ELSEVIER

Journal of Neuroimmunology 165 (2005) 83 – 91

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Effective combination of minocycline and interferon-β in a model of multiple sclerosis

Fabrizio Giuliani[a,c,1], Sue Anne Fu[a,c], Luanne M. Metz[a,c], V. Wee Yong[a,b,c,*]

[a]Department of Clinical Neurosciences, University of Calgary, Calgary, Alberta, Canada
[b]Department of Oncology, University of Calgary, Calgary, Alberta, Canada
[c]The Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada

Received 14 February 2005; accepted 22 April 2005

Abstract

The objective of the current study was to investigate whether minocycline improves the effect of an existing multiple sclerosis (MS) medication, interferon-β, on experimental autoimmune encephalomyelitis (EAE) in mice. When used at sub-optimal doses, neither medication affected EAE but their combination at these doses led to the significant alleviation of EAE disease severity scores and histological outcomes. In culture, the toxicity of T cells to neurons was alleviated by their prior exposure to minocycline or interferon-β and their combination further attenuated neuronal death. Collectively, these results suggest the utility of the combination of minocycline and interferon-β in MS.

Keywords: EAE; Multiple sclerosis; Neuroprotection

1. Introduction

The use of interferon-β and glatiramer acetate in MS has resulted in the decrease of relapse rate, reduction in the frequency of enhancing lesions in magnetic resonance imaging (MRI), and a slowing of progression of disability (The IFNB Multiple Sclerosis Study Group, 1993; Jacobs et al., 1996; Johnson et al., 2000; PRISMS-4, 2001; Comi et al., 2001). Despite the significant advances, not all patients have responded optimally to these immunomodulators. Further improvement in MS therapeutics is necessary, either with the advent of new medications or the combination of existing ones, to improve the prognosis of MS.

Minocycline is an oral tetracycline derivative with good penetration properties into the CNS. It is commonly used in the treatment of acne where a good safety record has been established in long term use (Seukeran et al., 1997; Shapiro et al., 1997). There is increasing recognition that minocycline has properties unrelated to its anti-microbial action. In particular, minocycline has multiple immunomodulatory activities, including the inactivation of microglia, and it has several neuroprotective and anti-apoptotic mechanisms (reviewed in Yong et al., 2004). Impressively, minocycline reduces the neuropathological consequences of animal models of stroke (Yrjanheikki et al., 1998; Arvin et al., 2002), Huntington's Disease (Chen et al., 2000), Parkinson's Disease (Du et al., 2001; Wu et al., 2002), amyotrophic lateral sclerosis (Kriz et al., 2002), Down's syndrome (Hunter et al., 2004) and spinal cord injury (Wells et al., 2003; Stirling et al., 2004).

We (Brundula et al., 2002) and others (Popovic et al., 2002; Nessler et al., 2002) have reported that minocycline attenuates disease activity in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Based on these findings, and of its neuroprotective capacity in alleviating axonal loss (Wells et al., 2003), we have tested oral minocycline in a Phase II trial of 10 patients with

* Corresponding author. University of Calgary, 3330 Hospital Drive, Calgary, Alberta, Canada T2N 4N1. Tel.: +1 403 220 3544; fax: +1 403 283 8731.
E-mail address: vyong@ucalgary.ca (V.W. Yong).
[1] Current address: Division of Neurology, University of Alberta, Edmonton, Alberta, Canada.

0165-5728/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2005.04.020

relapsing–remitting MS. We found that minocycline reduced MRI-detected gadolinium enhancements within 2 months of treatment (Metz et al., 2004); after 24 months of therapy, patients have remained clinically stable (Zabad et al., submitted for publication). As minocycline is an inexpensive drug and has multiple immunomodulatory and neuroprotective activities, this oral medication could be a useful adjunct to the current therapies in MS. Accordingly, we have observed that minocycline in combination with glatiramer acetate resulted in a greater attenuation of EAE disease activity than either drug used alone (Giuliani et al., 2005).

In this report, we have considered whether minocycline would act together with interferon-β in vitro and in EAE. This combination is advantageous for several reasons. First, both are immunomodulators with various effects on cells of the immune system. Second, while the interferons are thought to enter minimally into the central nervous system (Greig et al., 1988; Wills, 1990), minocycline penetrates into the brain and spinal cord (MacDonald et al., 1973; Carney et al., 1974) where it has the potential to exert immunomodulatory and neuroprotective activities within (Yong et al., 2004). Third, interferon-β was shown to be proteolytically cleaved by matrix metalloproteinases (MMPs), which are upregulated in MS (reviewed in Yong et al., 2001), and this cleavage was blocked by minocycline's inhibition of the activity of MMPs (Nelissen et al., 2003). Minocycline would thus be expected to prolong the bioavailability of interferon-β when both are used concurrently to treat patients with MS.

The main objective of the current study was to investigate whether minocycline improves the effect of interferon-β on EAE in mice. Because optimal doses of interferon-β alone would prevent disease activity in EAE, making it impossible to study the impact of combination treatment, we have used interferon-β at a sub-optimal dose that, alone, had no benefit in EAE. Similarly, minocycline was used at a sub-optimal dose so that any additive effect with interferon-β could be determined. Furthermore, as activated T lymphocytes kill neurons in culture (Giuliani et al., 2003), a finding of relevance to the neurodegeneration that occurs in MS (Ferguson et al., 1997; Trapp et al., 1998; Bjartmar and Trapp, 2001; Cifelli et al., 2002), we have tested whether the combination of interferon-β and minocycline reduces the capacity of T cells to promote neuronal death in culture. Our collective results demonstrate that the impact of the combination is better than either used individually, suggesting the potential of this combination treatment to improve the management of patients with MS.

2. Materials and methods

2.1. Induction and assessment of EAE

C57/BL6 mice of 8–12 weeks of age were used. EAE was induced using a single injection of 50 μg of myelin oligodendrocyte glycoprotein (MOG peptide, amino acid sequence 35-55, synthesized by the peptide facility of The University of Calgary), emulsified in 100 μl of complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan). The MOG injectate was distributed equally on both sides at the base of the tail subcutaneously. Pertussis toxin (0.3 μg/200 μl, List Biological Laboratories, Campbell, California) was injected intraperitoneally on days 0 and 2, with day 0 being the day of MOG immunization.

Mice were evaluated daily for clinical signs. We used a modified behavioural scoring system that assessed all four limbs and tail independently (Giuliani et al., 2005). For the tail, a score of zero reflects no symptoms; 1 represents a paralyzed tail while a score of 2 is given to a mouse with a fully paralyzed tail. For the hind or fore limbs, each assessed separately, zero signifies no symptoms; a score of 1 indicates a weak or funny walk; 2 represents a mouse that drags that limb which is still moving while a score of 3 denotes a fully paralyzed limb. The score from each limb and the tail was summed to obtain the daily disease score for that animal. An advantage of this scoring system is that it enables separate assessment of each limb because the right and left side may be neurologically different.

2.2. Treatment of mice

Both minocycline (HCl salt, Sigma, Oakville, Canada) and recombinant murine interferon-β (Serono, Geneva) were used at sub-optimal doses that barely affected the disease course since optimal doses would have adequately controlled disease activity making the impact of the combination difficult to assess. An optimal regimen of minocycline in mice (Brundula et al., 2002) is 50 mg/kg, twice daily for the first two days, then 50 mg/kg once daily the next 5 days, and 25 mg/kg per day thereafter. In a preliminary experiment, we determined that doses of recombinant murine interferon-β above 15,000 IU/mouse are effective to alleviate disease activity optimally (data not shown).

Thus, in this study, we used minocycline at 25 mg/kg daily and murine interferon-β at 7500 IU/mouse daily. Treatment was initiated 7 days following MOG immunization and medications were administered daily until mice were sacrificed.

2.3. Neuropathological analysis

Mice were overdosed with chloral hydrate and the entire spinal cord was then removed and immersed in 10% buffered formalin. Blocks consisting of lumbar-sacral cord for transverse sections and thoracic spinal cord for longitudinal sections were then embedded in paraffin. Sections were subsequently stained with haematoxylin-eosin and luxol fast blue for evidence of inflammation and demyelination, respectively. The second series of adjacent sections was processed with Bielchowsky silver stain to

identify axons. Histological scores of the degree of inflammation, demyelination and axonal loss in the spinal cord of each mouse were evaluated blind using a semi-quantitative system described previously (Giuliani et al., 2005). In brief, a score of zero indicated no disease and higher scores depict increasing degrees of pathology. For inflammation, which tended to progress from the meninges deeper into the parenchyma in increasingly sick mice, a grade of 0 refers to no infiltration of inflammatory cells, grade 1 represents foci of subarachnoid cell infiltration, grade 2 is diffuse subarachnoid infiltration, grade 3 has foci of parenchymal infiltration, while grade 4 denotes diffuse and widespread parenchymal infiltrates. For demyelination, a score of 0 indicates no demyelination; 1 refers to foci of demyelination that is superficial and proximal to the subarachnoid space, and that involves less than 25% of the lateral columns; 2 represents foci of deep parenchymal demyelination and that involves over 25% of the lateral columns; while a score of 3 denotes diffuse and widespread demyelination. For axonal loss, grade 0 is no axonal loss, grade 1 indicates the presence of foci of superficial axonal loss and which involves less than 25% of the lateral columns, grade 2 denotes foci of deep axonal loss and that encompasses over 25% of the lateral columns, while grade 3 has diffuse and widespread axonal loss.

2.4. T cell–neuronal co-cultures

Human fetal neurons were cultured to over 90% purity as described previously (Giuliani et al., 2003). Peripheral blood mononuclear cells were obtained from normal healthy adult donors and the T cells were activated by incubation with anti-CD3 for three days as described previously (Giuliani et al., 2003). Where T cells were left unactivated, these were not exposed to the anti-CD3 treatment. In the case of medication-treated T cells, cultures were exposed to the treatment three hours after the initiation of anti-CD3 exposure. The cells were treated with human interferon-β (Rebif®, 2000 IU/ml) or minocycline (50 μg/ml) for 3 days. The floating T cells were then collected, counted, and 50,000 cells from each treatment group were added to neurons in culture. Each treatment group consisted of four cultures of human neurons and experiments were repeated twice using different sets of neurons and human leukocytes. Twenty four hours after incubation of T cells with neurons, cultures were fixed with 4% paraformaldehyde and subjected to immunohistochemistry for microtubule-associated protein 2 (MAP-2) to detect neurons (Giuliani et al., 2003). The number of surviving neurons in culture was then counted in five defined fields per culture.

We note that minocycline inhibits the proliferation of anti-CD3 activated cells (Giuliani et al., in press) and that its effects become visible at the concentration of 10 μg/ml and are maximal at 100 μg/ml. These doses are not toxic to the T cells (Giuliani et al., in press). For the current experiments, cells were counted at the end of the 3 day incubation with minocycline, and an equivalent number (50,000) of these minocycline-pretreated cells, as with controls, were then added to neurons.

2.5. Statistical analysis

As the majority of the animal data consisted of ordinal scores in the different treatment groups, the Kruskal–Wallis nonparametric ANOVA test was employed, along with post hoc Dunn's multiple comparisons test. For the analysis of T cell–neuronal co-cultures, a parametric ANOVA test was used with Tukey's post hoc comparisons. For correlations, Spearman's nonparametric correlation test was used. Statistical significance was set at $p < 0.05$.

3. Results

3.1. The combination of minocycline and interferon-β improves the clinical severity of EAE in mice

Saline-treated mice developed EAE with symptoms appearing by 8–10 days following MOG immunization and developing to an average maximum severity of grade 6 where tail and hind limbs were affected (grades 2–3 on the commonly used 5 point scale). Treatment of mice with sub-optimal doses of minocycline or interferon-β alone did not alter disease severity but their combination at these doses led to marked attenuation of disease (Fig. 1A).

We tabulated the overall disease burden for each mouse; this is the sum of the daily disability score over the entire course of an experiment. Fig. 1B indicates that the overall burden of disease was alleviated in the combination group. When the maximum disease score achieved by each mouse was plotted, it was noted that this was also reduced by treatment of the combination of interferon-β and minocycline (Fig. 1C).

3.2. Combination treatment improves histological outcomes in EAE

After obtaining the disability profiles displayed in Fig. 1, mice were sacrificed for neuropathological assessments. Blinded analyses revealed that the degree of inflammation and demyelination was similar in mice given either individual treatment alone, used at sub-optimal doses, when compared to saline-treated EAE mice. Their combination, however, at sub-optimal doses resulted in a very marked decrease of inflammation, demyelination and axonal injury (Figs. 2A–C and 3).

We noted a correspondence between the degree and location of inflammation with axonal injury, in that regions with inflammation typically also contained axons of apparent reduced density. Thus, we plotted the degree of axonal injury and inflammation for each animal and such analysis for all the mice analysed in Fig. 2A–C revealed a

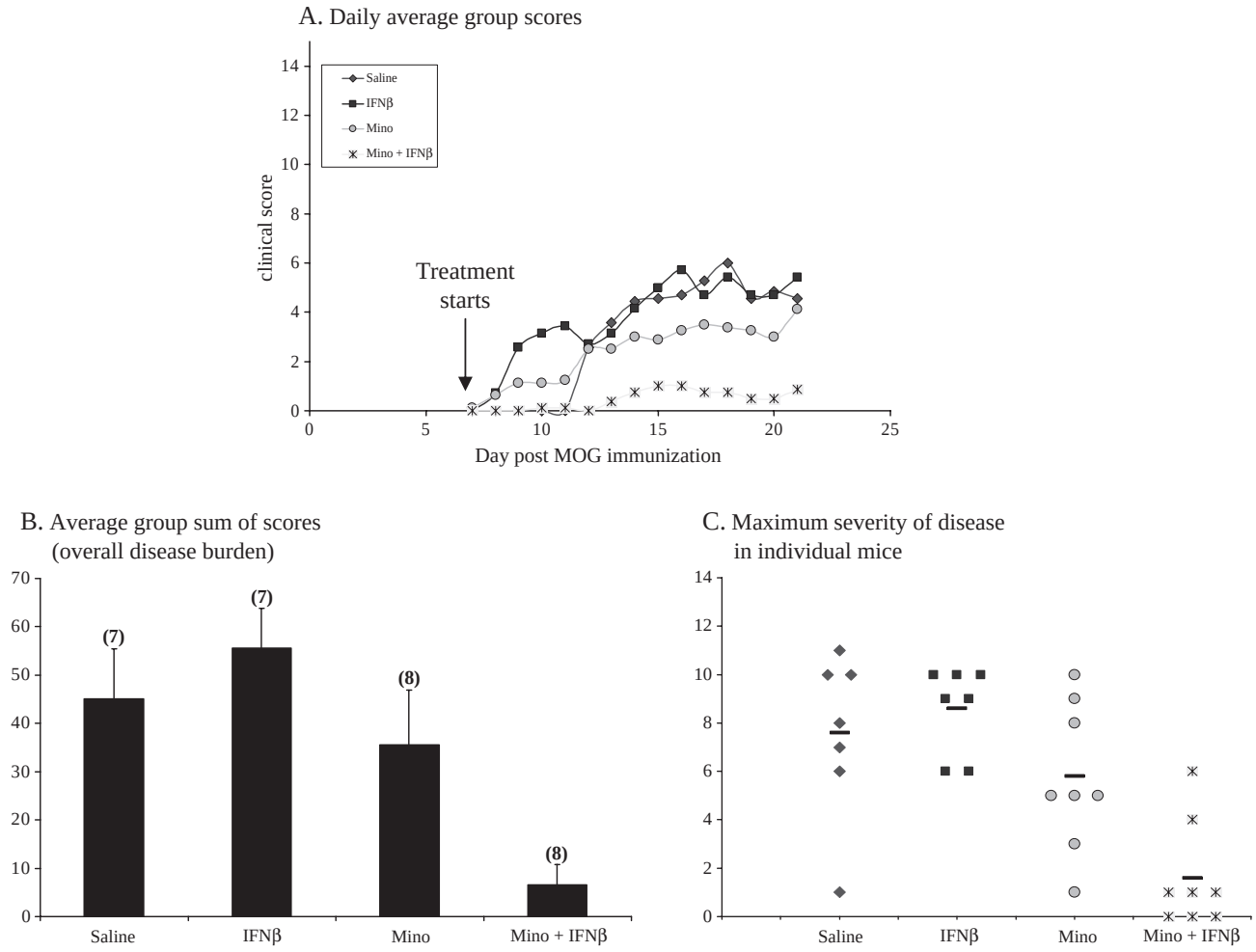

Fig. 1. The combination of minocycline and interferon-β (IFNβ) attenuates clinical severity of EAE. Panel A of mean daily group disease score shows that saline-treated mice developed EAE with an average maximum severity of grade 6 where tail and hind limbs were affected (Grades 2–3 on the commonly used 5 point scale). Treatment of mice with sub-optimal doses of minocycline or interferon-β alone did not alter disease severity but their combination at these doses led to the marked attenuation of disease (tail affected, but there was no hind limb involvement). There were 7 mice each in the saline and interferon-β groups while the minocycline alone or combination groups had 8 mice each. In B, the overall disease burden for each mouse is obtained from the sum of the disability scores tabulated daily over the course of an experiment. The bars represent the mean ± SEM for each group. In panel C, the maximum disease score achieved by each mouse over the course of the entire experiment is plotted, with each symbol representing an individual mouse. Combination versus saline: $*p<0.05$ (Kruskal–Wallis nonparametric ANOVA test). The trend of this figure (with the disease severity of combination group being significantly different from saline mice) was also reproduced across 2 other experiments involving 30–40 mice in each experiment.

very significant correlation (Fig. 2D) between the 2 parameters. The correlation of demyelination with inflammation was also significant but less marked ($r=0.882$, $p<0.0001$).

3.3. Killing of neurons by T cells in culture

Given the significant correlation between axonal injury and inflammation, and in line with our previous demonstration that activated T cells can kill neurons (Giuliani et al., 2003), we investigated whether the treatment of activated T cells with minocycline or interferon-β would alter their capacity to kill neurons. Fig. 4 demonstrates that T cells activated with anti-CD3 produced a marked loss of neurons (by 89% compared to untreated neuronal cultures) by 24 h. The treatment of activated T cells with interferon-β and minocycline alone resulted in some diminution of T cell toxicity to neurons (59% and 67% loss respectively) and this was further increased by the combination of the two medications (21% loss, not significant compared to untreated controls). The health of the neurons in response to the different treatment groups is also indicated in Fig. 5.

4. Discussion

There is increasing interest in combination therapy in MS to achieve a better therapeutic response (Weinstock-Guttman and Bakshi, 2004). Various combinations have been tested in animals (for example, Brod et al., 2000; Soos et al., 2002; Jolivalt et al., 2003; Weilbach et al., 2004) and in humans (for example, Calabresi et al., 2002; Vollmer et al.,

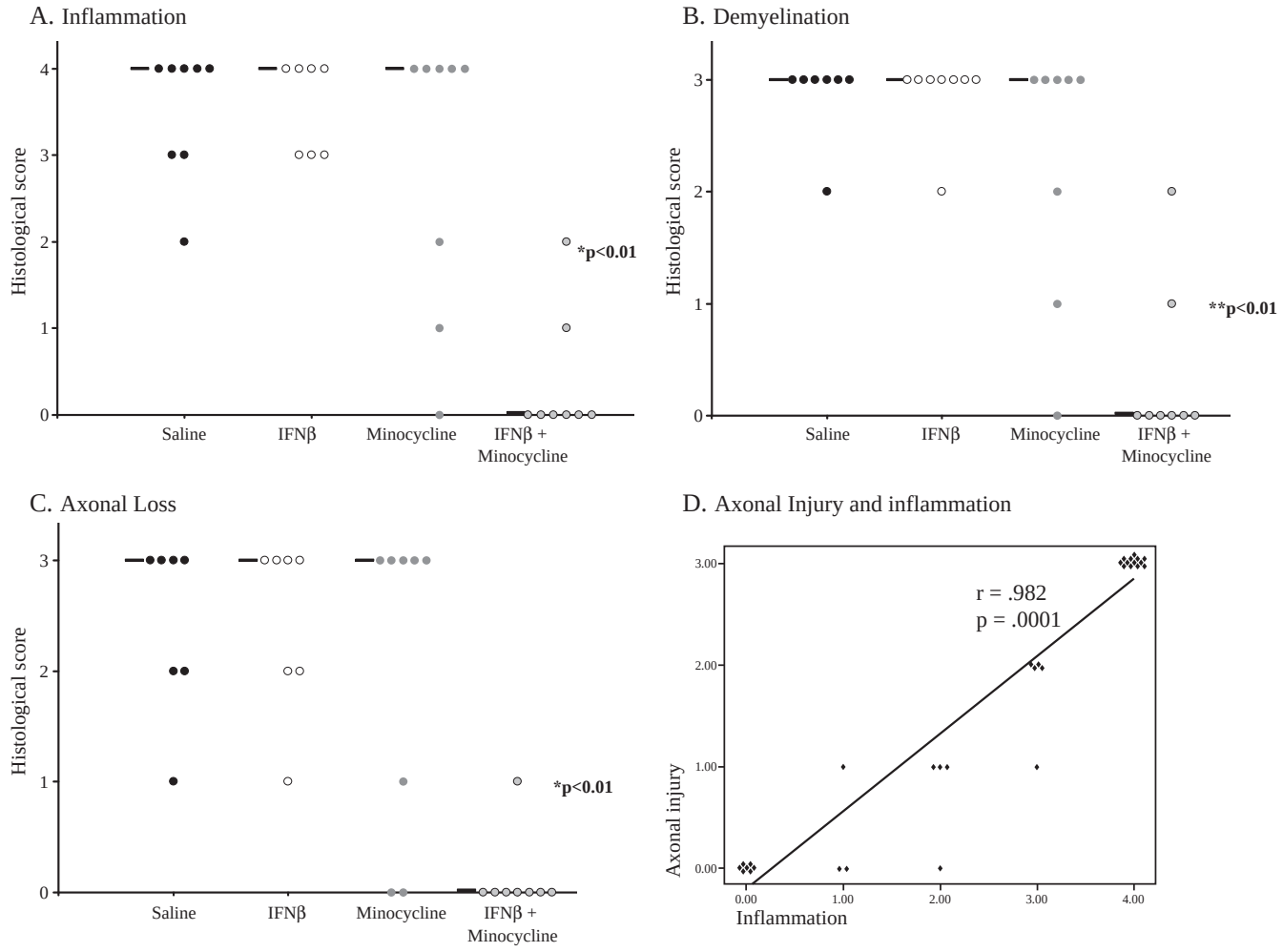

Fig. 2. The degree of histopathology in EAE is decreased by the combination of minocycline and interferon-β. Mice were sacrificed for histological analyses after obtaining the disability profiles shown in Fig. 1. Each point is the value from an individual mouse, analysed blind; the line drawn across each group represents the median. The combination treated mice had a much reduced index of inflammation (A), demyelination (B) and axonal injury (C) compared to that of all the other animals. Groups were analysed using Kruskal–Wallis nonparametric ANOVA test (compared to saline). In D, data from all the animals of the histological analyses from the previous panels was plotted to obtain a correlation coefficient between the degree of inflammation and the extent of axonal loss (using Spearman's nonparametric test).

2004) and there was a recently completed phase I clinical trial of the combination of interferon-β and glatiramer acetate in relapsing–remitting MS (Lublin et al., 2001). Investigators using the latter combination are recruiting subjects for a large multi-center Phase III clinical trial.

Combination therapy in MS is advantageous if one or both drugs are orally available, inexpensive, have complementary actions, and are not toxic with chronic usage. Minocycline meets many of these criteria as it has multiple immunomodulatory and neuroprotective activities (reviewed in Yong et al., 2004) and it has been shown to be safe in chronic long term oral use in the treatment of acne (Seukeran et al., 1997; Shapiro et al., 1997). Serious side effects such as systemic lupus erythrematosus and serum sickness can develop, but the incidence of this is low and in the order of 1 per million (Sturkenboom et al., 1999; Elkayam et al., 1999).

Thus, in this manuscript, we have tested the combination of interferon-β and minocycline in EAE. The results demonstrate that, when used at sub-optimal doses in which each drug alone did not affect EAE outcome, their combination at these doses significantly attenuated disease severity and the neuropathology of EAE. The mechanisms of the combination are likely multiple. At least one contributing mechanism is their effect on activated T cells such that the toxicity of T cells on neurons is significantly attenuated by the combination. This has relevance in the treatment of MS since axonal injury and neuronal loss are now considered hallmarks of the disease (Ferguson et al., 1997; Trapp et al., 1998; Bjartmar and Trapp, 2001; Cifelli et al., 2002). We speculate that in conditions in which activated T cells transmigrate into the CNS of MS patients, the prior exposure of the T cells to interferon-β and minocycline in the periphery makes these "modulated" cells less capable of inflicting neurotoxicity.

The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes implicated in the pathogenesis of MS

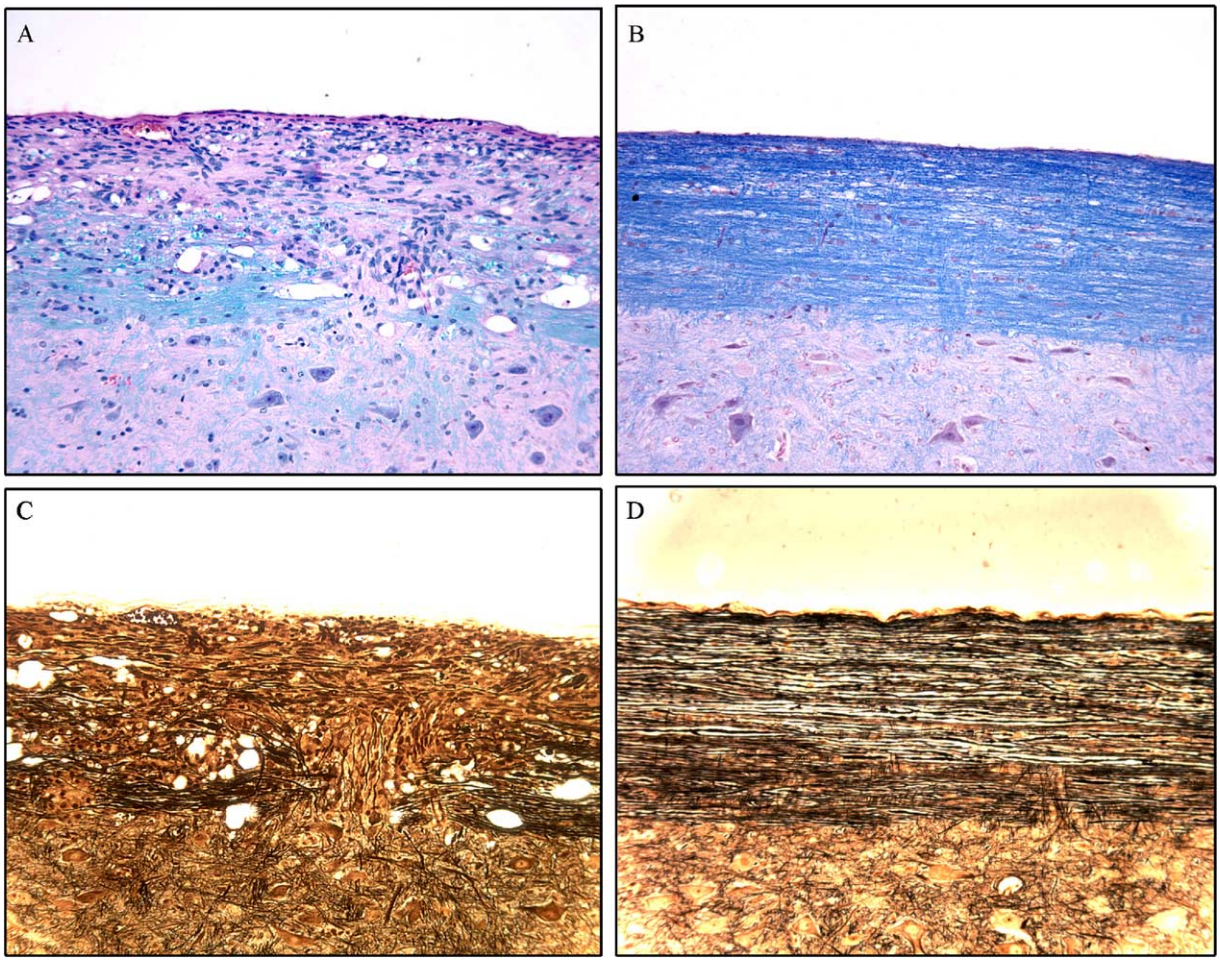

Fig. 3. Histological evidence of the efficacy of the combination of minocycline and interferon-β in EAE. Hematoxylin-eosin and luxol fast blue stained slides (A, B) show inflammation and demyelination in saline-treated mice (A) and a much reduced severity in the combination group (B). In panels C and D, Bielchowsky silver stained specimens indicate axonal injury in the saline (C) and a reduced amount in animals with the combination treatment (D).

(Kieseier et al., 1999; Yong et al., 2001; Rosenberg, 2002; Opdenakker et al., 2003; Fiotti et al., 2004). Detrimental consequences of MMP over-expression in MS may include the increased transmigration of leukocytes into the CNS (Rosenberg, 2002), enhanced neuroinflammation (Starckx et al., 2003), and demyelination (Matyszak and Perry, 1996) and axonal injury (Newman et al., 2001). Minocycline inhibits the production and activity of MMPs (Brundula et al., 2002), while interferon-β reduces MMP production (Stuve et al., 1996; Leppert et al., 1996); recently, it has been noted that interferon-β also inhibits MMP activity (Bartholome et al., 2001; Kieseier et al., 2004). Thus, the combination of minocycline and interferon-β may result in the greater reduction of excessive MMP activity than either alone. In addition, Nelissen et al. (2003) reported that MMP-9 can degrade interferon-β, an action that is inhibited by minocycline. Thus, the concurrent use of minocycline could extend the bioavailability of interferon-β.

Another important series of actions of interferon-β involves adhesion molecules that regulate the entry of leukocytes into tissues, such as vascular cell adhesion molecule-1 (VCAM-1) and leukocyte function antigen-1 (LFA-1). Several of these adhesion molecules are upregulated in MS (Dore-Duffy et al., 1995; Hartung et al., 1995) and this is attenuated by interferon-β treatment (Soilu-Hanninen et al., 1995; Gelati et al., 1999; Muraro et al., 2004). There is also increased levels of soluble VCAM-1 in the sera of MS patients treated with interferon-β (Calabresi et al., 1997; Kraus et al., 2004); this soluble form can bind to its ligand (the very late activating antigen-4, VLA-4) on circulating leukocytes to decrease their adhesion onto endothelial cells and thereby reduce their transmigratory capacity. We recently found that in MS patients treated with minocycline, the level of soluble VCAM-1 in their sera was increased compared to pre-treatment values (Zabad et al., submitted for publication). Thus, the additive effect of interferon-β and minocycline may involve mechanisms that regulate the interaction of leukocytes and endothelial cells prior to their influx into the CNS.

In summary, we have demonstrated that the combination of interferon-β and minocycline provided for a better outcome in EAE than either drug when used alone at sub-optimal doses. We were not able to perform experiments in which interferon-β and minocycline were given at optimal

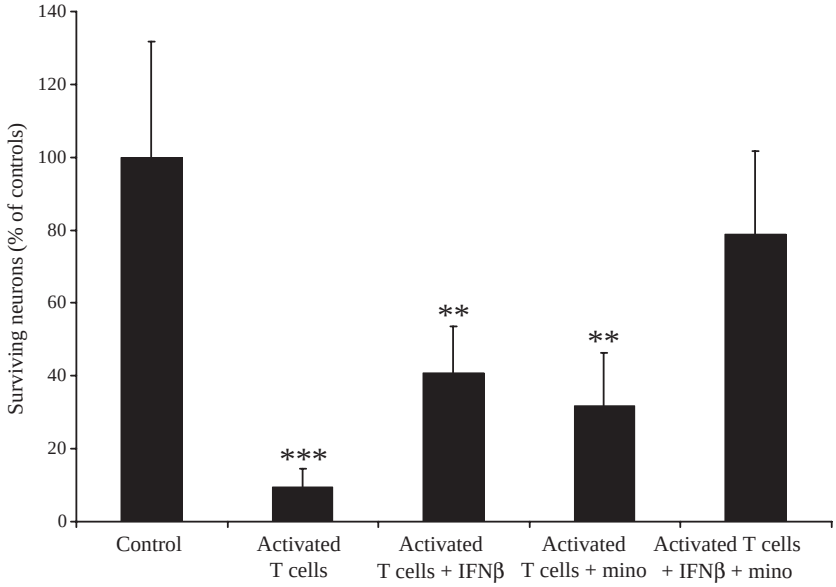

Fig. 4. The number of neurons in culture was counted and normalized to that in the control group, with 100% indicating no toxicity to neurons. On average, 640 neurons per culture in the control group were tabulated. The bars represent the mean ± SEM of 4 cultures per treatment group. Activated T cells are very potent mediators of neuronal death and this toxicity is alleviated by the treatment of activated T cells with interferon-β (Rebif®) or minocycline. The combination of these agents further alleviated cell death, resulting in survival of neurons that were not significantly different from control cultures. $^{**}p<0.01$, $^{***}p<0.001$ compared to controls (1 way ANOVA with Tukey's multiple comparisons).

doses individually to animals since it would not be possible then to address whether a combinational effect could occur. Nonetheless, the results infer that if used adequately at therapeutic doses in MS patients, the combination has the potential to be more effective than either one alone. We also found that the pre-treatment of activated T cells in vitro with minocycline or interferon-β attenuates the potential of these cells to produce cytotoxicity to neurons and this was further alleviated by the combination treatment. Overall, the combination of interferon-β and minocycline in MS appears

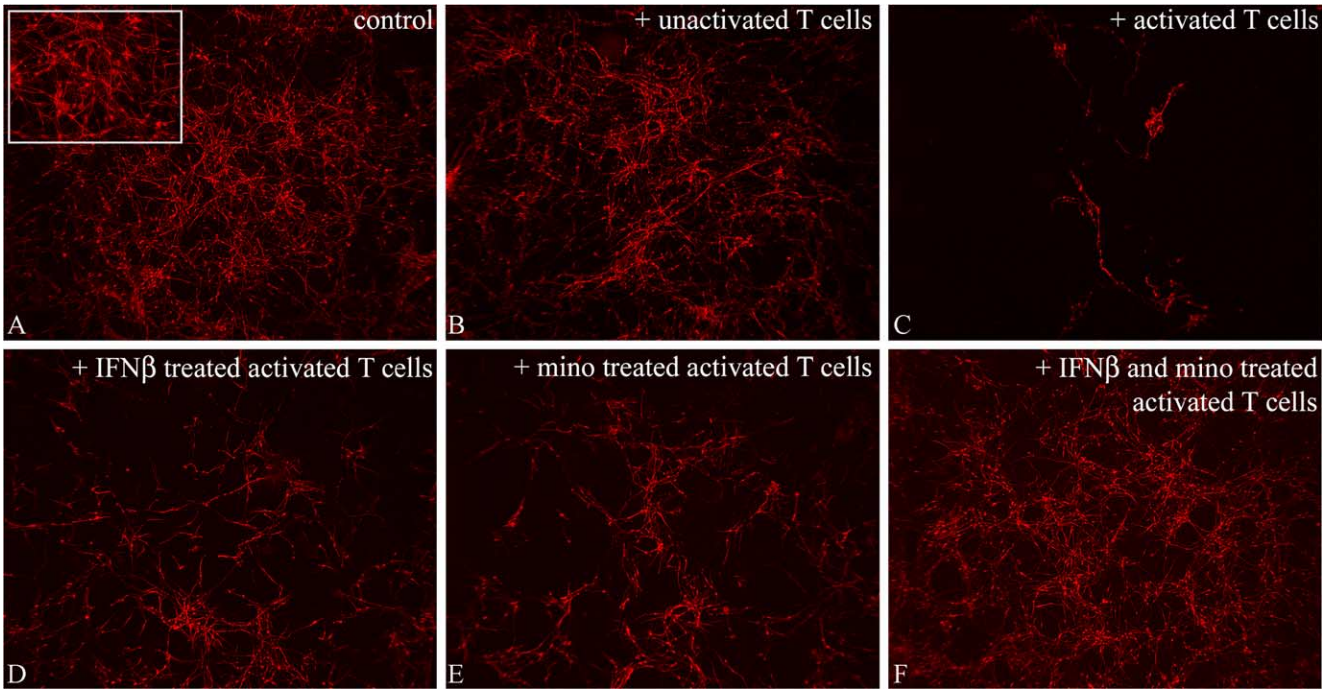

Fig. 5. MAP-2 staining depicts the toxicity of activated T cells to human neurons in culture. A: Control neurons not exposed to T cells, with the insert showing cells at higher magnification. B: Neurons exposed to unactivated T cells do not undergo death. C: When neurons are incubated with activated T cells, very few of them survive at 24 h. The toxicity of activated T cells is alleviated by their pre-treatment with recombinant human interferon-β (Rebif®) (D) and minocycline (E), and their combination further alleviates neuronal death (F).

promising and a clinical trial to test this would seem warranted.

Acknowledgements

This study was supported by operating grants from Serono Canada and the Interdisciplinary Health Research Team Program of the Canadian Institutes of Health Research. We thank Tammy Wilson and Yan Fan for expert technical help.

References

Arvin, K.L., Han, B.H., Du, Y., Lin, S.Z., Paul, S.M., Holtzman, D.M., 2002. Minocycline markedly protects the neonatal brain against hypoxic–ischemic injury. Ann. Neurol. 52, 54–61.

Bartholome, E.J., Van Aelst, I., Koyen, E., Kiss, R., Willems, F., Goldman, M., Opdenakker, G., 2001. Human monocyte-derived dendritic cells produce bioactive gelatinase B: inhibition by IFN-beta. J. Interferon Cytokine Res. 21, 495–501.

Bjartmar, C., Trapp, B.D., 2001. Axonal and neuronal degeneration in multiple sclerosis: mechanisms and functional consequences. Curr. Opin. Neurol. 14, 271–278.

Brod, S.A., Lindsey, J.W., Wolinsky, J.S., 2000. Combination therapy with glatiramer acetate (copolymer-1) and a type I interferon (IFN-alpha) does not improve experimental autoimmune encephalomyelitis. Ann. Neurol. 47, 127–131.

Brundula, V., Rewcastle, N.B., Metz, L.M., Bernard, C.C., Yong, V.W., 2002. Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. Brain 125, 1297–1308.

Calabresi, P.A., Tranquill, L.R., Dambrosia, J.M., Stone, L.A., Maloni, H., Bash, C.N., Frank, J.A., McFarland, H.F., 1997. Increases in soluble VCAM-1 correlate with a decrease in MRI lesions in multiple sclerosis treated with interferon beta-1b. Ann. Neurol. 41, 669–674.

Calabresi, P.A., Wilterdink, J.L., Rogg, J.M., Mills, P., Webb, A., Whartenby, K.A., 2002. An open-label trial of combination therapy with interferon beta-1a and oral methotrexate in MS. Neurology 58, 314–317.

Carney, S., Butcher, R.A., Dawborn, J.K., Pattison, G., 1974. Minocycline excretion and distribution in relation to renal function in man. Clin. Exp. Pharmacol. Physiol. 1, 299–308.

Chen, M., Ona, V.O., Li, M., Ferrante, R.J., Fink, K.B., Zhu, S., Bian, J., Guo, L., Farrell, L.A., Hersch, S.M., Hobbs, W., Vonsattel, J.P., Cha, J.H., Friedlander, R.M., 2000. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat. Med. 6, 797–801.

Cifelli, A., Arridge, M., Jezzard, P., Esiri, M.M., Palace, J., Matthews, P.M., 2002. Thalamic neurodegeneration in multiple sclerosis. Ann. Neurology 52, 650–653.

Comi, G., Filippi, M., Wolinsky, J.S., European/Canadian Glatiramer Acetate Study Group, 2001. European/Canadian multicenter, double-blind, randomized, placebo-controlled study of the effects of glatiramer acetate on magnetic resonance imaging-measured disease activity and burden in patients with relapsing multiple sclerosis. Ann. Neurol. 49, 290–297.

Dore-Duffy, P., Newman, W., Balabanov, R., Lisak, R.P., Mainolfi, E., Rothlein, R., Peterson, M., 1995. Circulating, soluble adhesion proteins in cerebrospinal fluid and serum of patients with multiple sclerosis: correlation with clinical activity. Ann. Neurol. 37, 55–62.

Du, Y., Ma, Z., Lin, S., Dodel, R.C., Gao, F., Bales, K.R., Triarhou, L.C., Chernet, E., Perry, K.W., Nelson, D.L., Luecke, S., Phebus, L.A., Bymaster, F.P., Paul, S.M., 2001. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A. 98, 14669–14674.

Elkayam, O., Yaron, M., Caspi, D., 1999. Minocycline-induced autoimmune syndromes: an overview. Semin. Arthritis Rheum. 28, 392–397.

Ferguson, B., Matyszak, M.K., Esiri, M.M., Perry, V.H., 1997. Axonal damage in acute multiple sclerosis lesions. Brain 120, 393–399.

Fiotti, N., Zivadinov, R., Altamura, N., Nasuelli, D., Bratina, A., Tommasi, M.A., Bosco, A., Locatelli, L., Grop, A., Cazzato, G., Guarnieri, G., Giansante, C., Zorzon, M., 2004. MMP-9 microsatellite polymorphism and multiple sclerosis. J. Neuroimmunol. 152, 147–153.

Gelati, M., Corsini, E., Dufour, A., Massa, G., La Mantia, L., Milanese, C., Nespolo, A., Salmaggi, A., 1999. Immunological effects of in vivo interferon-beta1b treatment in ten patients with multiple sclerosis: a 1-year follow-up. J. Neurol. 246, 569–573.

Giuliani, F., Goodyer, C.G., Antel, J.P., Yong, V.W., 2003. Vulnerability of human neurons to T cell-mediated cytotoxicity. J. Immunol. 171, 368–379.

Giuliani, F., Metz, L., Bar-Or, A., Yong, V.W., 2005. Synergistic activity of minocycline and glatiramer acetate in an animal model of multiple sclerosis. J. Neuroimmunol. 158, 213–221.

Giuliani, F., Hader, W., Yong, V.W., in press. Minocycline attenuates both T cell and microglia activity to impair cytokine production in T cell–microglia interaction. J. Leuk. Biol.

Greig, N.H., Soncrant, T.T., Wozniak, K.M., Rapoport, S.I., 1988. Plasma and tissue pharmacokinetics of human interferon-alpha in the rat after its intravenous administration. J. Pharmacol. Exp. Ther. 245, 574–580.

Hartung, H.P., Reiners, K., Archelos, J.J., Michels, M., Seeldrayers, P., Heidenreich, F., Pflughaupt, K.W., Toyka, K.V., 1995. Circulating adhesion molecules and tumor necrosis factor receptor in multiple sclerosis: correlation with magnetic resonance imaging. Ann. Neurol. 38, 186–193.

Hunter, C.L., Bachman, D., Granholm, A.C., 2004. Minocycline prevents cholinergic loss in a mouse model of Down's syndrome. Ann. Neurol. 56, 675–688.

Jacobs, L.D., Cookfair, D.L., Rudick, R.A., Herndon, R.M., Richert, J.R., Salazar, A.M., Fischer, J.S., Goodkin, D.E., Granger, C.V., Simon, J.H., Alam, J.J., Bartoszak, D.M., Bourdette, D.N., Braiman, J., Brownscheidle, C.M., Coats, M.E., Cohan, S.L., Dougherty, D.S., Kinkel, R.P., Mass, M.K., Munschauer III, F.E., Priore, R.L., Pullicino, P.M., Scherokman, B.J., Whitham, R.H., The Multiple Sclerosis Collaborative Research Group (MSCRG), 1996. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. Ann. Neurol. 39, 285–294.

Johnson, K.P., Brooks, B.R., Ford, C.C., Goodman, A., Guarnaccia, J., Lisak, R.P., Myers, L.W., Panitch, H.S., Pruitt, A., Rose, J.W., Kachuck, N., Wolinsky, J.S., Copolymer 1 Multiple Sclerosis Study Group, 2000. Sustained clinical benefits of glatiramer acetate in relapsing multiple sclerosis patients observed for 6 years. Mult. Scler. 6, 255–266.

Jolivalt, C.G., Howard, R.B., Chen, L.S., Mizisin, A.P., Lai, C.S., 2003. A novel nitric oxide scavenger in combination with cyclosporine A ameliorates experimental autoimmune encephalomyelitis progression in mice. J. Neuroimmunol. 138, 56–64.

Kieseier, B.C., Seifert, T., Giovannoni, G., Hartung, H.P., 1999. Matrix metalloproteinases in inflammatory demyelination: targets for treatment. Neurology 53, 20–25.

Kieseier, B.C., Archelos, J.J., Hartung, H.P., 2004. Different effects of simvastatin and interferon beta on the proteolytic activity of matrix metalloproteinases. Arch. Neurol. 61, 929–932.

Kraus, J., Bauer, R., Chatzimanolis, N., Engelhardt, B., Tofighi, J., Bregenzer, T., Kuehne, B.S., Stolz, E., Blaes, F., Morgen, K., Traupe, H., Kaps, M., Oschmann, P., 2004. Interferon-beta(1b) leads to a short-term increase of soluble but long-term stabilisation of cell surface bound adhesion molecules in multiple sclerosis. J. Neurol. 251, 464–472.

Kriz, J., Nguyen, M.D., Julien, J.P., 2002. Minocycline slows disease progression in a mouse model of amyotrophic lateral sclerosis. Neurobiol. Dis. 10, 268–278.

Leppert, D., Waubant, E., Burk, M.R., Oksenberg, J.R., Hauser, S.L., 1996. Interferon beta-1b inhibits gelatinase secretion and in vitro migration of human T cells: a possible mechanism for treatment efficacy in multiple sclerosis. Ann. Neurol. 40, 846–852.

Lublin, F., Cutter, G., Elfont, R., 2001. A trial to assess the safety of combining therapy with interferon beta-1a and glatiramer acetate in patients with relapsing MS. Neurology 56 (Suppl. 3): A148. (abstract).

MacDonald, H., Kelly, R.G., Allen, E.S., Noble, J.F., Kanegis, L.A., 1973. Pharmacokinetic studies on minocycline in man. Clin. Pharmacol. Ther. 14, 852–861.

Matyszak, M.K., Perry, V.H., 1996. Delayed-type hypersensitivity lesions in the central nervous system are prevented by inhibitors of matrix metalloproteinases. J. Neuroimmunol. 69, 141–149.

Metz, L.M., Zhang, Y., Yeung, M., Patry, D.G., Bell, R.B., Stoian, C.A., Yong, V.W., Patten, S.B., Duquette, P., Antel, J.P., Mitchell, J.R., 2004. Minocycline reduces gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis. Ann. Neurol. 55, 756.

Muraro, P.A., Liberati, L., Bonanni, L., Pantalone, A., Caporale, C.M., Iarlori, C., De Luca, G., Farina, D., Lugaresi, A., Gambi, D., 2004. Decreased integrin gene expression in patients with MS responding to interferon-beta treatment. J. Neuroimmunol. 150, 123–131.

Nelissen, I., Martens, E., Van den Steen, P.E., Proost, P., Ronsse, I., Opdenakker, G., 2003. Gelatinase B/matrix metalloproteinase-9 cleaves interferon-beta and is a target for immunotherapy. Brain 126, 1371–1381.

Nessler, S., Dodel, R., Bittner, A., Reuss, S., Du, Y., Hemmer, B., Sommer, N., 2002. Effect of minocycline in experimental autoimmune encephalomyelitis. Ann. Neurol. 52, 689–690.

Newman, T.A., Woolley, S.T., Hughes, P.M., Sibson, N.R., Anthony, D.C., Perry, V.H., 2001. T-cell- and macrophage-mediated axon damage in the absence of a CNS-specific immune response: involvement of metalloproteinases. Brain 124, 2203–2214.

Opdenakker, G., Nelissen, I., Van Damme, J., 2003. Functional roles and therapeutic targeting of gelatinase B and chemokines in multiple sclerosis. Lancet Neurol. 2, 747–756.

Popovic, N., Schubart, A., Goetz, B.D., Zhang, S.C., Linington, C., Duncan, I.D., 2002. Inhibition of autoimmune encephalomyelitis by a tetracycline. Ann. Neurol. 51, 215–223.

PRISMS-4, 2001. Long-term efficacy of interferon-beta-1a in relapsing MS. Neurology 56, 1628–1636.

Rosenberg, G.A., 2002. Matrix metalloproteinases and neuroinflammation in multiple sclerosis. Neuroscientist 8, 586–595.

Seukeran, D.C., Eady, E.A., Cunliffe, W.J., 1997. Benefit–risk assessment of acne therapies. Lancet 349, 1251–1252.

Shapiro, L.E., Knowles, S.R., Shear, N.H., 1997. Comparative safety of tetracycline, minocycline, and doxycycline. Arch. Dermatol. 133, 1224–1230.

Soilu-Hanninen, M., Salmi, A., Salonen, R., 1995. Interferon-beta down-regulates expression of VLA-4 antigen and antagonizes interferon-gamma-induced expression of HLA-DQ on human peripheral blood monocytes. J. Neuroimmunol. 60, 99–106.

Soos, J.M., Stuve, O., Youssef, S., Bravo, M., Johnson, H.M., Weiner, H.L., Zamvil, S.S., 2002. Cutting edge: oral type I IFN-tau promotes a Th2 bias and enhances suppression of autoimmune encephalomyelitis by oral glatiramer acetate. J. Immunol. 169, 2231–2235.

Starckx, S., Van den Steen, P.E., Verbeek, R., van Noort, J.M., Opdenakker, G., 2003. A novel rationale for inhibition of gelatinase B in multiple sclerosis: MMP-9 destroys alpha B-crystallin and generates a promiscuous T cell epitope. J. Neuroimmunol. 141, 47–57.

Stirling, D.P., Khodarahmi, K., Liu, J., McPhail, L.T., McBride, C.B., Steeves, J.D., Ramer, M.S., Tetzlaff, W., 2004. Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury. J. Neurosci. 24, 2182–2190.

Sturkenboom, M.C., Meier, C.R., Jick, H., Stricker, B.H., 1999. Minocycline and lupuslike syndrome in acne patients. Arch. Intern. Med. 159, 493–497.

Stuve, O., Dooley, N.P., Uhm, J.H., Antel, J.P., Francis, G.S., Williams, G., Yong, V.W., 1996. Interferon beta-1b decreases the migration of T lymphocytes in vitro: effects on matrix metalloproteinase-9. Ann. Neurol. 40, 853–863.

The IFNB Multiple Sclerosis Study Group, 1993. Interferon beta-1b is effective in relapsing–remitting multiple sclerosis: I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. Neurology 43, 655–661.

Trapp, B.D., Peterson, J., Ransohoff, R.M., Rudick, R., Mork, S., Bo, L., 1998. Axonal transection in the lesions of multiple sclerosis. N. Engl. J. Med. 338, 278–285.

Vollmer, T.L., Phillips, J.T., Goodman, A.D., Agius, M.A., Libonati, M.A., Giacchino, J.L., Grundy, J.S., 2004. An open-label safety and drug interaction study of natalizumab (Antegren) in combination with interferon-beta (Avonex) in patients with multiple sclerosis. Mult. Scler. 10, 511–520.

Weilbach, F.X., Chan, A., Toyka, K.V., Gold, R., 2004. The cardioprotector dexrazoxane augments therapeutic efficacy of mitoxantrone in experimental autoimmune encephalomyelitis. Clin. Exp. Immunol. 135, 49–55.

Weinstock-Guttman, B., Bakshi, R., 2004. Combination therapy for multiple sclerosis: the treatment strategy of the future? CNS Drugs 18, 777–792.

Wells, J.E., Hurlbert, R.J., Fehlings, M.G., Yong, V.W., 2003. Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. Brain 126, 1628–1637.

Wills, R.J., 1990. Clinical pharmacokinetics of interferons. Clin. Pharmacokinet. 19, 390–399.

Wu, D.C., Jackson-Lewis, V., Vila, M., Tieu, K., Teismann, P., Vadseth, C., Choi, D.K., Ischiropoulos, H., Przedborski, S., 2002. Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. J. Neurosci. 22, 1763–1771.

Yong, V.W., Power, C., Forsyth, P., Edwards, D.R., 2001. Metalloproteinases in biology and pathology of the nervous system. Nat. Rev., Neurosci. 2, 502–511.

Yong, V.W., Wells, J., Giuliani, F., Casha, S., Power, C., Metz, L.M., 2004. The promise of minocycline in neurology. Lancet Neurol. 3, 744–751.

Yrjanheikki, J., Keinanen, R., Pellikka, M., Hokfelt, T., Koistinaho, J., 1998. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. Proc. Natl. Acad. Sci. U. S. A. 95, 15769–15774.

Zabad, R.K., Metz, L.M., Todoruk, T.R., Zhang, Y., Mitchell, J.R., Yeung, M., Patry, D.G., Bell, R.M., Yong, W.V., submitted for publication. Minocycline in multiple sclerosis: continued clinical and MRI response is supported by increased Interleukin-12 p40 and soluble VCAM levels and reduced MMP-9 activity.