Methylthioadenosine Reverses Brain Autoimmune Disease

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Objective: To assess the immunomodulatory activity of methylthioadenosine (MTA) in rodent experimental autoimmune encephalomyelitis (EAE) and in patients with multiple sclerosis.

Methods: We studied the effect of intraperitoneal MTA in the acute and chronic EAE model by quantifying clinical and histological scores and by performing immunohistochemistry stains of the brain. We studied the immunomodulatory effect of MTA in lymphocytes from EAE animals and in peripheral blood mononuclear cells from healthy control subjects and multiple sclerosis patients by assessing cell proliferation and cytokine gene expression, by real-time polymerase chain reaction, and by nuclear factor-κB modulation by Western blot.

Results: We found that MTA prevents acute EAE and, more importantly, reverses chronic-relapsing EAE. MTA treatment markedly inhibited brain inflammation and reduced brain damage. Administration of MTA suppressed T-cell activation in vivo and in vitro, likely through a blockade in T-cell signaling resulting in the prevention of inhibitor of kappa B (IκB-α) degradation and in the impaired activation transcription factor nuclear factor-κB. Indeed, MTA suppressed the production of proinflammatory genes and cytokines (interferon-γ, tumor necrosis factor-α, and inducible nitric oxide synthase) and increased the production of antiinflammatory cytokines (interleukin-10).

Interpretation: MTA has a remarkable immunomodulatory activity and may be beneficial for multiple sclerosis and other autoimmune diseases.

Ann Neurol 2006;60:323–334

Methylthioadenosine (MTA) is a lipophilic sulfur-containing adenine nucleoside produced from *S*-adenosylmethionine, during the synthesis of the polyamines spermine and spermidine.[1] Besides its strong inhibitory effect on the polyamine biosynthesis, MTA has been shown to exert other potent and specific pharmacological effects on cellular function, such as control of hepatocellular proliferation, inhibition of the development of neoplastic liver lesions, protection from toxic liver injury, and modulation of the inflammatory response.[2] Indeed, we have recently reported that MTA prevented bacterial lipopolysaccharide (LPS)-induced lethality in mice, likely through the suppression of tumor necrosis factor-α (TNF-α) production and inducible nitric oxide synthase (iNOS) gene expression and by enhancing the expression of interleukin-10 (IL-10).[3]

Multiple sclerosis (MS) is an autoimmune disease affecting two million people around the world, mainly in well-developed countries.[4] MS represents a significant health and social burden because it affects young adults, many of whom suffer a significant disability, and because the cost of years with the disease is high. MS is diagnosed when only minor central nervous system (CNS) damage has already occurred, which suggests that if we stop the inflammatory process at the early phase of the disease, we can prevent most of the brain damage and future disability. Although in recent years it has been stressed that the progressive phase of MS can be considered a neurodegenerative process, even during this chronic phase, the major cause of axonal damage appears to be related to the immunopathology secondary to the autoimmune process.[5] Thus, controlling the autoimmune response into the brain

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Received Oct 26, 2005, and in revised form Mar 31, 2006. Accepted for publication Apr 22, 2006.

B.M. and H.H. made equal contribution to this work. F.J.C., M.A.A., and P.V. share senior authorship.

This article includes supplementary materials available via the Internet at http://www.interscience.wiley.com/jpages/0364-5134/suppmat

Published online Jun 19, 2006, in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.20895

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might be highly efficacious in preventing brain tissue damage and increase of disability, and may reduce the risk for progression toward the neurodegenerative phase.

Currently approved therapies for MS are immunomodulatory drugs, such as interferon-β (IFN-β) and glatiramer acetate, which are only partly effective, are administered parentally, and are often limited by side effects.[6] Close to 40% of patients are considered nonresponders,[7–9] and their disease continuously progresses leading to significant disability. Thus, the discovery of a new immunomodulatory agent that improves the control of the disease, which might be suitable and safe for polytherapy and for oral administration, will be of great medical interest. Here, we report that the small molecule MTA is an immunomodulatory compound able to prevent acute experimental autoimmune encephalomyelitis (EAE), but more important, it is able to ameliorate chronic-relapsing EAE by suppressing the T-cell activation process. Thus, MTA might be an effective therapy for treating MS and other autoimmune diseases.

Subjects and Methods

Patients

We studied 13 patients with MS fulfilling McDonald criteria.[10] They were 10 female and 3 male patients aged 46 ± 13 years old, with a disease duration of 14.1 ± 14 years, moderate disability (Expanded Disability Status Scale = 3.0 ± 1.4, range, 0–6.0; Multiple Sclerosis Functional Composite = 0.31 ± 0.43), and the following disease subtypes: 7 relapsing-remitting MS, 3 secondary progressive MS, 2 progressive-relapsing MS, and 1 primary progressive MS. Only three relapsing-remitting MS patients had undergone immunomodulatory therapy (IFN-β) at the time of the study. We also studied 10 healthy donors (6 female and 4 male donors; mean age, 37 ± 13 years). All patients were stable at time of assessment, and none were suffering a clinical relapse. Patients' consent was obtained according to the Declaration of Helsinki, and the study was approved by the ethical committee of University of Navarra.

Animals, Experimental Autoimmune Encephalomyelitis Induction, and Treatment

Studies were approved by the University of Navarra Committee on Animal Care. Female Lewis rats from Charles River or Dark Agouti (DA) rats from Harlan (6–8 weeks old; 175–200gm body weight) were immunized in both hind pads with 100μl of an emulsion of saline and incomplete Freund's adjuvant containing 75μg guinea pig myelin basic protein fragment 68-82 (Sigma, St. Louis, MO) or 100μg recombinant rat myelin oligodendrocyte glycoprotein (purified in our laboratory as described previously[11]) and supplemented with 4mg/ml *Mycobacterium tuberculosis* (H37Ra strain; Difco, Detroit, MI). Animals were weighed and inspected for clinical signs of disease on a daily basis by a blinded observer. Disease severity of EAE was assessed according to the following scale: 0 = normal; 0.5 = mild limp tail; 1 = limp tail; 2 = mild paraparesis of the hind limbs, unsteady gait; 3 = moderate paraparesis, voluntary movements still possible; 4 = paraplegia or tetraparesis; 5 = moribund state. Data shown for the clinical studies in acute EAE and chronic-relapsing EAE are representative of three and two independent experiments performed with the indicated number of animals, respectively.

MTA was prepared from *S*-adenosylmethionine (Europharma, Madrid, Spain) as described elsewhere.[12] Animals were treated with MTA (96μmol/kg body weight) or placebo (tris[hydroxymethyl]aminomethane buffer 100mM, pH 7.0) through daily intraperitoneal injection starting after immunization in the prevention trial (acute EAE) or after the end of the first relapse in the treatment trial (chronic-relapsing EAE). MTA dose was based on our previous experience treating LPS-induced mortality in mice.[3] At the end of the study, rats were anesthetized and perfused intracardiacally with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.6). Brains, spinal cord, and spleens were dissected and either fixed or frozen until use. Serum was obtained from all animals included in the study, and transaminases levels (alanine and aspartate aminotransferase) were measured. No significant differences in serum transaminases levels were observed between the different groups of animals at the end of the study.

Real-time Quantitative Polymerase Chain Reaction

Splenocytes from Lewis and DA rats obtained at the time of death were homogenized in RNA lysis buffer. Total RNA from rat splenocytes or human peripheral blood mononuclear cells (PBMCs) was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA) isolation system, including DNase treatment using the RNase-Free DNase Set (Qiagen). Total RNA (35μg) was reverse transcribed using the Reverse Transcription System (High Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA). The real-time reaction was conducted at 25°C for 10 minutes, followed by 37°C for 2 hours, and finally stored at 4°C. Primers and target-specific fluorescence-labeled TaqMan probes were purchased from Applied Biosystems (TaqMan Gene Expression Assays). We used the TaqMan Universal Master Mix (Applied Biosystems). Amplification of complementary DNA was performed on a DNA Engine Opticon 2 Real-Time System (MJ Research, Watertown, MA) using 0.9μM for each primer and 0.25μM for the probe and 20ng complementary DNA. The reaction conditions were an initial 2 minutes at 50°C, followed by 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Each sample was run in triplicate, and in each plate the target and the endogenous control were amplified in different wells. The expression of the different genes tested was quantified relative to the level of the housekeeping gene 18S rRNA.

Immunohistochemistry

Histological evaluation was done on paraformaldehyde-fixed, paraffin-embedded sections of brain and spinal cord. Sections (10 μm thick) were stained with hematoxylin and eosin and Luxol fast blue to assess inflammation and demyelination. We examined 20 consecutive sagittal sections from every region examined (brain, cervical, thoracic, and lumbar spinal cord) per rat for all animals of the study. Semiquantitative histological evaluation for inflammation and demyeli-

Table 1. *Results of the Prevention Trial (Acute Experimental Autoimmune Encephalomyelitis in Lewis Rats)*

Treatment	Rats, n	EAE Incidence	EAE Onset (days)	EAE Maximum Score	EAE Cumulative Score
MTA	20	5/20	14.5 ± 1.2	0.10 ± 0.21	2.5 ± 0.07
Placebo	27	26/27[a]	13.4 ± 0.8	1.75 ± 0.41[a]	134.5 ± 4[a]

Results are the sum of three different experiments.

[a] $p < 0.005$, comparison of either methylthioadenosine (MTA)-treated group with placebo-treated group.
EAE = experimental autoimmune encephalomyelitis.

nation was conducted and scored blindly using the following scale: 0 = normal; 1 = 1 to 3/section perivascular cuffs with minimal demyelination; 2 = 3 to 10 perivascular cuffs/section accompanied by moderate demyelination; 3 = widespread perivascular cuffing, extensive demyelination with large confluent lesions.[11]

Immunohistochemical procedures were performed on 10μm paraffin-embedded sections of brain and spinal cord as described previously.[11] Primary antibodies were added at the following dilutions: polyclonal rabbit anti–glial fibrillary acidic protein antibody, 1:1,000 (Dakocitomation, Glostrup, Denmark); mouse anti-amyloid β-precursor protein (APP) antibody, 1:100 (Zymed Laboratories, San Francisco, CA); mouse anti I-A (OX-6) antibody, 1:200 (Serotec, Bicester, United Kingdom); mouse anti–rat CD8 antibody, 1:250 (Serotec); iNOS antibody, 1:250 (Serotec); mouse anti–rat CD68 (ED1) antibody, 1:200 (Serotec); and mouse anti–rat CD43 (W3/13) antibody, 1:50 (Serotec). The specificity of the immunoreaction was determined by incubating sections without the primary antibodies or using the corresponding isotype controls (rabbit and mouse IgG; Vector Laboratories, Burlingame, CA), which yielded no immunoreactivity. Immunohistochemical findings were quantitated based on the average number of positively labeled cells per section. In brief, scores were determined by a blinded observer using the following scale: negative, no positive cells; +, rare, less than 5 positive cells/10× magnified microscope field; ++, 5 to 20 positive cells/10x×magnified microscope field; +++, more than 20 positive cells/10× magnified microscope field.[13]

In Vitro Immunological Studies

Human PBMCs and rat splenocytes from MTA- (n = 20) and placebo-treated (n = 27) Lewis rats were isolated by Ficoll Hypaque density centrifugation (Pharmacia, Gaithersburg, MD). In addition, splenocytes from naive, nonimmunized Lewis rats were obtained for in vitro assessment of the effect of MTA in cell proliferation. Splenocyte proliferation assay was performed as described previously.[11] Phytohemagglutinin (PHA), 3′-deazaadenosine (DZA) and adenosine-2-3-dialdehyde (AdOx), forskolin, and H89 were obtained from Sigma. At the end of all experiments, cellular viability was assessed by the trypan blue exclusion test as described previously,[3] and no significant differences were observed among the different treatments (always >95% cell viability). Cell viability also was examined by the determination of lactate dehydrogenase (LDH) activity in the culture medium at the end of each experiment, using the Cytotox 96 assay (Promega, Madison, WI). There were no significant differences in LDH activity between control cultures and the different treatments. LDH activity in the culture medium never exceeded 5% of total LDH activity, determined after total cell lysis by incubation with 1% Triton X-100 (Sigma) for 10 minutes at 37°C.

Western Blot Analysis

After the indicated treatments, PBMCs cells were lysed and Western blots were performed as described previously.[14] Immunodetection of inhibitor kappa B-α (IκB-α) and Ser-32 phosphorylated IκB-α was performed using the corresponding antibodies (sc-371 [Santa Cruz, Santa Cruz, CA] and 9241S [Cell Signaling Technology, Beverly, MA]). Equal loading of the gels and specificity of the effects were demonstrated by hybridizing membranes with an antibody specific for actin (Calbiochem-Novabiochem, Darmstadt, Germany).

Statistical Analysis

Statistical analyses were performed with the two-tailed Mann–Whitney *U* test for comparing EAE scores, χ^2 test for comparing disease incidence, Kaplan–Meier curves for differences in day of onset of acute EAE or differences in the onset of the second relapse in chronic-relapsing EAE, and contingency tables with Kendal's tau test for histological score comparison. *p* values less than 0.05 were considered to indicate a significant difference. The statistical evaluation was conducted using the SPSS 11.0 statistical program (SPSS, Chicago, IL).

Results

Methylthioadenosine Prevents Acute Experimental Autoimmune Encephalomyelitis in Lewis Rats

Beginning the same day as induction of EAE, Lewis rats were randomly assigned to treatment with daily MTA or placebo and assessed blindly. All but one of the placebo-treated animals developed neurological symptoms of acute EAE, consisting of progressive weight loss, limp tail, and mild-to-moderate paraparesis. First neurological symptoms were observed at day 12 with mean day of onset 13.4 ± 0.8 (n = 27). The maximum score individual animals reached during the time course of the experiment was 1.75 ± 0.41 (Table 1). By contrast, MTA-treated rats (n = 20) showed either no clinical signs or a markedly attenuated disease course, with lower disease incidence (5/20; $p < 0.005$) and lower maximum clinical scores (0.10 ± 0.21) with

respect to the placebo group ($p < 0.005$) (see Table 1; Fig 1A). These results are the sum of three different experiments.

Histological evaluation confirmed the observed clinical protection (see Fig 1B and Table 2). In MTA-treated rats, the number and size of the inflammatory infiltrates were reduced compared with placebo animals ($p < 0.05$; see Fig 1B). Compared with placebo animals, we found a decrease in the number of monocytes, T cells, iNOS-positive cells, and astrocyte activation in MTA-treated animals, and similar amounts of B cells ($p < 0.01$; see Table 2 and Fig 1C). Finally, APP staining of damaged axons was decreased in MTA-treated animals ($p < 0.01$; see Table 2 and Fig 1C).

Methylthioadenosine Ameliorates Ongoing Chronic-Relapsing Experimental Autoimmune Encephalomyelitis in DA Rats

Because immunomodulatory therapy starts in patients after suffering at least the first relapse, we assessed whether MTA was able to reverse or ameliorate the ongoing autoimmune attack in a chronic-relapsing model of MS. DA rats immunized with myelin oligodendrocyte glycoprotein developed moderate-to-severe

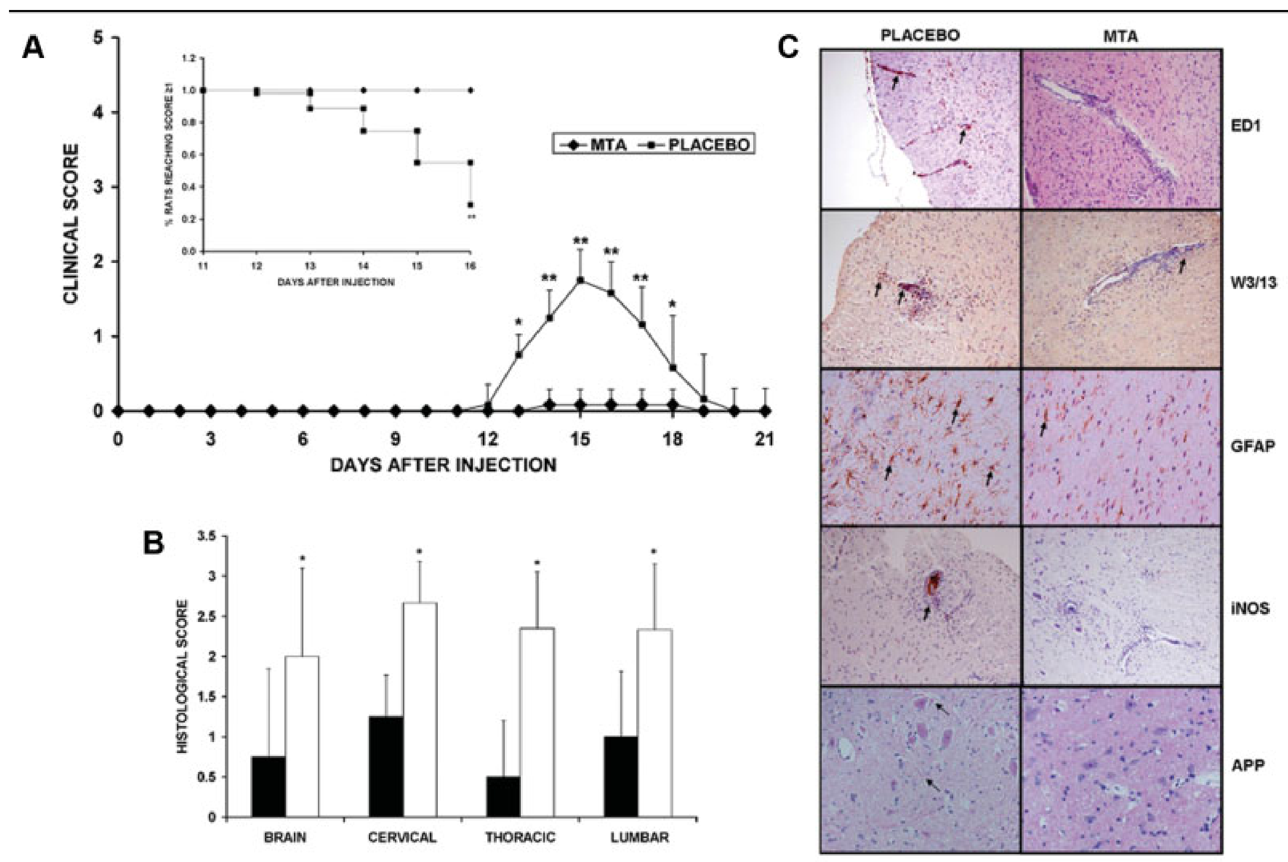

Fig 1. Methylthioadenosine (MTA) administration prevents acute experimental autoimmune encephalomyelitis (EAE). (A) MTA was administered intraperitoneally after immunization of Lewis rats with myelin basic protein (MBP; day 0). By day 12, placebo animals (n = 27) developed an acute relapse of moderate severity. By contrast, animals treated with MTA (n = 20) had almost a complete prevention of acute EAE. Results are expressed as the mean plus standard deviation of the clinical score (see Subjects and Methods). Differences between groups were compared with the Mann–Whitney U *test. *p < 0.05; **p < 0.01. Inset shows differences in day of onset evaluated using Kaplan–Meier curves. Animals treated with MTA have a delayed day of onset (p < 0.001, long-rank test). (B) Histological score of brain and spinal cord from placebo and MTA-treated animals analyzed at the end of the experiment. MTA-treated animals* (black bars) *had a lower histological score (see Subjects and Methods) either at the brain or spinal cord sections than placebo animals* (white bars) *(p < 0.05). The number and the extension of the inflammatory infiltrates and areas or demyelination were reduced in MTA-treated animals. (C) The inflammatory infiltrate in the spinal cord was composed by macrophages and activated microglial cells (ED1) and T lymphocytes (W3/13), with a decrease in the number of ED1- and W3/13-positive cells in MTA-treated animals. Enhanced astrocyte activation also was observed in brain sections from placebo-treated rats compared with animals that received MTA (glial fibrillary acidic protein [GFAP]). By contrast, inducible nitric oxide synthase (iNOS) was expressed by infiltrating inflammatory cells in spinal cord tissue in the placebo group (iNOS), but was significantly reduced in the MTA-treated group. Finally, APP expression was present in damaged axons in placebo animals (amyloid β-precursor protein [APP]), but was significantly decreased in MTA-treated animals. Representative immunohistochemical stainings are shown.*

Table 2. *Semiquantitative Analysis of Histological Findings in the Prevention Trial (Acute Experimental Autoimmune Encephalomyelitis in Lewis Rats)*

Treatment	CNS Region	iNOS$^+$	T Cells	B Cells	Macrophages	APP$^+$
MTA	Brain	Negative	+	+	++	+
	Cervical cord	+	++	Negative	+	Negative
	Thoracic cord	Negative	+	Negative	Negative	Negative
	Lumbar cord	Negative	+	+	+	Negative
Placebo	Brain	+++[a]	+++[a]	+	+++[a]	++[a]
	Cervical cord	++[a]	+++[a]	Negative	+++[a]	+[a]
	Thoracic cord	+[a]	++[a]	Negative	+[a]	+[a]
	Lumbar cord	+[a]	++[a]	+	+	+[a]

Differences in the semiquantitative immunohistochemical score were compared between groups using a contingency table and the Kendall's tau test for ordinal data.

[a] $p < 0.01$.

CNS = central nervous system; iNOS = inducible nitric oxide synthase; APP = amyloid β-precursor protein; MTA = methylthioadenosine.

chronic-relapsing EAE with an incidence close to 100%. Animals were randomly assigned to either the MTA or placebo group at the end of the first clinical relapse to assess its ability to prevent or ameliorate further relapses or chronic diseases. All placebo animals developed a severe second relapse with a mean clinical score of 3.12 ± 0.9. By contrast, development of the second relapse was prevented in 7 of 10 MTA-treated animals, whereas the other 3 animals developed a second but milder relapse ($p < 0.05$; Fig 2A and Table 3). These results are the sum of two different experiments. Histological analysis also showed that MTA-treated animals have less inflammatory infiltrates with a smaller amount of demyelinated areas in the brain and lumbar spinal cord ($p < 0.05$; see Fig 2B). Similar to the effect of MTA in acute EAE, inflammatory infiltrates in MTA-treated animals have a decrease of T cells, macrophages, and iNOS-expressing cells and less APP+ axons ($p < 0.01$; Table 4). Thus, MTA was able to prevent or ameliorate the autoimmune attack to the brain once it had already started, which mimics the clinical settings.

Immunomodulatory Effect of Methylthioadenosine in Rat Peripheral Immune Responses

To assess which was the effect of MTA in the peripheral immune response, we evaluated the proliferative responses against the immunizing antigen, or PHA, and the cytokine profile in spleen cells from placebo- and MTA-treated Lewis rats. Myelin basic protein–specific proliferative response was significantly lower in MTA-treated animals ($p < 0.05$; Fig 3A). In addition, MTA also inhibited in a dose-dependent fashion the proliferation of splenocytes isolated from naive Lewis rats when stimulated with PHA ($p < 0.05$; see Fig 3B), suggesting that MTA was acting at a critical step downstream of T-cell receptor (TCR) activation, but was not antigen-specific.

Gene expression of IL-2, IFN-γ, TNF-α, iNOS, and IL-10 was investigated by quantitative reverse transcriptase PCR at 9 and 16 days after immunization in splenocytes from placebo- and MTA-treated Lewis rats. We found that MTA-treated animals display a suppression of T helper 1 cell phenotype and an enhanced expression of the immunosuppressive cytokine IL-10 (see Fig 3C). The downregulation of iNOS in the peripheral immune cells also correlated with the diminished iNOS expression by inflammatory cells in the CNS (see earlier). These observations are in agreement with our previous findings in the LPS mouse model[3] and rule out a nonspecific effect of MTA on overall gene transcription activity. Interestingly, animals that received MTA and that were protected from the disease showed enhanced levels of IL-10 expression by day 9, after immunization, compared with placebo-treated rats (see Fig 3B). In contrast, in the placebo group, IL-10 upregulation was observed at day 16, concomitantly with the onset of the spontaneous recovery characteristic of this experimental model.[15] In addition, we also found a significant attenuation in proinflammatory cytokine gene expression in DA rats treated with MTA (data not shown).

Immunomodulatory Role of Methylthioadenosine in Peripheral Blood Mononuclear Cells from Healthy Subjects and Multiple Sclerosis Patients

To assess whether the immunomodulatory role of MTA found in rodent EAE was also present in humans suffering an autoimmune disease, we first studied in vitro the effect of MTA on T-cell activation and cytokine profile in PBMCs from healthy control subjects. Activation of the master transcription factor nuclear factor-κB (NF-κB) downstream of the TCR allows antigen-specific proliferation and maturation of lymphocytes into effector cells.[16,17] NF-κB activation involves phosphorylation and subsequent ubiquitin-mediated degradation of its inhibitor IκB.[17] We found that in the presence of MTA, the phosphorylation and

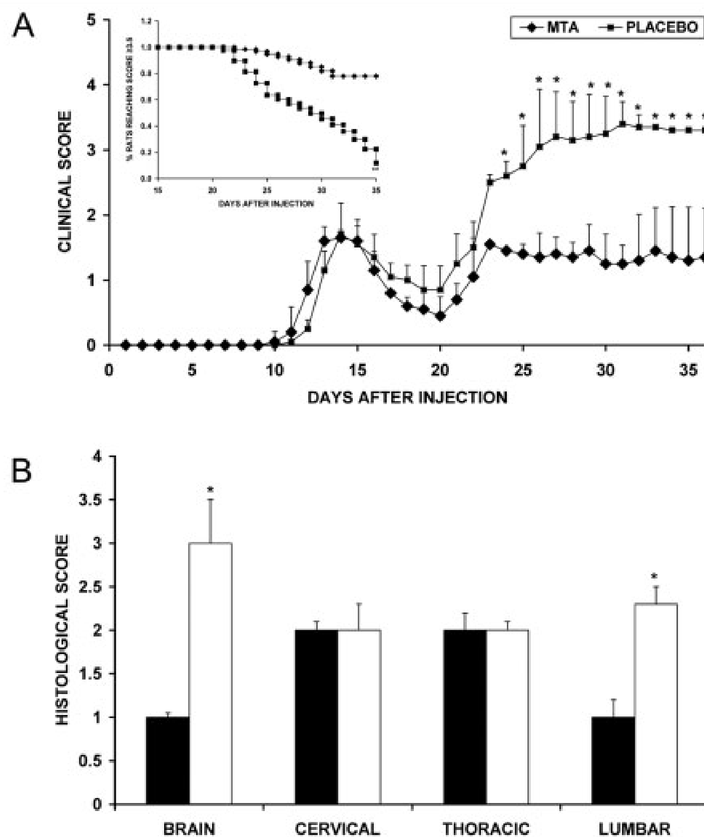

Fig 2. Methylthioadenosine (MTA) treatment ameliorates ongoing chronic-relapsing experimental autoimmune encephalomyelitis (EAE). (A) Chronic-relapsing EAE was induced in DA rats by immunizing with myelin oligodendrocyte glycoprotein (MOG). Animals developed the first relapse by day 12 and started to recover from such relapse by day 16. MTA (diamonds) *or placebo* (squares) *treatment was started after the remission of the first relapse (5 days after the onset of the first relapse, day 18, and were assessed for the presence of a second relapse in the following 18 days). Placebo animals (n = 10) developed a second moderate-to-severe relapse. By contrast, animals treated with MTA (n = 10) were either prevented from developing the second relapse or developed a mild one. Results are expressed as the mean plus standard deviation of the clinical score (see Subjects and Methods). Differences between groups were compared with the Mann–Whitney* U *test.* $^{*}p < 0.05$. *Inset shows differences in the progression of the disease between placebo- and MTA-treated animals after the first remission (*arrow, *day 18) evaluated using Kaplan–Meier curves. Animals treated with MTA had either no second relapse or a delayed one (*$p < 0.001$*, long-rank test). Results are the sum of two independent experiments. (B) Brain and spinal cord from placebo-* (white bars) *and MTA-treated* (black bars) *animals were analyzed at the end of the experiment (day 36). MTA-treated animals had a lower histological score at the brain and lumbar spinal cord level than placebo animals (*$p < 0.05$*).*

degradation of IκB-α elicited by PHA stimulation was almost completely prevented in human PBMCs (Fig 4A). This effect of MTA was dose-dependent (see Fig 4B). Similar observations were made when splenocytes from naive Lewis rats were stimulated with PHA in the presence of MTA (see Fig 4C), indicating that no

Table 3. Results of the Treating Trial (Chronic-Relapsing Experimental Autoimmune Encephalomyelitis in DA Rats)

Treatment	Rats, n	EAE Incidence, First Relapse	EAE Incidence, Second Relapse	EAE Onset (days)	EAE Maximum Score	EAE Cumulative Score
MTA	10	10/10	3/10	26 ± 6	1.8 ± 1.5	23 ± 20
Placebo	10	10/10	10/10[a]	21 ± 3[a]	3.6 ± 1.5[a]	50 ± 20[a]

Results are the sum of two different experiments.

[a] $p < 0.05$, comparison of either methylthioadenosine (MTA)-treated group with placebo-treated group.
EAE = experimental autoimmune encephalomyelitis.

species-specific differences existed regarding the response to MTA treatment. We also have examined whether the effect of MTA on PHA-mediated IκB-α degradation resulted in impaired NF-κB activation. For this, we tested the expression of three NF-κB target genes in human PBMCs that had been pretreated with MTA and then stimulated with PHA. The upregulation in the expression of TNF-α, IκB-α, and IFN-γ induced by PHA was significantly attenuated by MTA treatment (see Fig 4D). No significant toxicity was appreciated in MTA-treated cells. Together, these observations also suggest that the immunomodulatory effects of MTA in the EAE model could be due in part to the direct interaction of MTA with the NF-κB pathway in inflammatory cells.

MTA is structurally related to adenosine and is able to interact with adenosine purinergic receptors.[18] Adenosine is a potent endogenous modulator of the inflammatory response,[19] including neuroinflammation in experimental MS.[20] Signaling of the antiinflammatory effects of adenosine is mainly attributed to the elevation of cyclic adenosine monophosphate (cAMP) levels and the activation of its downstream effector protein kinase A (PKA).[19] Moreover, adenosine and cAMP are potent inhibitors of the NF-κB pathway downstream of immunoreceptors.[21] These facts, together with the previously described ability of MTA to increase cellular cAMP levels,[22] made it important to elucidate whether the currently described effects of MTA were dependent on the cAMP-PKA pathway. To this end, PBMCs from healthy subjects were either pretreated or not pretreated with forskolin, a drug that activates the cAMP-producing enzyme adenylyl cyclase, and then stimulated with PHA. In the presence of forskolin, the degradation of IκB-α elicited by PHA was significantly prevented (see Supplementary Fig S1A). This is in agreement both with previously described effects of this PKA activator on T-cell activation and NF-κB signaling[19,23] and with what has been observed in B cells on antigen receptor stimulation.[21] When forskolin pretreatment was performed in the presence of the PKA inhibitor H89, the effect of forskolin preventing PHA-induced IkB-α degradation was abrogated (see Supplementary Fig S1A). Once it was established that in our experimental model the cAMP-PKA pathway can modulate NF-κB signalling on TCR stimulation, we tested whether the effect of MTA could be mediated by the elevation of cAMP levels. For this purpose, PBMCs were pretreated with MTA in the presence or absence of H89, and then stimulated with PHA. We observed that the protective effect of MTA on IκB-α degradation was preserved when H89 was used (see Supplementary Fig S1A), implying that the cAMP effector PKA was not involved in the mechanism of action of MTA.

A well-known biochemical action of MTA is its ability to interfere with transmethylation reactions either directly or through the inhibition of *S*-adenosyl-L-homocysteine hydrolase.[1,2] There is increasing evidence indicating that methylation reactions, including protein arginine methylation, play a central role in T-lymphocyte activation and the cytokine response.[24–26] Given the key role of NF-κB activation in T-cell function, we wanted to know whether the effects of MTA on PHA-induced IκB-α phosphorylation and degradation could be mimicked by other inhibitors of transmethylation reactions. This was done by preincubating human PBMCs with either DZA or AdOx, two different *S*-adenosyl-L-homocysteine hydrolase inhibitors.[27] We observed that, similar to MTA, these two compounds significantly prevented the phosphorylation and degradation of IκB-α in PBMCs (see Supplementary Fig S1B). Collectively, these observations suggest that the immunomodulatory effects of MTA are independent from the cAMP-PKA pathway and could be mediated by the suppression of a methylation reaction involved in NF-κB activation.

Finally, we assessed whether the effect of MTA on

Table 4. Semiquantitative Analysis of Histological Findings in the Treating Trial (Chronic-Relapsing Experimental Autoimmune Encephalomyelitis in DA Rats)

Treatment	CNS Region	iNOS+	T Cells	B Cells	Macrophages	APP+
MTA	Brain	+	+	Negative	+	+
	Cervical cord	Negative	++	Negative	++	Negative
	Thoracic cord	Negative	++	Negative	++	+
	Lumbar cord	Negative	+	+	+	Negative
Placebo	Brain	+++[a]	+++[a]	+[a]	+++[a]	++[a]
	Cervical cord	+[a]	++	Negative	++	+[a]
	Thoracic cord	++[a]	++	Negative	++	+
	Lumbar cord	+[a]	++[a]	+	+++[a]	+[a]

Differences in the semiquantitative immunohistochemical score was compared between groups using a contingency table and Kendall's tau test for ordinal data.

[a]$p < 0.01$.

CNS = central nervous system; iNOS = inducible nitric oxide synthase; APP = amyloid β-precursor protein; MTA = methylthioadenosine.

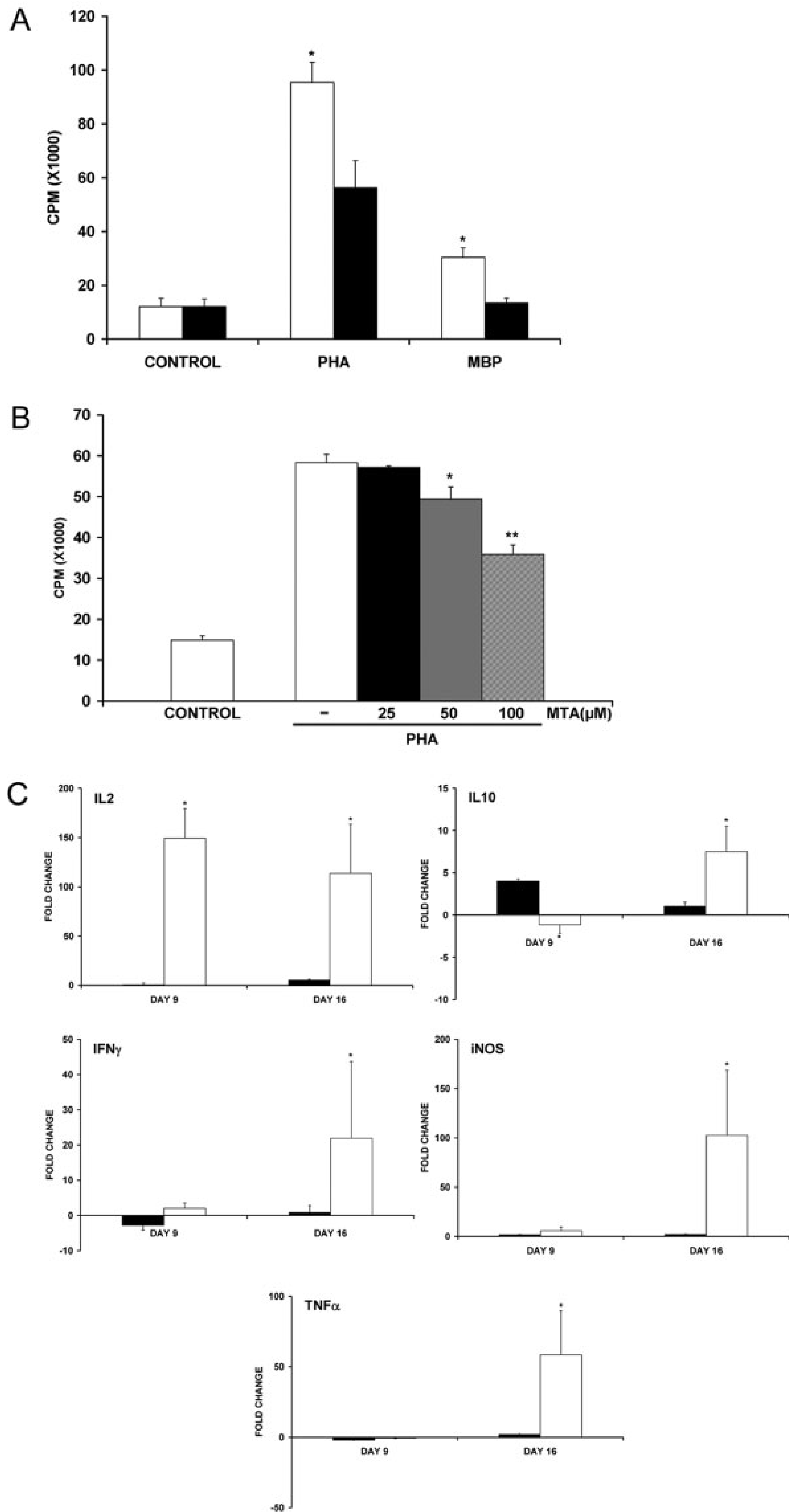

Figure 3

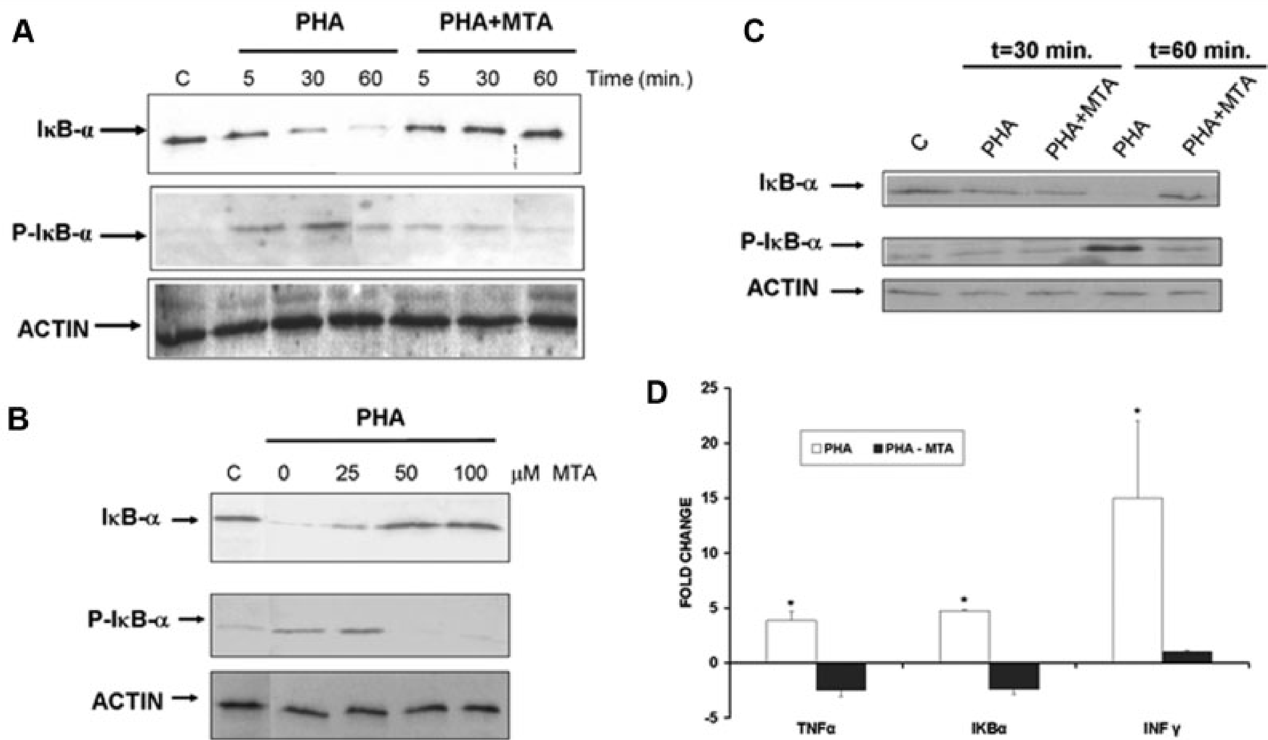

Fig 4. Methylthioadenosine (MTA) interferes with the activation of the nuclear factor-κB (NF-κB) pathway in human peripheral blood mononuclear cells (PBMCs) and rat splenocytes. (A) Effect of MTA treatment on phytohemagglutinin (PHA) induced inhibitor of kappa B (IκB-α) degradation and Ser-32 IκB-α phosphorylation. Human PBMCs were pretreated with 100μM MTA for 2 hours and then stimulated with PHA (10μg/ml) for the indicated periods of time. Control cells (C) received no treatments. Levels of total IκB-α and Ser-32 IκB-α phosphorylated were assessed by Western blotting. Representative blots of three different experiments performed with independent PBMCs preparations are shown. (B) Dose-dependent effect of MTA on PHA induced IκB-α degradation and Ser-32 IκB-α phosphorylation. Human PBMCs were pretreated with different concentrations of MTA for 2 hours and then stimulated with PHA (10μg/ml) for 30 minutes. Control cells (C) received no treatments. Levels of total IκB-α and Ser-32 IκB-α phosphorylated were assessed by Western blotting. Representative blots of three different experiments performed with independent PBMCs preparations are shown. (C) Effect of MTA treatment on PHA induced IκB-α degradation and Ser-32 IκB-α phosphorylation in rat cells. Splenocytes isolated from naive Lewis rats were pretreated with 100 μM MTA for 2 hours and then stimulated with PHA (10μg/ml) for the indicated periods of time. Control cells (C) received no treatments. Levels of total IκB-α and Ser-32 IκB-α phosphorylated were assessed by Western blotting. Representative blots of three different experiments performed with independent splenocyte preparations are shown. (D) Effect of MTA on the expression of NF-κB target genes (tumor necrosis factor-α [TNF-α], IκB-α, and interferon-γ [IFN-γ]) in human PBMCs stimulated with PHA. PBMCs were pretreated with 100μM MTA for 2 hours and then stimulated with PHA (10μg/ml). The expression of TNF-α and IκB-α was measured 6 hours after PHA stimulation, and IFN-γ 48 hours after PHA stimulation (time points at which maximal induction was observed). Gene expression was assessed by real-time polymerase chain reaction. Data are represented as fold change in gene expression levels of treated groups compared with untreated cells and correspond to three independent experiments performed in triplicate. *$p < 0.05$.*

Fig 3. Effects of methylthioadenosine (MTA) on the peripheral immune response during experimental autoimmune encephalomyelitis (EAE). (A) Myelin basic protein–specific (MBP; 10μg/ml) and mitogen (5μg/ml phytohemagglutinin [PHA]) proliferative response of splenocytes from placebo- (white bar) *or MTA-treated* (black bar) *Lewis rats obtained at time of death. Data are means ± standard error of the mean (SEM) of two independent experiments performed in triplicate.* *$p < 0.05$. *(B) MTA dose dependently inhibits the proliferation of splenocytes isolated from naive Lewis rats and stimulated with PHA (5μg/ml). Splenocytes were cultured for 2 hours with MTA before PHA addition. Data are means ± SEM of two independent experiments performed in triplicate.* *$p < 0.05$ *with respect to cells treated with PHA only;* **$p < 0.05$ *with respect to cells treated with 50μM MTA. (C) Gene expression profile of cytokines and inducible nitric oxide synthase (iNOS) assayed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) in the spleen of placebo-* (white bars) *and MTA-treated* (black bars) *Lewis rats at days 9 and 16 after immunization. Data are expressed as fold change in gene expression levels in the treated groups compared with untreated controls.* *$p < 0.05$. *IFN = interferon; IL = interleukin; TNF = tumor necrosis factor.*

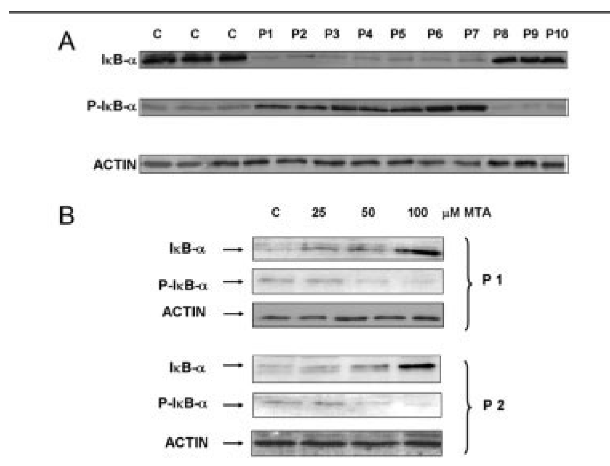

Fig 5. Inhibitor of kappa B (IκB-α) and phospho-IκB-α levels in peripheral blood mononuclear cells (PBMCs) from healthy subjects and multiple sclerosis (MS) patients and effect of methylthioadenosine (MTA) treatment. (A) Levels of IκB-α and P-Ser-32 IκB-α phosphorylation in PBMCs from MS patients and healthy control subjects. PBMCs were obtained under the same conditions from 3 healthy subjects (C1-3) and 10 MS patients (P1-10) and were analyzed by Western blotting. Equal loading was demonstrated using an antibody against Actin. (B) Dose-dependent effect of MTA levels of IκB-α and P-Ser-32 IκB-α in PBMCs obtained from two MS patients (P1 and P2) treated in vitro with the indicated concentrations of MTA for 2 hours. Equal loading was demonstrated using an antibody against actin. Representative blots are shown.

T-cell activation was preserved in PBMCs obtained from patients with MS. First, we observed that when compared with healthy control subjects, unstimulated PBMCs from 7 of 13 MS patients studied showed reduced levels of IκB-α protein and enhanced IκB-α phosphorylation (Fig 5A). This is in agreement with a recent publication in which PBMCs from patients with relapsing MS showed enhanced NF-κB DNA-binding activity.[28] Subsequently, we observed that treatment of PBMCs obtained from those MS patients with low levels of IκB-α ($n = 7$) with increasing concentrations of MTA resulted in a dose-dependent upregulation of IκB-α protein levels and the concomitant inhibition of IκB-α phosphorylation. Figure 5B shows two representative cases, and a similar response was obtained with PBMCs from the remaining five MS patients who displayed reduced levels of IκB-α (data not shown). MTA did not affect the IκB-α levels in PBMCs from MS patients with normal basal levels of IκB-α (data not shown). In summary, these observations indicate that MTA has an immunomodulatory effect both in rodents and humans, and that such effect is also present in immune cells from MS patients.

Discussion

We have shown that administration of the naturally occurring nucleoside MTA reduces the development of clinical signs in two models of EAE. Moreover, MTA treatment resulted in the prevention of acute EAE, but more importantly in the amelioration of the disease in the chronic-relapsing EAE model, which more closely resembles the clinical situation found in humans. Neural tissue damage was attenuated and inflammatory infiltrates were reduced in those animals that received MTA.

The mechanism of action of MTA is likely to be multifaceted. On one hand, MTA is known to exert direct antioxidant effects in vivo,[29] and an ability to attenuate the production of reactive oxygen species has been demonstrated to participate in the therapeutic effects of neuroprotective compounds in EAE models.[30] We believe, however, that more specific immunomodulatory effects are involved in the mechanism of action of MTA in EAE. We have previously demonstrated that MTA is a potent modulator of the innate immune response, preventing death in LPS-challenged mice.[3] Subsequently, it was shown by others that in vitro MTA treatment inhibited cytokine gene expression in isolated T lymphocytes and Jurkat T cells after TCR activation.[25,31] Here, we have provided evidence showing that MTA can modulate T-cell activation and TCR-conveyed signaling in vivo and in vitro, including the downregulation of proinflammatory cytokines and the enhancement of IL-10 production, effects that are likely related to the beneficial outcome of MTA administration in EAE.[30,32] Furthermore, our data suggest that modulation of NF-κB–mediated signaling may be central to the effects of this nucleoside on T-cell activation and cytokine production.[33] Indeed, we have shown that MTA inhibited the degradation of IκB-α on TCR activation in human PBMCs. On cellular stimulation (as occurs after TCR engagement), IκB proteins become phosphorylated by the IκB kinase complex, and these phosphorylations trigger the subsequent degradation of IκB proteins, which ultimately results in the release and nuclear translocation of NF-κB where it regulates the expression of its target genes.[17] We observed that MTA prevented the phosphorylation of IκB-α in PHA-stimulated human PBMCs, suggesting that one site of action of MTA could be located between the TCR and the IκB kinase complex. Interestingly, we also observed that PBMCs obtained from a significant number of MS patients displayed reduced IκB-α protein levels and enhanced IκB-α phosphorylation, suggesting that basal activation of the NF-κB pathway may be present in PBMCs from MS patients, as recently reported by others.[28] Currently, however, we do not have an explanation for the different levels of IκB-α protein found among the MS patients tested. Nevertheless, what we have consistently

observed is that MTA treatment of PBMCs from those MS patients with reduced IκB-α levels results in the attenuation of IκB-α phosphorylation and the recovery of IκB-α protein levels. Together, these data suggest that MTA has an immunomodulatory function and that, at least ex vivo, MTA can reverse the proinflammatory status of immune cells from MS patients.

Our findings also indicate that the effects of MTA are independent from the cAMP-PKA pathway, which is central to the antiinflammatory mechanism of the MTA analogue adenosine acting through the A_{2A} receptors.[19] Indeed, adenosine recently has been shown to suppress the activation of NF-κB in B lymphocytes through inhibition of IκB-α phosphorylation; however, contrary to our observations with MTA, these effects were dependent on PKA activation.[21] Nevertheless, the interaction of MTA with other adenosine receptors that mainly convey cAMP-independent immunomodulatory signals, such as the A_1 receptor, cannot be discarded.[18]

Lymphocytes appear to be highly dependent on transmethylation reactions for efficient activation and function.[26,34] As mentioned previously, one of the most prominent biochemical actions of MTA is the inhibition of methylation reactions.[1,2] Interference with arginine methylation at the amino terminus of the Nuclear Factor of Activated T Cells (NFAT) cofactor NIP45 has been proposed to mediate the inhibitory effect of MTA on cytokine gene expression in T lymphocytes.[25] Our observations indirectly suggest that activation of the NF-κB pathway on TCR stimulation also can be dependent on de novo methylation reaction. Supporting this notion is the finding that MTA effects on IκB-α phosphorylation and degradation were mimicked by DZA and AdOx, two different inhibitors of methylation reactions. The molecular pathway linking the TCR to NF-κB activation is complex, and so far is not completely known.[16,17] Of the many components of the cascade from the TCR to IκB kinase that could be potential targets of MTA, one likely candidate could be Vav-1, recently reported to undergo arginine methylation on T-cell activation.[35] Nevertheless, although suggestive, our in vitro experiments using transmethylation inhibitors do not allow us to conclude that the effects of MTA in the two models of EAE would be mediated exclusively through the impairment of a methylation reaction.

In our study, we focused on the role of MTA in T-cell activation. However, MTA might also have a role in the CNS innate immune system, mainly in microglia cells. In recent years, microglia have gained increasing attention in MS research, but also in the overall spectrum of neurodegenerative diseases, because they are the resident effector cells of the immune system, which might imply involvement in disease pathogenesis. Microglia have a dual role in the CNS. Microglia might amplify the effects of inflammation in the CNS and mediate cell degeneration. They might also have a protective effect of the CNS.[36] Recent evidences suggest that activated microglia might have a neuroprotective function (eg, by releasing neurotrophic factors or scavenging toxic compounds).[37,38] Because microglia need to be activated to exert their functions, we can hypothesize that some of the pathways involved in this process can be targeted by MTA, and thus modulate microglia activity and influence EAE outcome.

MTA is a well-tolerated drug, devoid of the unwanted effects of other methyltransferase inhibitors. It has been administered previously in both acute and chronic experimental models of liver injury and systemic inflammation, showing efficacy and a safe profile,[3] with an ID_{50} of 2.9± 0.4gm/kg (intramuscular) in rats.[29] In humans, MTA is also well tolerated. Twenty-eight individuals (21–48 years old) were treated with 100mg every 8 hours for 3 days or 600mg/day for 1 month without signs of toxicity.[39,40] Thus, MTA would be an excellent candidate drug to be tested in patients with MS because it is safe and is effective in preventing brain autoimmune attack. In addition, because MTA may have a pleiotropic mechanism of action in preventing T-cell activation, which is a critical step in autoimmune diseases, but without inducing immunosuppression, it appears an ideal candidate for combining with other immunomodulatory drugs to obtain a higher efficacy in stopping relapses and disease progression without increasing side effects. The immunomodulatory properties of MTA also provide a rationale for testing MTA in other autoimmune diseases in which the T-cell activation process plays a critical role, such as type 1 diabetes and rheumatoid arthritis.

This work was supported by the agreement between FIMA and the "UTE project CIMA," the Fundación Ramon Areces and the PIUNA program at the University of Navarra (P.V.), the Instituto de Salud Carlos III (C03/02, G03/015, and PI051098, C.B., E.R.G.T., F.J.C., M.A.A.), the Ministerio de Sanidad y Consumo (FIS PI020369, PI040819, and CP04/00123, E.R.G.T., C.B., M.S., M.A.A.), the Gobierno de Navarra (Ortiz de Landazuri, M.A.A., C.B., P.V.), the Ministerio de Educación y Ciencia (SAF 2004-03538 and SAF 2004-01855, M.A.A., F.J.C.), and fellowships from the Fundación Ramón Areces (B.M., H.H.).

We thank B. Fernandez, M. Mora, and C. Miqueo for their technical support. This work is dedicated to the memory of the neurologist E. Zaragoza, MD.

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