Ribavirin administration alters ectonucleotidase activities in experimental autoimmune encephalomyelitis

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Abstract. The role of extracellular purines and purinoreceptors in the pathophysiology of different neurological disorders is the focus of rapidly expanding area of research. Ectonucleotidases are the enzymes with multiple roles in extracellular nucleotides metabolism and regulation of nucleotide-based intercellular signaling. The aim of present study was to investigate the changes in the ATP, ADP and AMP hydrolyzing activities after ribavirin treatment in spinal cord during experimental autoimmune encephalomyelitis (EAE). Our results demonstrate that ribavirin itself had no significant effect on ectoenzyme activities, when tested *in vitro* and *in vivo* on spinal cord crude membrane preparation of intact animals. We observed significant increase in ATP, ADP and AMP hydrolyzing activity in the spinal cord crude membrane preparation in EAE animals at 15 days post immunization compared to control animals. The increase was registered at 28 days post immunization, as well. At same time points, ribavirin treatment decreased ATP, ADP and AMP hydrolyzing activity compared to EAE animals. In addition, no significant changes 8 days post immunization was observed between EAE-induced and ribavirin- treated EAE animals and these levels were similar to control level. Thus, we suppose that ribavirin-induced alteration in ectonucleotidase activities is rather due to its suppression of inflammation, than to its direct action on ATP, ADP and AMP hydrolysis.

Key words: Ribavirin — EAE — Rat spinal cord — Ectonucleotidase activity

Introduction

Purine nucleotides, such as ATP and adenosine, represent a ubiquitous class of extracellular signaling molecules crucial for normal functioning of the nervous system. Recently, extracellular nucleotides such as ATP and adenosine have become clearly recognized to play an important role in modulating immune processes (Dwyer et al. 2007). Extracellular ATP and ADP are important signaling molecules in inflammatory events operative through the activation of specific P2X and P2Y receptors that are expressed on many cell types (Yegutkin 2008). Activation of these receptors regulates lymphocyte and leukocyte functions such as cytokine secretion and/or migration (Langson et al. 2003; Jalkanen and Salmi 2008). On the other hand, adenosine, an end product of nucleotide hydrolysis, has potent anti-inflammatory and immunosuppressive properties (Haskó et al. 2005).

Levels of extracellular nucleotides and the generation of adenosine are tightly regulated by cell surface ectoenzymes known as ectonucleotidases (Zimmermann 2001). The most relevant ecto-enzymes involved in adenine nucleotide extracellular hydrolysis are NTPDase (E.C. 3.6.1.5, ecto-nucleoside triphosphate phosphohydrolase, ecto-NTPDase/CD39) and ecto-5'-nucleotidase (E.C. 3.1.3.5, CD73) (Colgan et al. 2006). NTPDase is a membrane-bound enzyme that

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hydrolyzes ATP and ADP to AMP, which is subsequently converted to adenosine by ecto-5'-nucleotidase (Birk et al. 2002; Leal et al. 2005). CD39 has been identified originally as a cell-surface glycoprotein expressed on activated B-cells after cell activation (Maliszewski et al. 1994). CD39 is also expressed on NK-cells and subsets of T-cells, vascular endothelial cells, macrophages, and dendritic cells (Kansas et al. 1991). Wang and Guidotti (1996, 1998) have shown that CD39 is expressed widely in the glial cells and neurons in the rat brain. CD73 is a 70-kDa cell surface enzyme expressed on many cell types including subsets of on subsets of B-cells and T-cells (Thompson et al. 1987; Yang et al. 2005), endothelial cells (Narravula et al. 2000), neurons (Maienschein and Zimmermann 1996) and epithelial cells (Strohmeier et al. 1997). Ecto-5'-nucleotidase activity is also found on astrocytes, oligodendrocytes, microglial cells and brain endothelial cells (Robson et al. 2006).

Multiple sclerosis (MS) is the most common chronic demyelinating disease of the CNS of unknown etiology and the major disabling neurological illness of young adults (Noseworthy et al. 2000). Several animal models have been applied for mimicking pathophysiological events in MS and experimental-induced autoimmune encephalomyelitis (EAE) is the best avaliable model so far (Gold et al. 2006). Early events in the pathogenesis of MS/EAE are associated with breakdown of blood brain barrier, massive infiltration of activated mononuclear cells to the CNS, production of different inflammatory mediators leading to demyelination (Compston and Coles 2002) and axonal loss (Bjartmar et al. 2003; Lisak 2007).

Recently, it was shown that CD39-null mice can spontaneously develop features of autoimmune diseases associated with Th1 immune deviation (Dwyer et al. 2007) and that CD73-null mouse are resistant to EAE (Mills et al. 2008), implying the significance of these enzymes in control of autoimmune response.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Virazol) is a synthetic guanosine analogue, an agent approved as a broad-spectrum antiviral drug (Sidwell et al. 1972). The mechanism of its action includes inhibition of inosine-monophosphate dehydrogenase (IMPDH), an enzyme necessary for de novo synthesis of purine nucleotides and causes interruption of cell metabolism by reducing cellular guanilate concentration (Weber et al. 2003). Earlier studies have shown that ribavirin exerts immunosuppressive activity (Peavy et al. 1980; Jolley et al. 1988; Heagy et al. 1991). We have recently described suppressive effect of ribavirin on the development of actively induced EAE in rats (Milicevic et al. 2003).

Extracellular ATP induces secretion of cytokines involved in the progression of neurodegenerative diseases, like IL-2, IFN-γ, IL-1-β and TNF-α from the activated lymphocytes (Langston et al. 2003; Bours et al. 2006), macrophages (Ferrari et al. 1997) and microglial cells (Hide et al. 2000), whereas adenosine inhibits the production of pro-inflammatory cytokines (Bours et al. 2006). The expression of pro-inflammatory cytokines is positively correlated to the disease induction and progression of EAE (Merril and Benveniste 1996) and it was shown that these cytokines induce upregulation of ecto-5'-nucleotidase on peritoneal macrophages (Savic et al. 1990) and NTPDase expression on lymphocytes (Duensing et al. 1994). We have shown in our previous study (Lavrnja et al. 2008) that ribavirin alters the balance between pro- and anti-inflammatory cytokines in favor of the latter (Lavrnja et al. 2008), which might contribute to the beneficial effect in EAE. However, the exact cellular and molecular mechanisms that underlie the suppression of EAE by of ribavirin have not been fully understood yet. Hence, the aim of this study has been to investigate whether previously described positive effects of ribavirin treatment during EAE could lead to alteration in ecto-enzyme activities *via* adenosine-mediated anti-inflammatory and neuroprotective effects.

Materials and Methods

Experimental animals

Female Dark Agouti (DA) rats, 8 weeks of age, were used throughout the experiments. The animals, obtained from the animal colony maintained at the Institute for Biological Research, were divided into groups of 5 per cage, matched by weight (130–160 g). They were housed under conventional conditions with laboratory chow and water *ad libitum,* and were watered by hand during the period of paralysis. Experimental protocols were approved by the Local Animal Care Committee and conformed to the recommendations given in "Guide for the Care and Use of Laboratory Animals" (National Academy Press, Washington D.C., 1996).

EAE was induced as described previously (Milicevic et al. 2003; Lavrnja et al. 2008). Briefly, rats were immunized by intradermal injection of 100 μl emulsion of rat spinal cord homogenate (50% w/v in saline) and complete Freund's adjuvant containing 1 mg/ml Mycobacterium tuberculosis (Sigma, St. Louis, MO, USA) divided in half and injected in both hind footpads.

Ribavirin (ICN Pharmaceutical Costa Mesa, CA, USA) was dissolved in saline and daily administered intraperitoneally (i.p., 30 mg/kg), from the day of immunization until the end of experiment. The dose of ribavirin was chosen on the basis of previous studies (Milicevic et al. 2003; Lavrnja et al. 2008). Control EAE rats received the equal volume of saline. All immunized animals irrespective of treatment were compared to intact, control rats.

Animals from EAE and ribavirin-treated and/or EAE rats were sacrificed by a decapitation on 8 dpi (day post immunization) (E8 and R8, respectively), 15 dpi (E15 and R15, respectively) or 28 dpi (E28 and R28, respectively).

Immunized animals were daily monitored for clinical signs of EAE, which were graded on scale of 0–5 as follows: 0 – no clinical signs; 1 – flaccid tail; 2 – hind limb paresis; 3 – hind limb paralysis; 4 – moribund state, 5 – death of the animal.

The influence of ribavirin administration on ectonucleotidases activity in intact animals was investigated in the independent experiment, where one group of intact animals was given ribavirin for 15 days at daily dose of 30 mg/kg i.p., while another group of intact animals served as control (data not shown).

Histochemistry

At sacrifice, lumbosacral regions of spinal cords were dissected and fixed in Bouin's solution for 48 h, then dehydrated in a series of alcohol, immersed in xylene and embedded in paraffin. Hematoxylin-eosin staining on 5 μm paraffin thick transverse sections of spinal cord sections was performed according to standard procedures.

Crude membrane preparation

After decapitation, lumbosacral segments of spinal cords were rapidly removed from EAE-induced, ribavirin-treated EAE and control, intact rats. Crude membrane preparation was isolated by homogenization of the obtained tissue in the buffer containing 0.32 mol/l sucrose and 0.005 mol/l Tris pH 7.4, followed by centrifugation at 1000 × *g* for 10 min at 4°C, as previously described for the brain tissue (Nedeljkovic et al. 2003). Resulted supernatant was centrifuged at 12,000 × *g* for additional 30 min, and pelleted membrane preparation was resuspended and homogenized in 0.005 mol/l Tris, pH 7.4. The protein contents were determined by the method of Markwell et al. (1978), using bovine serum albumin as a standard.

Ectonucleotidase assays

Rates of ATP, ADP and AMP hydrolysis were measured by colorimetric determination as previously described (Nedeljkovic et al. 1998). Briefly, the ATP and ADP hydrolyzing activity was measured in reaction mixture containing: 50 mmol/l Tris-HCl (pH 7.4), 0.5 mmol/l EDTA, 5 mmol/l $MgCl_2$, 1 mmol/l ATP or ADP and 20 μg of membrane preparations in a final volume of 200 μl. Reaction was started by the addition of ATP or ADP to the mixture and proceeded for 15 min. Medium for measuring AMP hydrolyzing activity contained: 100 mmol/l Tris-HCl (pH 7.4), 10 mmol/l $MgCl_2$, 1 mmol/l AMP and 50 μg of membrane preparations in a final volume of 200 μl. Reaction was started by the addition of AMP and allowed to proceed for 30 min. Incubations were carried out in a water bath at 37°C. Reactions were stopped by the addition of 3 mol/l perchloracetic acid. All samples were chilled on ice and used for the determination of liberated inorganic phosphate (Pi) (Penniall 1966).

In vitro assays for the estimation of ribavirin effect on ectonucleotidase activities

In these assays crude membrane preparations obtained from spinal cords of intact animals were used. Ribavirin was added to the reaction mixtures to reach 0.01, 0.1, 1 or 10 μg/ml final concentrations. ATP and AMP hydrolysis was then measured as described above.

Data analysis

All samples were run in duplicate in $n > 3$ independent determinations. The ATP and ADP hydrolyzing activities were expressed as μmol Pi/mg proteins/min ± SD. The AMP hydrolyzing activity was expressed as nmol Pi/mg proteins/min ± SD. Significance of differences between groups was determined using Student's *t*-test for paired samples. The values of $p \leq 0.05$ were considered statistically significant ($p \leq 0.05$; $p \leq 0.005$).

Results

Clinical assessment of EAE and the effect of ribavirin on EAE development

After active immunization with whole rat spinal cord homogenate in complete Freund's adjuvant, all rats (100%) in both EAE-induced and ribavirin-treated animals developed acute monophasic disease. In accordance with previous experiments (Milicevic et al. 2003; Lavrnja et al. 2008), first signs of EAE were observed at 9–10 days after the induction of disease in both groups, were increased and peaked at day 14–15 dpi and period of recovery was prolonged until day 22 Pi (data not shown). Rats treated with ribavirin developed milder form of disease as evaluated by mean maximal severity score, disease index and duration of disease in respect of EAE rats, thus confirming previously demonstrated beneficial effects of the drug (Milicevic et al. 2003; Lavrnja et al. 2008). Hematoxylin-eozin staining revealed massive infiltration in white matter in lumbosacral regions of EAE-induced animals in 15 dpi and inflammation persists until the end of experiment 28 dpi (data not shown). Ribavirin treatment reduces inflammation at both examined time points (data not shown).

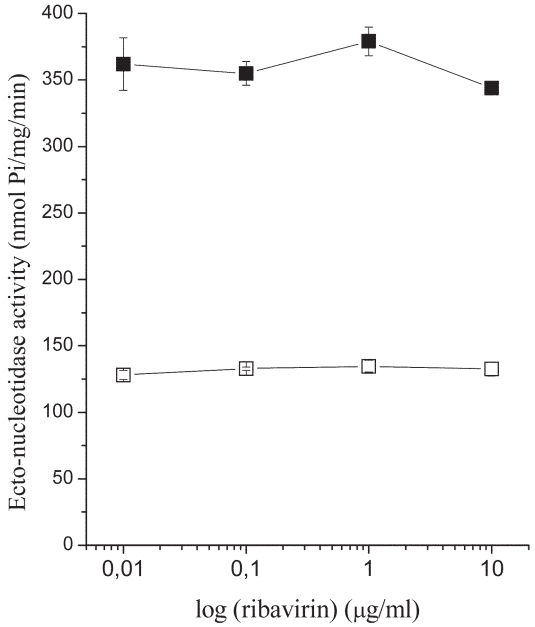

Figure 1. Effect of ribavirin *in vitro* on ATPase and AMPase hydrolysis in the crude membrane preparation of spinal cord. Ribavirin was added to the crude membrane preparation of spinal cord of control healthy animals and the results from 3 different experiments performed in duplicate of *in vitro* on ATPase (solid symbols) and AMPase (empty symbols) hydrolysis are shown. Ribavirin concentrations are given in μg/ml (0.01, 0.1, 1 and 10; logarithmic scale).

In vitro effect of ribavirin on ectonucleotidase activities in the spinal cord crude membrane preparation

In order to investigate *in vitro* effect of ribavirin on ectonucleotidase activities, we added the pure substance to the reaction mixture, in final concentrations of 0.01, 0.1, 1 and 10 μg/ml. Obtained results show that, at any concentration tested, ribavirin by itself did not significantly affect rates of ATP and AMP hydrolyses in the spinal cord crude membrane preparation of control healthy animals (Fig. 1).

Temporal changes in ATP, ADP and AMP hydrolyzing activities in spinal cords of EAE and ribavirin-treated EAE rats

Time-course changes in ATP, ADP and AMP hydrolysis in the spinal cord plasma membrane preparations obtained from control, EAE-induced and ribavirin-treated EAE animals are presented in Fig. 2. Levels of ATP-, ADP- and AMP-hydrolysis at 8 dpi in EAE-induced and ribavirin-treated EAE animals remain unchanged in respect to the corresponding control levels (Fig. 2). However, at 15 dpi, a significant increase in ATP (53%, $p < 0.001$), ADP (33%, $p < 0.001$) and AMP (43%, $p < 0.001$) hydrolysis in EAE-

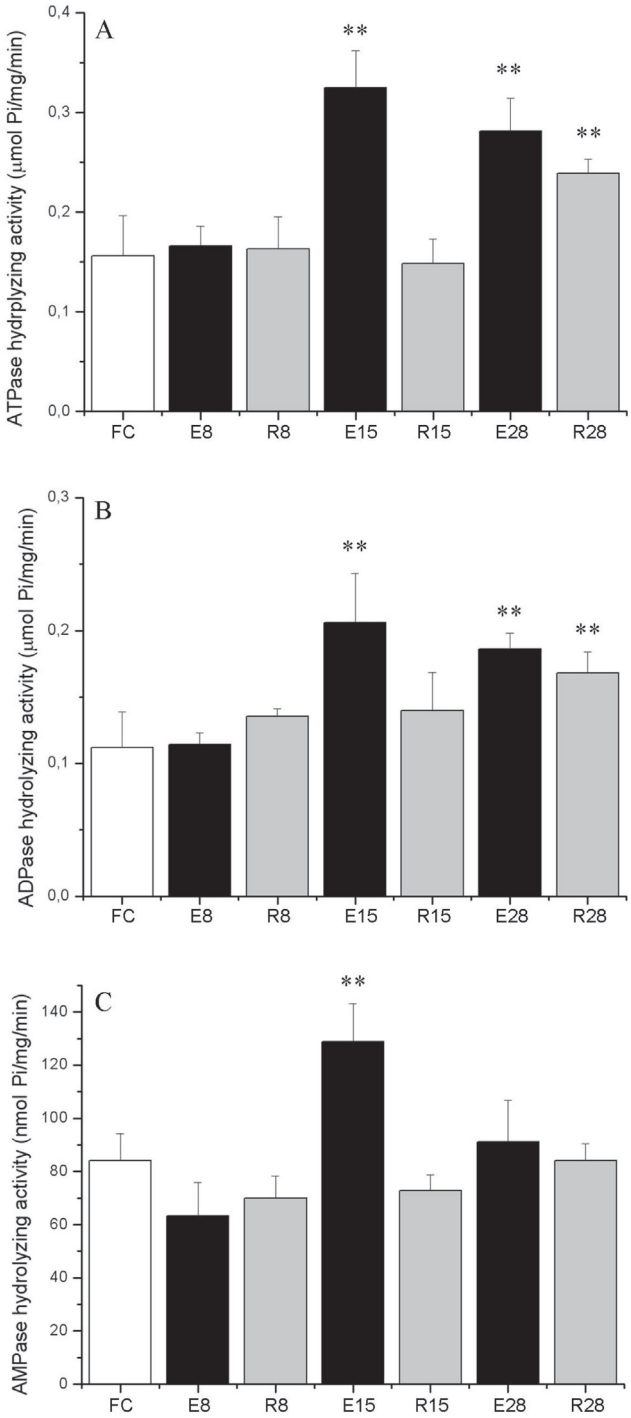

Figure 2. ATP (A), ADP (B) and AMP (C) hydrolyzing activities in the crude membrane preparations of lumbosacral segments of spinal cord of control (FC) (white bars), EAE (black bars) from 8, 15 and 28 days after immunization (E8, E15 and E28) and ribavirin-treated rats (gray bars) from 8, 15 and 28 days after immunization (R8, R15 and R28) ($n = 5$). The results are expressed as mean specific activities ± S.D. from 5 different experiments performed in duplicate. Activities obtained from EAE and ribavirin-treated animal rats were compared with control with significance levels (Student's *t*-test): ** $p < 0.005$.

induced animals was observed when compared to control group (Fig. 2). Similarly, at same time point, EAE group showed significant increase in ATP (50%, $p < 0.001$), ADP (48%, $p < 0.001$) and AMP (35%, $p < 0.001$) hydrolysis when compared to ribavirin-treated animals while no statistical difference was observed between ribavirin-treated EAE animals and control group. At 28 days after immunization, although ectonucleotidase activities in EAE-induced group decreased in relation to previous time point tested, ATP and ADP hydrolysis remained significantly increased in respect to the control group and ribavirin-treated group, ATP (42%, 33%, $p < 0.001$, respectively) and ADP (40%, 35%, $p < 0.001$, respectively) hydrolyzing activities. At same time point, AMP hydrolysis in EAE animals, although significantly decreased in respect to previous time point remained unchanged in respect to ribavirin-treated and control group (Fig. 2). No significant differences was observed in AMPase activity between ribavirin-treated EAE group in all examined times.

Discussion

In the present study we investigated the ability of ribavirin to interfere with the ectonucleotidase activities during EAE actively induced in highly susceptible DA rats (Stosic-Grujicic et al. 2004). Our results demonstrate the significant increase in ATP, ADP and AMP hydrolyzing activities in spinal cords of EAE-induced animals in the peak of disease. This increase was sustained until the end of disease. Since adenosine, the end product of ecto-enzymatic cascade has anti-inflammatory and neuroprotective effects, we analyze whether previously described beneficial effects of ribavirin in EAE (Milicevic et al. 2003) lies in alternation of ectonucleotidase activities in favor of generation of adenosine. In the present study, we have shown that ribavirin treatment significantly decreased ectonucleotidase activities to almost control levels in both examined time points.

The main findings of our study are that EAE animals express increased ectonucleotidase activities in spinal cord membrane preparations in respect to ribavirin-treated animals and the most prominent alterations coincide with clinical score and most pronounced histopathological changes (Lavrnja et al. 2008), that is in the peak of disease. Since extracellular ATP levels increase in injured tissue (Yegutkin 2008), it is possible that the up-regulation of ectonucleotidase activities in EAE animals is caused by inflammation, demyelination, axonal loss and reactive astrogliosis, common features observed in spinal cord after the induction of EAE, as we have shown in our previous studies (Milicevic et al. 2003; Stojkov et al. 2008). It has been previously shown that under inflammatory conditions, release of ATP can contribute to neurodegeneration in MS/EAE (Le Feuvre et al. 2002) and as it was already proposed, demyelination itself, could increase extracellular ATP levels and consequently lead to activation of ectonucleotidases (Spanevello et al. 2006). Thus, if inflammation and demyelination is a trigger for ectonucleotidase up-regulation, ribavirin could impede this cascade simply by preventing lymphocyte infiltration to CNS. Although the mode of action in the modulation of induced EAE is unknown, we proposed that ribavirin exerts these effects through inhibition of IMPDH and depletion of guanilate pools by inhibiting recruitment of activated lymphocytes, macrophage/microglia (Milicevic et al. 2003) and reactive astrocytes (Pekovic et al. 2005). Therefore, lower ectonucleotidase activities observed in the present study could occur as a result of ribavirin action on lymphocytes and glial cells, rather than as a result of direct action of this drug on these enzymes. Our results also demonstrated that no significant changes 8 days post immunization was observed between EAE-induced and ribavirin-treated EAE animals and these levels were similar to control level. Since no histopathological changes could be seen in both examined groups (data not shown), at this time point the absence of ectonucleotidase activities alteration confirms that it reflects tissue injury induced by the autoimmune attack.

We also tested the possibility of ribavirin to interfere with nucleotide hydrolysis. The *in vitro* effect of ribavirin on the hydrolysis of ATP and AMP was also studied. When added in the incubation mixture with the crude membrane preparation obtained from intact animals, ribavirin did not interfere neither with ATP nor AMP hydrolysis. Since ribavirin is a prodrug that must be converted to its nucleotide metabolites to exert activity (Parker 2005), we assumed that its active metabolite generated *in vivo* might be involved in decreased ectonucleotidase activities observed in this study. Therefore, we also investigated the effect of prolonged ribavirin treatment on intact animals and measured ATP and AMP hydrolyzing activities. Likewise, *in vivo* ribavirin treatment had no impact on ATP or AMP hydrolysis in the spinal cord crude membrane preparations of intact animals.

Recently, it was reported that CD73-generated adenosine restricts lymphocyte migration into draining lymph nodes (Takedachi et al. 2008), which is an absolute prerequisite for autoimmune damage to the CNS (Gold et al. 2006). On the contrary CD73 is required for efficient entry of lymphocytes into CNS during EAE (Mills et al. 2008) and lymphocyte-endothelial interaction regulates CD73 activity (Jalkanen and Salmi 2008), implying the importance of this enzyme in regulation of lymphocyte migratory pattern, an event shown to be critical step in the pathogenesis of MS/EAE (Engelhardt 2006). We previously hypothesized that ribavirin prevents the development of EAE in DA rats by inhibiting the proliferation of encephalitogenic cells in the lymph node draining the site of inoculation and affects migration of encephalitogenic T lymphocytes to the target tissue (Lavrnja et al. 2008). Therefore, the observed decrease

in ATP, ADP and AMP hydrolysis in ribavirin-treated animals in the peak and at the end of disease seems expected. Furthermore, it was previously shown that other IMPDH inhibitors like tiazofurin and mycophenolate mofetil attenuate clinical symptoms in EAE by affecting cell-cell interaction and suppressing glycosylation and the binding activity of various adhesion molecules (Tran et al. 2001; Stosic-Grujicic et al. 2002), thus participating in regulation of lymphocyte migratory pattern.

It was recently reported that CD39 is important component of $CD4^+/CD25^+/Foxp3^+$ T regulatory cells (Deaglio et al. 2007), acting through removal of proinflammatory ATP (Borsellino et al. 2007) and in concert with CD73 participates in generation of immunosuppressive adenosine (Dwyer et al. 2007), leading to the inhibition of T-cell proliferation and pro-inflammatory cytokine secretion (Bours et al. 2006; Kobie et al. 2006). Therefore, an observed increase in nucleotide hydrolysis in EAE animals could be related to compensatory response to inflammation, demyelinization and axonal loss decreasing ATP availability and consequently contributing to the production of adenosine and possibly recovery of disease.

It is proposed that CD73-generated adenosine appears to be beneficial in fight against inflammation (Thompson et al. 2004). For that reason, the primary therapeutic option with CD73 is to increase its enzymatic activity and expression. It was reported that IFN-β treatment, one of the most effective treatment in MS, increased expression of CD73 and consequently decrease the transmigration of lymphocytes through blood-brain barrier (Niemelä et al. 2008). Hence, the increased production of adenosine at least in part can contribute to the beneficial effect of IFN-β. Therefore, the observed decrease in ectoenzyme activities in ribavirin-treated animals seems unexpected. On the other hand, it is important to note that CD73 and extracellular generated adenosine are needed for the efficient migration of T-cells to CNS and mice lacking CD73 is resistant to the induction of EAE (Mills et al. 2008), implying that inhibition of CD73 could be valuable approach in treating MS.

In summary, we have shown that ribavirin can affect the ecto-nucleotidase pathway during EAE. Altered ectonucleotidase activities observed after ribavirin treatment are rather due to its suppression of inflammation and demyelination, than due to its direct action on ATP, ADP and AMP hydrolysis.

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