy hydrolase at 10 μM, did not inhibit the [3H]thymidine incorporation into murine T cells up to 10 μM. Wolos et al. (1993a) reported that MDL28842, the parent and active compound of 3'-deoxy-3'-fluoro-MDL28842, strongly inhibited the T-cell proliferation. These results indicate that the inhibition of T-cell proliferation in vitro is related to the inhibition of AdoHcy hydrolase.

Further evidence for the relation between AdoHcy hydrolase inhibition and an immunosuppressive effect was demonstrated by results from the DTH study. Ear swelling in DTH is primarily the result of in vivo functions of antigen-specific CD4+ T-cell response (Grabbe and Schwarz, 1998). DHCaA administered just before the efferent phase strongly inhibited DNF-induced ear swelling in mice, indicating that DHCaA is capable of inhibiting T-cell-dependent immune reactions. In fact, when AdoHcy hydrolase activities in the spleen of these mice were measured, it was found that AdoHcy hydrolase activity was substantially inhibited. The suppression in ear swelling and the AdoHcy hydrolase inhibition correlated well. As predicted, DHCeA, a weaker inhibitor of AdoHcy hydrolase, was found to be less effective than DHCaA in the DTH experiment. These results suggest that a correlation exists between AdoHcy hydrolase inhibition and anti-inflammatory activity in vivo.
The potency of 1 mg/kg DHCaA in the experiment of Fig. 3 was weaker than that of Fig. 2. The control value of ear swelling in Fig. 3 (average 308 μm) was much higher than that in Fig. 2 (average 234 μm). The condition of ear swelling might be relatively severe for estimation of drug efficacy in the experiment shown in Fig. 3.

The effectiveness of DHCaA in suppressing inflammation was tested in another series of experiments, i.e., PG/PS-A in rats. Previously, an oral dose of MDL28842 (1–5 mg/kg/day) was shown to have anti-inflammatory activity in collagen-induced mouse arthritis (Wolos et al., 1993c). However, the correlation between anti-inflammatory effect of MDL28842 and the local AdoHcy hydrolase inhibition was not clearly demonstrated. Our results from the PG/PS-A model clearly demonstrated the correlation between joint volumes, histopathological score, and AdoHcy hydrolase activities in joints and spleens. In particular, the degree of AdoHcy hydrolase inhibition in joints correlated with the joint swelling in a linear manner, i.e., the greater the inhibition of AdoHcy hydrolase, the lesser the extent of joint swelling observed. The dose-dependent reductions of histological scores by DHCA at 1-mg/kg dose reduced the histological score from 10.44 to 4.78, but did not reach the normal control level. This may be explained by the severe erosive tissue damage that can occur during the acute flare in joints with greater than 0.4-ml increase in paw volume. It has been previously observed that erosive changes and pannus formation can occur within 4 days after PG/PS injection (Skaleric et al., 1991; Wahl et al., 1994; Palombella et al., 1998).

The AdoHcy hydrolase activity in joints of vehicle control was weaker than that of DHCaA.
was approximately 2.5-fold higher than that of control, although the AdoHcy hydrolase activity in spleens of vehicle control was lower than that of control. These differences of AdoHcy hydrolase activities between joints and spleens might come from the differences of tissue components in joints (cell-poor tissue) and spleens (cell-rich tissue). In PG/PS-induced arthritis, chronic phase of inflammation is characterized by more exuberant synovial lining cell hyperplasia, infiltration of the sublining spaces with macrophages and T cells, and proliferation of the fibroblast-like cells in the sublining stroma (Yocum et al., 1986). The increasing number of cells might be the reason why AdoHcy hydrolase activity increased in joints of vehicle control.

Based on the clear correlation observed between AdoHcy hydrolase inhibitors and an immunosuppressive effect, it is proposed that the immunosuppressive effect of DHCaA is at least partially from its specific inhibitory activity against AdoHcy hydrolase. Several hypotheses have been proposed to address the mechanism of action of the anti-inflammatory effects by AdoHcy hydrolase inhibitors. It is known that AdoHcy inhibits phosphatidylinositol kinase, which is an enzyme responsible for second messenger signaling in cell activation (Pike and DeMeester, 1988). Because T-cell activation via the T-cell receptor requires the phosphatidylinositol-signaling pathway (Desai et al., 1990), inhibition of phosphatidylinositol kinase could inhibit T-cell activation. In addition, inhibition of AdoHcy hydrolase decreases the intracellular Hcy level. Hcy is the methyl acceptor in the conversion of 5-methyltetrahydrofolate to tetrahydrofolate (Cantoni, 1985, 1986). Tetrahydrofolate is required for purine and thymidylic synthesis (Boss, 1987). Hcy may also play a role in maintaining intracellular redox status (Hutter et al., 1997; Koch et al., 1998; Tyagi, 1998). The accumulation of AdoHcy or depletion of Hcy in T cells may therefore induce T-cell inactivation, which leads to the observed anti-inflammatory effects (Kim et al., 1982; Wolfson et al., 1986).

Another possible mechanism of the inhibitory effect of DHCaA in the PG/PS-A model may come from its ability to effectively inhibit lipopolysaccharide-induced tumor necrosis factor (TNF-α) production from macrophages. In a separate experiment, it was found that DHCaA in an oral dose of 1 mg/kg could significantly, or at 10 mg/kg could completely, inhibit lipopolysaccharide-induced TNF-α production in mice. The IC_{50} value for inhibition of TNF-α production was estimated to be approximately 1 mg/kg (data not shown). It is known that injection of PG/PS induces a large amount of TNF-α production, which causes inflammation and tissue damage (Fuseler et al., 1997). Inhibition of AdoHcy hydrolase in macrophages may have interrupted the normal signaling pathways due to changes in second messenger levels and intracellular redox status, and lead to inhibition of TNF-α production. Inhibition of TNF-α production even results in inhibition of IL-1β expression in the arthritic joints because IL-1β expression from macrophages is TNF-α-dependent. DHCaA at 0.3 and 1 mg/kg reversed liver injury and near completely inhibited the T-cell-dependent inflammation phase, indicating that the effectiveness of DHCaA in suppression of inflammation may come from both inhibition of TNF-α production from macrophages and inhibition of T-cell activation or migration. Previously, Lambert et al. (1995) reported that MDL28842 inhibited TNF-α synthesis from mouse macrophages in vitro. However, the precise mechanism by which MDL28842 inhibits TNF-α production from macrophages was not clearly explained.

In conclusion, the results from this study demonstrated that AdoHcy hydrolase inhibitor DHCaA had strong anti-inflammatory activity evidenced in DTH ear swelling and PG/PS-A animal models. This efficacy correlated well with AdoHcy hydrolase inhibition in local lesions. Therefore, AdoHcy hydrolase may be an effective drug design target for treatment of T-cell-dependent chronic inflammatory diseases, such as rheumatoid arthritis.

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