ELSEVIER

Journal of Neuroimmunology 158 (2005) 213–221

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Additive effect of the combination of glatiramer acetate and minocycline in a model of MS☆

Fabrizio Giuliani[a], Luanne M. Metz[a], Tammy Wilson[a], Yan Fan[a], Amit Bar-Or[b], V. Wee Yong[a,c,*]

[a]*Departments of Clinical Neurosciences University of Calgary, Calgary, Alberta, Canada*
[b]*Montreal Neurological Institute, McGill University Montreal, Quebec, Canada*
[c]*Oncology University of Calgary, Calgary, Alberta, Canada*

Received 28 August 2004; accepted 3 September 2004

Abstract

There have been significant advances in the treatment of multiple sclerosis (MS) in recent years, but further improvement in therapy is required as not all patients have responded optimally. An approach to enhancing MS treatment is to combine drugs that impact on different aspects of the disease process. We have described that the tetracycline derivative, minocycline, attenuates the severity of experimental autoimmune encephalomyelitis (EAE), a model of MS. Here, we have evaluated the combination of minocycline and glatiramer acetate (GA), a current therapy in MS, on the course of EAE in mice. This combination resulted in a significant reduction of disease severity and disease burden with attenuation of the inflammation, axonal loss and demyelination.

Keywords: Multiple sclerosis; Glatiramer acetate; Minocycline; EAE

1. Introduction

The management of patients with multiple sclerosis (MS) has improved significantly in the last decade with the introduction of the immunomodulators, interferon-βs and glatiramer acetate (GA). While many patients respond favourably to these drugs, some, however, have a less than ideal response. New medications and the rational combination of existing therapies may lead to a better therapeutic outcome in MS.

Minocycline is a semisynthetic derivative of tetracycline that is effective against gram-positive and -negative infections. It is commonly used in the treatment of acne where a good safety record for prolonged oral use has been established. In recent years, it has been appreciated that minocycline has several immunomodulatory activities, including the inhibition of inducible nitric oxide synthase (Yrjanheikki et al., 1998), caspase-1 and -3 (Chen et al., 2000) and mitogen-activated kinases (Du et al., 2001). Minocycline has also been found to attenuate microglia functions and glutamate excitotoxicity (Yrjanheikki et al., 1999; Tikka et al., 2001; Wu et al., 2002) and to inhibit apoptosis through preventing the mitochondrial permeability transition-mediated release of cytochrome *C* from mitochondria (Zhu et al., 2002). We have further described that minocycline reduces the level and activity of matrix metalloproteinase (MMP)-9 produced by T lymphocytes, and that this is correlated with the reduced ability of minocycline-treated lymphocytes to transmigrate a model of the blood–brain barrier (Brundula et al., 2002). MMPs are a family of proteases that are implicated in the influx of leukocytes into the CNS in both health and CNS inflammatory conditions, such as MS (Yong et al., 2001).

Given its multiple immunomodulatory activities, we have tested and found that minocycline attenuated the

☆ This study was funded by a research grant from Teva Neuroscience, Canada. VWY, LMM and A.B.O. have received honoraria or educational grants from Teva Neuroscience, Berlex Laboratories, Serono and Biogen.

* Corresponding author. University of Calgary, 3330 Hospital Drive, Calgary Alberta T2N 4NI, Canada. Tel.: +1 403 220 3544; fax: +1 403 283 8731.

E-mail address: vyong@ucalgary.ca (V. Wee Yong).

0165-5728/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2004.09.006

clinical severity and histological consequences of experimental autoimmune encephalomyelitis (EAE) in mice (Brundula et al., 2002), an observation that was also noted by Popovic et al. (2002) in rats. We have recently completed a baseline versus treatment trial of minocycline in relapsing–remitting MS, in which a significant and rapid decrease in the number and volume of gadolinium-enhancing lesions were found after treatment (Metz et al., 2004).

Whether minocycline by itself would be useful as a treatment in MS awaits confirmation in phase III trials. However, inasmuch as minocycline is orally bioavailable, is reasonably priced and has multiple immunomodulatory as well as neuroprotective activities (reviewed in Yong et al., revised), it may be a useful adjunctive therapy to immunomodulators that are currently approved for use in MS. In this study, we have evaluated whether the combination of minocycline and GA would result in a greater response in EAE compared to either alone. Because optimal doses of GA alone prevented disease activity altogether making it impossible to study combination treatment, we have used GA at suboptimal doses that, alone, showed minimal benefit in EAE. Similarly, minocycline was used at suboptimal doses so that any possible synergistic activity with GA could be determined. We demonstrate that the combination of these drugs resulted in a marked attenuation of disability, as well as a significant improvement in the neuropathological correlates of the disease. The mechanism involved may involve an increased shift of T lymphocytes towards a Th2 response compared to the single treatments alone. These results suggest the utility of the combination of minocycline and GA in MS.

2. Methods

2.1. Induction of EAE and grading of disability

C57/BL6 mice of 8 to 10 weeks of age at the start of each experiment were used. EAE was induced by using 50 μg of the immunogenic myelin oligodendrocyte glycoprotein (MOG) peptide (amino acids 35–55, synthesized by the Peptide Facility of the University of Calgary) emulsified in 100 μl of complete Freund adjuvant (Difco Laboratories, Detroit, Michigan); this was not supplemented with mycobacterium. Pertussis toxin (0.3 μg/200 μl, List Biological Laboratories, Campbell, CA) was injected intraperitoneally at days 0 and 2; day 0 in this manuscript refers to the day of MOG immunization.

Mice were weighed and evaluated daily. To assess the degree of disability of EAE symptoms, we considered that the normally used scale of 0 to 5 (e.g., see Brundula et al., 2002; Popovic et al., 2002) was inadequate inasmuch as this scored mice equally whether one or two hind or forelimbs were involved. For example, there could be quite marked differences between two mice designated as grade 2 EAE on the old scale; yet, one mouse might have two hind limbs impaired, while the other mouse might have only one hind limb involved. Thus, we developed a new scale to better differentiate functional outcomes. This scale goes from 1 to 15 and is the sum of the state of the tail and all of the four limbs. For tail function, a score of 0 referred to no symptoms, 1 was partial paralysis, while 2 was full paralysis. For the hind or forelimbs, each assessed separately, a grade of 0 referred to no symptoms, 1 was a weak and abnormal walk with that limb, 2 was a limb that was dragged but which still had movements, while the score of 3 was full paralysis of that limb. Thus, a fully paralysed quadriplegic animal would attain a score of 14. A mouse with tail and three limbs maximally involved (the fourth being normal) would have a score of 11; in the old scale, this would not have been differentiated easily from a fully paralysed mouse, as both would be scored 4. Death in the new scale is given a score of 15.

Another parameter that we have employed is the "sum of scores." This refers to the addition of each daily clinical score over the entire period of the experiment and is a reflection of the total burden of disease.

2.2. Treatment of mice

Given that one of the mechanisms of GA in MS is thought to involve the generation of GA-specific Th2 cells (Sela and Teitelbaum, 2001; Neuhaus et al., 2001; Yong, 2002) and as Th2-polarised cells require time to be generated in vivo, it was decided to give GA as a pretreatment to mice. This is congruent with other reports of GA in the alleviation of EAE, where GA was given prior to the induction of disease (Aharoni et al., 2000; Brod et al., 2000). We administered GA (Teva Pharmaceuticals, Israel) as a single subcutaneous injection before MOG immunization in 100 μl of incomplete Freund adjuvant (IFA). This formulation in IFA, which aids in the generation of an immune response, avoids the need for daily administration of GA to animals.

In preliminary dose-finding experiments, we administered a single dose (200, 500 μg or 2 mg) of GA in IFA to groups of three mice each 14 days prior to the administration of MOG. While saline-treated EAE control mice attained an average maximum disease score of 6 representing moderate EAE severity of hind limb impairment that did not ascend to the forelimbs, treatment with 2 mg/mouse of GA prevented the manifestation of EAE altogether. Two hundred and five hundred microgram per mouse of GA resulted in an average disease score that was between that of saline-treated and 2-mg GA mice (data not shown). Thus, the doses of 200 and 500 μg of GA were considered suboptimal in preventing EAE, and subsequent experiments utilised an intermediate of these doses, 250 μg/mouse (equivalent to 10 mg/kg). All subsequent experiments with GA utilised this suboptimal dose given once in IFA, 7 days before MOG treatment.

Minocycline (HCl salt, Sigma, Oakville, Canada) was administered at 25 mg/kg intraperitoneally to mice designated for this drug when the first mouse in an entire experiment began to develop signs of EAE. As noted in Fig. 1, this was 7 days post-MOG immunization. Minocycline was administered daily until mice were sacrificed. In previous experiments, we had determined that a fully effective dose regimen for minocycline in EAE would require 50 mg/kg twice a day for the first 2 days, 50 mg/kg once daily for the next 5 days and 25 mg/kg once daily thereafter (Brundula et al., 2002).

2.3. Neuropathological examinations

Anesthetized mice were subjected to a cardiac puncture to drain blood, 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

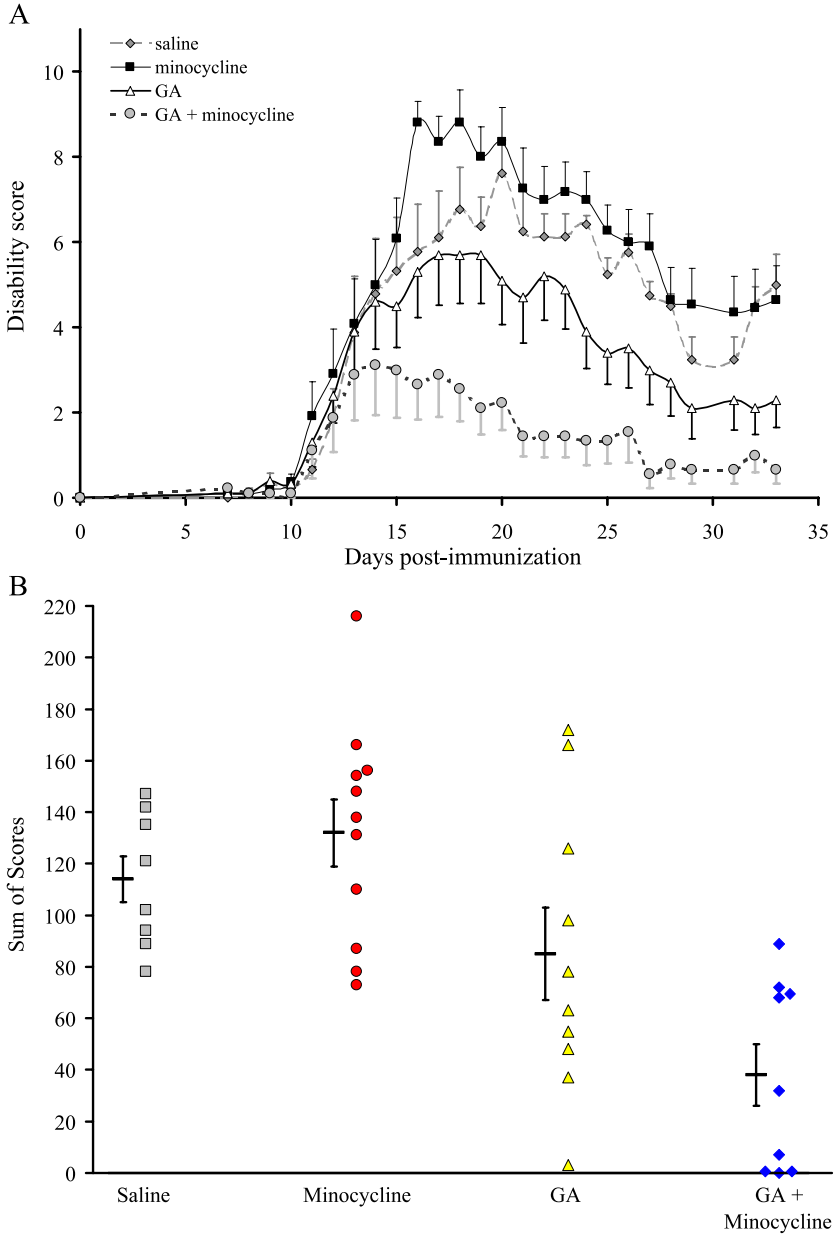

Fig. 1. The combination of GA and minocycline attenuated EAE. While suboptimal doses of minocycline or GA alone did not significantly alter disease severity, the combination of GA and minocycline at these suboptimal doses attenuated EAE severity over the course of the experiment (A). In panel (B), the overall disease burden for each mouse is represented as the sum of scores, which is the sum of the disability scores obtained daily over the course of the 33-day experiment. Each symbol is the sum of score for each mouse, and the mean±S.E.M. is displayed for each group. There were 8 mice in the saline-treated group, 11 in the minocycline alone group, 10 in the GA alone group and 9 in the GA plus minocycline group. Combination versus saline, $p<0.001$; combination versus minocycline, $p<0.001$; combination versus GA, $p<0.05$.

spinal cord for longitudinal sections were then embedded in paraffin. Two adjacent 6-μm thick sections were cut every 60 μm and placed on separate glass slides for a total of two series of 15 sections from each animal. The first series of sections was subsequently stained with hematoxylin, eosin and Luxol fast blue for evidence of inflammation and demyelination, respectively, and the second series of adjacent sections was processed with Bielschowsky silver stain to identify axons.

Histological scores of the degree of inflammation, demyelination and axonal loss in the spinal cord of each animal were evaluated blind, using a semiquantitative system where 0 was no disease and ascending numerical scores indicated increasing degrees of pathology. For inflammation in hematoxylin, eosin and Luxol fast blue-stained slides, because the inflammation tended to be concentrated in the meninges initially which then progressed deeper into the parenchyma towards the spinal cord grey matter 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. In the case of demyelination in hematoxylin, eosin and Luxol fast blue-stained slides, a score of 0 has 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 in Bielschowsky silver-stained slides, grade 0 has no evidence of axonal loss, grade 1 contains 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.

Histological analyses were performed on samples collected from the experiment listed in Fig. 1.

2.4. Cytokine analysis

Splenocytes and lymph node cells (LNC) cells were isolated 10 days post-MOG immunization from groups of mice administered with (1) vehicle, (2) minocycline (started 6 days postimmunization) only, (3) GA only (250 μg/mouse GA was given in IFA 7 days before MOG) or (4) minocycline plus GA (same dose regimen as in the individually treated groups). Cells were cultured in 96-well microtitre plates at a concentration of $4\times10_5$ cells ml^{-1} with the specific encephalitogenic peptide (MOG p35–55; 25 μg/ml) used for the immunization.

Supernatants from splenocytes and LNC were collected after 72 h, and protein levels of cytokines representative of Th1 (interferon-γ; IFNγ) or Th2 cytokines (interleukin-5; IL-5) were measured using the appropriate ELISA kits (Biosource, Camarillo, CA). The assay was performed following detailed instructions from the manufacturer. Results are shown as mean of triplicates ± S.D.

2.5. Statistical analyses

As the majority of the animal data collected in this manuscript consisted of numerical scores in four treatment groups, the Kruskal-Wallis nonparametric ANOVA test was employed, along with post hoc Dunn's multiple comparisons test. For cytokine analysis, a parametric ANOVA was used, with Tukey's post hoc test. Statistical significance was set at $p<0.05$.

3. Results

3.1. The combination of GA and minocycline alleviated EAE severity compared to either alone

We tested the combination of suboptimal doses of GA (250 μg/mouse) and minocycline (25 mg/kg) when used alone or in combination. Fig. 1A shows that in saline-treated mice, EAE of moderate severity was attained; the maximum disease severity of grade 7 (akin to grade 3 on the old EAE scale) indicated hind limb but not forelimb involvement. Treatment with a suboptimal dose of minocycline or GA by themselves did not affect EAE outcome. Interestingly, the combination of these suboptimal doses of GA and minocycline resulted in a significant reduction of EAE severity (Fig. 1A). This was apparent by day 14, which was 7 days after the initiation of minocycline treatment. The sum of scores was also plotted (Fig. 1B), and there was a significant decrease in disease burden in animals given the combination treatment compared to all other groups.

The benefit of the combined treatment over either alone was also noted in two other separate series of experiments involving 8–10 mice per treatment group in each series (data not shown).

3.2. GA and minocycline in combination decreases the neuropathology of EAE

The spinal cord of mice was removed for histological analyses following the completion of the behavioral assessments of Fig. 1. With respect to inflammation, saline-treated EAE mice had a mean histological score of 4, indicative of diffuse and widespread parenchymal infiltration. The degree of inflammation was slightly reduced in the minocycline or GA groups compared to control EAE mice, but this did not attain statistical significance (Fig. 2A). In contrast, in the combined GA plus minocycline group, inflammation remained confined to the subarachnoid space (histological score of 2).

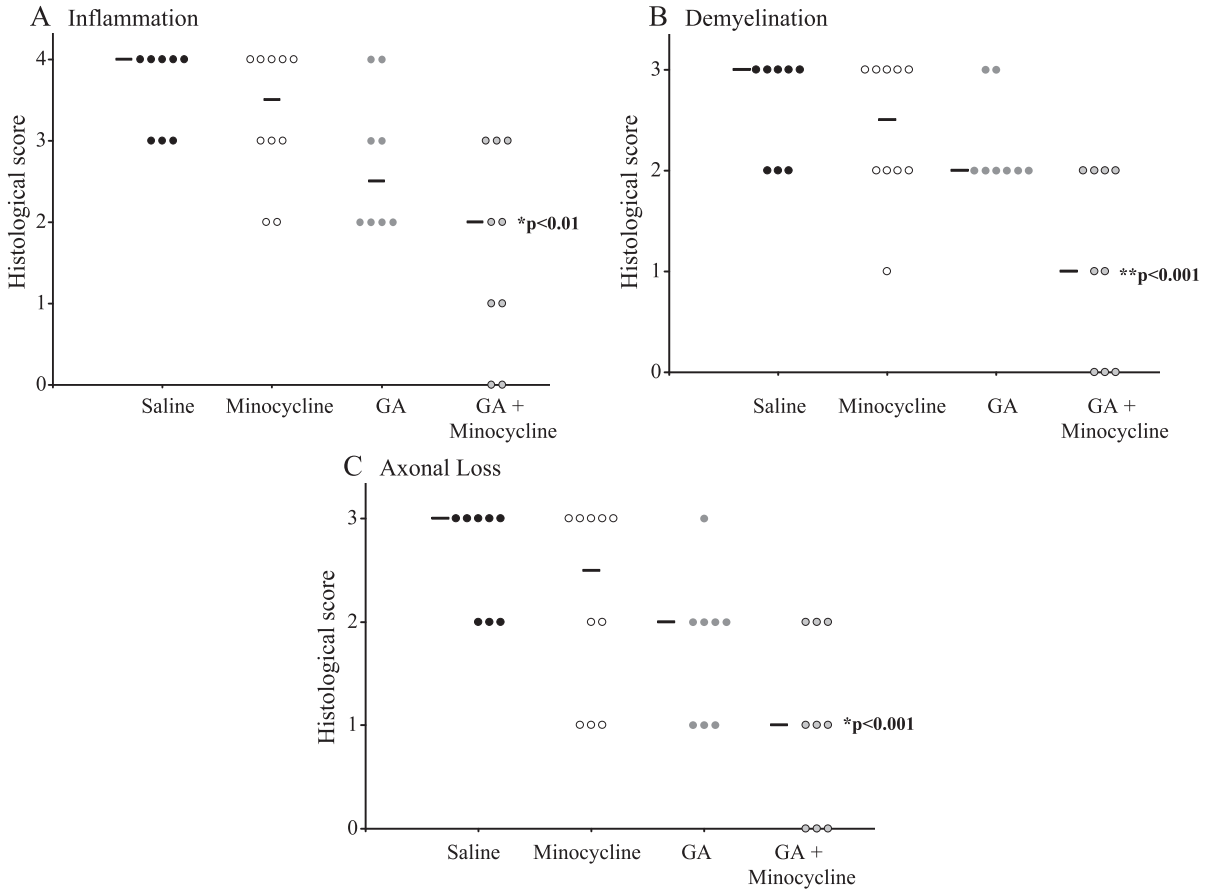

Fig. 2. The combination of GA and minocycline improves the neuropathology of EAE. The degree of inflammation (A), demyelination (B) and axonal loss (C) was graded semiquantitatively and in a blinded manner, as described in Methods. Each point refers to a single animal while the bar represents the median score for each group. Although modestly reduced, the values for GA or minocycline alone were not statistically different from that of saline controls. In contrast, as noted in the *p* values compared to saline controls, the combination of GA and minocycline decreased the inflammation, axonal loss and demyelination resulting from EAE.

The demyelination index (Fig. 2B) also revealed a beneficial outcome for the combination of GA plus minocycline, as mice had a median score of 1 that reflected foci of demyelination that were confined superficially near the subarachnoid space. In contrast, the median score for saline mice was 3, representing widespread and diffuse demyelination in the spinal cord. Mice given either minocycline or GA alone tended towards a reduced median demyelination score compared to saline controls, but this was not statistically significant from the saline control group.

Finally, the evaluation of axonal integrity demonstrated widespread loss (median score of 3) in saline-treated EAE mice, and this was attenuated by the combination of GA and minocycline which confined axonal loss to the superficial areas near the subarachnoid space (median score of 1; Fig. 2C). As with the demyelination index, mice given either minocycline or GA alone had a reduced median axonal loss score compared to saline-treated controls, but these values did not arrive at statistical significance.

Collectively, these results show that the combination of GA and minocycline reduced the neuropathological correlates of EAE, in correspondence with the reduction of disability score described earlier. Representative sections depicting the reduction of inflammation, demyelination and axonal loss by the combination treatment over saline controls are displayed in Fig. 3.

3.3. The combination of glatiramer acetate with minocycline promotes a Th2 shift

As EAE is considered a Th1-mediated disease, we examined whether Th regulation was altered during treatment with the combination of GA plus minocycline compared to GA or minocycline alone. Cells were collected from spleen and lymph nodes of MOG-EAE animals treated with saline, GA, minocycline or the combination of GA and minocycline. These cells were subsequently restimulated with MOG (25 μg/ml) in vitro. After 72 h, supernatants were collected and analyzed for IFNγ or IL-5 as markers, respectively, for a Th1 or a Th2 response. As shown in Fig. 4, cells isolated from mice treated with the combination of GA and minocycline had reduced secretion of IFNγ (Fig. 4A) and

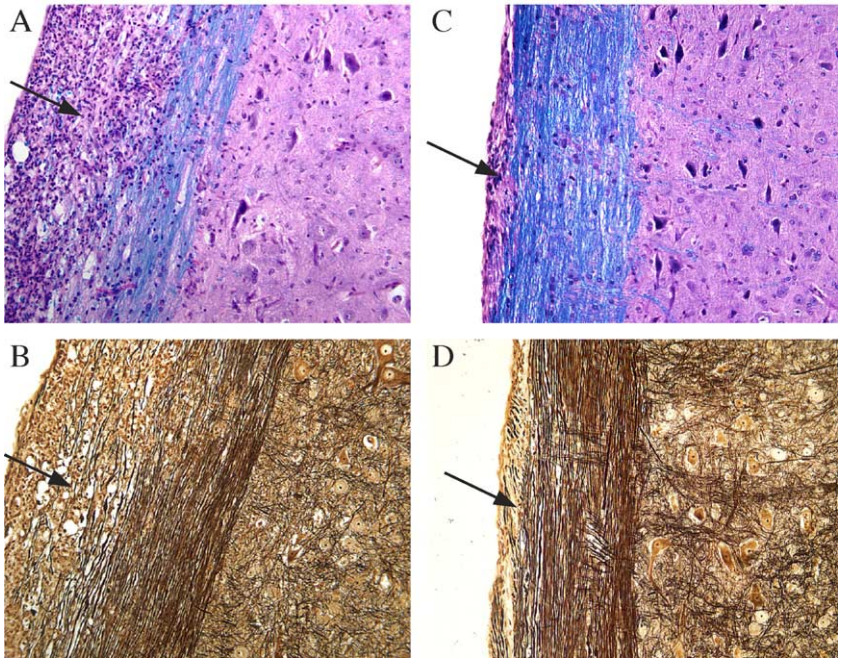

Fig. 3. Inflammation, demyelination and axonal loss are reduced by the combination of GA and minocycline. Displayed are representative results from saline control group (A and B) and from mice given the combination treatment (C and D). Note that while the inflammation and demyelination (arrows) is widespread in the saline group (A), this was confined to the superficial areas bordering the subarachnoid space in mice given GA and minocycline (C). Similarly, axonal disruption and loss assessed by Bielschowsky silver stain is more extensive in saline-treated mice (B) than in the spinal cord of mice treated with the combination of GA plus minocycline (D).

increased production of IL-5 (Fig. 4B), compared to cells from the saline-treated group, when restimulated with MOG in vitro. Cells from animals administered with the suboptimal doses of GA or minocycline alone did not respond differently from those of the saline-treated group. These results indicate that a mechanism for the combination of GA and minocycline in alleviating EAE is related to the promotion of the generation of Th2-biased cells.

4. Discussion

It is increasingly being recognised that combination therapy with existing or novel MS therapeutics may produce a more favourable clinical outcome than monotherapy in the disease and therefore represent the future of MS treatment. Combination therapies are being tested in animals (e.g., see Brod et al., 2000; Soos et al., 2002) and humans (e.g., see Calabresi et al., 2002), including a recently completed phase

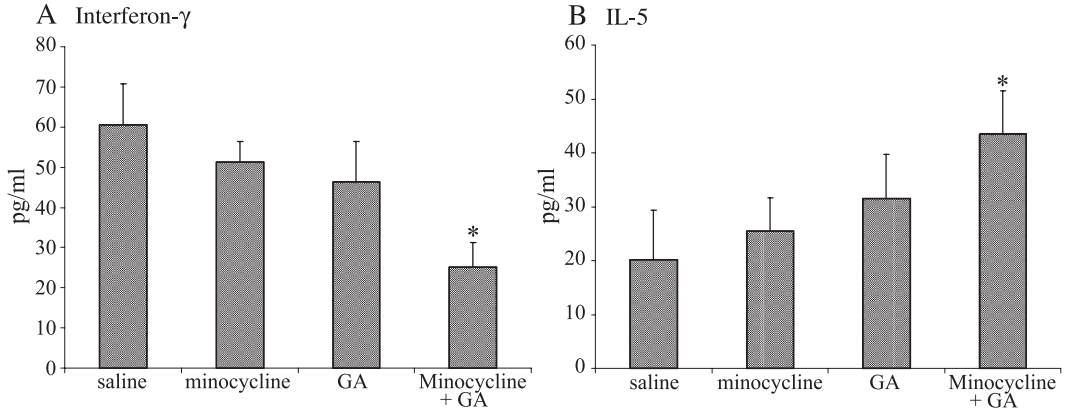

Fig. 4. The combination of GA plus minocycline promotes a Th2 bias of T cells. (A) Interferon-γ levels. (B) IL-5 levels. One-way ANOVA, Tukey's post hoc test compared to microglia plus activated T cells, *$p<0.05$.

I clinical trial of the combination of interferon-β and GA (Lublin et al., 2003), which represent two of the major approved immunomodulators in MS. Combination therapy in MS is advantageous if one or both drugs are orally available, reasonably priced, has no competing actions and has low toxicity with chronic usage. Because minocycline meets many of these criteria and appears to impact on multiple mediators of neurotoxicity (Yrjanheikki et al., 1998, 1999; Chen et al., 2000; Du et al., 2001; Tikka et al., 2001; Wu et al., 2002; Zhu et al., 2002), we have considered that this drug would be useful as a mono- or adjunct therapy in MS. In this regard, we described that minocycline attenuated EAE disease severity in mice (Brundula et al., 2002), and this was also noted in rat EAE by another group (Popovic et al., 2002). Furthermore, we have recently found that minocycline has neuroprotective properties, and that it reduced axonal loss following acute spinal cord trauma in mice (Wells et al., 2003). Axonal injury is now recognised to be a hallmark of MS (Ferguson et al., 1997; Trapp et al., 1998). Thus, minocycline could be useful in MS either as a monotherapy, the subject of a recently completed phase I clinical trial (Metz et al., 2004), or it could be used to supplement the activity of approved MS therapeutics.

In this manuscript, we have tested the possibility that minocycline may have additive activity with GA. A major mechanism of GA is thought to involve the generation of GA-specific T cells that are of the antiinflammatory Th2 subclass, which then attenuate peripheral and central inflammation through bystander suppression (Sela and Teitelbaum, 2001; Neuhaus et al., 2001; Yong, 2002). GA is not thought to directly affect the levels of MMPs or the trafficking of leukocytes into the CNS (Yong, 2002), which are characteristics of minocycline (Brundula et al., 2002). Thus, the combination of minocycline with GA may act by multiple mechanisms against the disease process in MS.

By testing minocycline and GA in isolation and then together, we first found that these drugs, used individually at suboptimal doses, did not markedly decrease disease severity. It is emphasized that a suboptimal dose of GA or minocycline was used in the combination experiments, as optimal doses of either would have completely prevented mice from developing EAE and thus would not have been useful in evaluating efficacy in combination experiments. However, the combination at these suboptimal doses resulted in a significant attenuation of disease, either when evaluated clinically or by neuropathological criteria. It is noteworthy that the combination of minocycline and GA decreased not only neuro-inflammation but also axonal loss and demyelination. A mechanism accounting for the additive effect is the increased generation of Th2-polarised cells in the combination-treated animals. Inasmuch as minocycline is not known to increase Th2 bias while GA is (Yong, 2002), we would propose that the presence of minocycline facilitates the formation of GA-specific Th2 cells in vivo. It is likely that other mechanisms may also be involved, and these remain to be defined.

In considering safety issues, minocycline has been generally found to be innocuous in the chronic treatment of patients with acne (Seukeran et al., 1997; Shapiro et al., 1997; O'Dell, 1999; Sturkenboom et al., 1999). While serious drug reactions can occur, including serum sickness-like reactions and drug-induced lupus (Elkayam et al., 1999), the incidence of these is very rare (1.6 cases per million exposure) and tends to be found in subjects of African descent, a population more prone to hypersensitivity reactions. Also, there is clinical and biochemical resolution of adverse events upon drug withdrawal (Gough et al., 1996; Akin et al., 1998). In our baseline versus treatment trial of its use in MS, minocycline was found to be safe, well tolerated and rapidly reduced MRI enhancing activity (Metz et al., 2004).

An issue that has been raised is whether the effective doses of minocycline in rodents (ordinarily 50 mg/kg) are high when compared to the standard 100-mg bid dose used in humans for the treatment of acne. Animals often require higher per kilogram doses of drugs than humans due to the higher rate of liver metabolism in small animals, particularly rodents. Indeed, it has been found that the half-life of minocycline in rodents is about 2–3 h (Andes and Craig, 2003; Colovic and Caccia, 2003) while that in humans is about 15 h (Saivin and Houin, 1988). Furthermore, we have conducted pharmacokinetic studies in which we have found that the disparate doses given to mice (50 mg/kg, intraperitoneal) and humans (100 mg, oral) achieve similar steady-state concentrations (5–10 μg/ml) in the serum (Wells, Simon, Lyons, Metz, Yong, manuscript in preparation). Thus, the higher doses of minocycline used in animal studies appear to correspond well to the lower doses given to humans.

Another consideration is that minocycline is commonly administered orally to humans while most animal studies have utilised minocycline given through parenteral routes. Indeed, Nessler et al. (2002) found that intraperitoneal but not oral minocycline was effective against adoptive transfer EAE. This likely reflects pharmacokinetic considerations in mice that favor better absorption of drug through parenteral rather than the oral route. Indeed, intravenous administration of minocycline resulted in a more predictable serum concentration and a lower dose requirement than intraperitoneal injection (Fagan et al., 2004; Xu et al., 2004), and it is possible that even higher doses will need to be given to animals in oral dosing compared to parenteral routes. Finally, it has been questioned whether the intraperitoneal injection of minocycline, which has a pH of 5, results in a stress response that then mediates the action of minocycline (Nessler et al., 2002). We do not think that this is the case inasmuch as, at least in spinal cord injury, intraperitoneal minocycline is neuroprotective under conditions in which the intraperitoneal injections of acidified saline (pH 5) or methylprednisolone did not ameliorate the trauma outcome (Wells et al., 2003).

We currently do not know the entire spectrum of mechanisms accounting for the increased efficacy of minocycline and GA when used in combination. Minocycline has wide-ranging actions including the inhibition of production and activity of MMPs, the lowering of levels of free radicals and interleukin-1β, the antagonism of glutamate excitotoxicity and the regulation of apoptosis (Yrjanheikki et al., 1998, 1999; Chen et al., 2000; Du et al., 2001; Tikka et al., 2001; Wu et al., 2002; Zhu et al., 2002; Brundula et al., 2002). It is possible that all these mechanisms combined with the Th2 bias induced by GA operate concordantly to increase the threshold required to produce inflammation and neuropathology in EAE. In this manuscript, we have shown that the combination of GA with minocycline can induce an increase of IL-5 and a decrease in IFNγ secretion compared to GA or minocycline alone. Furthermore, as minocycline (Wells et al., 2003) and GA (Kipnis et al., 2000; Schori et al., 2001; Kipnis and Schwartz, 2002) have both been shown to have neuroprotective activities in conditions of CNS compression injuries, these agents might have synergistic activity within the CNS to alleviate neurodegeneration. The decrease in axonal loss and demyelination (Fig. 2) would be consistent with this postulate. Alternatively, minocycline, following promotion of the generation of GA-reactive Th2 cells, may enhance the survival of these cells and their subsequent entry into the CNS. Clearly, the mechanisms whereby minocycline promotes the activity of GA deserve attention in future studies.

As discussed earlier, a proposed mechanism for GA in MS is the generation of GA-specific Th2 lymphocytes that then traffic into the CNS to suppress inflammation through bystander suppression (Sela and Teitelbaum, 2001; Neuhaus et al., 2001; Yong, 2002). Thus, if an activity of minocycline is the decrease of leukocyte trafficking into the CNS, might this not inhibit the entry of GA-specific Th2 cells into the CNS and thereby decrease the efficacy of GA? While we have no evidence that this occurs, it should be noted that Th2-polarised cells may employ different mechanisms from other leukocyte subsets, including the proinflammatory Th1 lymphocytes, to transmigrate into the CNS. Indeed, one recent study showed that the migration of Th2 lymphocytes but not cells of the Th1 subclass was attenuated by a function-blocking antibody to a chemokine, macrophage chemoattractant protein -1 (Biernacki et al., 2001). Similarly, the motility of Th1 but not Th2-polarised cells was inhibited by blocking CD44 and intercellular adhesion molecule-1 or its ligand, LFA-1 (Katakai et al., 2002). Overall, our results do not suggest that minocycline had impaired the transmigration of GA-specific Th2 cells into the CNS.

In summary, we have demonstrated that the combination of GA and minocycline provided for a better outcome in EAE than either drug when used alone at suboptimal doses. These results suggest that the addition of minocycline to the therapeutic regimen of the approved MS drug, GA, may result in an even better response in the disease. We propose that a clinical trial to test the effect of the combination of GA and minocycline in MS is warranted; such a trial is currently ongoing in our center.

References

Aharoni, R., Teitelbaum, D., Leitner, O., Meshorer, A., Sela, M., Arnon, R., 2000. Specific Th2 cells accumulate in the central nervous system of mice protected against experimental autoimmune encephalomyelitis by copolymer 1. Proc. Natl. Acad. Sci. U. S. A. 97, 11472–11477.

Akin, E., Miller, L.C., Tucker, L.B., 1998. Minocycline-induced lupus in adolescents. Pediatrics 101, 926.

Andes, D., Craig, W.A., 2003. Animal model pharmacokinetics and pharmacodynamics: a critical review. Int. J. Antimicrob. Agents 19, 261–268.

Biernacki, K., Prat, A., Blain, M., Antel, J.P., 2001. Regulation of Th1 and Th2 lymphocyte migration by human adult brain endothelial cells. J. Neuropathol. Exp. Neurol. 60, 1127–1136.

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., 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.

Chen, M., Ona, V.O., Li, M., Ferrante, R.J., Fink, K.B., Zhu, S., et al., 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.

Colovic, M., Caccia, S., 2003. Liquid chromatographic determination of minocycline in brain-to-plasma distribution studies in the rat. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 791, 337–343.

Du, Y., Ma, Z., Lin, S., Dodel, R.C., Gao, F., Bales, K.R., et al., 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.

Fagan, S.C., Edwards, D.J., Borlongan, C.V., et al., 2004. Optimal delivery of minocycline to the brain: implication for human studies of acute neuroprotection. Exp. Neurol. 186, 248–251.

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

Gough, A., Chapman, S., Wagstaff, K., Emery, P., Elias, E., 1996. Minocycline induced autoimmune hepatitis and systemic lupus erythematosus-like syndrome. BMJ 312, 169–172.

Katakai, T., Hara, T., Sugai, M., Gonda, H., Nambu, Y., Matsuda, E., et al., 2002. Chemokine-independent preference for T-helper-1 cells in trans-endothelial migration. J. Biol. Chem. 277, 50948–50958.

Kipnis, J., Schwartz, M., 2002. Dual action of glatiramer acetate (Cop-1) in the treatment of CNS autoimmune and neurodegenerative disorders. Trends Mol. Med. 8, 319–323.

Kipnis, J., Yoles, E., Porat, Z., Cohen, A., Mor, F., Sela, M., et al., 2000. T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: possible therapy for optic neuropathies. Proc. Natl. Acad. Sci. U. S. A. 97, 7446–7451.

Lublin, F., Baier, M., Cutter, G., et al., 2003. Results of the extension of a trial to assess the longer term safety of combining interferon beta-1a and glatiramer acetate. Neurology 58 (Suppl. 3), A85.

Metz, L.M., Zhang, Y., Yeung, M., et al., 2004. Minocycline reduces gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis. Ann. Neurol. 55, 756.

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

Neuhaus, O., Farina, C., Wekerle, H., Hohlfeld, R., 2001. Mechanisms of action of glatiramer acetate in multiple sclerosis. Neurology 56, 702–708.

O'Dell, J.R., 1999. Is there a role for antibiotics in the treatment of patients with rheumatoid arthritis? Drugs 57, 279–282.

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.

Saivin, S., Houin, G., 1988. Clinical pharmacokinetics of doxycycline and minocycline. Clin. Pharmacokinet. 15, 355–366.

Schori, H., Kipnis, J., Yoles, E., WoldeMussie, E., Ruiz, G., Wheeler, L.A., et al., 2001. Vaccination for protection of retinal ganglion cells against death from glutamate cytotoxicity and ocular hypertension: implications for glaucoma. Proc. Natl. Acad. Sci. U. S. A. 98, 3398–3403.

Sela, M., Teitelbaum, D., 2001. Glatiramer acetate in the treatment of multiple sclerosis. Expert Opin. Pharmacother. 2, 1149–1165.

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.

Soos, J.M., Stuve, O., Youssef, S., Bravo, M., Johnson, H.M., Weiner, H.L., et al., 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.

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

Tikka, T., Fiebich, B.L., Goldsteins, G., Keinanen, R., Koistinaho, J., 2001. Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J. Neurosci. 21, 2580–2588.

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.

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.

Wu, D.C., Jackson-Lewis, V., Vila, M., Tieu, K., Teismann, P., Vadseth, C., et al., 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.

Xu, L., Fagan, S.C., Waller, J.L., 2004. Low dose intravenous minocycline is neuroprotective after middle cerebral artery occlusion–reperfusion in rats. BMC Neurol. 4, 7.

Yong, V.W., 2002. Differential mechanisms of action of interferon-beta and glatiramer acetate in MS. Neurology 59, 802–808.

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. The promise of minocycline in neurology. Revised for Lancet Neurology

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.

Yrjanheikki, J., Tikka, T., Keinanen, R., Goldsteins, G., Chan, P.H., Koistinaho, J., 1999. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc. Natl. Acad. Sci. U. S. A. 96, 13496–13500.

Zhu, S., Stavrovskaya, I.G., Drozda, M., Kim, B.Y., Ona, V., Li, M., et al., 2002. Minocycline inhibits cytochrome *c* release and delays progression of amyotrophic lateral sclerosis in mice. Nature 417, 74–78.