Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the human central nervous system (CNS), which culminates in progressive neurological deterioration. Experimental autoimmune encephalomyelitis (EAE) is the best existing animal model of MS. Distinctions of MS/EAE pathogenesis are as follows: compromising of the blood–brain barrier, infiltration of the CNS by myelin reactive CD4+ T cells and macrophages (1), activation of CNS cells (microglia and astrocytes), and demyelination and axonal loss (2). EAE is a helpful tool for investigating immunological aberrations involving several CNS cell groups (astrocytes, microglia, and oligodendrocytes). Glial activation is a well-known characteristic of many CNS diseases; therefore the focus of this study was to describe the astrocyte reaction following the EAE onset in immunized and ribavirin treated Dark Agouti rats.

Astrocytes are the major glial cell within the CNS and have a number of important physiological properties related to CNS homeostasis. They are involved in the regulation of brain microenvironment, and upon inflammation, astrocytes and microglia are activated and can act as immune competent cells (3). The astroglial production of trophic factors and elimination of neurotoxins are likely to fulfill important protective and reparative functions during CNS infection. In addition, astrocytes could, in concert with microglial cells, regulate the recruitment and activity of infiltrating hematogenous cells through their expression of cytokines, proteases, protease inhibitors, adhesion molecules, and extracellular matrix com-
ponents (4). It has been shown that they have significant functions in the inflammatory and immune responses in MS (5). Under pathological circumstances, reactive astrocytes could have beneficial roles in defense and repair. However, MS/EAE glial activation also involves microglia, and their interaction with astrocytes may contribute to secondary nerve-cell damage (6). In addition, reactive glial cells release inflammation-promoting mediators and oxidative radicals (7). Astrocytes and microglia enter into a communication via cytokines (3), and they are involved in the balance of destructive vs. protective actions that characterizes the pathogenesis of neurological disorders (4). For these reasons, considerable effort is being directed toward understanding the etiology of reactive gliosis and identification of substances that could modulate proliferation of glial cells.

Currently, therapeutic strategies for MS mainly target the immunological aspects. Despite significant advances, many patients do not respond optimally to these drugs (8) and further improvement in MS therapeutics is necessary, either through the development of new medication or combinations of the existing ones.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Virazol) is a synthetic guanosine analogue and displays well-known antiviral activity against several RNA viruses involved in neurological damage in humans and animals (9 – 11); and it is used to treat influenza, parainfluenza, herpes, Lassa fever, measles, chicken pox, and respiratory syncytial virus (RSV) (12). Ribavirin is already in clinical practice against hepatitis C virus / human immunodeficiency virus co-infected patients (13). Ribavirin is a potent inhibitor of type II IMPDH enzyme, which is expressed in activated lymphocytes (14, 15). After enzymatic conversion in sensitive cells, ribavirin acts through its active metabolite. Ribavirin is metabolized mainly into RTP (RBV-triphosphate), RDP (RBV-diphosphate), and RMP (RBV-monophosphate). RMP blocks the target enzyme at the IMP binding site (16). Generally, ribavirin is well tolerated. The most common side effect after prolonged administration of ribavirin is frequently associated with hemolytic anemia, starting at dose of 50 mg/kg in rats, whose severity correlates with dose level. However, this effect is reversible, and disappears upon reduction or cessation of treatment (17 – 19).

Concerning obscure data about ribavirin’s effect on reactive astrogliosis in EAE, the present paper is focused on astrocytes and their response to ribavirin treatment during various time points post-immunization. Since the hallmark of reactive astrocytes is the upregulation of glial fibrillary acidic protein (GFAP) and vimentin, in this study we investigated the effect of ribavirin treatment on tissue and gene expression pattern of vimentin and GFAP in EAE rats.

**Materials and Methods**

**Induction and treatment of EAE**

Inbred two-month-old female Dark Agouti rats were maintained at the animal facility of the Institute for Biological Research “Sinisa Stankovic” (Belgrade, Serbia) in accordance with institutional guidelines. Animals were housed (three to five rats per cage) under conventional conditions with laboratory chow and water ad libitum and given water 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).

Animals were randomly organized into the following groups (n = 6 within each group): control group (C), intact rats; EAE group, EAE was induced as described previously (20). Briefly, rats were immunized by intraperitoneal 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. In the Ribavirin group (R), EAE rats were treated with ribavirin (ICN Pharmaceuticals, Costa Mesa, CA, USA), which was dissolved in saline and daily administered intraperitoneally (30 mg/kg), from the onset of immunization until the end of the experiment (at 28th day post immunization, 28 dpi). The dosage of ribavirin (30 mg/kg) used in our study was chosen on the basis of earlier reports (19 – 21), which have shown that there were no drug-related mortalities or overt signs of toxicity, except a slight decrease in hematocrit. EAE-untreated rats received an equal volume of saline. After the induction of EAE, the rats were daily monitored for their neurological signs.

**Clinical evaluation**

Rats were examined, weighed, and scored daily in a double-blind manner for signs of EAE for up to 28 days after immunization. The scores were graded according to the clinical severity of neurological symptoms on a scale of 0 – 5, with 0.5 points for intermediate clinical findings: grade 0, no abnormality; grade 0.5, partial loss/reduced tail tone, assessed by inability to curl the distal end of the tail; grade 1, tail atony; grade 1.5, slightly/moderately clumsy gait, impaired righting ability, or combination; grade 2, hind limb weakness; grade 2.5, partial hind limb paralysis; grade 3, complete hind limb paralysis; grade 3.5, complete hind limb paralysis and fore limb weakness; grade 4, tetraplegic; grade 5, moribund state.
or death. Scores of the two investigators, who were kept unaware of the protocol, were averaged. Data were plotted as daily mean clinical score, calculated by averaging the individual scores obtained for all animals in a particular treatment group.

**Tissue preparation, histology, and immunohistochemistry**

After the animals were sacrificed, lumbosacral regions of spinal cords were dissected and fixed in Bouin solution for 48 h, and then dehydrated in a series of alcohol, immersed in xylene, and embedded in paraffin. The spinal cord tissue was obtained from EAE (n = 3) and ribavirin-treated rats (n = 3) at 15 dpi (E15 and R15) and at 28 dpi (E28 and R28).

Five micrometers of transversal lumbosacral sections (L1 – L5) were cut and five consecutive slices were placed on the same glass slide. Histological and immunohistological data were determined on 5 – 7 sections per rats. A series of sections was stained with hematoxylin and eosin (H&E) and Luxol Fast Blue (LFB) to detect inflammatory infiltrates and demyelination. Immunohistochemistry was performed on 5-μm-thick paraffin coronal sections of the spinal cord. To optimize immunohistological staining, the following microwave antigen retrieval technique was used: The sections were immersed in citrate buffer, pH 6.0, in a microwave oven at 700 W for 10 min and cooled to room temperature. Then, immunostaining was routinely performed. After washing in PBS, the sections were blocked by 10% normal rabbit serum (for GFAP staining) or 10% normal goat serum (for vimentin and SMI-31). Sections were then routinely stained for glial markers using the following primary antibodies: rabbit anti-GFAP (1:500; Dako, Glostrup, Denmark), mouse anti-vimentin (1:200, Dako), and mouse anti-SMI-31 (1:1000; Steinberger, Lutherville, MD, USA). Sections were incubated with appropriate peroxidase-linked secondary antibody (1:200, Dako). Bound antibodies were visualized with 3,3’-diaminobenzidine-tetrahydrochloride (DAB, Dako). The specificity of the staining was tested on the sections in the second dish by omission of the primary specific antibodies. No immunoreactive products were found on these sections. After dehydration and clearing, sections were mounted with DPX Mounting medium (Fluka, Buchs, Switzerland) and examined under a Zeiss Axiovert microscope (Carl Zeiss GmbH, Vienna, Austria).

For the semi-quantification of our results, the astroglial reaction was rated using a 5-point grading scale: 4 (strong), 3 (moderate), 2 (mild), 1 (partial/weak), 0 (nil). A distinct GFAP staining without astrocyte hypertrophy was rated as mild, and an intense GFAP staining with astrocyte hypertrophy was rated as strong (22).

**Tissue processing for gene expression studies**

Rats were deeply anesthetized with Zoletil (30 mg/kg, i.p.) and lumbosacral regions of spinal cords (n = 3 per group) were rapidly frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total cellular RNA was isolated from the spinal cord tissue using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) as per manufacturer’s instructions. RNA purity and concentration were insured by gel visualization and spectrophoto metrically by calculating the ratio between the absorbance at 260 and 280 nm. The absorbance ratio for all samples ranged between 1.8 and 2.1. RNA was transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions.

**Real time PCR**

TaqMan PCR reactions were performed with Assay-on-Demand Gene Expression Products (Applied Biosystems) for GFAP (Assay ID Rn00566603_m1). Reactions were performed in a 25-μl reaction mixture containing 1 × TaqMan Universal Master Mix with AmpErase UNG, 1 × Assay Mix (Applied Biosystems) and the cDNA template (10 ng of RNA converted to cDNA). PCR reactions were carried out in the ABI Prism 7000 Sequence Detection System (Applied Biosystems) at 50°C for 2 min, at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and at 60°C for 1 min. The experimental threshold was calculated from the mean baseline fluorescence signal from cycles 3 to 15, plus 10 standard deviations. Each sample was run in triplicate and a mean value of each Ct triplicate was used for further calculation. A reference, endogenous control, was included in every analysis to correct the differences in inter-assay amplification efficiency, and the expression of each gene was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression. The obtained results were analyzed by RQ Study Add ON software for 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System) with a confidence level of 95% (P < 0.05).

The cDNA products were used for quantitative real-time PCR carried out with the SYBR Green PCR Master Mix gene expression assay (Applied Biosystems) according to the manufacturer’s instructions. Reactions were performed on an ABI Prism 7000 apparatus. Results were presented as the difference in the number of cycles to reach the detection threshold (Ct, cycle at threshold), using GAPDH (Invitrogen Life Technologies) as the internal reference standard (ΔCt = Ctcytokine – CtGAPDH). ΔCt was presented as relative fold change in gene expression (mean ± S.E.M.). The following primer pairs (Invitrogen Life Technologies) were used: GAPDH (157 bp product): forward, TGGACCTCATGGCCTACAT and reverse, TGGAGGATGCTGGTTGTTTG.
GGATGGAATTGTGAGGGAGA; vimentin (87 bp product): forward, CGTACGTCAGCAATATGAAAGTG and reverse, TCAGAGAGGTCAGCAAACTTGGA. GAPDH and vimentin were amplified at an annealing temperature of 60°C for 40 cycles; TNF-α (209 bp product): forward, CTCCCAGAAAAGCAAGCAAC and reverse, CGAGCAGGAATGAGAAGGG; IL-10 (105 bp product): forward, GCTCAGCACTGCTATGTGTC and reverse, GTCTGGCTGACTGGGAAGTG; transforming growth factor beta (TGF-β): forward, ACGGTGATGCGGAAGCAC and reverse, CCCTGCCCTACATTGGG.

Data analysis
All data were collected and analyzed by researchers kept unaware of the treatments received by the animals, including the induction of disease and reagents used. Results were presented as the mean ± S.E.M. Significance of difference between the data obtained for different groups was determined by Student’s t-test. The values of $P \leq 0.05$ and $P \leq 0.005$ were considered statistically significant.

Results

Ribavirin treatment suppresses 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 (20, 23), first signs of EAE were observed at 8 – 9 days after the induction of disease in both groups, peaked at 12 – 15 dpi, and the period of recovery was prolonged until 23 dpi (Fig. 1). EAE rats treated with ribavirin developed a milder form of the disease, thus confirming previously demonstrated beneficial effects of the drug compared to untreated EAE rats (20, 23). In addition, the group of animals that received only Complete Freund adjuvant sacrificed on 15 and 28 dpi compared to intact-physiological control revealed no statistically significant difference (data not shown).

Ribavirin decreases infiltration in EAE rats
H&E staining revealed massive infiltration in white matter in lumbosacral regions of EAE induced animals at 15 dpi (Fig. 2A) and inflammation persists until the end of the experiment at 28 dpi (Fig. 2B). Ribavirin treatment reduces inflammation at both examined time points (Fig. 2: C, D).

Demyelination and axonal loss were ameliorated by ribavirin treatment during EAE
Large area of axonal loss in lateral funiculus and part of the gracile fasciculus were observed in EAE rats 15 dpi (Fig. 3: A, C), followed by the presence of a large plaque of demyelination in the same areas of axonal loss (Fig. 3: B, D). These changes were more prominent at 28 dpi and areas of myelin pallor were seen in EAE rats (Fig. 3: F, H) that coincide with areas of increased axonal loss (Fig. 3: E, G). Axonal loss and demyelination were markedly attenuated after ribavirin treatment at 15 dpi (Fig. 3: I, J) and 28 dpi (Fig. 3: K, L).

Effects of ribavirin on GFAP+ expression in lumbosacral spinal cord during EAE
Immunohistochemical analysis of 5-μm-thick paraffin lumbosacral sections of the spinal cord of EAE rats obtained at the peak of disease (15 dpi) revealed a huge number of GFAP+ astrocytes in all analyzed regions (Fig. 4). GFAP immunoreactivity in white matter contained moderately labeled fibrous astrocytes processes, sending processes in deep white matter to the pial surface where they formed a portion of glia limitans (Fig. 5: A, C). In spinal cord gray matter, immunostaining was of moderate density with small cell bodies and long, thin extensions (Fig. 5E). However, after ribavirin treatment, partial/weak signal of GFAP+ fibrous astrocytes was found throughout white matter and gray matter (Fig. 5: B, D, F).

GFAP immunoreactivity was increased at the end of the disease (28 dpi) in both examined groups of rats (Fig. 4). The strong intensity of GFAP+reactive astrocytes were registered in the vicinity of areas of demyelination in ventral (Fig. 6A) and lateral columns (Fig. 6C) of
white matter. Simultaneously, in the same areas of R28 rats (Fig. 6: B, D) only fibrous astrocytes with thin processes were noticed. Interestingly, in spinal cord sections of both E28 (Fig. 6E) and R28 (Fig. 6F) groups, the strong intensity of GFAP immunohistochemistry with huge number of hypertrophic astrocytes was seen throughout the gray matter.
Response of vimentin cells to ribavirin treatment after 15 and 28 dpi

Increase in vimentin expression was also noted, following a time course similar to that of GFAP. In the spinal cord of E15 rats, the vimentin+ astrocytes with large cell body and thin processes were found only in lateral columns of white matter (Fig. 7: A, C). At the same time point, immunohistochemical labeling for vimentin in ribavirin-treated rats was reduced (Fig. 7B), and vimentin+ astrocytes with small bodies and thin, long processes (Fig. 7D) resembling fibrous astrocytes were found only around lateral columns. At the end of the disease, the number of vimentin+ astrocytes was decreased, as well as the size of the cell body (Fig. 7: E, G). Moreover, in R28 rats, vimentin staining was additionally decreased (Fig. 7: G, H), and only a few vimentin+ astrocytes could be detected.

Ribavirin affects GFAP and vimentin mRNA expression in spinal cords of EAE animals

Changes in GFAP and vimentin mRNA expression in lumbosacral regions of spinal cords were investigated by real time PCR analysis 15 and 28 days after the induction of disease and after ribavirin treatment (Fig. 8). GFAP and vimentin mRNA levels in the EAE group and riba-

Fig. 4. The semi-quantitative analysis of astroglial reaction in EAE (n = 5) and ribavirin (n = 5) treated rats. The intensity of GFAP immunohistochemistry was rated using the 5-point grading scale: 4, strong; 3, moderate; 2, mild; 1, partial/weak; 0, nil in E15 and E28 (black dots), R15 and R28 (white dots). Dots represent number of animals expressing GFAP reactivity graded on the scale.

Fig. 5. Identification of astrocytes with GFAP antibody in 5-μm-thick paraffin sections of the lumbosacral region (L2) of spinal cord in EAE and ribavirin-treated rats 15 dpi. In the ventral column (A) of E15 animals, many radially-oriented astrocytes were detected, while in the lateral column (C, arrow) and in gray matter (E, arrow), there was an abundance of reactive astrocytes. In R15 animals, low expression of GFAP was observed in ventral (B) and lateral column (D, arrow) of white matter and in gray matter (F, arrow). Scale bars: 50 μm.
Ribavirin and Astrogliosis in EAE

Virin-treated animals were compared to control values (C). GFAP mRNA expression in the E15 group was increased by approximately 25% \( (P < 0.05) \), while in the ribavirin-treated group, we noticed a decrease of mRNA levels, which was not statistically significant. However, when transcriptional levels of GFAP mRNA of the R15 group were compared to those in the E15 group, a statistically significant decrease (30%, \( P < 0.005 \)) was observed. At the end of the disease (28 dpi), levels of GFAP mRNA in E28 and R28 were almost 2-fold increased compared to C. Strikingly, this increase of GFAP mRNA was also noticed when comparison was done vs. E15 (1.5-fold, \( P < 0.005 \)) and vs. R15 (2-fold, \( P < 0.005 \)). Nevertheless, no difference between transcriptional levels of GFAP in the E28 and R28 group was found.

Vimentin mRNA expression increases significantly \( (P < 0.005) \) in all examined groups: E15 (2.5-fold), R15 (2.2-fold), E28 (3.8-fold), and R28 (1.8-fold) compared to C (Fig. 8). However, comparison of vimentin mRNA levels in E15 vs. R15 groups revealed no statistically significant difference. In contrast, at the end of the disease, expression of vimentin mRNA in the ribavirin-treated group was reduced to half of the value in the E28 group.

**TGF-β, IL-10, and TNF-α mRNA expression in spinal cords of EAE and ribavirin-treated animals**

TGF-β mRNA expression increases significantly \( (P < 0.05) \) in E15 compared to C. (Fig. 9). Comparison of TGF-β mRNA levels in E15 vs. R15 groups and E28 vs. R28 revealed no statistically significant difference. IL-10 mRNA expression increases significantly \( (P < 0.005) \) in E15 and R15, \( (P < 0.05) \) in R28 compared to C (Fig. 9). No difference between transcriptional levels of IL-10 in the E15 and R15 group was found. However, IL-10 mRNA expression showed significant difference \( (P < 0.005) \) between E15 vs. E28 and R15 vs. R28.

TNF-α mRNA expression increases significantly \( (P < 0.005) \) in E15, while in R15 we noticed a decrease of mRNA levels that was statistically significant \( (P < 0.05) \) when compared to E15, although R15 showed no difference when compared to C. Also, a significant difference \( (P < 0.005) \) was observed in E15 vs. E28 (Fig. 9).
Discussion

In this study we have for the first time provided evidence that administration of ribavirin in EAE-induced animals suppressed reactive astrogliosis at the peak of the disease, what is considered to be the hallmark of EAE. On the other hand, at the end of the disease, in both EAE and ribavirin-treated groups, the increased number of reactive astrocytes was observed.

Although a role of T cells, particularly in their ability to initiate disease in EAE and MS, has been well documented (24), other cell types including astrocytes, microglia, and macrophages are also critical for the evolution and maintenance of neuroinflammation via mechanisms such as reactivation of T cells, production of proinflammatory cytokines, and phagocytosis of myelin (25). Astrocytes play a crucial role in the maintenance of neuronal structure and function. During MS/EAE, astrocytes undergo a process of proliferation and extensive hypertrophy of cell bodies, accompanied by angiogenesis and thickening and elongation of its processes (26). Astrogliosis is also characterized by rapid synthesis of intermediate filaments (vimentin and/or nestin), finally forming glial scar (25).

The present study confirms our previous findings that ribavirin reduced clinical signs, inflammation, demyel-
and axonal loss during EAE (20, 23). Thus, our objective in the present series of experiments was to evaluate whether ribavirin would affect the astroglial response after induction of EAE in Dark Agouti rats.

The prominent feature of MS/EAE is the accumulation of enlarged, multipolar GFAP immunoreactive astroglia within and at the margins of the inflammatory demyelinating lesions. After accumulation of hypertrophic astroglia at the margins of the lesions, the lesions themselves

**Fig. 8.** Ribavirin effects on GFAP (A) and vimentin (B) gene expression. We analyzed mRNA expression in the spinal cords of C, E15, E28, R15, and R28 animals using TaqMan for GFAP and SYBR Green real time PCR for vimentin; GAPDH was used as the internal reference standard. The data are presented as the mean ± S.E.M. and were assessed from 3 independent experiments, each run in triplicate. Dotted lines indicate mean values in intact, control animals (C) ± S.E.M. (gray areas). **P < 0.005: significance of the difference between test and control groups; ""P < 0.005: significance of the difference between E15 vs. R15 or E28 vs. R28; """"P < 0.005: significance of the difference between E15 vs. E28 or R15 vs. R28. Level of significance was analyzed by Student’s t-test.

**Fig. 9.** Ribavirin effects on TGF-β (A), IL-10 (B), and TNF-α (C) gene expression. mRNA expression was analyzed in the spinal cords of C, E15, E28, R15, and R28 animals using SYBR Green real time PCR; GAPDH was used as the internal reference standard. The data are presented as the mean ± S.E.M. and were assessed from 3 independent experiments, each run in triplicate. Dotted lines indicate mean values in intact, control animals (C) ± S.E.M. (gray areas). *P < 0.05, **P < 0.005: significance of the difference between test and control groups; #P < 0.05: significance of the difference between E15 vs. R15; ++P < 0.005: significance of the difference between E15 vs. E28 or R15 vs. R28. Level of significance was analyzed by Student’s t-test.
remained depleted of oligodendroglia, suggesting that migration of oligodendroglial lineage cells into the lesions was retarded by the intense perilesional gliosis (4). In our study, we observed radially-oriented astrocytes in ventral columns of white matter in spinal cords in EAE animals at the peak of disease, sending processes to the pial surface forming glia limitans. Also, in lateral columns at the same time point we observed hypertrophic astrocytes adjacent to the active demyelinating lesion. In addition, an increase in vimentin expression was also noted, following a time course similar to that of GFAP. Vimentin is the cytoskeletal component responsible for maintaining cell integrity (27), and it is expressed in immature astrocytes early in the development of the CNS and replaced with GFAP during maturation (28). Therefore, the increased mRNA expression of GFAP and vimentin, followed by increased GFAP and vimentin tissue expression at the peak of disease in EAE rats was expected. Ribavirin treatment significantly reduced the number of reactive astrocytes at the peak of the disease in the spinal cord, probably via inhibition of IMPDH, a key enzyme in the de novo purine nucleotide synthetic pathway. IMPDH inhibition leads to depletion of the guanylate and ATP pools (14). Therefore, by depleting the intracellular content of guanosine nucleotides, ribavirin can act as a powerful proliferation inhibitor in proliferating cell types (16), including astrocytes (29). In addition, it has been shown to arrest the cell cycle in the G0/G1 phase and induce cell differentiation and growth suppression (16). These data are consistent with formerly published reports that ribavirin down-regulates process of reactive astrogliosis after brain injury (29).

Hypertrophic astrocytes were observed adjacent to the demyelinating lesion in ventral and lateral columns of white matter in spinal cords of EAE animals at the end of the disease, while vimentin-expressing cells were evident in those areas with elongated and think branches. It is interesting to note that ribavirin treatment did not reduce the number of GFAP+ astrocytes or the level of mRNA GFAP at the end of the disease, while the number of vimentin+ astrocytes and vimentin mRNA expression were markedly reduced. These dual effects of ribavirin could be explained with the fact that ribavirin manifests its effect only on highly proliferating (immature) cells and has no, or very slight, effect on mature cells (our unpublished in vitro data). Reactive astrocytes could be beneficial in the control of injury and their protective effect was associated with production of several growth factors that may be protective to neurons (30). Moreover, it was shown that reactive astrocytes may form scar-like perivascular barriers that restrict the influx of leukocytes into CNS parenchyma and protect CNS function during peripherally initiated, acquired immune inflammatory responses in the CNS (31). Also, the increase in GFAP immunoreactivity in the gray matter, in both examined groups at the end of disease was more prominent in this area, where neurons were thought to be in danger. This increase in GFAP immunoreactivity could play a role in protecting neurons and that neuronal death occurs when the severity of cellular derangement exceeds the protective action of astrocytes. Therefore, the reactive GFAP+ and vimentin+ astrocytes observed at the end of the disease in the immunized and ribavirin-treated group, accompanied by increased GFAP mRNA expression, may not be detrimental per se and may be involved in the recovery from disease.

Furthermore, it is well documented that astrocytes in MS plaques express high levels of constitutive nitric oxide (NO) synthase (25), producing NO and superoxide radicals, which may directly or indirectly damage oligodendrocytes and axons (5). The mechanisms underlying ribavirin inhibitory action on astrocytes might therefore include inhibition of NO production and angiogenesis (32), as it was previously shown for other IMPDH inhibitors (33).

We have shown that ribavirin intensively suppresses proinflammatory response during EAE, so another possibility is that ribavirin mediates the astroglial response during EAE via modulation of pro- and/or anti-inflammatory cytokines production. Namely, it is known that activation of astrocytes may facilitate their synthesis and release of proinflammatory cytokines (3, 34, 35). In line with these findings, ribavirin-induced reduction of mRNA TNF-α expression after EAE induction, may contribute to down regulation of astrocytes activation and prevent myelin destruction, as we showed previously by cytokine immunohistochemistry (23).

Astrocytes in MS/EAE seem to play dual roles in destruction and protection. Activated astrocytes secrete the anti-inflammatory cytokine IL-10 (26), which inhibits the microglia antigen-presenting function, T cell proliferation, and cytokine synthesis by CD4+ T cells. TGF-β is expressed by all glial cells, including astrocytes in vivo and in vitro (36) and is implicated in the remyelination process (37). It deactivated microglia by down-regulating the expression of molecules associated with antigen presentation and production of proinflammatory cytokines, NO, and oxygen free radicals (38). We have shown that mRNA IL-10 and TGF-β are markedly elevated at the peak of the disease and reduced at the end of the disease in both examined groups. Those results are consistent with previously shown immunohistochemistry data obtained on spinal cords in rats during EAE (23).

In the absence of the drugs or procedures which would efficiently establish specific tolerance to the target autoantigen(s), therapeutic strategy for treatment of MS
is still based on a selection of drugs with the best risk/benefit ratio. Therefore, the approach to initiating first-line preventative therapies must focus on individualizing treatment strategies. Taken together, this makes ribavirin a possible candidate for early treatment of EAE/MS.

In summary, the present study indicates that ribavirin may have the ability to attenuate astrocyte proliferation in the peak of the disease and could modulate the astrogial response to EAE during the time-course of the disease by affecting GFAP and vimentin expression at the mRNA level. Considering the ability of astrocytes to act as proinflammatory mediators, ribavirin may be regarded as a promising agent for preventing and treating neuroinflammation.

Acknowledgments

This work was supported by Serbian Ministry of Science, grant No. III 41014.

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