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Neuroscience Research 47 (2003) 201–207
Neuroscience Research
www.elsevier.com/locate/neures

Axonal damage is reduced following glatiramer acetate treatment in C57/bl mice with chronic-induced experimental autoimmune encephalomyelitis

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Received 20 March 2003; accepted 9 June 2003

Abstract

Glatiramer acetate (GA) is efficacious in reducing demyelinating-associated exacerbations in patients with relapsing-remitting multiple sclerosis (RRMS) and in several experimental autoimmune encephalomyelitis (EAE) models. Here we report that GA reduced the clinical and pathological signs of mice in chronic EAE induced by myelin oligodendrocyte glycoprotein (MOG). GA-treated mice demonstrated only mild focal inflammation, and less demyelination, compared with controls. Moreover, we also found minimal axonal disruption, as assessed by silver staining, antibodies against amyloid precursor protein (APP) and non-phosphorylated neurofilaments (SMI-32), in the GA-treated group. In conclusion, our study demonstrated for the first time that axonal damage is reduced following GA treatment in C57/bl mice with chronic MOG-induced EAE.

Keywords: Axonal damage; Glatiramer acetate; Experimental autoimmune encephalomyelitis (EAE); Multiple sclerosis (MS); Myelin oligodendrocyte glycoprotein (MOG)

1. Introduction

Multiple sclerosis (MS) is a chronic disabling autoimmune neurological disorder targeting the white and gray matter of the central nervous system (CNS). It often has an initial relapsing-remitting course, which later progresses into a secondary progressive mode. The etiology of MS has not yet been fully elucidated, but it is believed that immunological mechanisms and axonal damage operate in disease initiation and progression (Stinissen et al., 1997; Rieckmann and Mauser, 2002). The autoimmune attack includes auto-reactive lymphocytes and local inflammatory response that causes demyelination, and oligodendrocyte death. Recently, axonal damage and axonal loss, first described by Charcot (Charcot, 1868) have been emphasized by a novel technique using *N*-acetylaspartate (NAA), a compound found exclusively in neurons and used in magnetic resonance imaging (MRI) (Matthews et al., 1998; Pike et al., 1999) and also by unique antibodies against amyloid precursor protein A4 (APP) and non-phosphorylated neurofilaments (SMI-32) (Ferguson et al., 1997; Trapp et al., 1998). In experimental autoimmune encephalomyelitis (EAE), the animal model of MS, a variable amount of demyelination can be induced, depending on the method of sensitization, the species and strain used (Lassmann and Wekerle, 1998). Axonal insult has been previously reported in acute and chronic EAE in rodents (De Stefano et al., 1999; Trapp et al., 1999; Silber and Sharief, 1999; Kornek et al., 2000; Offen et al., 2000) and in non-human primates (Mancardi et al., 2001).

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0168-0102/03/$ - see front matter
doi:10.1016/S0168-0102(03)00217-7

Glatiramer acetate (GA), previously known as copolymer 1 (Weiner, 1987), is the acetate salt of the random polymerization of four amino acids (L-Glutamic acid, L-Lysine, L-Alanine, and L-Tyrosine). Treatment with GA in EAE models in various species either prior to, concomitant with or after a challenge (Teitelbaum et al., 1997) using spinal cord homogenates, myelin basic protein (MBP), proteolipid protein (PLP, Teitelbaum et al., 1996) and myelin oligodendrocyte glycoprotein (MOG, Ben-Nun et al., 1996) showed marked improvement in clinical signs and immunological parameters. Moreover, GA has been approved in North America since 1996 for the treatment of relapsing-remitting MS (RRMS). In the US pivotal trial, treatment with GA has been shown to sustain reduction of clinical disease activity in humans with RRMS (Johnson et al., 1995, 1998, 2001). A recent study of 239 patients with RRMS showed that GA can lower the proportion of new MS lesions evolving into permanent "black holes", seen as permanent T1 hypointense lesions, reflecting a beneficial effect on the events leading to irreversible tissue disruption (Filippi et al., 2001).

The mode of action of GA has not been fully elucidated but it is known to competitively inhibit the effect of CNS myelin antigens, thought to be important in the pathogenesis of MS, through MHC blockade (Racke et al., 1992; Teitelbaum et al., 1992; Fridkis-Hareli et al., 1994). This effect is unlikely to occur in EAE or in MS and is found only in vitro, as GA is quickly degraded to oligopeptides at the injection site (Teitelbaum et al., 1988). An additional mechanism involved in GA immunomodulatory activity is the shift of Th1 to Th2 cells (specific suppressors T-cells), which are known to be deficient in MS (Teitelbaum et al., 1997). These GA specific T-cells migrate through the Blood Brain Barrier (BBB) to the CNS (Aharoni et al., 1998), whereupon reactivation by myelin antigens, secrete anti-inflammatory cytokines (Neuhaus et al., 2001). Although the immune response to GA has been intensively examined, its possible effects on neurons and axons have been examined by only a few studies reporting GA's neuroprotective features, shown in optic nerve injury, glutamate toxicity, and in acute and chronic motor neuron diseases models (Kipnis et al., 2000; Schori et al., 2001; Angelov et al., 2003). In addition, activated GA reactive T-cells were shown to secrete significant amounts of brain derived neurotrophic factor (BDNF), a neurotransmitter that plays a major role in neuronal survival (Ziemssen et al., 2002). We, therefore, studied the possible role of GA in reducing the insult of neurons and axons in MOG-induced EAE mice, a chronic model which appears to resemble the clinical course of progressive MS better than the other disorders induced by auto-antigens (Kerlero de Rosbo et al., 1995).

2. Materials and methods

2.1. Animals

6–8-week-old C57/bl mice ($n = 10–12$ each group) weighing 20 g were obtained from Harlan Laboratories, Israel. The animals were housed in standard conditions: constant temperature ($22 \pm 1\ ^{\circ}C$), humidity (relative, 25%) and a 12-h light:12-h dark cycle, and were allowed free access to food and water. The animals and protocol procedures were approved and supervised by the Animal Care Committee at the Rabin Medical Center.

2.2. Induction of EAE

EAE was induced by immunization with the peptide encompassing amino acids 35–55 of rat MOG. MOG synthesis was carried out by the Weizmann Institute Synthesis Unit, using a solid-phase technique on a peptide synthesizer (Applied Biosystems Inc., Foster City, CA). Mice were injected subcutaneously at one site in the flank with a 200 μl emulsion containing 300 μg MOG in complete Freund's adjuvant (CFA) and 200 μg of heat-inactivated *Mycobacterium tuberculosis* (Sigma, Israel). An identical booster was given at one site of the other flank 1 week later.

2.3. Neurological evaluation of EAE

Following the encephalitogenic challenge, mice were observed daily and clinical manifestations of EAE were scored as follows: 0, no clinical symptoms; 1, loss of tail tonicity; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, paralysis of four limbs; 5, total paralysis; 6, death (Mendel et al., 1998).

2.4. Treatment

GA (Copaxone®; Teva Pharmaceutical Industries, Ltd., Petah Tiqva, Israel) (25 mg/kg) was given together with the MOG injections on days 1 and 8 in the same syringe and with identical CFA-dose of the control group.

2.5. Histopathology

Spinal cords from GA-treated and control mice were dissected 25 days after immunization with pMOG 35-55, fixed in 10% buffered formalin and embedded in paraffin. Five micron thick sections were stained with Hematoxylin and Eosin (H&E), and Luxol Fast Blue (LFB). These methods were used to assess myelin loss (Luna, 1968a). Bielshowesky's silver impregnation was used to evaluate axonal integrity (Luna, 1968b).

2.6. Immunohistochemistry

Paraffin sections were exposed to monoclonal antibodies against mouse non-phosphorylated neurofilament H (Clone SMI-32, Sternberger Monoclonals, Baltimore, USA, (Trapp et al., 1998) and monoclonal antibodies against APP (Clone 22C11, Chemicon, Kornek et al., 2000; Mancardi et al., 2001) to assess the amount of axonal loss. Shortly, sections were deparaffinized, blocked with 3% normal goat serum, and incubated with SMI-32 at a dilution of 1:1000, at room temperature for 30 min. They were then washed and incubated with fluorescence-labeled antibodies (Cy-3 donkey anti-mouse, Jackson, USA) and visualized with fluorescence microscopy. The sections that were pre-incubated with anti-APP antibodies were washed out and incubated with goat anti-mouse IgG at room temperature for 30 min, and then with ColonoPAP for 30 min. APP-positive axons were visualized with 3,3′-diaminobenzidine (DAB).

2.7. Microscopes

All slides of histopathology and immunocytochemistry were analyzed under an Olympus BX52TF microscope (Olympus, Tokyo, Japan). DP50 microscope digital camera system (Olympus) was used to capture all the pictures. VIEWFINDERLITE™ software (Olympus), with the digital camera attached to the microscopes, was used to acquire images. STUDIOLITE™ software (Olympus) was used to edit and analyze the images captured.

2.8. Image analysis technique

Image analysis was performed using the NIH public domain program, Image-J for PC (http://rsb.info.nih.gov/nih-image), and Adobe's PHOTOSHOP. The analysis was used to quantify areas stained by immunohistochemistry (for anti-SMI-32 and APP antibodies). Spinal cords of six animals taken from each experimental group were used for the analysis. The spinal cord sections were photographed in a series of six frames (a total of 36 pictures for each group), and two examiners preformed a double blind assessment.

2.9. Statistical analysis

The differences in the clinical severity of EAE following pMOG 35-55-induction between the control and GA-treated mice groups were evaluated daily using Student's two-tailed *t*-test.

3. Results

The MOG-treated mice developed severe clinical manifestations, starting on days 11–14, increasing in severity by days 18–20, and maintained until sacrifice at 40 days. All animals were affected and complete hind limb paralysis (clinical score of 4.1 ± 0.4) was observed in most animals. In contrast, the GA-treated mice showed mild and delayed clinical signs (0.3 ± 0.1, $P < 0.001$) (Fig. 1a, 80% being disease free Fig. 1b). The beneficial effect in GA-treated animals was stable over time and sustained until sacrifice at 40 days following MOG injection.

To evaluate the effects of GA treatment on inflammation and demyelination, spinal cords were examined by histological methods using light microscopy, 35–45 days after immunization.

H&E and LFB staining revealed marked multifocal and lymphohistiocytic inflammation in the white matter, which was both perivascular and diffuse, and myelin loss in the MOG-treated mice, especially around inflamed areas. In contrast, in most sections from GA-treated mice, there was less inflammation and myelin

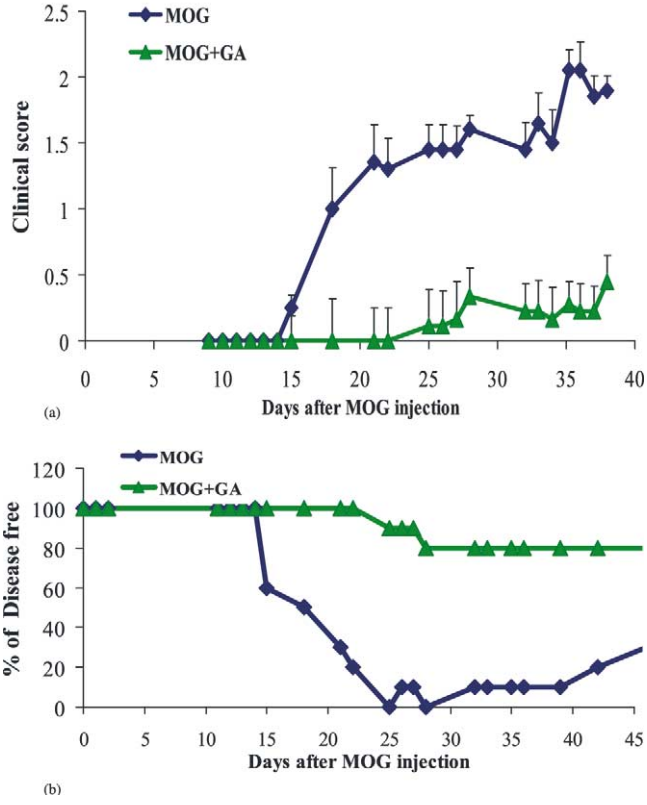

Fig. 1. Induction of EAE in C57/bl mice with pMOG 35-55. Ten control and ten glatiramer-treated mice were injected with pMOG 35-55 in CFA supplemented with *M. tuberculosis* (Mt). The mean daily clinical score ± S.E.M. is shown for each group of mice (0, no clinical symptoms; 1, loss of tail tonicity; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, paralysis of four limbs; 5, total paralysis; 6, death) (a). Percent of disease free animals (b).

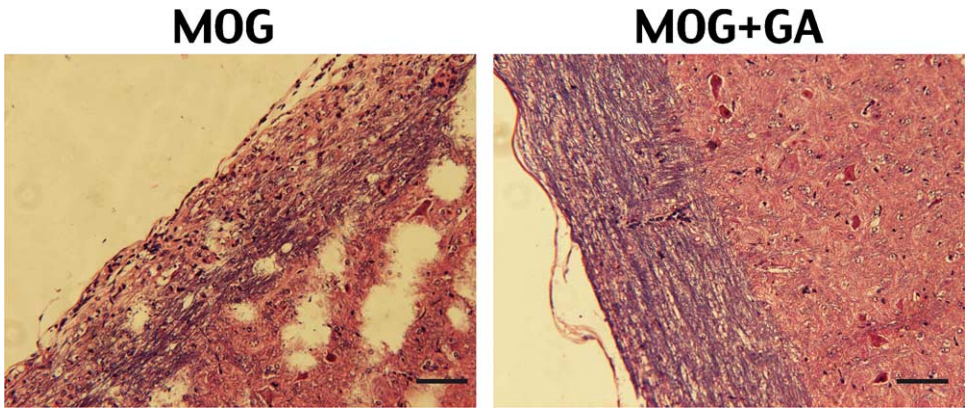

Fig. 2. Histopathology of spinal cord longitudinal sections from control and GA-treated mouse with pMOG 35-55-induced EAE H&E and LFB staining demonstrated areas of inflammation and loss of myelin in the control mouse (MOG). In contrast, MOG+GA-treated mouse show very mild inflammation with myelin integrity. Original magnification of all pictures $\times 100$. The scale bars represents 500 μm.

sheets were persevered (Fig. 2). Furthermore, axonal damage in spinal cords was qualitatively assessed by Bielshowesky's silver staining. Severe axonal damage was found in the spinal cord sections of the immunized control mice, whereas those from the GA-treated group showed minimal axonal impairment, occurring only in regions surrounding foci of inflammation (Fig. 3).

Qualitative assessment of axonal injury in the spinal cord was performed using immunohistochemistry for non-phosphorylated neurofilaments (SMI-32) and APP. As was demonstrated by previous studies, antibodies directed against SMI-32 and APP in normally myelinated regions of the brain provide sensitive markers for axonal damage (Trapp et al., 1998; Pitt et al., 2000; Kornek et al., 2000; Werner et al., 2001). As seen in Fig. 4, SMI-32 (A) and APP (B) immunostaining in spinal cord sections were positive and indicated high level of axonal injury in control mice compared with undamaged axons in GA-treated mice. Image analysis was performed on twenty slides from six mice in each group in order to quantify axonal damage in spinal cords. We found an 85% reduction in the area stained by the SMI-32 antibodies (Fig. 5A, $P < 0.003$), and 63% reduction in the area stained by anti-APP antibodies (Fig. 5B, $P < 0.001$) in the GA-treated group, compared with controls. When second antibody was used alone, no staining was observed (data not shown).

4. Discussion

Our study shows that GA-treated C57/bl mice are highly resistant to MOG-induced chronic EAE. Incidence of the disease and its clinical severity were markedly reduced in GA-treated mice, as compared with controls. Moreover, histological examination of CNS tissues demonstrated that GA treatment reduced the severity of inflammation and demyelination. Using silver staining, we observed marked axonal damage in MOG-induced EAE mice. Furthermore, immunohistochemical examination with SMI-32 antibodies in these animals showed that the heavy neurofilaments, the most important proteins in axon fibers, undergo dephosphorylation with impairment of axonal integrity. This was further confirmed by APP staining, indicating neuronal damage that is highlighted by the inability to promote

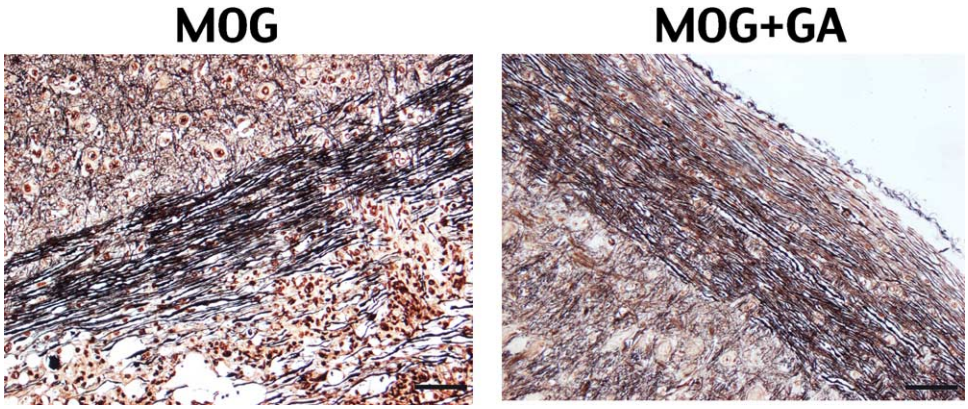

Fig. 3. Bielshowsky staining of the same area indicates axonal loss in the control mouse (MOG) whereas in the MOG+GA-treated mouse there was normal integrity of axons. Original magnification of all pictures $\times 100$. The scale bars represents 500 μm.

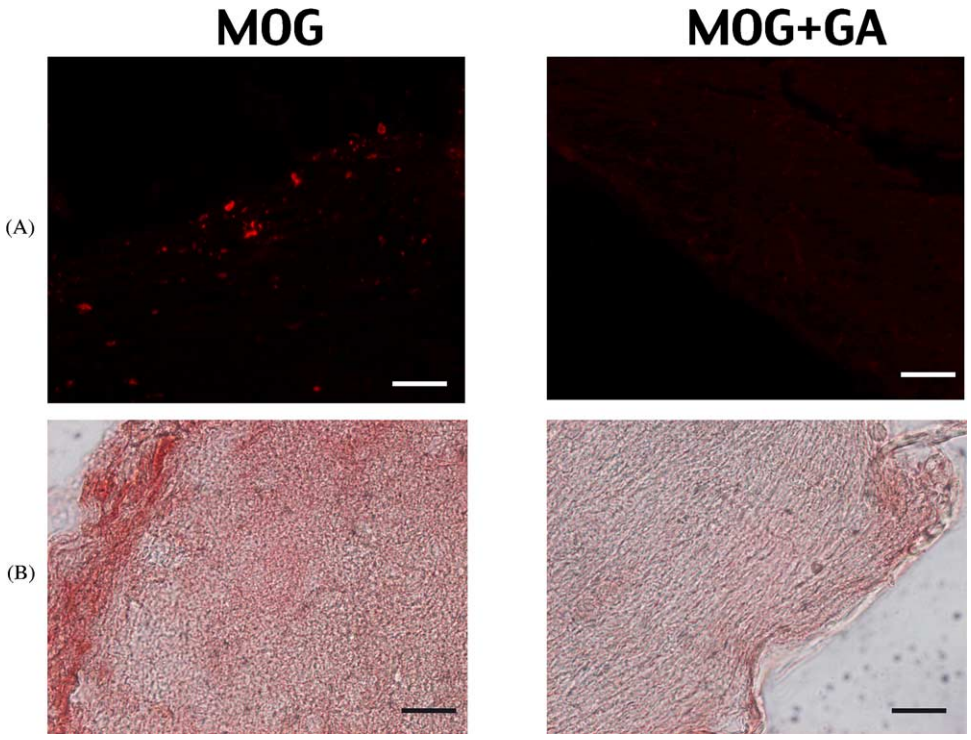

Fig. 4. Qualitative assessment of axonal damage. Immunoreactivity for abnormally dephosphorylated neurofilament H (SMI-32) (A), and anti-APP antibodies (B) in the MOG and MOG+GA-treated mouse with pMOG 35-55-induced EAE. The scale bars in Panels A and B represents 500 μm.

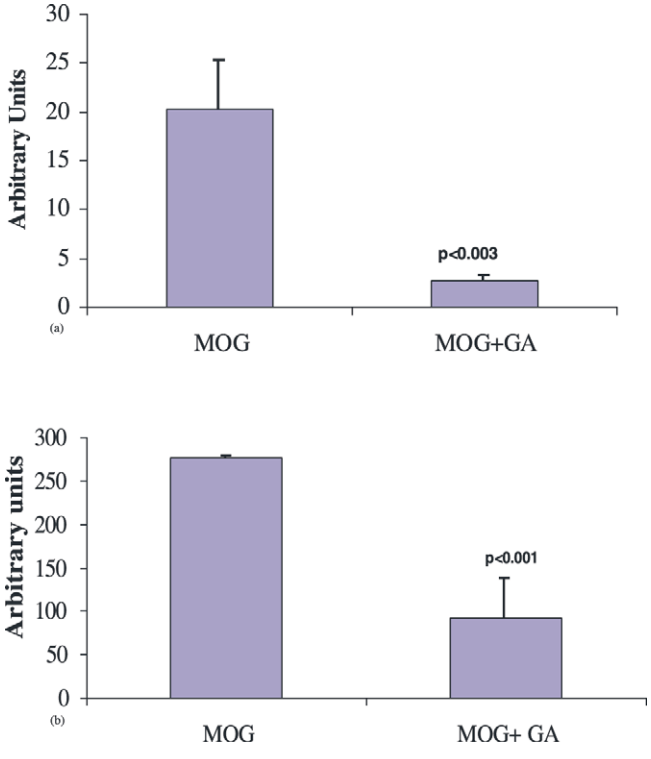

Fig. 5. Semi-quantitative assessment of axonal damage as processed by the Image Analysis Technique. Abnormally dephosphorylated neurofilament H (SMI-32) (A), and anti-APP antibodies (B) in the MOG and MOG+GA-treated mouse with pMOG 35-55 induced EAE. Results are shown in arbitrary units, representing the pixel area.

nerve impulses. In contrast, the GA-treated mice had a smaller amount of abnormal dephosphorylation, suggesting that GA effectively protected against axonal damage. Moreover, improvement in the clinical score shown following treatment, correlated with the reduced axonal damage.

Axonal damage and axonal loss is a well-known event in MS, described as early as 1868 by Charcot. Evidence of axonal damage in human MS lesions has been confirmed by a number of studies (Raine and Cross, 1989; Trapp et al., 1998; Noseworthy et al., 2000) using anti-APP and anti-SMI-32 antibodies. APP is a membrane-spanning glycoprotein transported from neurons, where it is normally present, to axons, by fast anterograde axonal transport. In the case of axonal damage, APP accumulates in axons and, therefore, it is a sensitive indicator of axonal injury (Kornek et al., 2000). Normally, neurofilaments are phosphorylated in axons, but in cases of axonal damage dephosphorylation of the neurofilaments occurs, and, therefore, it will react with the anti-SMI-32 antibodies (Kornek et al., 2000). Indeed, APP and SMI-32 were found within active MS lesions, characterized by a high degree of inflammation, and in active borders of less acute demyelinating areas (Trapp et al., 1998).

The exact mechanism by which GA exerts its observed beneficial effect in EAE and MS is not fully understood, but it has been proposed that it competes with MBP for MHC class II presentation, and inhibits

MBP-specific T-cell activation (Teitelbaum et al., 1992, 1997; Racke et al., 1992; Fridkis-Hareli et al., 1994). However, observations in EAE models suggest that its effect is not restricted to the MBP antigen but can also inhibit EAE induced by PLP and MOG (Ben-Nun et al., 1996). The effectiveness of GA in several EAE models may also result from the induction of antigen-specific suppressor T-cells (Th2) that penetrate the CNS and cross-react with MBP (Teitelbaum et al., 1997). The cross-reaction with MBP results in the secretion of protective cytokines (IL-4, IL-5, IL-6, IL-10, IL-13) which down-regulate local inflammation through the suppression of local inflammatory MBP-specific and non-specific T-cells by a mechanism termed 'bystander suppression' (Teitelbaum et al., 1996; Aharoni et al., 1998). By virtue of its mechanism of action, GA is also reported to have neuroprotective features (Kipnis et al., 2000; Schori et al., 2001; Angelov et al., 2003). For example, in a mouse model of acute and chronic degenerative conditions, the authors demonstrated that active therapeutic vaccination with GA protected motor neurons (Angelov et al., 2003). Furthermore, GA reactive T-cells activated by their specific antigen secrete significant amounts of BDNF, a well-known neurotransmitter involved in neuronal survival (Ziemssen et al., 2002). GA might induce neuroprotection by inhibiting NF-κB activation and chemokine production (such as TNF-alpha), which are part of the inflammatory process (Li et al., 2001), and by secreting of other neurotrophic substances by reactive T cells. In conclusion, our study demonstrates for the first time that axonal damage was reduced following GA treatment in chronic MOG-induced EAE. However, we cannot exclude that GA exerts its action through an anti-inflammatory mechanism. Although the mechanism for axonal damage prevention is not clearly understood, it is speculated that the reduction of the inflammatory process inhibits the release of neurotoxic substances, such as glutamate, and TNFα (Pitt et al., 2000; Smith et al. 2000; Li et al., 2001; Werner et al., 2001). These results can provide an additional insight on the processes by which GA exerts its observed beneficial effect. Updated information on the diverse mechanisms and timing of axonal degeneration combined with a better understanding of the MS pathology and GA's mechanism of action should contribute to the development of new combination therapies aimed at slowing down RRMS disease progression.

Acknowledgements

This work was performed in partial fulfillment of the requirements for a Ph.D. degree of Yossi Gilgun-Sherki, Sackler Faculty of Medicine, Tel Aviv University, Israel. Supported, in part, by the Israel Ministry of Health (D.O.) and the National Parkinson Foundation, USA (E.M.).

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