# Targeting leukocyte MMPs and transmigration
## Minocycline as a potential therapy for multiple sclerosis

Veronika Brundula,[1] N. Barry Rewcastle,[2] Luanne M. Metz,[1] Claude C. Bernard[4] and V. Wee Yong[1,3]

Departments of [1]*Clinical Neurosciences,* [2]*Pathology and*
[3]*Oncology, University of Calgary, Canada and*
[4]*Neuroimmunology Laboratory, La Trobe University,*
*Bundoora, Melbourne, Australia*

*Correspondence to: V. Wee Yong, Departments of*
*Oncology and Clinical Neurosciences, 3330 Hospital*
*Drive, Calgary, Alberta T2N 4N1, Canada*
*E-mail: vyong@ucalgary.ca*

## Summary
**Multiple sclerosis is characterized by the infiltration of leukocytes into the CNS. As matrix metalloproteinases (MMPs) facilitate the passage of leukocytes across matrix barriers, we tested the hypothesis that targeting MMPs could attenuate neuro-inflammation. We report that minocycline, a widely used generic drug with a good safety record, inhibited MMP activity, reduced production of MMP-9 and decreased the transmigration of T lymphocytes across a fibronectin matrix barrier. In addition, minocycline was efficacious against both mild and severe experimental autoimmune encephalomyelitis (EAE) in mice, an animal model of multiple sclerosis. When severe EAE was produced, minocycline pre-treatment delayed the course of the disease: when maximal disease activity occurred in vehicle-treated EAE mice, minocycline animals were relatively normal and had minimal signs of inflammation and demyelination in the CNS. When tested in mice afflicted with mild EAE, minocycline attenuated the clinical severity of disease throughout the course of treatment. These results indicate that minocycline may constitute a safe and inexpensive therapy for multiple sclerosis.**

**Keywords**: experimental autoimmune encephalomyelitis; lymphocyte; metalloproteinases; multiple sclerosis; neuro-inflammation

**Abbreviations**: CFA = complete Freund's adjuvant; EAE = experimental autoimmune encephalomyelitis; ECM = extracellular matrix; FCS = foetal calf serum; HRP = horseradish peroxidase; MMP = matrix metalloproteinase

## Introduction

Multiple sclerosis is an inflammatory demyelinating disease of the CNS that affects nearly 1 million people worldwide. It is the most common cause of non-traumatic disability in young adults in North America (Weinstock-Guttman and Cohen, 1996). While the introduction of glatiramer acetate and three recombinant forms of interferon-β have markedly altered treatment of early stages of multiple sclerosis (Noseworthy *et al*., 2000), improvement of multiple sclerosis therapeutics is warranted. These drugs are only effective in reducing disease activity in relapsing forms of multiple sclerosis, and many patients do not respond to treatment (Hohlfeld, 1997).

The hallmarks of multiple sclerosis include multifocal perivascular mononuclear cell infiltrates in the CNS, oligodendrocyte loss and demyelination, and variable axonal damage. The pathogenesis of multiple sclerosis is thought to be related to the recruitment of autoreactive T lymphocytes to the CNS, which then mediate injury (Hohlfeld, 1997; Noseworthy *et al*., 2000). Thus, preventing inflammatory

cells from infiltrating the CNS is a reasonable therapeutic strategy to control multiple sclerosis.

The entry of leukocytes into the CNS is dependent on several factors, including the expression of matrix metallo-proteinases (MMPs), which degrade the extracellular matrix (ECM) proteins of the basal lamina that surrounds blood vessels (Yong *et al*., 2001). Several lines of evidence implicate MMPs as pathogenic factors in multiple sclerosis and its animal model, experimental autoimmune encephalo-myelitis (EAE) (reviewed in Yong, 1999; Yong *et al*., 2001). MMPs (particularly MMP-7, -9 and -12) are elevated in the brain of multiple sclerosis individuals and animals afflicted with EAE. MMP-9 is detected in the CSF of all multiple sclerosis patients but is absent in controls (Leppert *et al*., 1998). In serum samples, where MMP-9 levels are significantly increased in multiple sclerosis compared with healthy individuals, MMP-9 content is higher during clinical relapse of multiple sclerosis relative to periods of stability (Lee *et al*., 1999; Lichtinghagen *et al*., 1999). Furthermore, an increased

MMP-9 level in the serum of multiple sclerosis patients correlates with gadolinium-enhancing activity on MRI at that time (Lee *et al.*, 1999) or 1 month after (Waubant *et al.*, 1999). In further support of a pathogenic role for MMP-9, young mice genetically deficient for MMP-9 are relatively resistant to EAE compared with age-matched controls (Dubois *et al.*, 1999). Finally, one mechanism by which interferon-β appears to reduce multiple sclerosis disease activity is by inhibiting the production and release of MMP-9 by T cells (Leppert *et al.*, 1996; Stuve *et al.*, 1996). Relapsing–remitting multiple sclerosis patients that are treated with interferon-β have reduced levels of MMP-9 in their serum (Trojano *et al.*, 1999) or peripheral blood mononuclear cells (Galboiz *et al.*, 2001). We have shown that while interferon-β inhibits the release of MMP-9, it does not affect the enzymatic activity of MMP-9 (Stuve *et al.*, 1996). Thus, targeting MMP enzyme activity directly, either alone or in combination with interferon-β or other treatments, could constitute a novel approach to the treatment of multiple sclerosis. While several hydroxamate-based inhibitors that affect MMP enzyme activity have been demonstrated to attenuate disease in EAE (reviewed in Yong, 1999; Yong *et al.*, 2001), it would be advantageous to employ an MMP activity inhibitor that is already in clinical use for other indications, since this would fast-track its potential utility in people with multiple sclerosis.

Minocycline is a semi-synthetic analogue of tetracycline used to treat microbial infections such as acne. Over 6 million people have been treated in the UK alone, for an average duration of 9 months, with minimal toxicity (Seukeran *et al.*, 1997). It is considered to be a safe drug (Seukeran *et al.*, 1997; Shapiro *et al.*, 1997; O'Dell, 1999; Sturkenboom *et al.*, 1999). Minocycline, probably because of its chelating property, has been found to be an inhibitor of MMP activity (Golub *et al.*, 1984; Paemen *et al.*, 1996). For this study, we have characterized the activity of minocycline on MMPs, and have tested the hypothesis that minocycline inhibits the transmigration of T cells *in vitro* and delays or prevents signs of EAE in mice.

## Methods
### Isolation of cells
Mononuclear cells were isolated from the blood of healthy volunteers by Ficoll–Hypaque centrifugation as described previously (Stuve *et al.*, 1996). Monocytes were adhered to plastic for 3 h in serum-free AIM-V® medium (Gibco-BRL, Burlington, Canada) and the floating cells were then collected for study. To characterize the leukocyte subsets, the following antibodies and flow cytometry were utilized (BD Pharmingen, San Diego, Calif., USA): anti-human Leu-4 (CD3) fluoroisothiocyanate (FITC) for T lymphocytes; Simultest™ CD3/CD4 (Leu™-4/3a) for CD4+ cells; Simultest™ CD3/CD8 (Leu™-4/2a) for CD8+ cells; Simultest™ CD3/CD16+CD56 (Leu™-4/3a) for natural killer

(NK) cells; Simultest™ LeucoGATE™ CD45/CD14 (anti-Hle-1/Leu™-M3) for monocyte/macrophages; and CD19 (SJ25C1) phycoerythrin for B cells.

### Transmigration assays
Fibronectin-coated chambers with pore size 3 μm (Collaborative Biomedical Products, Bedford, Mass., USA) were used as described previously (Stuve *et al.*, 1996; Uhm *et al.*, 1999). Cells were suspended at $1 \times 10^6$ cells/ml in AIM-V with 2.5% foetal calf serum (FCS) and 500 μl were added to the upper chamber. The bottom chamber contained 500 μl of AIM-V medium with 10% FCS. The higher concentration of FCS in the lower chamber served as a directional gradient for transmigration (Stuve *et al.*, 1996). After a specified time of migration (see Results), or 24 h if not otherwise indicated, cells in the bottom chamber were counted using a Coulter counter ($Z_1$) to obtain the number of transmigrated T cells. The latter was expressed either as such, or as a percentage of the total cells added to the top chamber (when cells in both the top and bottom chambers were Coulter counted). In minocycline-treated cultures, cells were incubated with minocycline-HCl (Sigma, Oakville, Canada) for 15 min and then transferred directly to the top compartment of the Boyden chamber.

### MMP determinations
Gelatin zymography (Stuve *et al.*, 1996) was used to assess the ability of minocycline or interferon-β to inhibit the enzyme activity of gelatinases (MMP-2 and -9). Supernatants from baby hamster kidney cells transfected to overexpress human MMP-2 and -9 (from Dylan Edwards, University of East Anglia, UK) were electrophoresed on a 10% SDS gel containing 1 mg/ml gelatin. The gel was washed and incubated overnight on a shaker at room temperature with rinse buffer containing 2.5% Triton X-100, 50 mM Tris pH 7.5 and 5 mM $CaCl_2$ to wash off the SDS and to allow the gelatinases to renature. Subsequently, the gel was incubated in reaction buffer (50 mM Tris pH 7.5, 5 mM $CaCl_2$) for 18 h at 37°C, in order for proteinases to degrade the gelatin. To test whether minocycline or interferon-β (recombinant interferon-β-1b, provided by Dr Gary Williams, Berlex Laboratories, Richmond, Calif., USA) inhibits MMP enzyme activity, these were added to the rinse and reaction buffers during the development of the zymograms. Each gel was then stained with Coomassie Blue for 4 h and destained (1 : 3 : 6 of acetic acid : methanol : water) in order to reveal the expression of clear bands (zone of gelatin degradation) against a dark background.

To quantitate the protein content of MMP-9, Western blot analysis was used. T cell media were concentrated using Centricon concentrators 10 (Amicon, Beverly, Mass., USA). The total protein concentration of the samples was deter-

**Table 1** *Severity of inflammation in vehicle- or minocycline-treated EAE mice*

| Mice | Grade when killed | Left cord | | | Right cord | | | Total score of lesions |
|---|---|---|---|---|---|---|---|---|
| | | Ant | Lat | Post | Ant | Lat | Post | |
| Vehicle #1 | 5 | 1 | 1 | 0 | 2 | 2 | 0 | 6 |
| Vehicle #2 | 4.5 | 2 | 2 | 1 | 1 | 1 | 1 | 8 |
| Vehicle #3 | 4 | 2 | 2 | 1 | 1 | 2 | 0 | 8 |
| Vehicle #4 | 5 | 3 | 2 | 0 | 3 | 2 | 0 | 10 |
| Vehicle #5 | 4.5 | 2 | 2 | 1 | 2 | 2 | 1 | 10 |
| Mino #1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mino #2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mino #3 | 5 | 2 | 2 | 1 | 2 | 3 | 1 | 11 |
| Mino #4 | 4.5 | 1 | 1 | 0 | 1 | 1 | 0 | 4 |
| Mino #5 | 4.5 | 2 | 2 | 1 | 2 | 2 | 1 | 10 |

The five vehicle-treated EAE mice, and the minocycline-treated ('Mino') mice #1 and #2 were killed at Days 19–21 after MOG immunization. Minocycline-treated mice #3 to #5 were killed at Day 27 when disease was full-blown. The anterior (Ant), lateral (Lat) and posterior (Post) columns of the right and left cord were evaluated blind, using a scale from 0 (no inflammation) to 3 (severe inflammation deep in the CNS parenchyma and which approaches grey matter areas), as described in the text.

mined by the Bradford Coomassie Brilliant Blue method (Bio-Rad, Hercules, Calif., USA), using bovine serum albumin as a standard. Ten micrograms of total protein per sample were resolved on a 10% polyacrylamide gel, and transferred overnight to Immobilon-P (Millipore, Bedford, MA, USA) in transfer buffer (Tris 25 mM, glycine 192 mM, methanol 20%) at 4°C and 30 V. The blots were incubated in blocking buffer [5% skimmed milk, 0.05% Tween-20 in Tris-base saline (TBS)] for 1 h at room temperature. Membranes were then incubated with 2 µg/ml of mouse anti MMP-9 (Ab-2) antibody (Calbiochem, Oncogene Research Products, Mass., USA) in blocking buffer for 1 h at room temperature, followed by three 5 min washes in washing buffer TBS-T (0.05% Tween-20 in TBS). The secondary antibody, a horseradish peroxidase (HRP)-conjugated goat anti-mouse (IgG + IgM), was used at a dilution of 1 : 5000 in blocking buffer and incubated for 1 h at room temperature, followed by four 5 min washes with TBS-T. Blots were developed by the enhanced chemiluminescence (ECL) method according to manufacturer's instructions (Amersham-Pharmacia Biotech, Baie d'Urfe, Canada).

## Myelin oligodendrocyte glycoprotein (MOG) EAE

Two regimens were used to obtain either severe or mild EAE in 12-week-old C57BL/6 female mice (Jackson Laboratory, Bar Harbor, Maine, USA). To generate severe EAE, mice were injected subcutaneously at the back of the tail and 7 days later in the flanks with 300 µg of MOG$_{35–55}$ peptide (Bernard *et al.*, 1997) emulsified in 100 µl of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich., USA) containing an additional 4 mg/ml of *Mycobacterium tuberculosis* (H37Ra). Mice were injected intraperitoneally with 300 ng of reconstituted lyophilized pertussis toxin (List Biological Laboratories, Campbell, Calif., USA) in 200 µl of

PBS. The pertussis toxin injection was repeated after 48 h (Liu *et al.*, 1998).

To obtain mild EAE, only one injection of MOG$_{35–55}$ peptide emulsified in 100 µl of CFA was given. Furthermore, the dose was reduced from 300 to 25 µg, and *M. tuberculosis* was not supplemented to the CFA. Pertussis toxin continued to be administered as with the severe EAE regimen.

Animals were treated intraperitoneally daily, beginning on the day of MOG induction, with minocycline or vehicle (saline). Seven to nine animals per group were used. Control EAE mice were injected with 200 µl saline daily, while the minocycline EAE mice received 50 mg/kg twice a day for the first 2 days, once daily for the next 5 days, followed by 25 mg/kg for the subsequent days until they were killed. These doses were chosen to approximate those reported to decrease infarct size in rats following focal or global ischaemia (Yrjanheikki *et al.*, 1998, 1999). In a last group of mice, treatment with intraperitoneal minocycline (25 mg/kg) daily was initiated only from Day 10 post-immunization, when clinical disease was beginning to be apparent in mice.

All mice were weighed on a daily basis. Severity of EAE was graded according to Liu *et al.* (1998). These were: 0, no disease; 1, limp tail; 2, partial paralysis of one or two hind limbs; 3, complete paralysis of hind limbs; 4, hind limb paralysis and fore limb paraparesis; 5, moribund. The animal experiments were approved by the Animal Care Committee of the University of Calgary.

For histological analyses, anaesthetized mice were perfused with 40 ml of cold saline and the CNS was dissected. The sacral part of the spinal cord was immersed in 4% paraformaldehyde overnight and embedded in paraffin wax, cross sectioned at 6–8 µm and stained with haematoxylin–eosin and Luxol fast blue for evidence of inflammation and demyelination, respectively. Optic nerves were fixed in 2.5%
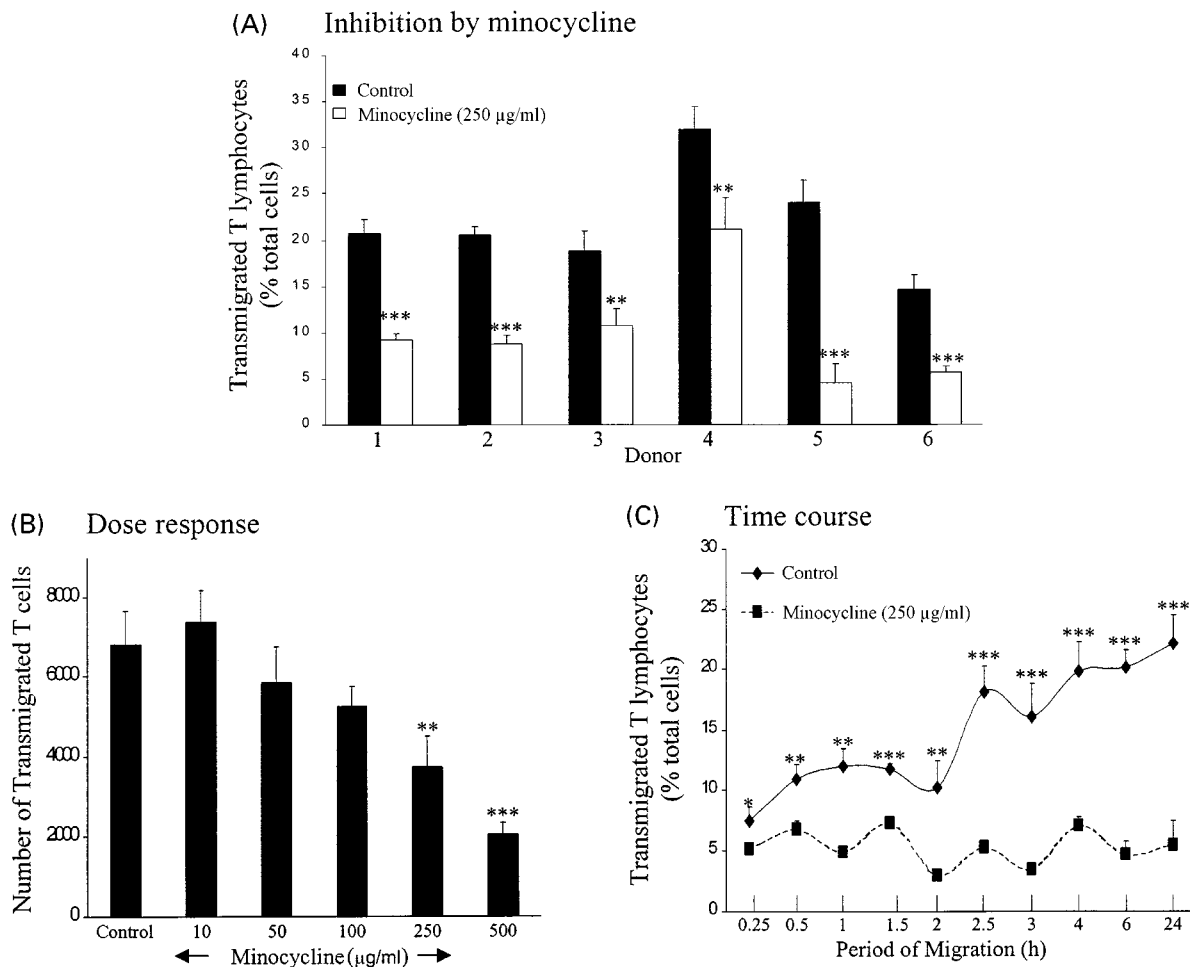
## (A)    Inhibition by minocycline



## (B)    Dose response

## (C)    Time course



**Fig. 1** Minocycline inhibits the transmigration of T lymphocytes across a fibronectin barrier. (**A**) minocycline inhibits the transmigration of T cells from six donors over a 24 h period. (**B**) Inhibition by minocycline was concentration dependent, in that only 250 and 500 μg/ml attenuated the transmigration of cells. (**C**) The time course of inhibition by minocycline was rapid, and was manifest within 15 min. All values are mean ± standard deviation of triplicate samples. Results were analysed using the unpaired Student's *t*-test (**A** and **C**) or ANOVA with Bonferroni's post-ANOVA comparisons (**B**). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

glutaraldehyde, embedded in epon, sectioned at 2 μm, and stained with toluidine blue.

To conduct a semi-quantitative evaluation of the extent of CNS inflammation in response to minocycline treatment, a cross-section of the sacral spinal cord from each mouse was stained with haematoxylin–eosin. A neuropathologist (N.B.R.) blinded to the sections then assessed the degree of inflammation of both the right and left cord sections, which were further subdivided into anterior, lateral and posterior columns. Since 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 grading of 0–3 was used to describe the ascending severity of lesions in the CNS parenchyma. These are: 0, no inflammation; 1, an inflammatory infiltrate in the superficial grey matter; 3, inflammation that reached the grey matter regions; and 2, intermediate location between

1 and 3. In general, the majority of the infiltrates were of a fairly uniform size, regardless of their location. The total disease score was then obtained by summing the grading obtained at each location (see Table 1).

## Multi-probe RNase protection assays

To determine the impact of minocycline treatment on the expression of MMPs in EAE, a multi-probe RNase protection assay that simultaneously detects nine MMPs (Vecil *et al.*, 2000) was used. A probe for a fragment of the house-keeping ribosomal protein, RPL32-4A, served as an internal loading control. Total RNA was isolated from the sacral cord of mice, and 15 μg total RNA was used for analyses as described previously (Vecil *et al.*, 2000). The multiprobe MMP set was courtesy of Dr Iain Campbell, Scripps Research Institute, La Jolla, Calif., USA.

## Statistics

When multiple groups were analysed simultaneously, the group comparison of one-way ANOVA (analysis of variance) with Bonferroni *post hoc*, or the non-parametric Kruskal–Wallis test with Dunn's multiple comparisons, were used. When two groups were analysed, the unpaired Student's *t*-test or the non-parametric Mann–Whitney *U*-test was employed. Statistical significance was set at $P < 0.05$.

## Results

### Minocycline attenuates T cell migration across a fibronectin barrier

While some variability was evident across donors, an average of $22 \pm 6\%$ (mean $\pm$ standard deviation) T cells from six normal individuals transmigrated the fibronectin barrier of the Boyden chamber over a 24 h period (Fig. 1A). A single administration of minocycline (250 µg/ml), applied to cells 15 min before they were transferred to Boyden chambers, inhibited the transmigration of T cells from all donors (Fig. 1A). The response to minocycline was dose-dependent with >50% inhibition at 250 and 500 µg/ml (Fig. 1B). The inhibition of T cell transmigration by minocycline was an early process (Fig. 1C), with the difference between controls and minocyline-treated cells being apparent within 15 min in the transmigration assay.

To address whether the inhibition of transmigration was due to a toxic effect of minocycline, we measured cell viability using Trypan blue exclusion. After 1 h of treatment with 250 µg/ml of minocycline there were no Trypan blue-positive cells. After 24 h, $5.3 \pm 0.6\%$ of cells were Trypan blue-positive in the minocycline group, similar to controls ($4.9 \pm 0.7\%$). Therefore, since inhibition of migration occurred within 15 min, and since increased cell death did not result from incubation with minocycline, the action of minocycline on cell migration did not appear to be due to non-specific cytotoxicity.

Minocycline did not preferentially inhibit the transmigration of any particular subset of lymphocytes. Fresh lymphocyte populations from normal human donors were composed of CD3$^+$ T cells ($83.3 \pm 5.1\%$), CD56/16$^+$ NK cells ($14.5 \pm 7.4\%$) and CD19$^+$ B cells ($2.5 \pm 1.6\%$) (mean $\pm$ standard deviation, of four different mononuclear cell preparations assayed by flow cytometry). Twenty-four hours after cells were added to the top compartment of the Boyden chamber, equal proportions of cells from each sub-population were present in the lower chamber when quantitated by flow cytometry.

### Minocycline not only inhibits MMP enzymatic activity, but also reduces the production of MMP-9

A requirement for transmigration of leukocytes across fibronectin is the protease-dependent degradation of fibronectin. Previous work has implicated MMPs as being the family of proteases responsible for fibronectin degradation (Leppert *et al.*, 1996). Indeed, we have demonstrated that TIMP-1 (tissue inhibitor of metalloproteinase-1), a specific inhibitor of MMPs, and phenanthroline, a non-specific metalloproteinase inhibitor, reduced T cell migration across fibronectin (Stuve *et al.*, 1996; Uhm *et al.*, 1999).

By using a modified zymography protocol, where minocycline was added to the reaction buffer during the development of in-gel gelatinolytic activity, we corroborated previous findings (Paemen *et al.*, 1996) that minocycline is an inhibitor of gelatinase (MMP-2 and -9) activity (Fig. 2A). Interestingly, the two concentrations of minocycline that had a marked effect on MMP enzyme activity, 250 and 500 µg/ml, were those that significantly attenuated lymphocyte transmigration (Fig. 1B). In contrast, interferon-β was not a direct inhibitor of gelatinase activity (Fig. 2A).

We investigated whether minocycline had an effect on MMP release. Since MMPs are secreted proteins, we tested for the presence of MMP-9 protein in the medium of cultured T lymphocytes using a Western blot assay. Interferon-β-treated T lymphocytes were used as a positive control, since our previous work had documented this drug to be an inhibitor of MMP-9 production (Stuve *et al.*, 1996). Figure 2B reveals that while interferon-β-treated cells had decreased levels of MMP-9, minocycline further reduced the level of MMP-9 protein produced by T lymphocytes. This reduction of lymphocyte MMP-9 protein content by minocycline is probably the result of an effect on MMP-9 transcripts, since the level of mRNA encoding MMP-9 was also decreased following minocycline treatment compared with controls (data not shown).

We tested whether minocycline also affects other MMPs. MMP-12 is a significant product of leukocytes, and it was noted that the levels of MMP-12 protein and transcripts in these cells were also decreased by minocycline treatment (data not shown).

These results confirm that minocycline is an inhibitor of MMP activity, and also decreases lymphocyte production of MMP-9.

### Minocycline delays the onset and course of disease in mice exposed to a severe EAE-inducing regimen

We tested the hypothesis that minocycline would affect T cell transmigration into the CNS parenchyma and ameliorate EAE. We utilized myelin MOG$_{35-55}$ as the immunogen, and C57BL/6 as the mouse strain, as this would produce a chronic non-remitting disease model as reported previously (Bernard *et al.*, 1997; Liu *et al.*, 1998). A severe immunogenic protocol was used to address in an unambiguous manner whether minocycline could alter the course of EAE. Control mice subjected to

## A  Minocycline inhibits MMP enzymatic activity



MMP-9
MMP-2

Interferon-β    Control    10    50    250    500
Minocycline (μg/ml)
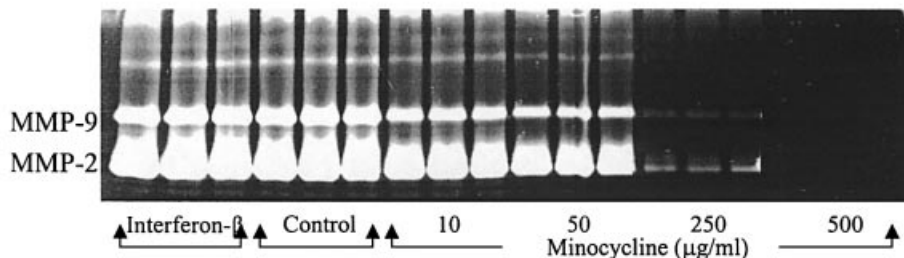
## B  Minocycline decreases MMP-9 protein level: Western blot

Interferon-β 1000 U/ml    0 | + | 0 | + | 0 | + | 0 | +
Minocycline 250 μg/ml     0 | 0 | + | + | 0 | 0 | + | +

MMP-9

24 h          48 h
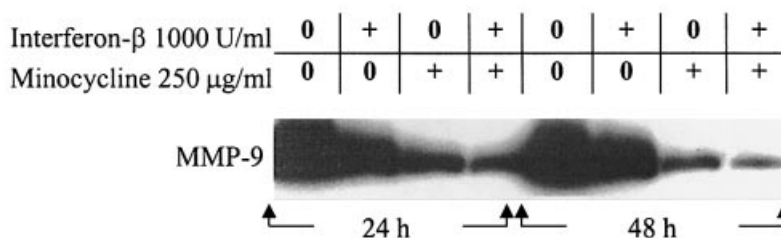
**Fig. 2** Minocycline not only inhibits MMP enzyme activity, but also decreases the production of MMP-9. An equal aliquot of MMP proteins was added to each lane and gels were incubated with different concentrations (triplicate lanes) of minocycline, interferon-β (1000 IU/ml) or without drugs (control), during the development of the zymogram. (**A**) Direct inhibition of the activity of MMP-2 or -9 is indicated by the decreased gelatinolytic bands compared with controls. (**B**) T cells were incubated in the presence (+) or absence (0) of 250 μg/ml minocycline, 1000 IU/ml interferon-β or both, for 24 and 48 h. The conditioned media of cells were resolved by 10% SDS–PAGE and immunoblotted against the zymogen form of MMP-9 as described in Methods. The control (no interferon-β or minocycline) bands are not overexposed as they were in the linear range of detection. The results show that minocycline reduces the level of MMP-9 secreted by T cells, and, indeed, that minocyline is more potent in this regard than interferon-β.

MOG inoculum developed signs of EAE after 12 days of induction, and the severity of disease progressively increased (Fig. 3A). By 19–20 days post-induction, control MOG animals were paralysed (Grade 4), and had to be killed for ethical reasons. In contrast, minocycline-treated mice at Days 19–20 had a mean EAE score of between 1 and 1.5 (Fig. 3A). Indeed, many minocycline-treated animals at Day 19 had no obvious symptoms of disease (Fig. 3B). Unfortunately, minocycline-treated mice, presumably because of the strong immunogenic protocol, continued to worsen, and Grade 4 EAE was attained 6–8 days later.

In order to determine whether minocycline was delaying EAE by arresting infiltration of leukocytes into the CNS parenchyma, histological analyses were performed on Days 19–21. Figure 4A shows extensive inflammation in the sacral spinal cord of MOG EAE mice compared with that from normal mice. Notably, at these time points, minocycline-treated mice had minimal, if any, infiltration of leukocytes into the CNS parenchyma (Fig. 4A and Table 1).

Because of the closely packed bundles of myelinated axons in the optic nerve, and since optic neuritis is a common feature of multiple sclerosis, this region was examined for demyelination. Figure 4B reveals extensive demyelination in control MOG EAE animals. In contrast, sections from minocycline-treated animals showed no demyelination. It is noted, however, that when minocycline-treated mice developed Grade 4 or 5 EAE, some 6–8 days after control mice had reached this disease score, inflammation (Table 1) and demyelination (data not shown) were qualitatively similar to those of control EAE mice shown in Fig. 4. Thus, minocycline delayed the onset and course of disease, but mice eventually succumbed to the severe EAE-inducing regimen. Histologically, when minocycline-treated mice were healthy, inflammation in the sacral cord was not evident, consistent with the hypothesis that minocycline decreases leukocyte infiltration. However, in minocycline-treated mice that eventually succumbed to severe EAE, this corresponded to significant inflammation in the sacral cord (Table 1).
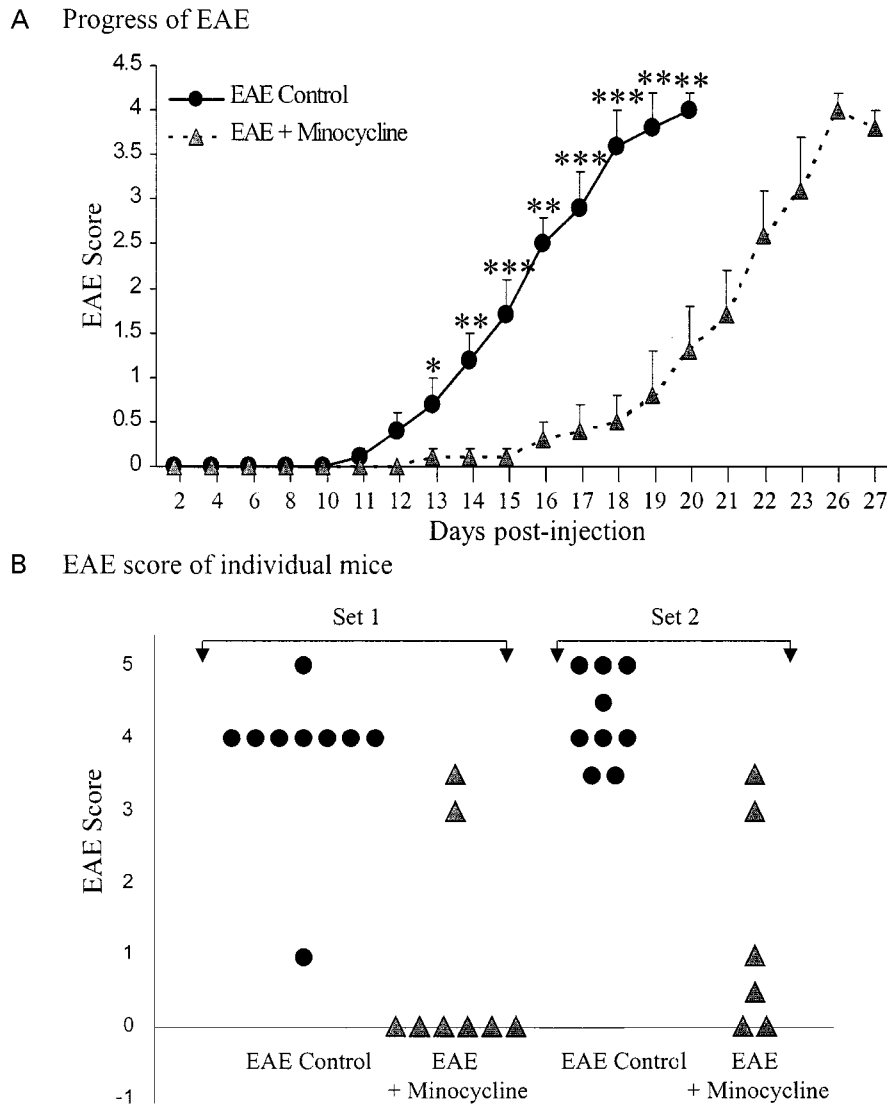
A   Progress of EAE



B   EAE score of individual mice



**Fig. 3** Minocycline delays the course of severe EAE. (**A**) Each plotted value represents mean $\pm$ standard error of the mean of between six and nine mice. The disease course for minocycline treatment versus control EAE has been reproduced in another series of experiments involving six to nine mice each. Note that while control EAE mice were in Grade 4 EAE (paralysis) by 19–20 days post-induction, and had to be euthanized, minocycline-treated mice at Days 19–20 had an average EAE score of between 1 and 1.5. Mann–Whitney $U$-test compared with the same time point in minocycline mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (**B**) the individual score of control or minocycline EAE mice at Day 19 has been plotted. While a minority of minocycline-treated mice had EAE scores >3, most did not have any obvious signs (0 score) at this point. In contrast, nearly all control EAE mice had scores of $\geqslant 4$.
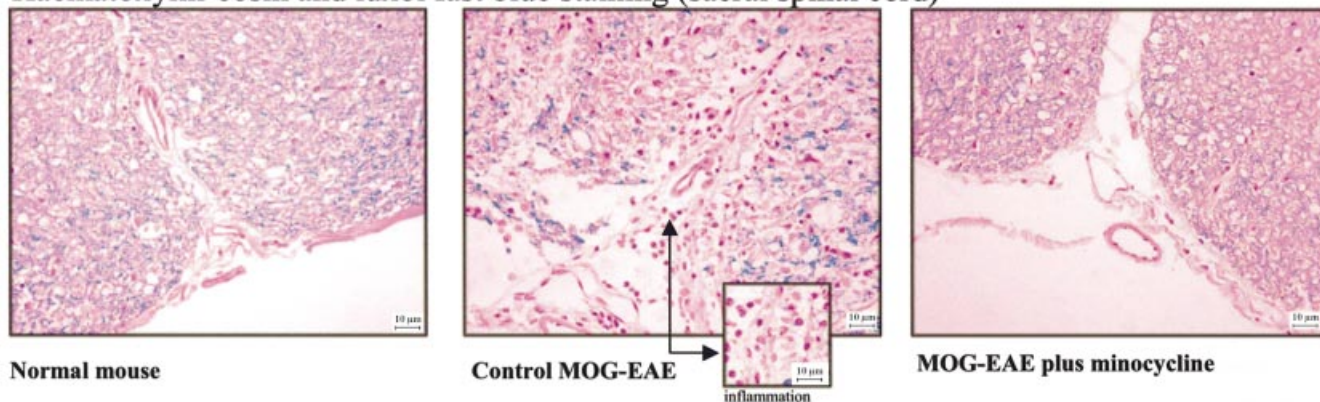
The spinal cords of selected mice were analysed for MMP transcript expression to determine whether the rise of specific MMPs is correlated with the evolution of the pathology of EAE. Figure 5 shows that a prominent induction of MMP-12 transcript is obtained when mice developed EAE symptoms; levels of MMP-9 and -14 were also increased when compared with those in normal uninduced mice. Minocycline-treated mice, prior to the occurrence of symptoms, had an MMP profile similar to that of normal mice (Fig. 5). However, in minocycline-treated mice that subsequently succumbed to

disease, elevations of MMP-9, -12 and -14 were also observed (unpublished observations).

## Minocycline attenuates the clinical severity of EAE in mice exposed to a mild EAE-inducing regimen

Most drug trials in the EAE literature involve treating EAE animals that have a maximum clinical activity of Grade 2

## Haematoxylin-eosin and luxol fast blue staining (sacral spinal cord)



**Normal mouse**    **Control MOG-EAE** inflammation    **MOG-EAE plus minocycline**

## Toluidine blue staining (optic nerve)



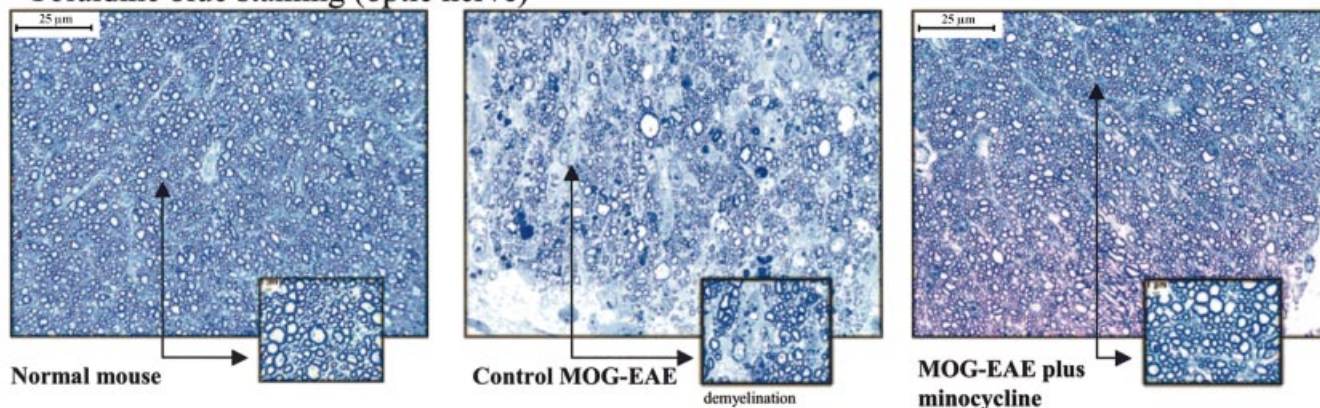**Normal mouse**    **Control MOG-EAE** demyelination    **MOG-EAE plus minocycline**

**Fig. 4** Minocycline prevents inflammation and demyelination at a time point when control EAE mice had maximal disease activity. Mice were killed at Days 19–21 post-EAE induction. While control MOG EAE sections (taken from a Grade 4 mouse, with Grade 4 representing the clinical score of most of the mice in set 1) revealed extensive inflammation in the spinal cord, and severe demyelination in the optic nerve, the sections from minocycline-treated mice (from a Grade 0 mouse, with Grade 0 being the clinical score of most mice in the minocycline group at this point) were in many ways similar to those from normal mice, although some oedema could be observed. The boxed areas are higher magnification frames from the regions indicated by arrows. (*Top*) Haematoxylin–eosin and Luxol fast blue staining (sacral spinal cord); (*Bottom*) toluidine blue staining (optic nerve).

EAE. We evaluated whether minocycline could affect EAE outcomes in mice that were subjected to a mild EAE-inducing regimen. Under such experimental conditions, mice developed clinical signs on Days 10–11 after MOG inoculation. Disease peaked at Grade 2 between Days 15 and 16 and was maintained at this level thereafter (Fig. 6). When minocycline was administered to mice from the time of MOG inoculation, the entire clinical course and severity of disease was attenuated (Fig. 6). On average, minocycline-treated mice had limpness of tail and no apparent involvement of the hind limbs (Grade 1), while vehicle-treated MOG animals had hind limb impairment (Grade 2).

Significantly, when minocycline treatment was initiated 10 days post-MOG inoculation, at a time when symptoms were beginning to be apparent in mice, severity of disease was also subsequently attenuated. The effect of minocycline initiated at Day 10 (i.e. no loading dose) was almost immediate, in that a separation in clinical score

from control EAE mice was beginning to be apparent 2 days afterwards (Fig. 6).

## Discussion

A pathogenic role for MMPs in multiple sclerosis has been suggested from several lines of evidence (reviewed in Yong, 1999; Yong *et al.*, 2001). The mechanisms by which MMPs are detrimental include their use by leukocytes to degrade the basement membrane that surrounds blood vessels so as to gain entry into the CNS parenchyma (reviewed in Kieseier *et al.*, 1999; Yong *et al.*, 2001). This process impairs the integrity of the blood–brain barrier, and may account for the close association between serum MMP-9 levels and the activity of gadolinium-enhanced MRI (Lee *et al.*, 1999; Waubant *et al.*, 1999). Within the CNS, another means by which the aberrant expression of MMPs may induce disease is by converting the pro-forms of several inflammatory
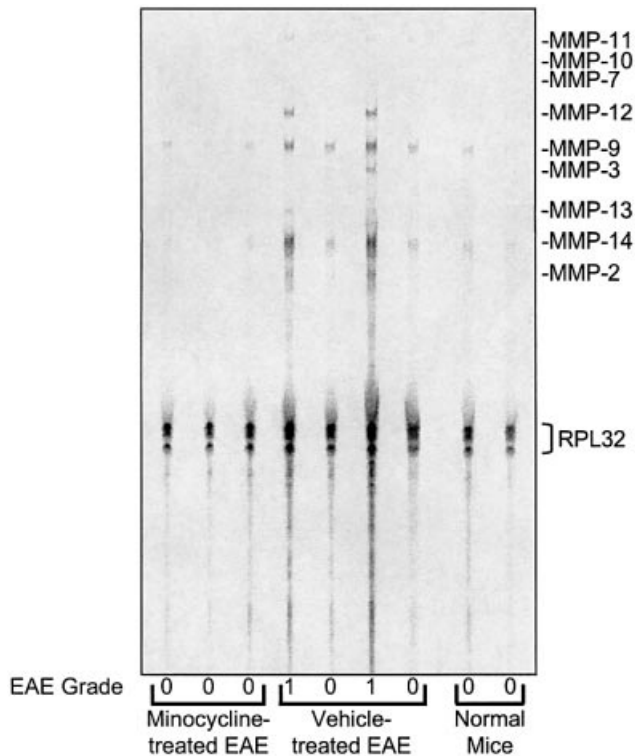
**Fig. 5** MMP profile in EAE. The spinal cords of mice were subjected to RNase protection assays as described previously (Vecil *et al.*, 2000). Mice were taken for analyses 9 days after the first MOG immunization of the severe EAE-inducing protocol. The spinal cords of two uninduced normal mice were also examined. In vehicle-treated EAE mice in which symptoms were developing (Grade 1), a clear induction of MMP-12 was obtained. A rise in transcripts encoding MMP-9 and -14 was also found. Minocycline-treated mice were asymptomatic at this point and had an MMP profile similar to that of normal mice.

molecules, such as tumour necrosis factor-α (TNF-α), into their active species; the result would be the propagation of CNS inflammation. Yet another potentially undesirable role for MMPs is the degradation of myelin, and indeed, the injection of MMPs into the brain produces demyelination (Anthony *et al.*, 1998) or axonal injury (Newman *et al.*, 2001). Opdenakker and colleagues have shown that the fragments of MMP-mediated digestion of myelin basic protein are encephalogenic when injected into mice, thereby generating a cascade of demyelinating and pro-inflammatory events in the CNS as a result of aberrant MMP expression (reviewed in Opdenakker and Van Damme, 1994).

In view of these observations, it is reasonable to suggest the targeting of MMPs as a therapeutic strategy in the treatment of multiple sclerosis (Rosenberg, 2001). In EAE, several hydroxamate-based inhibitors of MMPs have already been demonstrated to have efficacy (reviewed in Yong, 1999). The finding that interferon-β decreases the production of MMP-9 by lymphocytes (Leppert *et al.*, 1996; Stuve *et al.*, 1996) further suggests that targeting MMPs is a key step in the treatment of multiple sclerosis. Thus, we have sought to

identify inhibitors of MMP enzyme activity that may be used alone, or in combination with interferon-β or other therapies, in the treatment of multiple sclerosis. We selected minocycline for study because it has a long-term clinical track record (Seukeran *et al.*, 1997; Shapiro *et al.*, 1997; O'Dell, 1999; Sturkenboom *et al.*, 1999) and a good safety profile that should fast-track its potential use in multiple sclerosis patients.

We have confirmed reports that minocycline is a direct inhibitor of the activity of MMPs (Golub *et al.*, 1984; Paemen *et al.*, 1996). The activity of both MMP-9 and -2 was inhibited (Fig. 2), although the significance of the latter is not clear at present. Furthermore, we discovered that minocycline has other actions that should bode well for its use in multiple sclerosis. In this regard, minocycline inhibits the production of MMP-9; indeed, minocycline was more potent than interferon-β at the concentrations tested (Fig. 2B). Collectively, in a functional assay, minocycline decreased the migration of all subsets of lymphocytes across a fibronectin barrier. *In vivo*, minocycline delayed the onset of severe EAE produced by MOG, and significantly increased the average time (by 6–8 days) for animals to become paralysed (Grade 4 EAE) or moribund (Grade 5) compared with non-treated EAE animals. However, we were unable to prevent mice from eventually achieving Grade 4/5 EAE with minocycline. This may be because of the strong immunogenic protocol used in that study. Indeed, when milder EAE (Grade 2) was generated, minocycline treatment attenuated the clinical severity of disease throughout the course of drug administration, even when the drug was initiated after disease onset (i.e. without initial loading dose).

The finding that minocycline given from the time of MOG inoculation can delay the attainment of Grade 4/5 EAE, or suppress Grade 2 EAE, is relevant to modifying the next relapse in relapsing–remitting multiple sclerosis. Furthermore, the result that minocycline injected from disease onset attenuates disease severity indicates that this drug may suppress ongoing disease activity in patients that present with symptoms.

There are other immuno-modulatory properties of minocycline that may favour its use in multiple sclerosis. Kloppenburg *et al.* (1995) reported that when human T cells were treated with minocycline, there was a dose-dependent inhibition of T cell proliferation, decreased interleukin (IL)-2 responsiveness and reduced production of IL-2, interferon-γ and TNF-α. Minocycline also suppressed the proliferation of murine thymocytes that was induced by IL-1 (Ingham, 1990). Moreover, in patients with rheumatoid arthritis, minocycline improves laboratory parameters of disease activity, especially the acute-phase reactants and rheumatoid factor levels in the serum (Kloppenburg *et al.*, 1996). More recently, minocycline has been found to inhibit the activation of microglia (Tikka and Koistinaho, 2001; Tikka *et al.*, 2001), and this has been postulated to be the mechanism by which it is neuroprotective in focal or global ischaemic models of stroke (Yrjanheikki *et al.*, 1998, 1999).
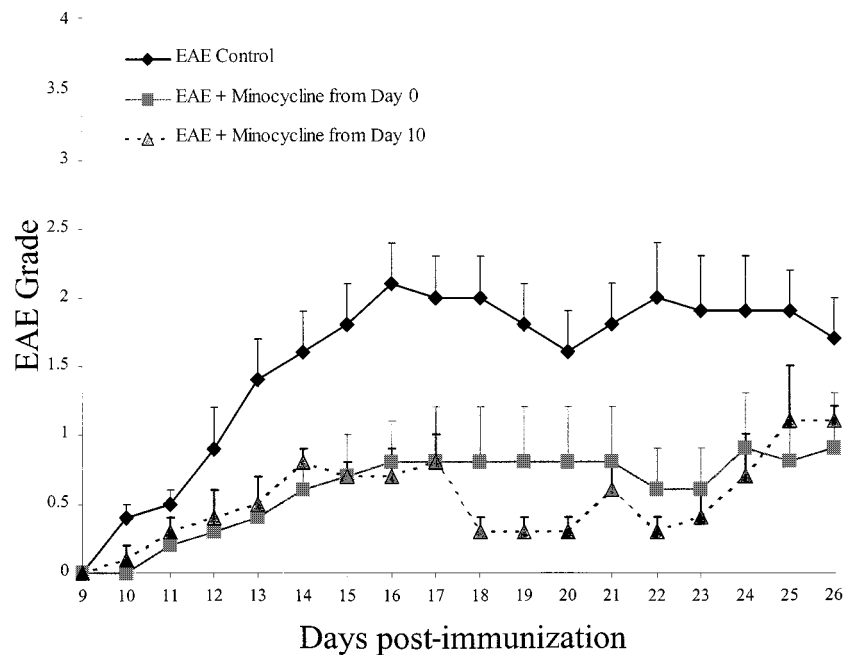
**Fig. 6** Minocycline attenuates the severity of EAE in mice subjected to a mild EAE-inducing regimen. Animals were treated intraperitoneally daily, beginning on the day of MOG induction, with minocycline (squares) or saline (diamonds). A third group received minocycline only from Day 10 of MOG immunization (triangles). The last injection of drug or vehicle occured at Day 22. Each value represents mean ± standard error of the mean of between seven and 10 mice. Statistical analyses (Kruskal–Wallis test with Dunn's multiple comparisons, $P < 0.05$) show that the group of mice treated with minocycline from the day of MOG inoculation was significantly different from control EAE mice from Day 10 onwards. For animals given minocycline from Day 10, statistical difference from control EAE mice was achieved from Day 16 onwards. These statistical differences are not plotted on the graph in order to avoid cluttering of the data.

Minocycline has also been reported to inhibit the expression of caspase-1 and -3, and to delay mortality in a transgenic mouse model of Huntington's disease (Chen *et al.*, 2000). In a model of Parkinson's disease, minocycline protected against MPTP-induced neurotoxicity (Du *et al.*, 2001).

It should be noted that another tetracycline antibiotic, metacycline, has been tested as a combination therapy with D-penicillamine for 1 year in patients with secondary progressive multiple sclerosis. No improvement in clinical score was apparent and severe toxicity including proteinuria and leukopenia was observed (Dubois *et al.*, 1998). The outcome of metacycline alone in multiple sclerosis was not assessed.

In considering the potential utility of minocycline in multiple sclerosis, two properties of this drug deserve further discussion: pharmacokinetics and safety. Oral minocycline is well absorbed (95–100%) and its small size (494 Da) and lipophilic nature result in ready access to the CNS, independent of blood–brain barrier inflammation (Saivin and Houin, 1988; Ingham, 1990). These characteristics are advantageous when considering that the currently used multiple sclerosis drugs, glatiramer acetate and interferon-βs, are administered via injections, and are not thought to

enter the CNS. By its presence within the CNS, minocycline could act on leukocytes already within the CNS parenchyma. In addition, by inhibiting MMP activity within the CNS, minocycline could contribute to the preservation of the myelin sheath, since MMPs can degrade myelin (Anthony *et al.*, 1998) and provide for a pro-inflammatory environment within the CNS (Kieseier *et al.*, 1999). Conversely, because the extension of processes from the OL soma during myelin formation may require MMP activity (Oh *et al.*, 1999), the presence of minocycline within the CNS could impair remyelination.

With respect to the safety profile of minocycline, this second-generation tetracycline has been available for over 30 years, and, in the UK alone, over 6.5 million people have been treated with it, mostly for acne, for an average of 9 months. Indeed, because antibiotic resistance is low with minocycline compared with other tetracyclines and antimicrobials, it is the most widely prescribed systemic antibiotic for acne. Given its widespread use, the adverse events and their frequency have been well described. In general, minocycline is considered a safe drug in humans (Seukeran *et al.*, 1997; Shapiro *et al.*, 1997; O'Dell, 1999; Sturkenboom *et al.*, 1999). Although serious drug reactions can occur, including hypersensitivity

syndrome reaction, serum sickness-like reaction, drug-induced lupus and single organ dysfunction (Elkayam *et al.*, 1999), the incidence of these is very rare (1.6 cases per million exposures) 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 after withdrawal of the drug (Gough *et al.*, 1996; Akin *et al.*, 1998). Nonetheless, it is important to be aware of these side effects in future clinical trials. It is noted that another tetracycline, doxycycline, is employed chronically to treat gum diseases and it has a good safety record (Thomas *et al.*, 1998; Caton *et al.*, 2000).

The doses of minocycline that are used here to treat EAE are comparable to those that have been used to ameliorate disease in models of stroke, Huntington's disease and Parkinson's disease (Yrjanheikki *et al.*, 1999; Chen *et al.*, 2000; Du *et al.*, 2001). How these doses translate into clinical doses for humans remains to be addressed, although rodents in general will require a larger dose of a given drug than humans, because of their larger liver mass per kg basis. When 120 mg/kg was administered to mice by oral gavage, a concentration of 0.32 μg/g of minocycline was detected in the brain 8 h later (Du *et al.*, 2001).

In summary, the results in this manuscript demonstrate that minocycline impacts on several factors considered to be detrimental in multiple sclerosis: MMP activity, MMP level and leukocyte transmigration. Furthermore, efficacy of minocycline in an animal model of multiple sclerosis, EAE, was demonstrated. Since the cost of minocycline is only 5–10% of glatiramer acetate or interferon-β, and given its safety record in humans and oral bioavailability, it appears prudent to evaluate the efficacy of minocycline as a novel therapeutic in multiple sclerosis.

**References**

Akin E, Miller LC, Tucker LB. Minocycline-induced lupus in adolescents. Pediatrics 1998; 101: 926.

Anthony DC, Miller KM, Fearn S, Townsend MJ, Opdenakker G, Wells GM, et al. Matrix metalloproteinase expression in an experimentally-induced DTH model of multiple sclerosis in the rat CNS. J Neuroimmunol 1998; 87: 62–72.

Bernard CC, Johns TG, Slavin AJ, Ichikawa M, Ewing C, Liu J, et al. Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. [Review]. J Mol Med 1997; 75: 77–88.

Caton JG, Ciancio SG, Blieden TM, Bradshaw M, Crout RJ, Hefti AF, et al. Treatment with subantimicrobial dose doxycycline improves the efficacy of scaling and root planing in patients with adult periodontitis. J Periodontol 2000; 71: 521–32.

Chen M, Ona VO, Li M, Ferrante RJ, Fink KB, Zhu S, et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med 2000; 6: 797–801.

Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, et al. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proc Natl Acad Sci USA 2001; 98: 14669–74.

Dubois B, D'Hooghe MB, De Lepeleire K, Ketelaer P, Opdenakker G, Carton H. Toxicity in a double-blind placebo-controlled pilot trial with D-penicillamine and metacycline in secondary progressive multiple sclerosis. Mult Scler 1998; 4: 74–8.

Dubois B, Masure S, Hurtenbach U, Paemen L, Heremans H, van den Oord J, et al. Resistance of young gelatinase B-deficient mice to experimental autoimmune encephalomyelitis and necrotizing tail lesions. J Clin Invest 1999; 104: 1507–15.

Elkayam O, Yaron M, Caspi D. Minocycline-induced autoimmune syndromes: an overview. [Review]. Semin Arthritis Rheum 1999; 28: 392–7.

Galboiz Y, Shapiro S, Lahat N, Rawashdeh H, Miller A. Matrix metalloproteinases and their tissue inhibitors as markers of disease subtype and response to interferon-β therapy in relapsing and secondary-progressive multiple sclerosis patients. Ann Neurol 2001; 50: 443–51.

Golub LM, Ramamurthy N, McNamara TF, Gomes B, Wolff M, Casino A, et al. Tetracyclines inhibit tissue collagenase activity: a new mechanism in the treatment of periodontal disease. J Periodontal Res 1984; 19: 651–5.

Gough A, Chapman S, Wagstaff K, Emery P, Elias E. Minocycline induced autoimmune hepatitis and systemic lupus erythematosus-like syndrome. Br Med J 1996; 312: 169–72.

Hohlfeld R. Biotechnological agents for the immunotherapy of multiple sclerosis. Principles, problems and perspectives. [Review]. Brain 1997; 120: 865–916.

Ingham E. Modulation of the proliferative response of murine thymocytes stimulated by IL-1, and enhancement of IL-1 beta secretion from mononuclear phagocytes by tetracyclines. J Antimicrob Chemother 1990; 26: 61–70.

Kieseier BC, Seifert T, Giovannoni G, Hartung HP. Matrix metalloproteinases in inflammatory demyelination: targets for treatment. [Review]. Neurology 1999; 53: 20–5.

Kloppenburg M, Dijkmans BA, Breedveld FC. Antimicrobial therapy for rheumatoid arthritis. [Review]. Baillieres Clin Rheumatol 1995; 9: 759–69.

Kloppenburg M, Dijkmans BA, Verweij CL, Breedveld FC. Inflammatory and immunological parameters of disease activity in rheumatoid arthritis patients treated with minocycline. Immunopharmacology 1996; 31: 163–9.

Lee MA, Palace J, Stabler G, Ford J, Gearing A, Miller K. Serum gelatinase B, TIMP-1 and TIMP-2 levels in multiple sclerosis: a longitudinal clinical and MRI study. Brain 1999; 122: 191–7.

Leppert D, Waubant E, Burk MR, Oksenberg JR, Hauser SL. 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 1996; 40: 846–52.

Leppert D, Ford J, Stabler G, Grygar C, Lienert C, Huber S, et al. Matrix metalloproteinase-9 (gelatinase B) is selectively elevated in CSF during relapses and stable phases of multiple sclerosis. Brain 1998; 121: 2327–34.

Lichtinghagen R, Seifert T, Kracke A, Marckmann S, Wurster U, Heidenreich F. Expression of matrix metalloproteinase-9 and its inhibitors in mononuclear blood cells of patients with multiple sclerosis. J Neuroimmunol 1999; 99: 19–26.

Liu J, Marino MW, Wong G, Grail D, Dunn A, Bettadapura J, et al. TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. Nat Med 1998; 4: 78–83.

Newman TA, Woolley ST, Hughes PM, Sibson NR, Anthony DC, Perry VH. T-cell- and macrophage-mediated axon damage in the absence of a CNS-specific immune response: involvement of metalloproteinases. Brain 2001; 124: 2203–14.

Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. [Review]. New Engl J Med 2000; 343: 938–52.

O'Dell JR. Is there a role for antibiotics in the treatment of patients with rheumatoid arthritis? [Review]. Drugs 1999; 57: 279–82.

Oh LY, Larsen PH, Krekoski CA, Edwards DR, Donovan F, Werb Z, et al. Matrix metalloproteinase-9/gelatinase B is required for process outgrowth by oligodendrocytes. J Neurosci 1999; 19: 8464–75.

Opdenakker G, Van Damme J. Cytokine-regulated proteases in autoimmune diseases. [Review]. Immunol Today 1994; 15: 103–7.

Paemen L, Martens E, Norga K, Masure S, Roets E, Hoogmartens J, et al. The gelatinase inhibitory activity of tetracyclines and chemically modified tetracycline analogues as measured by a novel microtiter assay for inhibitors. Biochem Pharmacol 1996; 52: 105–11.

Rosenberg GA. Matrix metalloproteinases in multiple sclerosis: is it time for a treatment trial? [Review]. Ann Neurol 2001; 50: 431–3.

Saivin S, Houin G. Clinical pharmacokinetics of doxycycline and minocycline. [Review]. Clin Pharmacokinet 1988; 15: 355–66.

Seukeran DC, Eady EA, Cunliffe WJ. Benefit–risk assessment of acne therapies [letter]. Lancet 1997; 349: 1251–2.

Shapiro LE, Knowles SR, Shear NH. Comparative safety of tetracycline, minocycline, and doxycycline. [Review]. Arch Dermatol 1997; 133: 1224–30.

Sturkenboom MC, Meier CR, Jick H, Stricker BH. Minocycline and lupus like syndrome in acne patients. Arch Intern Med 1999; 159: 493–7.

Stuve O, Dooley NP, Uhm JH, Antel JP, Francis GS, Williams G, et al. Interferon beta-1b decreases the migration of T lymphocytes in vitro: effects on matrix metalloproteinase-9. Ann Neurol 1996; 40: 853–63.

Thomas JG, Metheny RJ, Karakiozis JM, Wetzel JM, Crout RJ. Long-term sub-antimicrobial doxycycline (Periostat) as adjunctive management in adult periodontitis: effects on subgingival bacterial population dynamics. Adv Dent Res 1998; 12: 32–9.

Tikka TM, Koistinaho JE. Monocycline provides neuroprotection against N-methyl-D-asparate neurotoxicity by inhibiting microglia. J Immunol 2001; 166: 7521–33.

Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J. Minocycline a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J Neurosci 2001; 21: 2580–88.

Trojano M, Avolio C, Liuzzi GM, Ruggieri M, Defazio G, Liguori M, et al. Changes of serum sICAM-1 and MMP-9 induced by rIFNbeta-1b treatment in relapsing–remitting MS. Neurology 1999; 53: 1402–8.

Uhm JH, Dooley NP, Stuve O, Francis GS, Duquette P, Antel JP, et al. Migratory behavior of lymphocytes isolated from multiple sclerosis patients: effects of interferon beta-1b therapy. Ann Neurol 1999; 46: 319–24.

Vecil GG, Larsen PH, Corley SM, Herx LM, Besson A, Goodyer CG, et al. Interleukin-1 is a key regulator of matrix metalloproteinase-9 expression in human neurons in culture and following mouse brain trauma in vivo. J Neurosci Res 2000; 61: 212–24.

Waubant E, Goodkin DE, Gee L, Bacchetti P, Sloan R, Stewart T, et al. Serum MMP-9 and TIMP-1 levels are related to MRI activity in relapsing multiple sclerosis. Neurology 1999; 53: 1397–401.

Weinstock-Guttman B, Cohen JA. Newer versus older treatments for relapsing–remitting multiple sclerosis. [Review]. Drug Safety 1996; 14: 121–30.

Yong VW. The potential use of MMP inhibitors to treat CNS diseases. Exp Opin Invest Drugs 1999; 8: 255–68.

Yong VW, Power C, Forsyth P, Edwards DR. Metalloproteinases in biology and pathology of the nervous system. [Review]. Nat Rev Neurosci 2001; 2: 502–11.

Yrjanheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho J. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. Proc Natl Acad Sci USA 1998; 95: 15769–74.

Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci USA 1999; 96: 13496–500.