UNIVERSITY OF CALGARY

INHIBITING T CELL MIGRATION AS A THERAPEUTIC STRATEGY FOR MULTIPLE SCLEROSIS: MINOCYCLINE AND ITS MECHANISMS OF ACTION

by

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ABSTRACT

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) with no cure. MS is characterized by the infiltration of lymphocytes and monocytes into the CNS leading to an undesirable inflammatory response. Arresting inflammatory cells from infiltrating the CNS could lead to the amelioration of MS. Matrix metalloproteinases (MMPs) are extracellular matrix (ECM) degrading proteinases thought to facilitate the entry of leukocytes into the CNS in MS. This thesis tested the hypothesis that targeting MMPs could constitute an experimental approach to ameliorate CNS inflammation. Minocycline, a tetracycline that inhibits MMP activity, decreased adhesion and migration of T cells in vitro. Mechanisms of action included MMP enzyme inhibitory activity and inhibition of MMP-9 production. Minocycline delayed the onset of MOG EAE (experimental allergic encephalomyelitis) compared to non-treated or interferon-β (IFNβ) treated animals and increased the average time for animals to become paralyzed. Minocycline may improve the prognosis of patients with MS.

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DEDICATION

I dedicate this thesis to all patients with Multiple Sclerosis

This is for you

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ABBREVIATIONS

ANOVA	Analysis of variance
BBB	Blood brain barrier
BHK	Baby hamster kidney cells
CFA	Complete Freund's adjuvant
CMT	Chemically modified tetracyclines
CNS	Central nervous system
CSF	Cerebrospinal fluid
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
HRP	Horse-radish peroxidase
ICAM	Intercellular cell adhesion molecule
LFA	Leukocyte function antigen
IFN	Interferon
IL	Interleukin
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMPs	Matrix metalloproteinases
MOG	Myelin oligodendrocyte glycoprotein

MRAC-PM2	Mouse renal adenocarcinoma
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
PFA	Paraformaldehyde
PLP	Proteolipid lipoprotein
RA	Rheumatoid arthritis
RR-MS	Relapsing remitting MS
TCR	T cell receptor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of MMPs
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
VCAM	Vascular cell adhesion molecule
VLA-4	Very late antigen-4

CHAPTER 1

INTRODUCTION

1.1 Multiple Sclerosis

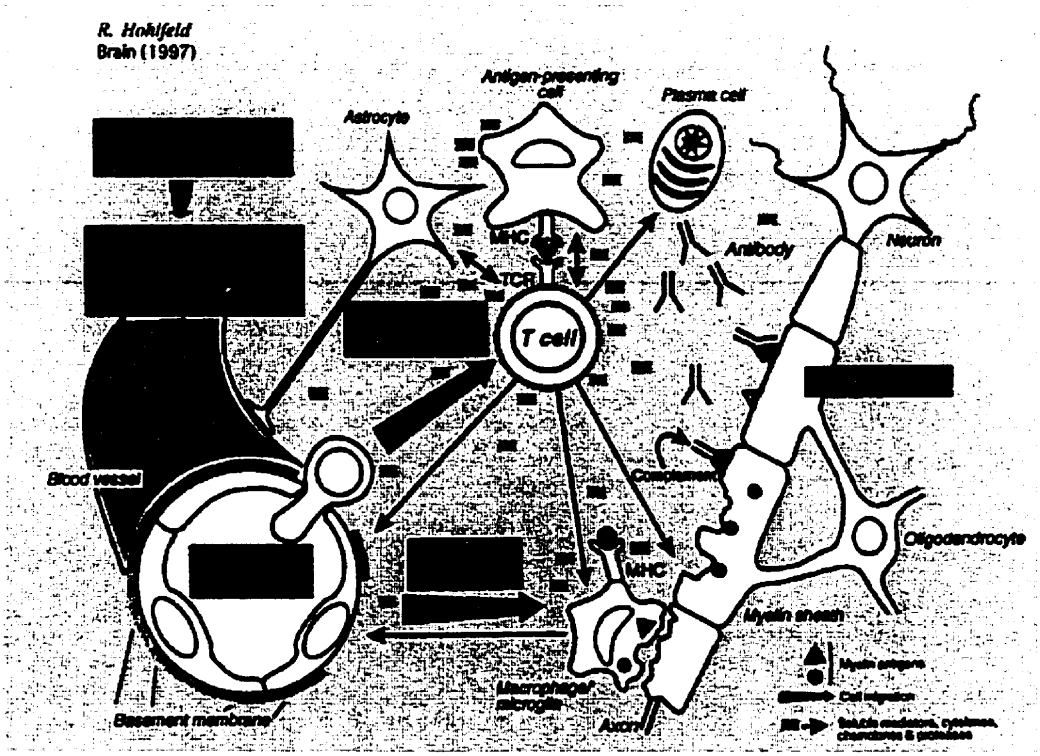
1.1.1 WHAT IS MULTIPLE SCLEROSIS?

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). The age of onset occurs typically between 20 to 50 years of age, and it targets more women than men (2:1). MS affects nearly one million people worldwide (Steinman, 2000) and it is the most common cause of non-traumatic disability in young adults in North America (Weinstock-Guttman and Cohen, 1996). Patients with MS manifest several distinct clinical patterns. The pattern of exacerbations followed by recovery is referred to as relapsing remitting MS (RR-MS), and is the most common form of the disease. Gradually progressive deterioration, without acute attacks or recovery, is seen in about 40% of patients and is referred to as chronic progressive MS. Chronic progression may occur later in the disease following an initial relapsing-remitting course (secondary progressive MS), or may occur from the onset of the disease in 15% of patients (primary progressive MS) (Paty and McFarland, 1998; Weinstock-Guttman and Cohen, 1996).

Even though the etiology and pathogenesis of MS remain an enigma, MS is considered to be an immune-mediated disease of the CNS. Multifocal perivascular mononuclear cell infiltrates in brain white matter and demyelination constitute hallmarks of MS. Acute lesions are characterized by lymphocyte and macrophage infiltrates, by macrophages filled with myelin debris, oligodendrocyte loss and breakdown of associated myelin sheaths, and astrocyte proliferation (Ffrench-Constant, 1994). Early in the course of the disease there may be considerable remyelination (i.e. repair) but as oligodendrocytes are lost remyelination becomes impossible. While demyelination is the principal feature of MS, there may be axonal interruption of varying extent and substantial astrogliosis at those sites where myelin is lost (Arnason, 1999; Lucchinetti et al, 1996). In chronic lesions there is little inflammatory

activity; the myelin sheaths and oligodendrocytes are absent and demyelinated axons are separated by a dense network of astrocytes processes. Sites of myelin loss, known as plaques, are scattered throughout the white matter of the optic nerve, cortex, periventricular regions, brain stem, and spinal cord. Plaques in critical locations determine the type of disability and/or symptomatology. Thus, vision may be compromised if there is a plaque in the optic nerve and spasticity may occur if there are plaques in the descending pyramidal tracts. Many cortical plaques are clinically silent. Common early manifestations in MS are optic neuritis, paresthesias, mild sensory or motor symptoms in a limb or cerebellar incoordination, while the end stage is characterized by unsteadiness of gait, incontinence, and paralysis (Arnason, 1999; Ffrench-Constant, 1994; Kumar et al, 1992).

Fig. 1.1.1 Crucial steps in multiple sclerosis pathogenesis



Pre-existing autoreactive T cells are activated outside the CNS. The activated T cells traverse the BBB and are locally reactivated when they recognize "their" antigen on the surface of local antigen-presenting cells. The activated T cells secrete cytokines that stimulate microglia cells and astrocytes, recruit additional inflammatory cells, and induce production by plasma cells. Anti-myelin antibodies and activated macrophage/microglia cells are thought to cooperate in demyelination (adapted from Hohlfeld, 1997).

1.1.2 IMMUNOPATHOGENESIS OF MS

While almost impossible to arrange chronologically in a disease with as protracted and varied course as MS, the immunopathologic data gathered thus far points to the CNS damage and myelin breakdown as being immune mediated (Raine, 1994). Specifically, MS is perceived as an autoimmune disease, where activated autoreactive CD4+ T cells are recruited to the CNS (Fig. 1.1.1), and, upon CNS specific antigen recognition they respond by releasing a cascade of cytokines ultimately leading to further mononuclear cell accumulation, inflammation and tissue damage (Hartung and Rieckmann, 1997).

It is known that myelin reactive T cells are present in normal blood being part of the normal T cell receptor (TCR) repertoire (Ben-Nun et al, 1981; Schluesener and Wekerle, 1985), but in order to traverse through the blood brain barrier (BBB) and penetrate into the CNS parenchyma, these T cells have to be activated. Indeed, any activated T cells are capable of entering the CNS irrespective of antigen specificity (Hickey, 1991; Wekerle et al, 1991). So, how do these autoreactive CNS specific T cell clones become activated in MS?

Despite much effort devoted to the search of the cause of MS, its etiology remains unknown. One of the first hypothetical events in MS pathogenesis is the activation of T cells in the periphery. How this initial activation occurs in MS patients is not known. Molecular mimicry, dual T cell receptor expression and activation by superantigens during bacterial or viral infection have been suggested and are reviewed by Hohlfeld (1997). Whatever the exact mechanism of the initial activation of autoreactive T cells and their subsequent reactivation during relapses, it is likely that this activation occurs outside the CNS. Magnetic resonance imaging (MRI) findings in MS demonstrate that brain and spinal cord lesions often occur concurrently (Thorpe et al, 1996) which strongly implicates a systemic trigger for disease activity.

Activated T cells have the ability to penetrate the CNS; however, nonspecifically activated T cells soon exit the CNS, and T cells that recognize CNS antigens remain. It has been suggested that myelin specific T cells remain in the perivascular location and serve to recruit non-antigen specific activated T cells into the CNS via cytokine production and adhesion

molecule expression (Lou et al, 1997) and thereby orchestrate inflammatory events (Raine, 1994).

The T cells found in MS lesions are activated and express interleukin (IL)-2 receptors (Hofman et al, 1986). By cytokine secretion within the CNS parenchyma, these T cell blasts may induce local glial cells to express major histocompatibility complex (MHC) products and to act as antigen presenting cells. Indeed, active demyelination in MS is accompanied by an inflammatory infiltrate in which CD4+ T cells and class II MHC+ macrophages predominate. Therefore, it is believed that T-cell receptors respond to antigen(s) presented by MHC class II molecules on macrophages/microglia and astrocytes. This interaction results in stimulation of helper T cells, and subsequent cytokine secretion, T cell proliferation, and B cell and macrophage activation (Ffrench-Constant, 1994; Wekerle et al, 1987).

There is an extensive literature describing the presence of pro-inflammatory cytokines in MS. Cytokines such as Interferon (IFN)γ and tumor necrosis factor (TNF)α are elevated in the cerebrospinal fluid (CSF) and serum of MS patients (Perella et al, 1993). IL-2 and IL-2 receptor are expressed within MS plaques. IL-1 is commonly found in astrocytes and microglial cells. TNFα has been localized to astrocytes at the edge of lesions and microglia within the lesions, as well as in endothelial cells. TNFβ has been found in microglial cells at the margin of lesions. IFNγ has been described on microglia, astrocytes and endothelial cells. Regulatory or anti-inflammatory cytokines including IL-10 and transforming growth factor (TGF)β have also been localized in MS lesions, and they usually correlate with periods of remission in MS (reviewed in Raine, 1994).

Evidence for B cell activation comes from the presence of immunoglobulins synthesized within the CNS, resulting in the characteristic finding of oligoclonal CSF bands. There is a critical antibody response directed to myelin and evidence of the complement cascade activated with membrane attack complexes appearing in the spinal fluid (Steinman, 1996).

Demyelination occurs in a highly edematous CNS parenchyma in which CNS elements are suspended in a greatly increased extracellular space and dissociated myelin debris.

Macrophages appear to phagocytose large pieces of myelin sheath. It is not clear though, if macrophages attack the myelin or they are just scavengers of cellular debris of MS lesions. Nonetheless, ongoing demyelination seems to be dependent upon the presence of Ia+ macrophages in MS (Traugott et al, 1983) which suggests a pathogenic role for monocyte/macrophages in MS.

In summary (Fig. 1.1.1), it is the concerted attack where T cells promote an inflammatory response in the CNS parenchyma, with the consequent production of cytokines, mononuclear cell recruitment and activation, that leads to areas of demyelination impairing saltatory conduction along the axon and producing the pathophysiologic defects.

1.1.3 EVIDENCE FOR AUTOIMMUNE T CELLS IN THE PATHOGENESIS OF MS

There are numerous lines of immunological evidence that suggests that T cells are central to the pathogenesis of MS. First, in the acute MS lesion, active demyelination is accompanied by an inflammatory infiltrate in which αβ CD4+ T cells predominate. Lesion progression has been associated with the presence of CD4+ (T4+) cells (Traugott et al, 1983). Besides, chronic **silent** lesions contain few T cells, and in non-inflammatory, non-MS conditions, are only rarely encountered and, when present, may be due to normal immunosurveillance mechanisms or ante-mortem infection rather than being a disease related feature (Raine, 1991). Second, specific T cells for different myelin antigens are present in the CSF of MS patients in higher quantity than in blood, suggesting a proliferative response of specific activated clones in the CNS (Bellamy et al, 1985).

Even though these observations indicate a role for T cells in MS, they remain circumstantial and a causative role for T cells in the pathogenesis of MS, however suggested, has been difficult to establish. It was not until the development of a T cell mediated neuroinflammatory animal model that the concept of autoimmunity was used to explain some of the pathogenic mechanisms in MS.

In 1933, Rivers et al described the induction of an inflammatory demyelinating disease affecting the CNS after sensitization of monkeys with CNS tissue. In 1949 Olitsky and Yager were able to

induce demyelinating disease or experimental allergic encephalomyelitis (EAE) in mice using homogenates of the CNS in adjuvants. Hallmarks of the disease were T cell infiltration and focal demyelination, very much alike to that seen in MS. The fact that EAE could not be transferred to naïve recipients by humoral anti-myelin antibodies, but by activated myelin specific CD4+ T lymphocytes (Paterson, 1960), showed clearly a pathogenic central role for T cells in EAE and therefore very likely in MS.

1.1.4 EAE: AN ANIMAL MODEL OF MS

EAE is an animal model for autoimmune diseases of the CNS and is induced by generating T cell mediated immunity to various CNS antigens. The clinical signs and lesions closely parallel those observed in MS and, as a result, EAE has become a widely used model for MS (Goverman and Brabb, 1996). Signs of EAE are generally manifested in an ascending manner, starting with loss of tail tonus and progressing to hind and forelimb paralysis (Goverman and Brabb, 1996; Glabinski et al, 1997). Following exposure to select CNS antigens, the onset of attack in EAE typically occurs between 14 and 24 days postimmunization and is associated with a 10% loss in body weight within one day of development (Glabinski et al, 1997).

MS and EAE resemble each other closely in their pathological changes (reviewed in Wekerle, 1993). First and foremost are mononuclear infiltrates, mainly concentrated around postcapillary microvessels of the CNS white matter. The cells contained within these infiltrates are predominantly lymphocytes (mainly CD4+, some CD8+ cells, and few B cells) and monocyte/macrophages. Second, there is a marked disturbance of the BBB, with edema formation and deposition of fibrin and other plasma proteins. Third, the lesions exhibit typical activation of local glia. Many astrocytes are activated and manifest signs of increased content of cytoskeletal glial fibrillary acidic protein (GFAP). In addition, the local microglia cells are activated. With their strong expression of MHC I and II antigens they may be difficult to distinguish from infiltrating blood borne macrophages.

Probably the greatest difference between EAE and MS lies in our understanding of the events responsible for disease induction. While in EAE it is known that immunization with any of several myelin antigens emulsified in adjuvant is responsible for disease induction, the triggering antigen for MS remains unknown. In 1962, using the mouse EAE as a model, Einstein et al identified myelin basic protein (MBP), which comprises 30% of CNS myelin, as an encephalitogenic antigen in CNS tissue. In 1951, proteolipid protein (PLP) which comprises 50% of CNS myelin, was identified (Folch and Lees, 1951). Since then, other CNS specific antigens such as myelin associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG) have been purified and the EAE model has been dissected and highly studied in a search for insights into pathogenic mechanisms as well as therapeutic approaches that could be applied to MS. It is now clear that the course of the disease and histological features of EAE vary depending on the antigen used for the immunization and the strain of mouse or rat used (Berger et al, 1997). For example, MBP 1-11 EAE in H-2^u mice or MBP 68-84 in Lewis rats can produce an acute EAE, where animals exhibit a single episode of paralytic disease from which they recover. PLP 139-151 EAE in H-2^s mice is a relapsing remitting form of EAE, where animals experience a moderate to severe initial episode of disease followed by remission and one or more relapses. MOG 33-55 EAE in H-2^b is a chronic EAE, where animals get progressively worse and never recover (reviewed in Schmidt, 1999; Kuchroo and Weiner, 1998).

Which is the best representative animal model for MS has been debated over the past decades (Wekerle, 1994). It is clear that no one EAE model represents all aspects of human MS. Nonetheless, different EAE systems have been of invaluable help for studying diverse defined aspects of the pathogenesis of MS. Particularly, MOG EAE has been considered to be a good model for MS. Even though the antigen(s) responsible for the autoimmune response in MS is (are) not known, there is a predominant T cell response to MOG in patients with MS. Moreover, anti-MOG antibodies with demyelinating activity are present in the CSF of such patients (Kerlero de Rosbo et al, 1993; Sun et al, 1991; Genain et al, 1999). There are also some important features in MOG EAE that better mimic those in MS. In contrast with MBP, PLP or MAG EAE models, demyelination is notorious in MOG EAE. This is thought to be due to the presence of anti-MOG antibodies (reviewed in Bernard et al, 1997). MOG is a specific antigen of the CNS myelin, while MBP, PLP and MAG are also

located in other tissues such as peripheral myelin (MBP and MAG) and the thymus (MBP and PLP). Moreover demyelination of the optic nerve can be observed after MOG EAE, which is very similar to the optic neuritis associated with MS. MOG EAE was the MS animal model of choice for this thesis.

1.2 Treatments for MS

1.2.1 PROVEN USEFUL TREATMENTS

While there is no cure for MS, the disease does respond in modest ways to treatment with anti-inflammatory and immune modulating drugs. Again, the fact that improvement is observed in some treated individuals indicates that inflammation in the CNS of persons with MS contributes to the disease process.

Corticosteroids are the most commonly used treatment for the relapses of MS. Their precise mechanism of action in MS is unknown, but they have numerous anti-inflammatory and anti-edema effects that could be beneficial to MS patients. Methylprednisolone has been reported to reduce intrathecal immunoglobulin synthesis and to decrease specifically the levels of anti-MBP antibodies, as well as the number of T cells, in the CSF (Troiano et al, 1985; 1987). Both corticotrophin and costicosteroids decrease the duration of clinical relapses in MS, accelerating recovery from MS exacerbations (Barnes et al, 1985).

Corticosteroids do not prevent new attacks and do not influence the overall disease progression. Conversely, interferons, specifically IFNβ, and copolymer 1 are now used in the treatment of MS to alter disease course. On the basis of results obtained from large multicenter clinical trials, the two forms of recombinant IFNβ, IFNβ-1a (Avonex® and Rebif®) and IFNβ-1b (Betaseron®), and copolymer 1 (Copaxone) were approved by the US Food and Drug Administration (FDA) for the treatment of RR-MS (reviewed in Arnason, 1999; Weinstock-Guttman and Cohen, 1996).

IFNβ-1a and –1b administered subcutaneously lead to a reduction by about 1/3 of the number of exacerbations, the total lesion load on MRI and the number of new lesions were smaller, and there was a significant effect in the progression of disability (Weinstock-Guttman and Cohen, 1996). Both forms of IFNβ are being tested in chronic progressive MS. The preliminary results show encouraging effects (Polman et al, 1995).

Copolymer 1 is a mixture of random synthetic polypeptides composed of 4 amino acids: L-alanine, L-glutamic acid, L-lysine and L-tyrosine. Copolymer 1 also reduces the number of exacerbations by about 30% (Arnason, 1999).

1.2.2 EXPERIMENTAL THERAPIES

Although much progress has been made during the last years, it is clear that the therapy of MS needs to be improved. A series of different approaches are being studied and under active research including, T cell vaccination with irradiated autologous MBP reactive T cells; monoclonal anti-CD4+ depletion; monoclonal anti-TNFα; inhibition of type IV phosphodiesterase; promotion of remyelination by growth factors and transplantation of myelin producing oligodendrocytes (reviewed in Hohlfeld, 1997; Noseworthy, 1999).

1.2.3 T CELL MIGRATION INTO THE CNS: THERAPEUTIC APPROACH

Since MS is an inflammatory disease, all the regulatory steps involved in leukocyte transmigration and trafficking are of interest in the development of new therapies. Accepting the premise that lymphocytes and monocytes are pathogenic in MS, it is hypothesized that arresting leukocyte trafficking into the CNS will ameliorate MS.

As stated before, T cells seem to be central to the pathogenesis of MS. An important pathologic feature is the transmigration of lymphocytes across the BBB into the CNS. This is a multistep process dependent on tethering, rolling, cell adhesion, chemotaxis and degradation of the extracellular matrix (ECM) proteins that constitute the basal lamina of the BBB (reviewed by

Springer, 1994). Numerous attempts at inhibiting T cells from entering the CNS have been made, by targeting each of the regulated steps for leukocyte infiltration and migration.

The first requirement for lymphocytes to enter the CNS is their interaction with the vascular endothelial cells that cover the BBB. These interactions involve cell adhesion molecules. Members of the selectin family, such as P-, E-, and L-selectin, are responsible for the low affinity interactions occuring during the tethering and rolling phase of leukocyte recruitment. β2 integrins (CD11/CD18) are involved in the adhesion step, by mediating high affinity binding to their receptors intercellular cell adhesion molecule (ICAM)-1 and ICAM-2, members of the immunoglobulin family (Springer, 1990). A monoclonal antibody against ICAM-1 suppresses MBP induced EAE in Lewis rats but has only a minor effect in EAE mediated by adoptive T cell transfer (Archelos et al, 1993). Treatment with monoclonal antibodies against the counter-receptors of ICAM-1, the β2 integrins leukocyte function antigen (LFA)-1 and the MAC-1 adhesion molecules, delayed onset, and diminished the severity, of T cell transfer EAE in mice (Gordon et al, 1995). In a different study, however, EAE was augmented by another anti-LFA monoclonal antibody (Welsh et al, 1993), illustrating the fact that the outcome of a therapeutic approach is by no means obvious.

An alternative pathway for lymphocyte recruitment is constituted by α4-integrins, which can mediate rolling, tethering, and adhesion (Alon et al, 1995; Johnston et al, 1996). α4β1, and α4β7 can bind to vascular cell adhesion molecule (VCAM)-1 in the endothelial cells, as well as to ECM components including fibronectin. In 1992, Yednock et al (1992) reported the prevention of T cell transfer EAE in Lewis rats by a single intraperitoneal injection of a monoclonal antibody directed against α4β1 integrin. In guinea pigs, actively induced EAE can also be prevented and reversed with a monoclonal antibody against α4 integrin (Kent et al, 1995). In an MS clinical trial, the anti-α4 antibody, Antegren®, shows a significant reduction in the number of new active lesions on MRI in the first 12 weeks of treatment. Nonetheless, after 24 weeks of treatment, there was no significant improvement (Tubridy et al, 1999).

It is probable that a unique and single therapy for MS will not be sufficient. Indeed, due to the extreme variety and distinct factors that govern the process of MS, its treatment will more likely be a combination or cocktail of drugs with different mechanisms of action. Arresting T cell

transmigration to the CNS seems to be a necessary and fundamental approach as preventive and modifying therapy for MS.

Once the activated lymphocytes have extravasated, they still must pass through a barrier of ECM proteins that constitute the basal lamina of the BBB. Matrix metalloproteinases (MMPs) play a key role in the penetration of this barrier, allowing the activated lymphocytes to gain access into the CNS parenchyma. MMPs have been detected in MS lesions and are considered to contribute in many ways to the pathogenesis of MS, constituting a good target for therapy in MS.

1.3 MMPs in the pathogenesis of MS

1.3.1 WHAT ARE MMPS?

MMPs are a family of zinc-containing endoproteinases that digest specific components of the ECM, thus contributing to matrix equilibrium and structural integrity. There are at least 20 mammalian species known (Fig. 1.3.1). MMPs appear indispensable for ECM degradation; however, their finely tuned regulation is of critical importance. Any increase in enzymatic activity will likely result in tissue destruction or cell invasion. Thus, MMPs are not only involved in important physiologic conditions but are thought to contribute to the pathogenesis of some diseases (Table 1.3.1) (Yong, 1999). For instance, degradation of the ECM by metalloproteinases is a critical phenomenon in cancer invasion and metastasis (Westermarck et al, 1999; Masumori et al, 1994); in rheumatoid arthritis the presence of MMPs is associated with the destruction of the collagen in the cartilage (reviewed in Harris, 1990). In adult periodontitis MMPs inhibitors reduce bone-type collagen degradation fragments in the gingival crevicular fluid, preventing the destruction of the periodontum (Golub et al, 1997).

MMP activity is strictly regulated at three different levels: gene transcription, proenzyme activation, and inhibition by tissue inhibitors of metalloproteinases (TIMPs). At the transcriptional level, cytokines such as TNFα and IL-1, as well as chemokines including macrophage inflammatory protein (MIP)-1α, MIP-1β, Rantes, induce the production of proMMP-9 in CD4+

cells (Johnatty et al, 1997). MMPs are secreted as inactive zymogens that require activation by proteolytic cleavage (Fig. 1.3.2). MMPs themselves and other proteinases, such as plasmin, are known to modulate this process. The activated forms are subject to inhibition by TIMPs, which are expressed ubiquitously in the extracellular milieu and form a complex of 1:1 stoichiometry with the endoproteinases (Kieseier et al, 1999).

Fig 1.3.1. Members and structure of the MMP family (adapted from Yong (1999) with permission)

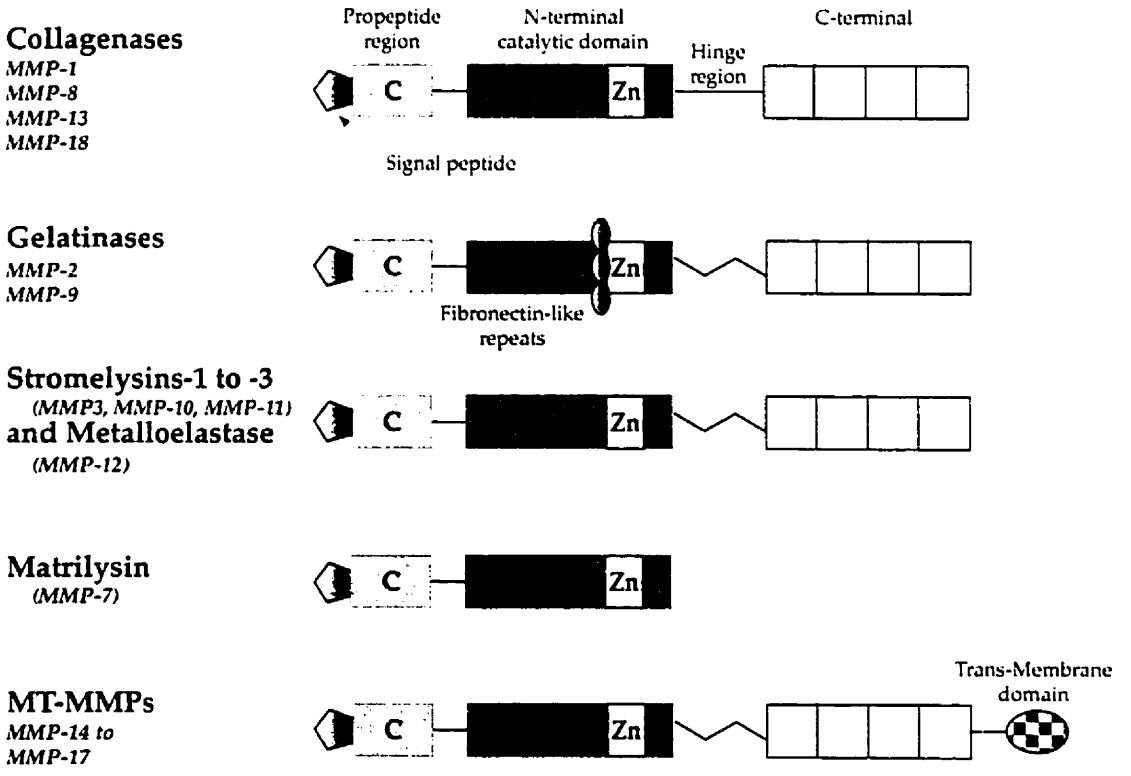
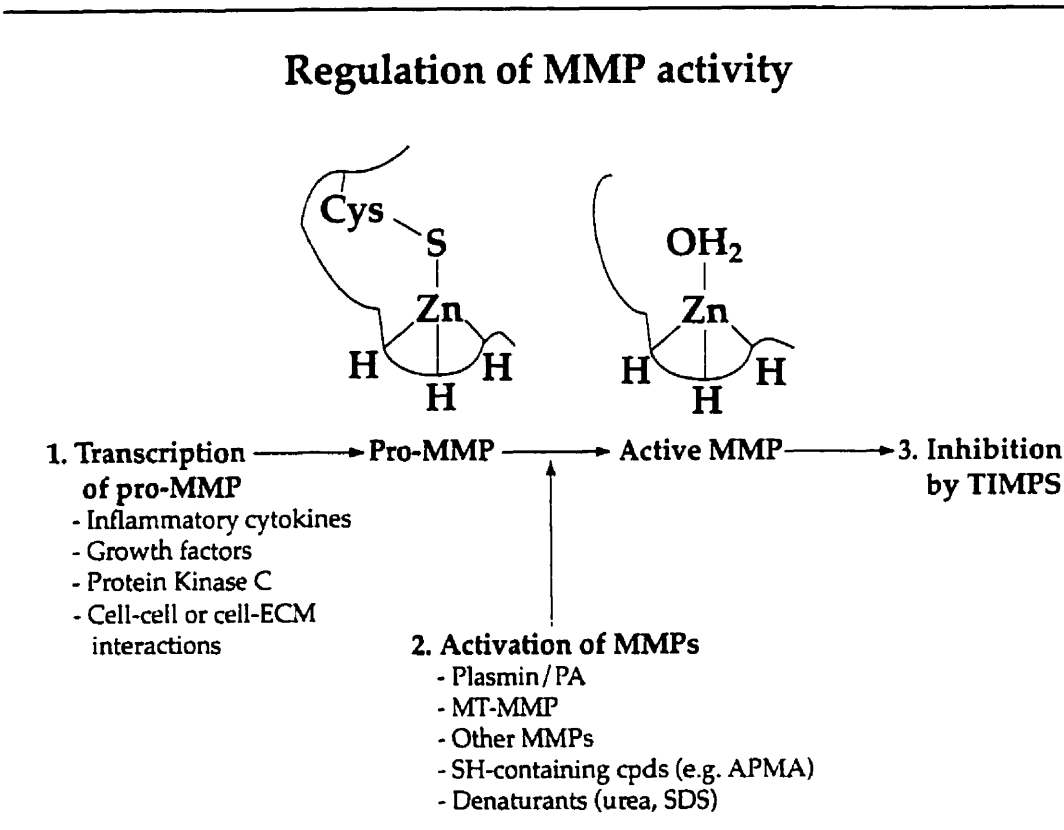


Table 1.3.1. Physiology of MMPs (Yong, 1999)

Normal processes	Pathologic processes
Ovulation	Cancer metastasis
Blastocyst implantation	Rheumatoid Arthritis
Embryogenesis	Periodontal disease
Bone growth and remodelling	Alzheimer's disease
Angiogenesis	Gastric ulcer and liver cirrhosis
Neuronal migration and neurite extension	Atherosclerosis
Wound healing	Fibrotic lung disease

Fig 1.3.2 Means by which the activity of MMPs are regulated (reviewed and adapted from Yong (1999) with permission)



1.3.2 MMPs: POSSIBLE PATHOGENIC MECHANISMS IN MS

MMPs, specifically MMP-9, are found in the cerebrospinal fluid of MS patients (Cuzner et al, 1978; Gijbels et al, 1992; Rosenberg et al, 1996; Leppert et al, 1998). Evidence suggests that MMPs are involved in BBB breakdown in MS. Raised CSF levels of MMP-9 are associated with a disturbed BBB, as demonstrated by gadolinium-enhanced MRI. In MS patients, MMP-9 levels were selectively elevated during clinical relapses in the CSF (Leppert et al, 1998) and in the serum (Lee et al, 1999). In addition treatment with high dose methylprednisolone, a drug known to downregulate the transcription of MMPs, reduced both gadolinium enhanced MRI activity and CSF levels for MMP-9. This correlative studies with MS patients suggests that the enhancement of proteolytic activity would more likely disrupt the basal lamina around capillaries and thereby pave the way for inflammatory cells into the CNS.

Potential pathogenic roles of MMPs have been evaluated in animals. MMPs are associated with BBB opening (Rosenberg et al, 1998), since the injection of MMPs into the rat brain increases capillary permeability which can be prevented by TIMP-2 (Rosenberg et al, 1992). Intracerebral stereotaxic injection of MMP-7, -8 or -9 in rats provokes recruitment of leukocytes and BBB breakdown. In addition, MMP-7 and -9 induce loss of myelin staining (Anthony et al, 1998). BBB leakage, T cell infiltration and myelin loss can all be reduced by treatment with BB-1101, an inhibitor of MMPs (Matyszak and Perry, 1996).

MMPs have been postulated to be the major group of proteinases that could be involved in the degradation of the ECM (reviewed in Yong et al, 1998b). The expression of MMPs, particularly MMP-7, -9 and -12, by perivascular leukocytes in MS and EAE, is thought to contribute to their infiltration into the CNS, since leukocytes are shown to depend on MMPs to penetrate barriers in vitro (Xia et al, 1996; Leppert et al,1995). Moreover, using an elegant in vitro model of the BBB, lymphocytes treated with inhibitors of MMPs were found to be able to adhere to and diapedise between endothelial cells but were then unable to penetrate the next barrier consisting of an artificial basement membrane matrix (Grasser et al, 1998). Therefore, migration and penetration of lymphocytes and monocytes into the CNS parenchyma seems to be mediated by MMPs.

It has been reported that the brains of patients with MS contain cells that are up-regulated for various MMPs, and that these are mainly lymphocytes and macrophages (Maeda and Sobel, 1996;

Cuzner et al, 1996). Cossins et al (1997) and Ozenci et al (1999) reported the expression of MMP-9 by infiltrating leukocytes and MMP-7 by macrophages in the CNS parenchyma while Anthony et al (1997) showed the up-regulation of MMP-7 also in T cells as well as in macrophages localized in perivascular cuffs.

Since the trafficking of lymphocytes and monocytes into the CNS parenchyma seems to be mediated by MMPs, it can be hypothesized that the application of an inhibitor of MMPs would reduce leukocyte infiltration into the CNS and thus ameliorate EAE. Indeed, it has been demonstrated that specific chemical inhibitors of MMPs can prevent or ameliorate EAE (Liedtke et al, 1998; Kieseier et al, 1999). Gijbels et al (1994) reported for the first time that an MMP inhibitor, the hydroxamate GM6001, when administered daily to rats with EAE either from the time of disease induction or from the onset of clinical symptoms, suppressed the development or reversed clinical EAE. In 1995, Hewson et al showed that another hydroxamate MMP inhibitor, Ro31-9790, reduced the clinical severity of adoptively transferred EAE, and prevented the onset in 90% of animals. BB-1101, a broad spectrum MMP inhibitor, also reduced weight loss and severity of EAE. Moreover, inhibition of MMPs by oral treatment with d-penicillamine suppressed murine EAE (Norga et al, 1995).

MMPs are not only implicated in the degradation of the ECM components but they can also degrade myelin proteins which can contribute to the disruption of the myelin sheath. Moreover, it has been shown that MMPs can degrade myelin basic protein into fragments that are encephalitogenic, contributing to the pathogenesis of EAE or MS (Chandler et al, 1997; Opdenakker et al, 1994). Therefore, secreted MMPs by infiltrating leukocytes could not only degrade the ECM and facilitate the trafficking and penetration into the CNS, but MMPs may also directly damage the myelin that surrounds axons thereby impairing nervous conduction and deriving the neurological deficits that characterize MS and EAE.

Several matrix metalloproteinase enzymes can cleave pro-TNFα (26 kDa membrane-anchored protein) to the mature biologically active form (17 kDa soluble protein). In addition to being pro-inflammatory, TNFα can damage oligodendrocytes and myelin both in vitro and in vivo, and has been implicated in the pathology of MS and EAE (reviewed in Yong et al, 1998b; 1999). Similarly, there are a number of cell surface molecules whose shedding is facilitated by metalloproteinases,

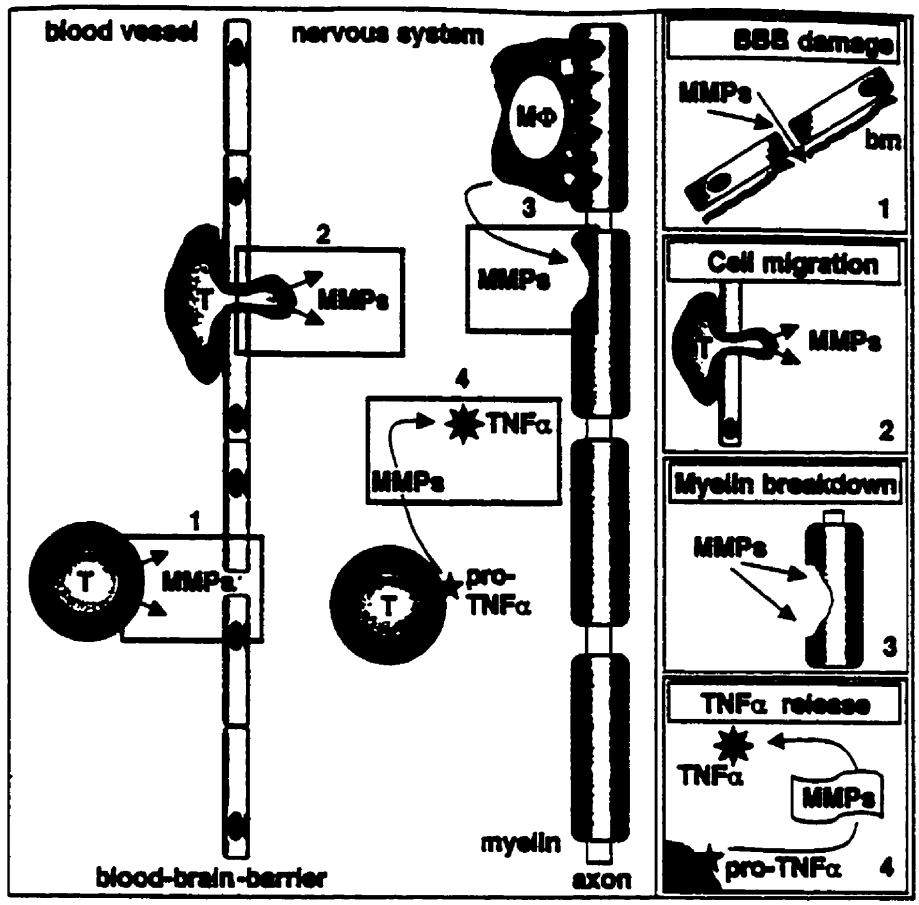
including other cytokines such as TGFα, cytokine receptors such as TNFR1, TNFR2 and IL6Rα, adhesion molecules including L-selectin, and others such as Fas ligand (reviewed in Chandler et al, 1997). Therefore another possible mechanism by which MMPs could be pathogenic is by removal/activation of cell surface cytokine/receptors contributing further to the inflammatory milieu.

In summary, MMPs could be involved in several different deleterious processes in the pathogenesis of inflammatory demyelination in MS: (1) opening of the BBB by disruption of ECM components of the basement membrane; (2) migration of the inflammatory cells across the BBB into the parenchyma; (3) direct degradation of the myelin sheath; (4) enhancement of the release of active TNFα and/or other pathogenic ligands (Fig. 1.3.3). Therefore, inhibiting MMPs production and/or enzymatic activity could be a good therapeutic approach for MS. Indeed, one of the mechanisms of action attributed to IFNβ is the inhibition of MMP expression in lymphocytes (Stuve et al, 1996). As stated before, MMPs inhibitors such as hydroxamates and d-penicillamine have shown encouraging beneficial effects on EAE. However, the only clinical study in MS patients with a combination of d-penicillamine and metacycline showed toxicity (Dubois et al, 1998). Nonetheless, using nontoxic MMPs inhibitors could be favorable to MS patients, and such a drug could be minocycline, a tetracycline that inhibits MMP enzymatic activity. Because of its antibiotic effect minocycline is a treatment for infectious diseases such as acne; indeed minocycline has been in long term clinical use of acne with minimal toxicity. In consequence, it can be hypothesized that the use of minocycline as an inhibitor of MMP activity could ameliorate MS.

1.3.3 MINOCYCLINE AS AN INHIBITOR OF MMP ENZYMATIC ACTIVITY

Minocycline is a semi-synthetic analog of tetracycline that has been shown to have anti-inflammatory properties. In patients with rheumatoid arthritis, minocycline has been shown to have beneficial effects (Kloppenburg et al, 1995a). The anti-inflammatory properties of minocycline in two rat models of rheumatoid arthritis have also been examined by Sewell et al (1996). Administration of oral minocycline significantly decreased ($P < 0.01$) the incidence of arthritis in both adjuvant and collagen rat arthritis. The use of minocycline and other

Fig. 1.3.3 MMPs in the pathogenesis of MS



MMPs could be involved in several different deleterious processes in the pathogenesis of inflammatory demyelination in MS: (1) opening of the BBB by disruption of ECM components of the basement membrane; (2) migration of the inflammatory cells across the BBB into the parenchyma; (3) direct degradation of the myelin sheath; (4) enhancement of the release of active TNFα and/or other pathogenic ligands (modified from Kieseier et al, 1999).

tetracyclines have also been considered in cancer. Minocycline suppresses the invasion and metastatic potential of MRAC-PM2 cells in vitro. Intraperitoneal administration of 500 μg per mouse minocycline reduced the number of metastatic nodules in the lung when MRAC-PM2 cells were injected intravenously (Masumori et al, 1994). Finally, other diseases where minocycline and other tetracyclines have shown promising results are non-infectious forms of dermatitis and in periodontitis (reviewed in Vanheusden et al, 1998; Kloppenburg et al, 1995a; 1996b). The beneficial properties of minocycline and other tetracycline-derivatives have been associated with their ability to suppress in vivo and in vitro mammalian MMP activity (Golub et al, 1984; Zucker et al, 1985).

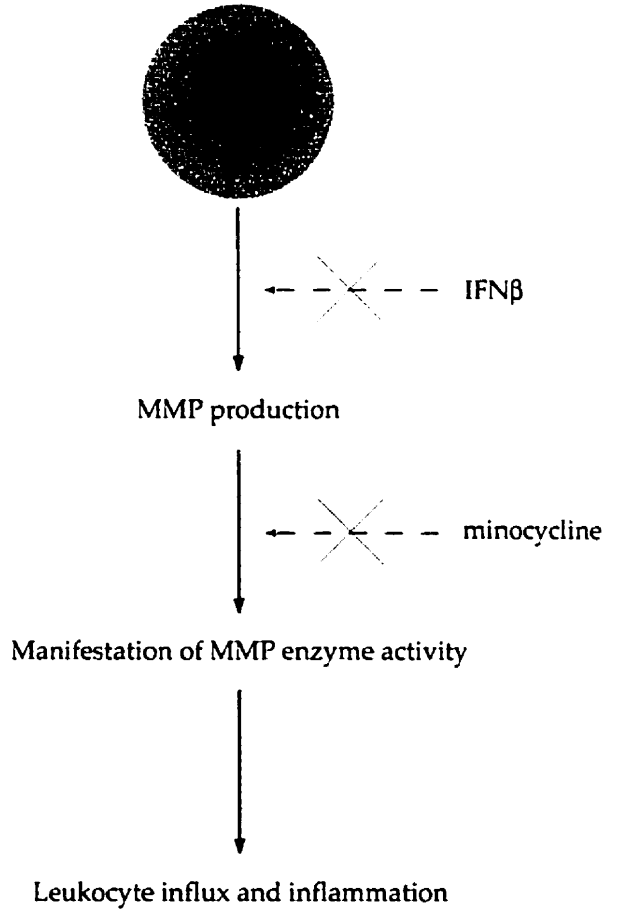
The effect of minocycline on leukocyte migration has been studied in polymorphonuclear cells. Minocycline can reduce polymorphonuclear chemotaxis significantly at a concentration as low as 1 μg/ml (Ueyama et al, 1994). **The first aim of this thesis was to address whether lymphocyte chemotaxis could be affected by minocycline in vitro, while the second aim dealt with the mechanisms by which minocycline achieved this effect.**

Minocycline has also been shown to have immunomodulating activity. Minocycline inhibits human neutrophil functions such as red blood cell lysis, neutrophil-associated collagenolysis, superoxide anion synthesis, degranulation and migration (Sugita et al, 1995; Gabler et al, 1991; Glette et al, 1984). Both minocycline and tetracycline suppress murine thymocyte co-mitogenesis induced by IL-1 at ~2 and 4 μg/ml respectively (Ingham, 1990). Studies done on human T cell clones derived from the synovium of a rheumatoid arthritis patient showed that when T cells were activated via the T cell receptor/CD3 complex, they were suppressed functionally by minocycline, resulting in a dose-dependent inhibition of T cell proliferation and reduction in production of IL-2, IFNγ, and TNFα. Besides an inhibition of IL-2 production, minocycline exerted its effect on T cell proliferation by induction of a decreased IL-2 responsiveness (Kloppenburg et al, 1995b). Thus minocycline, in addition to suppressing MMP activity, seems to have immunosuppressor activity, at least for lymphocytes and neutrophils. Collectively, these activities could be beneficial in the treatment in MS.

1.3.4 IFNβ AS AN INHIBITOR OF MMP PRODUCTION

As mentioned before, IFNβ is a drug currently used to treat MS. It has been shown to reduce the number of relapses in relapsing-remitting MS, as well as the frequency of lesion formation detected by magnetic-resonance imaging (Paty et al, 1993). Even though the mechanisms by which IFNβ ameliorate MS remain debated, the drug has immunomodulatory activity. It has been shown to suppress T cell proliferation and IFN-γ production (Noronha et al, 1993; Rudick et al, 1993) and to interfere with the antigen presentation process. Dhanami et al (1990) have shown that IFNβ can decrease the production of TNFα and TGFβ. It was recently discovered in our laboratory that IFNβ decreased the transmigration of lymphocytes across an artificial BBB. This inhibition was correlated with a downregulation of the lymphocyte production of MMP-9 (Stuve et al, 1996; 1997). Our laboratory has postulated that a mechanism of action of IFNβ is the inhibition of the production of MMPs leading to the reduction in transmigration of lymphocytes across an ECM barrier (reviewed in Yong et al, 1998a). Clinically, patients treated with IFNβ have decreased serum levels of MMP-9 (Trojano et al, 1999). Because IFNβ is already used as a treatment for MS, a combination of minocycline as an inhibitor of MMP **activity**, with IFNβ as an inhibitor of the **production** of MMP, may become a more effective treatment for MS (Fig. 1.3.4)

Fig. 1.3.4 Strategy to improve the efficacy of IFNβ in MS: by combining an inhibitor of MMP production with inhibitors of MMP enzyme activity



1.4 Hypothesis and specific Aims

The central hypothesis that was tested in this thesis was that minocycline would inhibit T lymphocyte migration through mechanisms that involved MMPs. Furthermore I tested the hypothesis that attenuating T cell infiltration into the CNS parenchyma by minocycline would ameliorate EAE and that a combination of IFNβ and minocycline, with different mechanisms of action on MMPs, would derive a better therapeutic response in EAE.

To test this hypothesis, 3 specific aims were formulated. Aim 1 sought to demonstrate that minocycline would inhibit T cell migration in vitro. Specific Aim 2 was designed to determine mechanisms of the inhibitory action of minocycline on T cell migration. Finally, Specific Aim 3 tested the combined efficacy of IFNβ and minocycline in vitro on T cell migration and as a treatment for MOG EAE.

CHAPTER 2

METHODS

2.1 In vitro experiments

2.1.1 T CELL ISOLATION

Human mononuclear cells were isolated from blood of healthy donors by the method of Ficoll-Hypaque (Stuve et al, 1996) and washed 3 times with PBS. In brief, fresh blood was diluted 1:2 and centrifuged on Ficoll-Hypaque at 1800 rpm for 30 minutes. The interphase was extracted and washed 3 x 10 minutes at 1200 rpm with PBS. Monocytes were adhered to plastic for 3 hours in AIM-V (Gibco/BRL) serum free culture medium and floating T cells were collected. During this process of differential adhesion T cells were activated by the addition of 1 ng/ml OKT3, an ascitic antibody against CD3 (provided by Dr Jack Antel, Montreal). This method of purification provided a lymphocyte population composed of CD3+ T cells (83.3 $\pm$ 5.1 %), CD56/16+ Natural Killer (NK) cells (14.5 $\pm$ 7.4 %) and CD19+ B cells (2.5 $\pm$ 1.6 %) (Mean $\pm$ SD, n=4 different mononuclear cell preparation assayed by flow cytometry). Other mononuclear cell preparations were not treated with OKT3 and are referred to as non-activated T lymphocytes.

2.1.2 DIRECT CELL FLUOROCYTOMETRY

Five hundred thousand cells were resuspended in 100 µl AIM-V and incubated with 20 µl of primary antibody conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) for 20 minutes at 4° C in the dark. Two washes with PBS were performed and cells were finally resuspended in 500 µl of PBS for flow cytometric analysis by the fluorocytometer facility. Antibodies (Becton Dickinson, CA) utilized were: Anti-human Leu-4 (CD3) 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 NK cells, Simultest™ LeucoGATE™ CD45/CD14 [Anti-Hle-1/Leu™-M3]) for monocyte/macrophages, CD19 (SJ25C1) PE for B cells, CD25 (Anti-IL-2R) PE for activated T cells and, Simultest™ control γ_1/γ_{2a} (IgG1/IgG2a) as isotype control.

2.1.3 MIGRATION ASSAY SYSTEMS

2.1.3.1 Boyden Chambers. Three μm pore size fibronectin (FN) coated chambers (Collaborative Biomedical Products, Bedford, MA) were used. The bottom chamber contained 500 μl of AIM-V medium with 10% fetal calf serum (FCS). Cells were resuspended at 1 x 10^6 cells/ ml in AIM-V with 2.5% FCS and 500 μl were added to the upper chamber. After 6 or 24 hours of incubation (specified in Results) cells in the bottom chamber were counted using a Coulter counter (Z_1) and expressed as a % of the initial cell seeding density or as number of transmigrated T cells.

2.1.3.2 Transwell polycarbonate assay. Three μm pore polycarbonate membrane (Fisher Scientific, Corning, NY), without any ECM coating or with 25 μg/ml fibronectin coat, were also used to assess lymphocyte migration. The bottom chamber contained AIM-V plus 10% FCS while the upper chamber had 500000 cells suspended in 200 μl of AIM-V plus 2.5% FCS.

2.1.4 ADHESION ASSAY

Sixteen-well chambers (Gibco) were coated with fibronectin (25 μg/ml; 100 μl/well). One x 10^5 T cells were added per well and incubated for one hour at 37° C. Cells were washed and fixed in 4% paraformaldehyde (PFA) and stained for F-actin with PE-phalloidin. Following staining, cell numbers were counted in six fields at 400X using an immunoflurescence microscope.

2.1.5 ZYMOGRAPHY

Gelatin-substrate gel electrophoresis has been used to determine the level of MMP-2 and -9 (Uhm et al, 1998; Stuve et al, 1996). In brief, serum free AIM-V medium was collected from T cells after a defined culture period and mixed with 4X gel loading buffer (200mM Tris pH 6.8, 4% SDS, 0.1% bromophenol blue, 40% glycerol). The samples 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 pH7.5 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 pH7.5 and 5mM $CaCl_2$) for 18 hours at 37° C, in order for proteinases to degrade the gelatin. Each gel was then stained with Coomasie blue for 4 hours 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. The molecular weight identified the MMPs species and this has been previously confirmed by western blot and immuno-depletion experiments (Uhm et al, 1998).

2.1.6 WESTERN BLOT ANALYSIS FOR MMP-9

When required, media were concentrated using Centricon concentrators # 10 (Amicon, Beverly, MA) according to the manufacturer's instructions. The total protein concentration of the samples was determined by the Bradford Coomasie Brilliant blue method (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard. Ten µg of total protein/sample were resolved on a 10% polyacrylamide gel, and transferred overnight to Immobilon-P (Millipore) in transfer buffer (Tris 25mM, glycine 192 mM, methanol 20%) at 4° C and 30 volts. The blots were incubated in blocking buffer {5% skim milk (Carnation), 0.05% Tween-20 in Tris Base Saline (TBS)} for one hour at room temperature. Membranes were then incubated with 2 µg/ml of mouse anti MMP-9 (Ab-2) antibody (Calbiochem, Oncogene Research Products, MA) in blocking buffer for 1 hour at room temperature, followed by 3 x 5 minutes washes in washing buffer TBS-T (0.05% Tween-20 in TBS). The secondary antibody, a horse-radish peroxidase (HRP) conjugated goat anti mouse (IgG + IgM) (Jackson Lab), was used at a dilution 1:5000 in blocking buffer and incubated for one hour at room temperature, followed by 4 x 5 minutes washes with TBS-T. Blots were

developed by the ECL method according to manufacturer instructions (Amersham-Pharmacia Biotech).

2.1.7 CROSS LINKING ANTI-β1 EXPERIMENT

Twenty four well plates were incubated with 10 μg/ml of rabbit anti-mouse antibody in 500 μl RPMI over night at 4°C and washed 2 times before the activation assay. Cells were isolated from healthy human controls (as described in section 2.1.1) and some samples were incubated for 30 minutes with minocycline 250 μg/ml. Ten μl of mouse anti-β1 antibody ascitic (Gibco/BRL) was added per 1.2 x 10^6 cells/ml RPMI and cells were transferred to rabbit anti-mouse plated wells for 5 minutes activation. Cells were rapidly collected, centrifuged and lysed in lysis buffer (1% Triton X 100, 150 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, PMSF, NA_2MoO_4, Na_3VO_4, NaF, 1 μg/ml Aprotinin, Leupeptin, 1 μM Pepstatin).

2.1.8 WESTERN BLOT ANALYSIS FOR TYROSINE PHOSPHORYLATED PROTEINS

Lysates were resolved on a 10% polyacrylamide gel, and transferred overnight to Immobilon-P (Millipore) in transfer buffer (Tris 25mM, glycine 192 mM, methanol 20%) at 4° C and 30 volts. The blots were incubated in blocking buffer (5% BSA, 0.01% Tween-20, 0.05% Nonidet P-40 in TBS) for one hour at room temperature. Membranes were then incubated with 1:1000 of mouse anti phospho-tyrosine (4G10) antibody (UBI, CA) in blocking buffer for 1 hour at room temperature, followed by 3 x 5 minutes washes in washing buffer TBS-T-NP (0.01% Tween-20, 0.05% Nonidet P-40 in TBS). The secondary antibody, an HRP conjugated goat anti mouse (IgG + IgM) (Jackson Lab), was used at a dilution 1:5000 in blocking buffer and incubated for one hour at room temperature, followed by 4 x 5 minutes washes with TBS-T-NP. Blots were developed by the ECL method (Amersham-Pharmacia Biotech).

2.1.9 MULTI-PROBE RNASE PROTECTION ASSAY (RPA)

This assay permited the simultaneous analysis of 9 different MMPs in the same sample. The assay conditions had been previously standardized by Pagenstecher et al (1998). Briefly, total RNA was isolated from cells by the method of Trizol® (Gibco). Ten µg per sample were heated to 95° C and hybridized with [α- ^{33}P]UTP labeled antisense probe set at 56° C for 16 hours. After 1.5 hours digestion at 30° C with RNAse A (80 µg/ml) and RNAse T1 (250 U/µl) mix (Pharmingen), the protected fragments were treated with proteinase K (10 mg/ml), SDS (4%) and yeast tRNA (2 mg/ml) for further 30 minutes at 37° C. The protected RNA duplexes were extracted (phenol-chloroform), precipitated (ammonium acetate-ethanol) and finally dissolved in 80% formamide. After denaturation for 3 minutes at 95° C, the samples were resolved on a 6% polyacrylamide sequencing gel. Dried gels were analyzed by a phosphorimager (Molecular Dynamics).

2.2 In vivo experiments

2.2.1 MOG EAE

Twelve weeks old C57BL/6 female mice (Jackson Lab, MA) were injected subcutaneously at the back of the tail and 7 days later in the flanks with 300 µg of MOG35-55 peptide (provided by Dr Claude Bernard, Sydney) emulsified in 100 µl of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI) containing an additional 4 mg/ml of *Mycobacterium tuberculosis* (H37Ra) (Difco Laboratories, Detroit, MI). Mice were injected intraperitoneally with 300 ng of reconstituted lyophilized Petussis toxin (List Biological Laboratories, Campbell, CA) in 200 µl of PBS. The Pertussis toxin injection was repeated after 48 hours. All animals were used in accordance with the guidelines of the Canadian Council on Laboratory Animal Care.

2.2.2 TREATMENTS

Animals were treated intraperitoneally beginning on the day of induction on a daily basis in 200 µl of PBS. Ten animals per group were used. Treatments were as follows:

Group 1 → **Control** non-treated animals (PBS i.p. once daily);

Group 2 → **Minocycline** (50 mg/kg i.p. twice a day for the first two days; once daily for the next five days; and 25 mg/kg for the subsequent days);

Group 3 → **IFNβ** (375000 U/kg i.p. once daily);

Group 4 → **Combination** of minocycline and IFNβ (as described per individual group).

It should be noticed that the dose of IFNβ used is in concordance with that reported in the literature (Yu et al, 1996). The dose of minocycline was adapted from reports that 50 mg/kg decreased infarct size in experimental ischemia in rats (Yrjanheikki et al, 1998; 1999).

2.2.3 CLINICAL EVALUATION

Mice are weighed on a daily basis. Severity of EAE was graded according to Bernard et al (1997). Briefly, 0, no disease; 1, loss of weight and 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.

2.2.4 HISTOLOGICAL EXAMINATION

Anesthetized mice were perfused with 40 ml of cold PBS and the CNS was dissected. The sacral part of the spinal cord was immersed in 4% PFA over night and embedded in paraffin wax, cross sectioned at 6 to 8 microns and stained with haematoxylin-eosin and Luxol fast blue for evidence of inflammation and demyelination, respectively. Optic nerves were taken

and incubated in 2.5 % glutaraldehyde to be embedded in epon, sectioned at 2 microns, and stained with toluidine blue.

2.3 Statistical Methods

When multiple groups were analyzed simultaneously, the group comparison of one way analysis of variance (ANOVA) with Bonferroni post-hoc was used. When two groups were analyzed, the unpaired Student's t-test was employed. Statistical significance was set at $p < 0.05$.

CHAPTER 3

RESULTS

3.1 Aim 1:

Does minocycline arrest T cell migration in vitro?

3.1.1 MINOCYCLINE ATTENUATES T CELL MIGRATION IN VITRO

Fibronectin coated 3 μm pore size Boyden chambers were used to assess the transmigration of lymphocytes. Over a 24 hour period, 22.8 ± 2.2% of OKT3 activity T cells transmigrated across the fibronectin barrier, while 14.7 ± 1.5% of T cells transmigrated if they were not activated. A single administration of minocycline (250 μg/ml) inhibited the transmigration of T cells whether these were OKT3 activated or non-activated (Fig. 3.1.1). The response to minocycline was dose dependent with over 50% inhibition at 250 and 500 μg/ml (Fig. 3.1.2). A time course study (Fig. 3.1.3) indicated that the inhibition of T cell transmigration by minocycline is an early process which occurs by 15 minutes.

While a correlation between pH and transmigration of T cells has been reported (Taub et al, 1995), tetracycline did not affect the pH of the culture medium (as measured by pH indicator paper). To address if the inhibition was due to a toxic effect of minocycline, we measured cell viability by the method of trypan blue exclusion. After 1 hour of treatment with 250 μg/ml of minocycline there were no trypan blue-positive cells, while after 24 hours, 5.3 ± 0.6 % (mean ± SD) of cells were trypan blue positive in the minocycline group; however, this value at 24 hours was not different from controls (4.9 ± 0.7 %). Since inhibition of migration occurs already by 15 minutes of treatment, the action of minocycline on cell migration does not appear to be due to non-specific cytotoxicity.

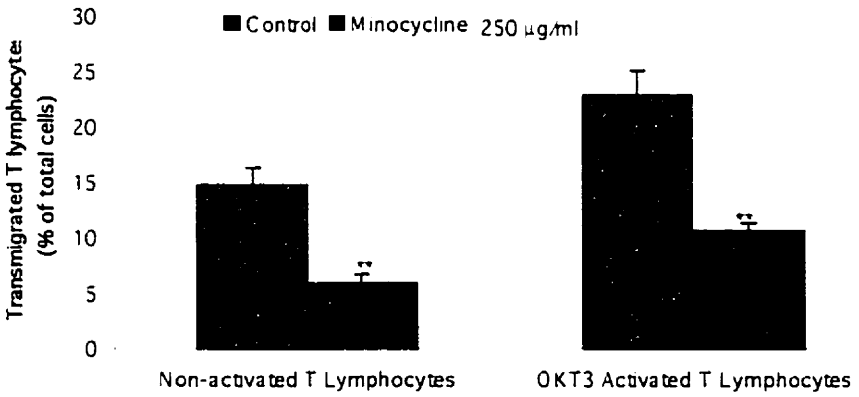
3.1.2 MINOCYCLINE INHIBITORY EFFECT IS NOT SPECIFIC TO A SUBSET OF CELLS

Because the population of lymphocytes generally isolated from human donors was composed of 14.5 ± 7.4 % of CD56/16+ natural killer cells, it was possible that minocycline could inhibit the transmigration of just that population. To study if the inhibitory action of minocycline was specific to a subset of leukocytes, I characterized the cells {T cells (both CD4+ and CD8+), NK cells (CD16+56) or activated cells that expressed the IL-2 receptor (CD25+)} that transmigrated. Specifically, 24 hours after cells were added to the top compartment of the Boyden chamber, with or without minocycline, cells in the lower chamber were analyzed by flow cytometry for the different subpopulations present. Comparisons of the subsets between minocycline and control groups (Table 3.1.1) revealed that no specific populations were preferentially inhibited by minocycline, since no specific subset was drastically reduced in amounts in the lower chamber in the minocycline group

compared to controls. The results suggest that the inhibition of transmigration by minocycline occurred for all cellular subsets.

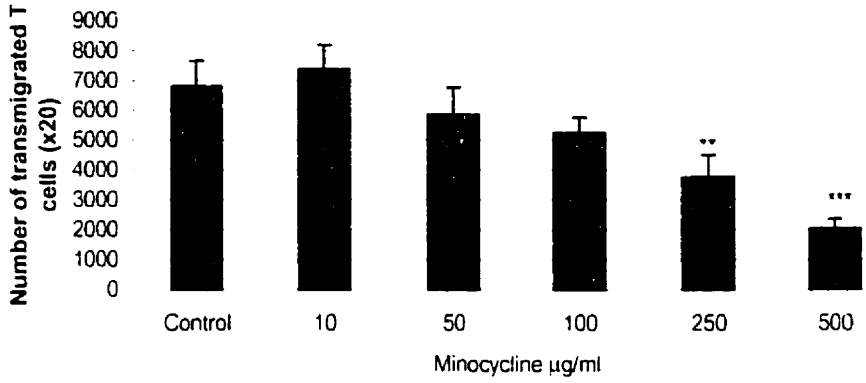
In summary, I established in this Aim the finding that minocycline inhibits T cell migration in vitro, and that this is not due to any non-specific cytotoxicity.

Fig. 3.1.1. Minocycline attenuates the transmigration of T lymphocytes.



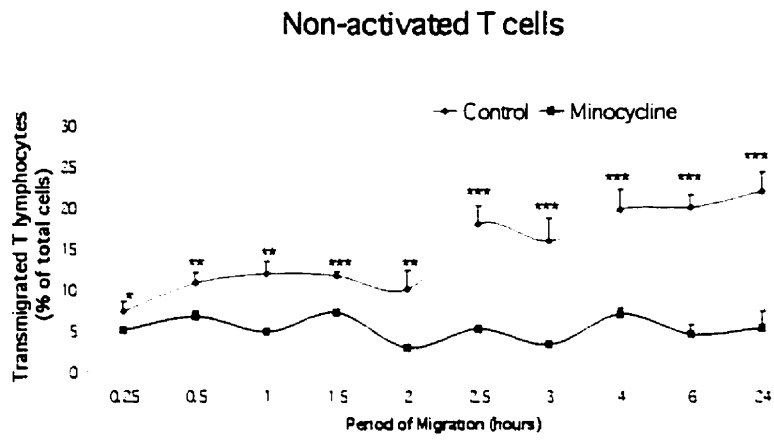
Five hundred thousand cells, unactivated or treated with 1 ng/ml OKT3 for 72 hours, were placed in the top chamber, and the number of cells in the lower chamber was counted using a Coulter counter after 24 hours. Cells were incubated with minocycline 15 minutes before the migration assay and then transferred directly to the top compartment of the Boyden chamber. A 3 µm pore size Boyden chamber coated with fibronectin was used. Values are mean ± SD of triplicate samples. Student's t-test compared to control **$p \leq 0.01$.

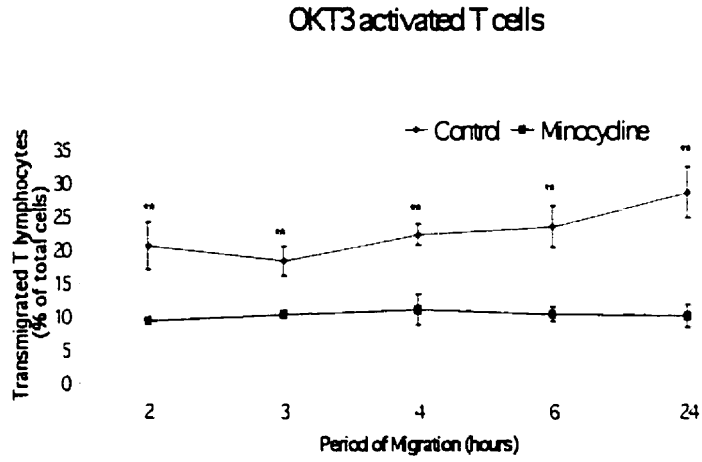
Fig. 3.1.2. Dose response of inhibition of T cell transmigration by minocycline.



Migration assay in Boyden chamber after 24 hours of incubation. Minocycline was incubated 15 minutes before the assay. Values are mean ± SD of triplicate samples. One-way ANOVA test with Bonferroni Post Hoc against control **$p<0.01$, ***$p<0.001$. Please note that the extent of transmigration was tabulated as the number of transmigrated cells (i.e. number of cells in the lower chamber) if an identical aliquot of initial cell population was added to chambers in all experimental groups (as in Fig 3.1.2). However, when comparing transmigration between 2 different samples (e.g. fig. 3.1.1) where the initial seeding of cells might be slightly varied, then the number of cells in the top and bottom compartments of each Boyden chamber was counted, and the number of transmigrated cells then expressed as a % of the initial seeding density.

Fig. 3.1.3. Time course of inhibition of T cell transmigration by minocycline.





Non-activated or OKT3 activated T cells were incubated with minocycline (250 μg/ml) 15 minutes before the Boyden chamber migration assay. Minocycline inhibition of transmigration across the fibronectin barrier is an early process (by 15 minutes), which is maintained for the 24 hours of experimentation. Values are mean ± SD of triplicate samples. Student's t-test compared to the corresponding control sample $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Table 3.1.1. The inhibition of T cell transmigration is not selective to a specific T cell subset.

	Experiment 1		Experiment 2	
	Control	Minocycline	Control	Minocycline
CD3+	76.7	74.5	77.9	74.4
CD4+	60.3	61.1	59.6	62.6
CD8+	41.4	36.6	36.9	35.2
CD16+56	13.0	19.5	15.6	15.8
CD25+	27.3	19.1	16.9	21.3

OKT3 activated T cells were incubated with minocycline (250 μg/ml) 15 minutes before the addition to the upper compartment of a Boyden chamber migration assay. After 24 hours cells in the bottom chambers were collected, immunolabeled for their respective cell-type specific markers, and analyzed by flow cytometry. Values are percentage of positive cells in the population.

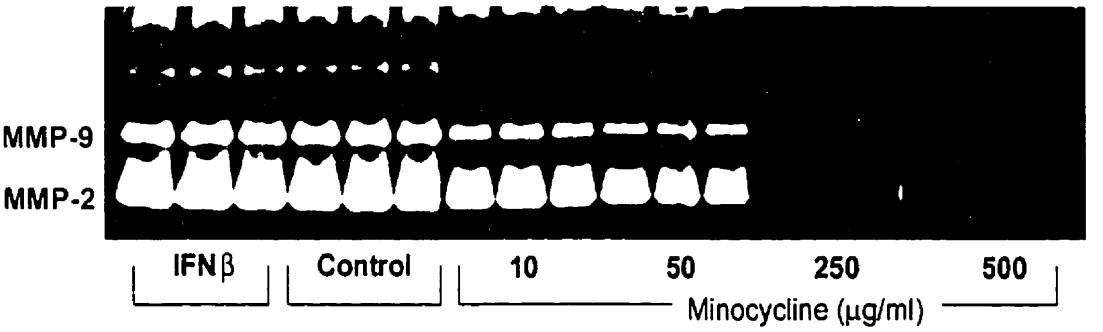
3.2 Aim 2:

What are the mechanisms by which minocycline inhibits T cell migration in vitro?

3.2.1 MINOCYCLINE INHIBITS MMP ENZYMATIC ACTIVITY

As stated in my first aim, minocycline inhibits T cell migration through a fibronectin barrier. Once adhered to the fibronectin substrate, and, in order to go through the 3 μm pore (Boyden chamber system), T cells have to be able to degrade the fibronectin barrier. This is a process dependent on MMPs (Leppert et al, 1996; Xia et al, 1996), and TIMP-1, a specific inhibitor of MMPs, and 100 μM phenanthroline, a non-specific metalloproteinase inhibitor, reduce T cell migration (Uhm et al, 1999). Indeed, I corroborated the findings of Paemen et al (1996) that minocycline is an inhibitor of gelatinase activity (Fig. 3.2.1) of MMPs.

Fig 3.2.1 Minocycline inhibits MMP enzymatic activity.

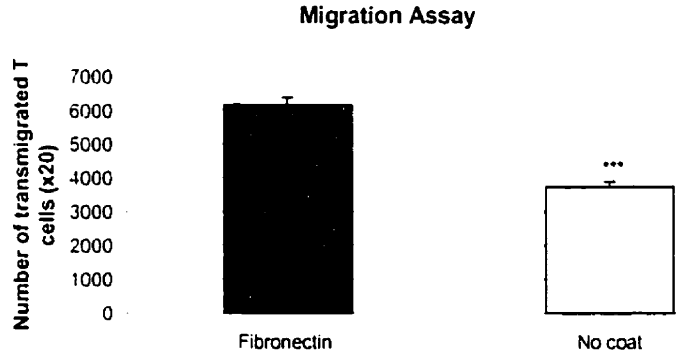


A mixture of MMP-2 and -9, obtained from the conditioned medium of BHK (baby hamster kidney) cells transfected with human MMP-2 and –9 (compliments of Dylan Edwards, University of East Anglia, UK) was resolved by 10% SDS-PAGE impregnated with gelatin. Gels were then incubated with different concentrations of minocycline, IFNβ (1000 U/ml) or without drugs (control), during the development of the zymogram. A direct effect on inhibiting the activity of MMP-2 or -9 is indicated by the decreased gelatinolytic bands compared to control.

3.2.2 MINOCYCLINE INHIBITS T CELL ADHESION ON FIBRONECTIN

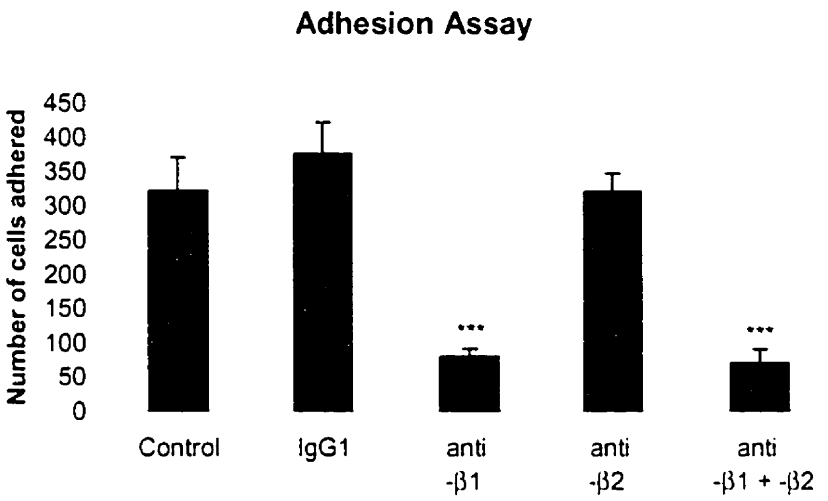
Besides its barrier function, fibronectin acts as an adherence substrate. An initial step for T cells to migrate is to adhere to fibronectin. Indeed, in the Boyden chamber system, fibronectin facilitates T cell transmigration compared to a barrier that is not coated (Fig. 3.2.2). Adhesion of T cells on fibronectin is mediated by integrins, specifically β1 integrins (Fig. 3.2.3). It was possible that minocycline caused inhibition of migration by interfering with integrin activity. Therefore, I performed an adhesion assay on fibronectin. As seen in Fig 3.2.4 the addition of minocycline (250 μg/ml) 15 minutes before the assay inhibited T cell adherence (Fig. 3.2.4).

Fig. 3.2.2 Fibronectin facilitates T cell transmigration.



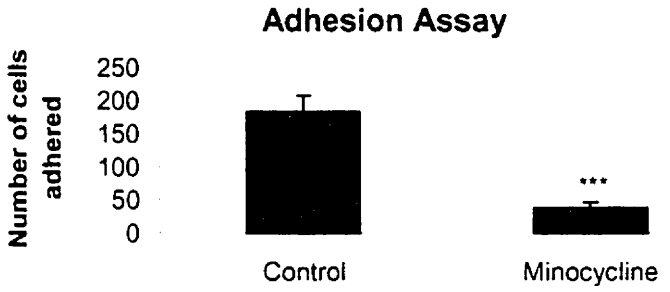
Migration assay performed in 3 μm pore size transwell chamber after 24 hours of incubation. Chambers were coated with 25 μg/ml fibronectin or were uncoated. Values are mean ± SD of triplicate samples. Student's t-test compared to fibronectin coated sample ***$p<0.001$.

Fig. 3.2.3 Adhesion of T cells on fibronectin is mediated by β1 integrins.



One hour adhesion assay on fibronectin. Sixteen-well chambers (Gibco/BRL) were coated with fibronectin (25 μg/ml; 100 μl/well). T cells were incubated with 10 μg/ml of IgG1 isotype control, or 1:100 dilution of an ascitic anti-β1 antibody (Gibco/BRL, clone P4C10), or 10 μg/ml of anti-β2 antibody (Immunotech, clone 7E4) 30 minutes before the adhesion assay. One x 10^5 cells were added per well and incubated for one hour at 37° C. Cells were washed and fixed in 4% PFA and stained for F-actin with PE-phalloidin. Following staining, cell numbers were counted in six fields at 400X from 2 chambers. Values are mean ± SD of triplicates. One-way ANOVA test with Bonferroni Post Hoc. Values compared against control ***$p \leq 0.001$.

Fig. 3.2.4 Minocycline inhibits adhesion on fibronectin.

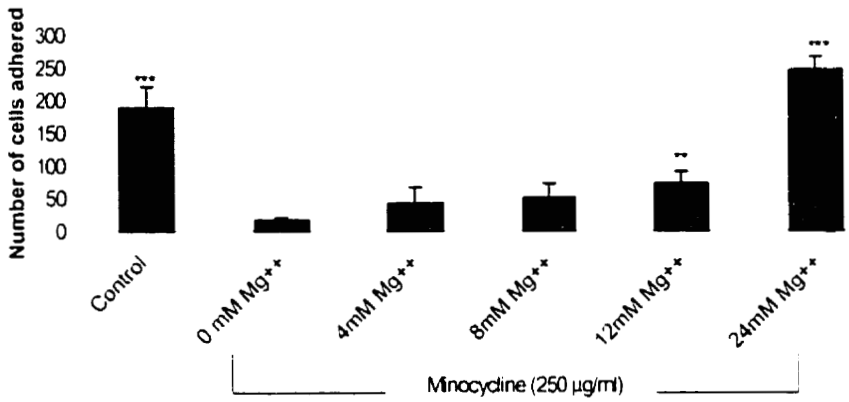


One hour adhesion assay on fibronectin. T cells were incubated with minocycline (250 µg/ml) 15 minutes before the adhesion assay. Values are mean ± SD of triplicates. Student's t-test compared to control ***$p<0.001$.

3.2.3 Mg++ CAN OVERCOME THE INHIBITORY EFFECT OF MINOCYCLINE ON T CELL ADHESION

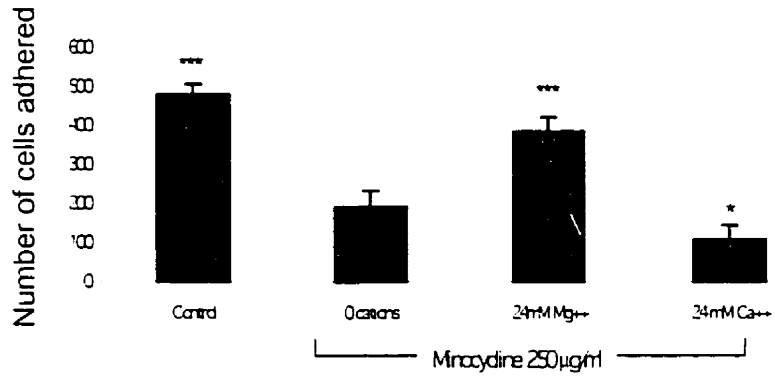
The proper function of integrins is dependent on the presence of cations such as Mn++ and Mg++ (Gahmberg et al, 1998). Because minocycline is a chelator of heavy metals (Paemen et al, 1996; Berthon et al, 1983), a possible mechanism by which minocycline was inhibiting adhesion was by sequestering Mg++ or Mn++. To test this possibility, a competition assay was performed. Minocycline inhibitory effect on adhesion could be overcome by increasing the concentration of Mg++ (Fig. 3.2.5). The addition of Ca++ decreased further T cell adhesion (Fig. 3.2.6) and the effect of Mn++ could not be assessed since it appeared to be toxic to the cells (about 85% of unfixed cells took up propidium iodide).

Fig. 3.2.5 The inhibitory action of minocycline can be overcome with Mg++.



One hour adhesion assay on fibronectin. Non-activated T cells were incubated with minocycline (250 µg/ml) and $MgCl_2$ 15 minutes before the adhesion assay. Values are mean ± SD of triplicate samples. One-way ANOVA test with Bonferroni Post Hoc. Values compared against minocycline 250 µg/ml (0 mM Mg++) $^{**}p<0.01$, $^{***}p<0.001$.

Fig. 3.2.6 The inhibitory action of minocycline cannot be overcome by Ca++.

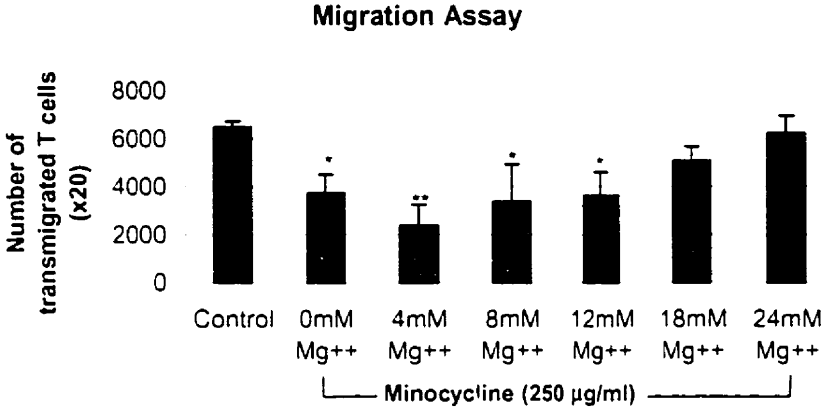


One hour adhesion assay on fibronectin. Non-activated T cells were incubated with minocycline (250 µg/ml) and $MgCl_2$ or $CaCl_2$ 15 minutes before the adhesion assay. Values are mean ± SD of triplicate samples. One-way ANOVA test with Bonferroni Post Hoc. Values compared against minocycline 250 µg/ml (0 mM cations: i.e. no further addition of cations to the MMV basal culture medium) *p<0.05, ***p<0.001.

3.2.4 Mg++ CAN OVERCOME THE INHIBITORY EFFECT OF MINOCYCLINE ON T CELL MIGRATION

To assess if the Mg++ chelating property of minocycline was a mechanism by which minocycline was inhibiting T cell transmigration, we performed a migration assay to determine whether the effect of minocycline could be competed by Mg++. As shown in Fig. 3.2.7 increasing Mg++ concentrations could overcome the inhibitory effect of minocycline.

Fig 3.2.7 The inhibitory action of minocycline on T cell transmigration can be overcome with Mg++.



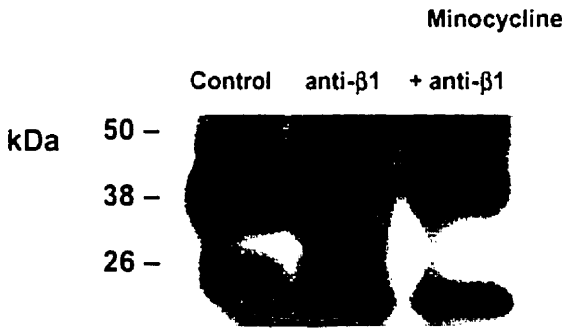
Migration assay performed in 3 µm pore size Boyden chamber coated with fibronectin. Non-activated T cells were incubated with minocycline (250 µg/ml) and $MgCl_2$ 15 minutes before the migration assay. Values are mean ± SD of triplicate samples. One-way ANOVA test with Bonferroni Post Hoc against control *p<0.05, **p<0.01.

3.2.5 MINOCYCLINE DOES NOT INTERFERE DIRECTLY WITH β1 SIGNALING

In order to determine if minocycline could inactivate the β1 integrin chain into a conformation that would not be able to signal, a crosslinking experiment and analysis of tyrosine phosphorylated proteins (as a marker of signal transduction) was performed. Minocycline did not inhibit β1 signaling, suggesting that minocycline does not compete for the epitope recognized by this mouse anti-human β1 integrin chain antibody from Gibco (Fig. 3.2.8).

In summary I have confirmed in this Aim that minocycline inhibits the gelatinase activity of MMPs. Minocycline also interferes with the adhesion of cells on fibronectin, although it does not inhibit directly the ability of the β1 integrin chain to signal. The inhibitory effect of minocycline on adhesion and T cell transmigration was competed out by increasing the concentration of Mg++, suggesting that an important mechanism of minocycline is by chelation of Mg++ required for the proper functioning of integrins. In Aim 3, I reveal further activities of minocycline, including inhibiting the production of MMPs.

Fig 3.2.8. Minocycline does not interfere with β1 signaling.



T cells were untreated (control) or were cross-linked with anti-β1 antibody. In the minocycline + anti-β1 group, T cells were incubated with minocycline (250 μg/ml) for 30 minutes before the anti-β1 antibody (10μg/ml) was added. Activation time with the anti-β1 antibody was 5 minutes. Cell lysates were immunoblotted with anti-phosphotyrosine antibody (4G10) as described in methods

3.3 Aim 3:

Will the combination of IFNβ and minocycline

decrease T cell migration in vitro?

Are the mechanims of action of

IFNβ and minocycline different?

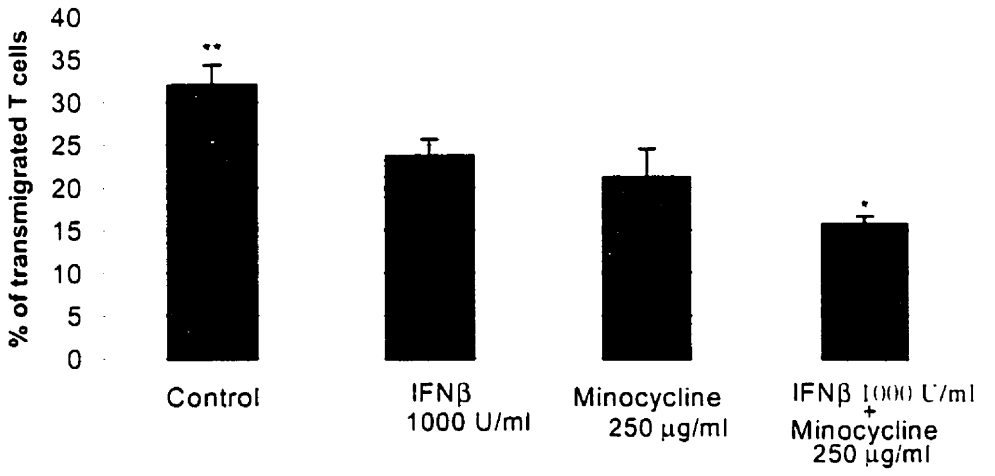
Will the drugs (separate and together) prevent MOG EAE?

3.3.1 MINOCYCLINE INCREASES THE EFFICACY OF IFNβ IN THE INHIBITION OF T CELL TRANSMIGRATION

IFNβ decreases the transmigration of T cells after 72 hours of treatment through mechanisms dependent on MMP-9 production (Stuve et al, 1996, 1997). Since minocycline inhibited the enzymatic activity of MMPs (Fig. 3.2.1) it was hypothesized that the combination of IFNβ and minocycline would decrease further T cell transmigration.

To address whether IFNβ and minocycline would act in combination in the reduction of T lymphocyte migration, migration experiments were performed in the presence of both drugs together or separately. In these series of experiments T cells were incubated in IFNβ (1000 U/ml) for 72 hours, while minocycline (250 μg/ml) was added 15 minutes before the cells were placed in fibronectin-coated Boyden chambers. As shown in Fig. 3.3.1 minocycline increased the efficacy of IFNβ in the inhibition of T cell transmigration.

Fig. 3.3.1. Minocycline increases the efficacy of IFNβ in the inhibition of T cell transmigration.



Migration assay performed in 3 μm pore size Boyden chamber coated with fibronectin. Cells were counted after 6 hours of transmigration. Values are mean ± SD of triplicate samples. One-way ANOVA test with Bonferroni Post Hoc. Values compared against IFNβ *$p<0.05$, **$p<0.01$.

3.3.2 MINOCYCLINE DECREASES MMP-9 EXPRESSION

The use of zymograms has previously revealed that minocycline acted through a different mechanism than IFNβ, since, as shown in fig. 3.2.1, minocycline inhibited MMP activity, while IFNβ did not show any effect. In this laboratory, Stuve et al (1997) have shown that IFNβ was able to inhibit T cell transmigration likely through the reduction of MMP-9 expression. Since I postulated that minocycline acted differently than IFNβ it was important to investigate the effect of minocycline on MMP production. Moreover, minocycline's long term effects on T cells would be important in an in vivo situation. As a first approach, I employed an RPA to determine the levels of transcripts encoding several MMPs. It was noted that of several potential MMP members, T cells express predominantly MMP-9 (gelatinase B), MMP-14 (MT1-MMP) and MMP-1 (collagenase-1). Figure 3.3.2 shows an inhibition of specifically MMP-9 (gelatinase B) transcript levels by minocycline (250 μg/ml) compared to control. In contrast, IFNβ (1000 U/ml) decreased in general the levels of all the MMPs expressed by T cells.

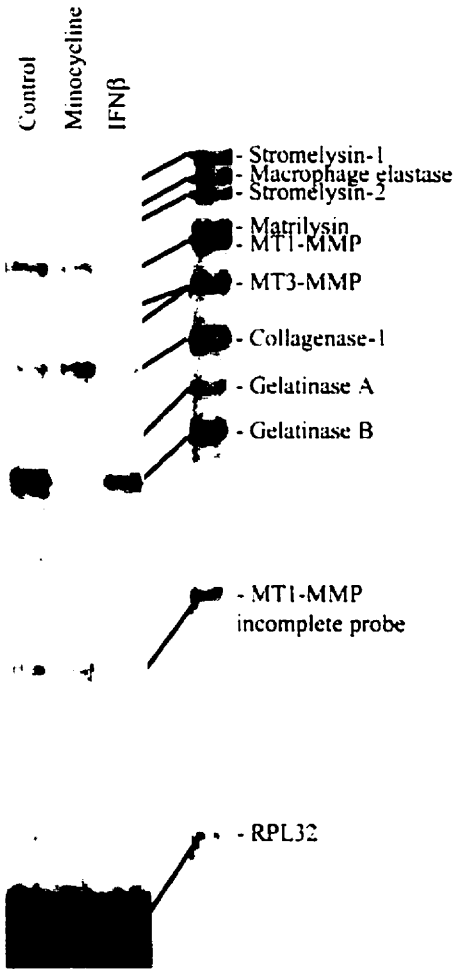
I tested for the presence of protein in the supernatant of cultured cells, specifically of MMP-9, by western blot. Minocycline decreased the level of MMP-9 protein (Fig. 3.3.3). Moreover, at the concentrations used its efficacy was greater than IFNβ.

Altogether, the differences between the two drugs (Table 3.3.1) indicated that minocycline in combination with IFNβ should enhance their individual efficacy in arresting T cells transmigration in vivo. Furthermore, it was predicted that minocycline would have greater efficacy on EAE compared to IFNβ.

Table 3.3.1. Effects of minocycline and IFNβ on T cells

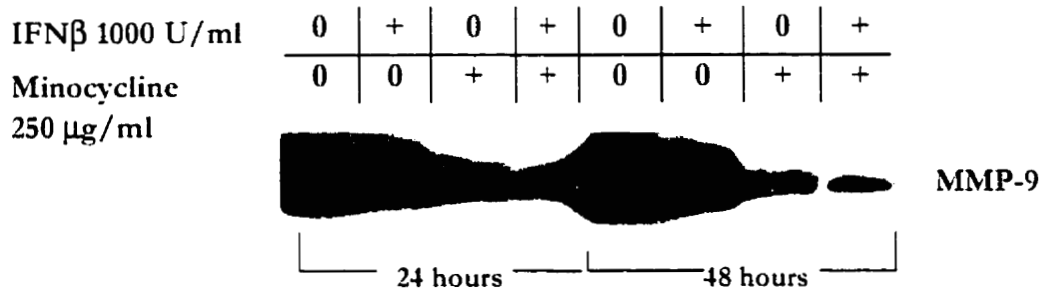
Minocycline	IFNβ
Decreases MMP-9 mRNA	Decreases MMP mRNA levels in general
Inhibits MMP enzymatic activity	No effect on MMP enzymatic activity
Reduces adhesion on fibronectin	No effect on adhesion to fibronectin

Fig. 3.3.2. Minocycline decreases MMP-9 RNA level.



T cells were incubated with Minocycline (250 μg/ml) or IFNβ (1000 U/ml) for 24 hours and then collected into an RNA extraction solution, Tryzol® (Gibco). Total RNA was extracted following the manufacturer's detailed instructions. Ten μg of RNA per lane were subjected to an RNAse protection assay and products were resolved using a sequencing gel.

Fig. 3.3.3 Minocycline decreases MMP-9 protein level.



Non-activated T cells were incubated in the presence (+) or absence (0) of 250 μg/ml minocycline or 1000U/ml IFNβ or the combination for 24 and 48 hours. The supernatant was resolved by 10% SDS-PAGE gel and immunoblotted aganst the zymogen form of MMP-9 as described in methods.

3.3.3 MINOCYCLINE DELAYS THE ONSET OF MOG EAE

As stated in the introduction, minocycline, by its inhibition of collagenolytic and gelatinolytic activity, is thought to be beneficial in rheumatoid arthritis and periodontitis. Minocycline has multiple immunomodulating activities (see Introduction) and physiologically has been considered as an anti-inflammatory agent. Because EAE is a T cell mediated disease, and because I have demonstrated that minocycline decreases T cell transmigration in vitro, I tested the hypothesis that minocycline would affect T cell transmigration into the CNS parenchyma and ameliorate EAE.

MOG EAE was induced in 12 week old C57BL/6 female mice as described in methods and animals were monitored over a period of 28 days. In order to prevent EAE, treatments (Table 3.3.2) were administered intraperitoneally beginning on the day of induction on a daily basis in 200 µl of PBS. Ten animals per group were used.

Table 3.3.2. Treatments applied to MOG EAE

Group 1	→	**Control** non-treated animals (PBS i.p. once daily);
Group 2	→	**Minocycline** (50 mg/kg i.p. twice a day for the first two days; once daily for the next five days; and 25 mg/kg for the subsequent days);
Group 3	→	**IFNβ** (375000 U/kg i.p. once daily);
Group 4	→	**Combination** of minocycline and IFNβ (as described per individual group).

Animals were examined clinically in a daily basis; some animals were sacrificed at day 21 for histological analysis. As seen in Fig. 3.3.4 control mice subjected to MOG innoculum developed signs of EAE by 12 days of induction, and the severity of disease progressively increased. By 18-20 days post-induction control MOG animals were paralyzed (grade 4). Minocycline delayed the onset of EAE for 8 days compared to non-treated animals. IFNβ prevented the onset of disease for 3 days, but after 20 days post-induction animals were as sick as non-treated controls. A drop in weight generally accompanies the onset of EAE as shown in panel B (Fig. 3.3.4). One animal from each minocycline treated group died for unknown reasons, presumably due to drug toxicity.

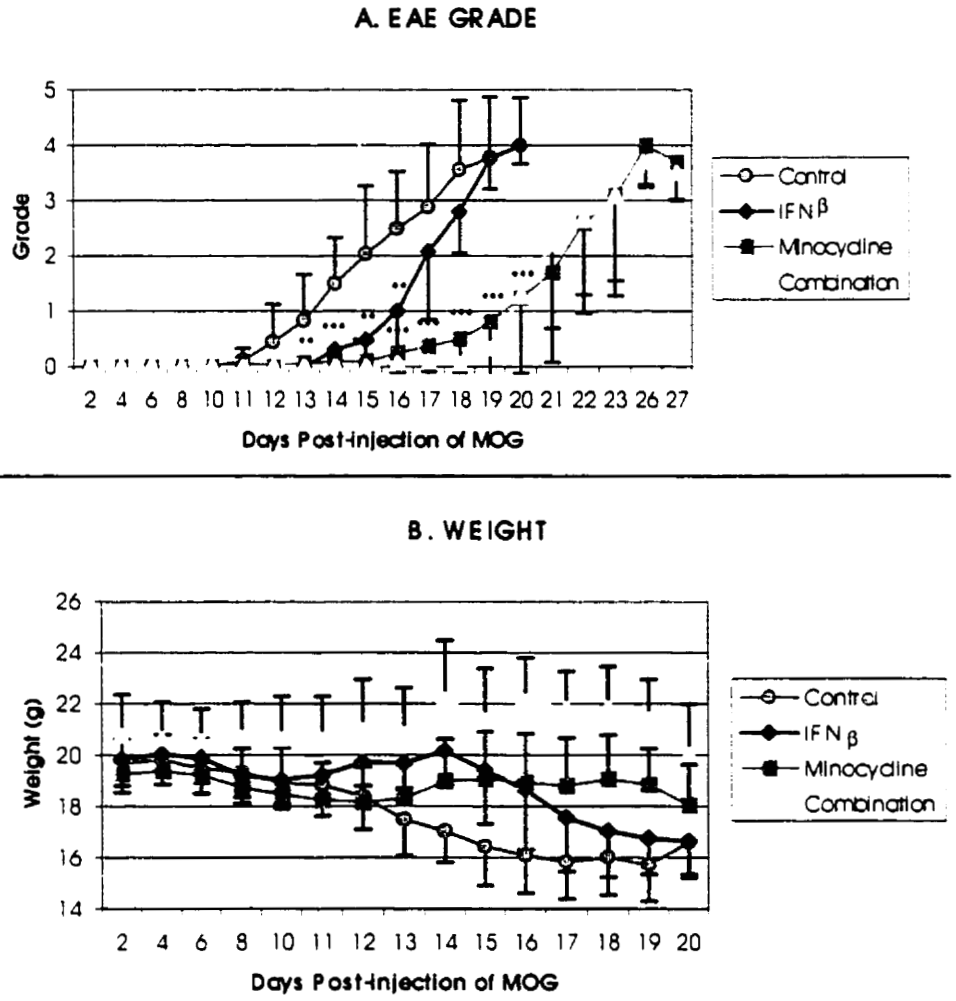
In order to determine if minocycline was delaying EAE by arresting infiltration into the CNS parenchyma histological analysis were performed at day 21. Fig. 3.3.5 shows representative cross sections of the sacral spinal cord stained for Luxol Fast Blue of MOG EAE animal and healthy control. As observed in EAE afflicted animals, there is leukocyte infiltration. Moreover, the loss in blue staining reflects some demyelination.

Minocycline treated animals (alone or in combination) showed no mononuclear infiltration into the CNS parenchyma at day 21. In contrast, IFNβ or non-treated EAE animals showed an extensive infiltration of leukocytes into the CNS parenchyma (Fig 3.3.6). This histological finding correlates with the clinical manifestations. Minocycline could prevent infiltration of leukocytes into the CNS and presumably delayed therefore the clinical onset of EAE.

One of the advantages of the MOG EAE model is the production of demyelination in the optic nerve which is an early symptom in many cases of MS. Cross sections of the optic nerve were taken at day 21 and examined for demyelination. Fig 3.3.7 shows signs of demyelination as well as reactive glial cells in the parenchyma in MOG EAE animals. Sections observed from minocycline treated animals showed a healthy parenchyma (Fig 3.3.8) where the axons examined remained myelinated and there were no signs of reactive glial cells.

In summary, minocycline delayed the onset of EAE for 8 days compared to non-treated animals. Moreover, the average time for minocycline treated animals to become paralyzed was 8 days longer than non-treated or IFNβ treated EAE animals. The clinical signs correlated with the histological signs, suggesting that minocycline could prevent leukocyte infiltration into the CNS parenchyma. Overall minocycline showed a greater efficacy in preventing EAE than IFNβ.

Fig. 3.3.4. Minocycline delays the onset of MOG EAE.

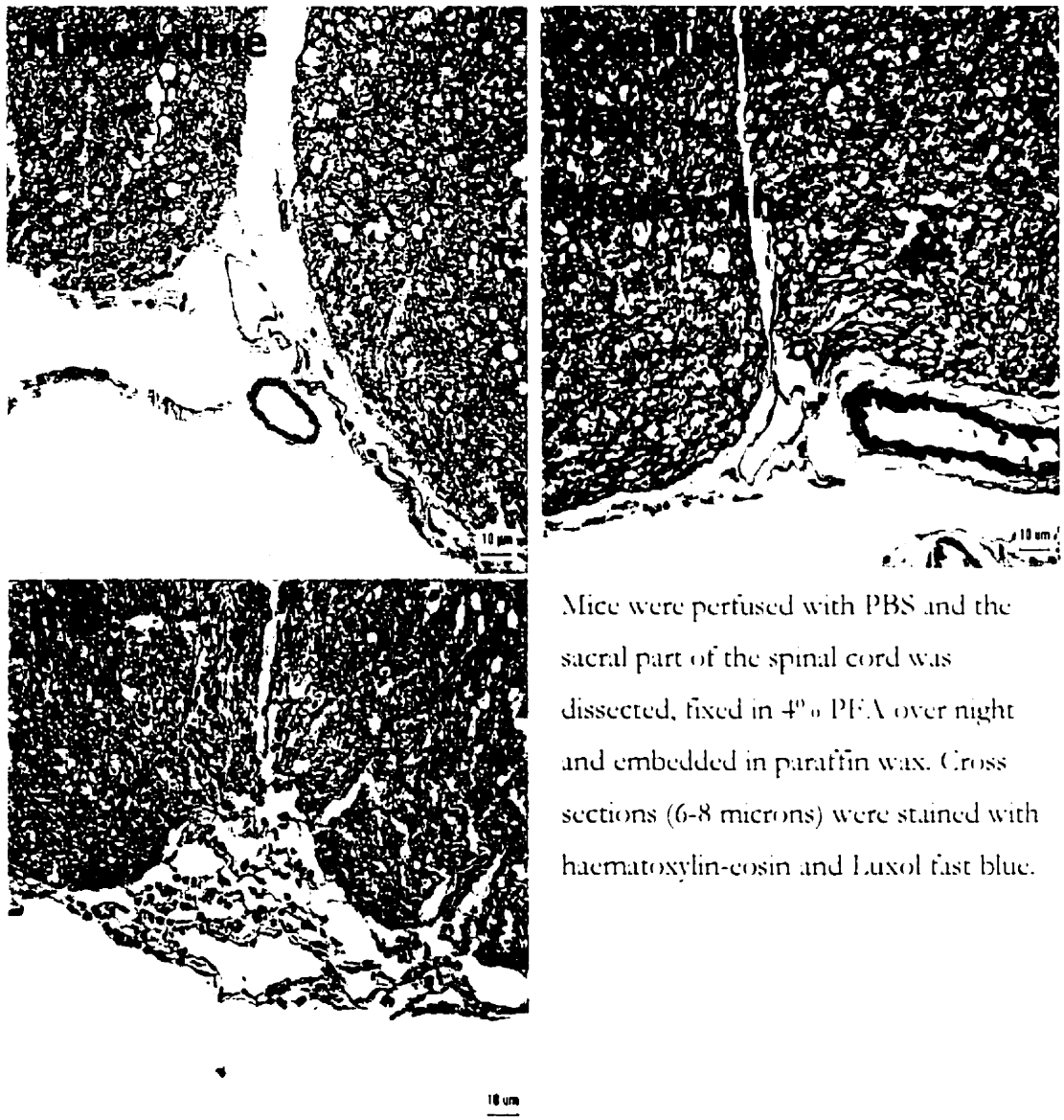


Panel A shows severity of EAE graded according to Bernard et al (1997) (see methods). Briefly, 0, no disease; 1, loss of weight and limp tail; 2, partial paralysis of one or two hind limbs; 3, complete paralysis of hind limbs; 4, hind limb paralysis and fore limb paraparesis; 5, moribund. Panel B shows the animal weights. Values are mean ± SD of about 6-10 mice. One-way ANOVA test with Bonferroni Post Hoc. Values compared against control $^{**}p<0.01$, $^{***}p<0.001$.

Fig 3.3.5 Histological section of MOG EAE compared to a healthy animal

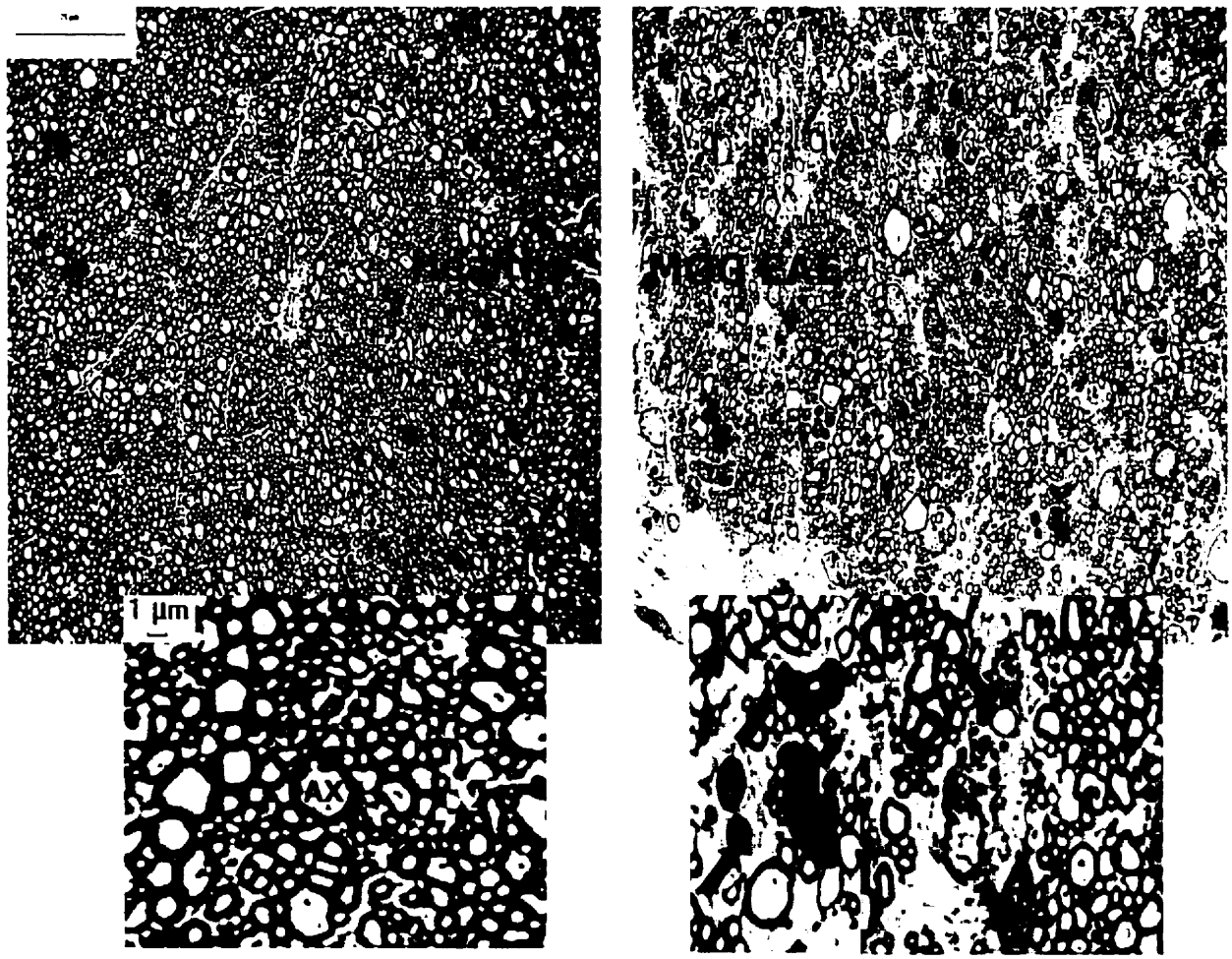
Mice were perfused with PBS and the sacral part of the spinal cord was dissected, fixed in 4% PFA over night and embedded in paraffin wax. Cross sections (6-8 microns) were stained with haematoxylin-eosin and Luxol fast blue for evidence of inflammation and demyelination, respectively. A view of the spinal cord of a MOG EAE mouse is shown so that the presence of leukocytes can be better appreciated.

Fig 3.3.6 Minocycline prevents leukocyte infiltration into the CNS parenchyma



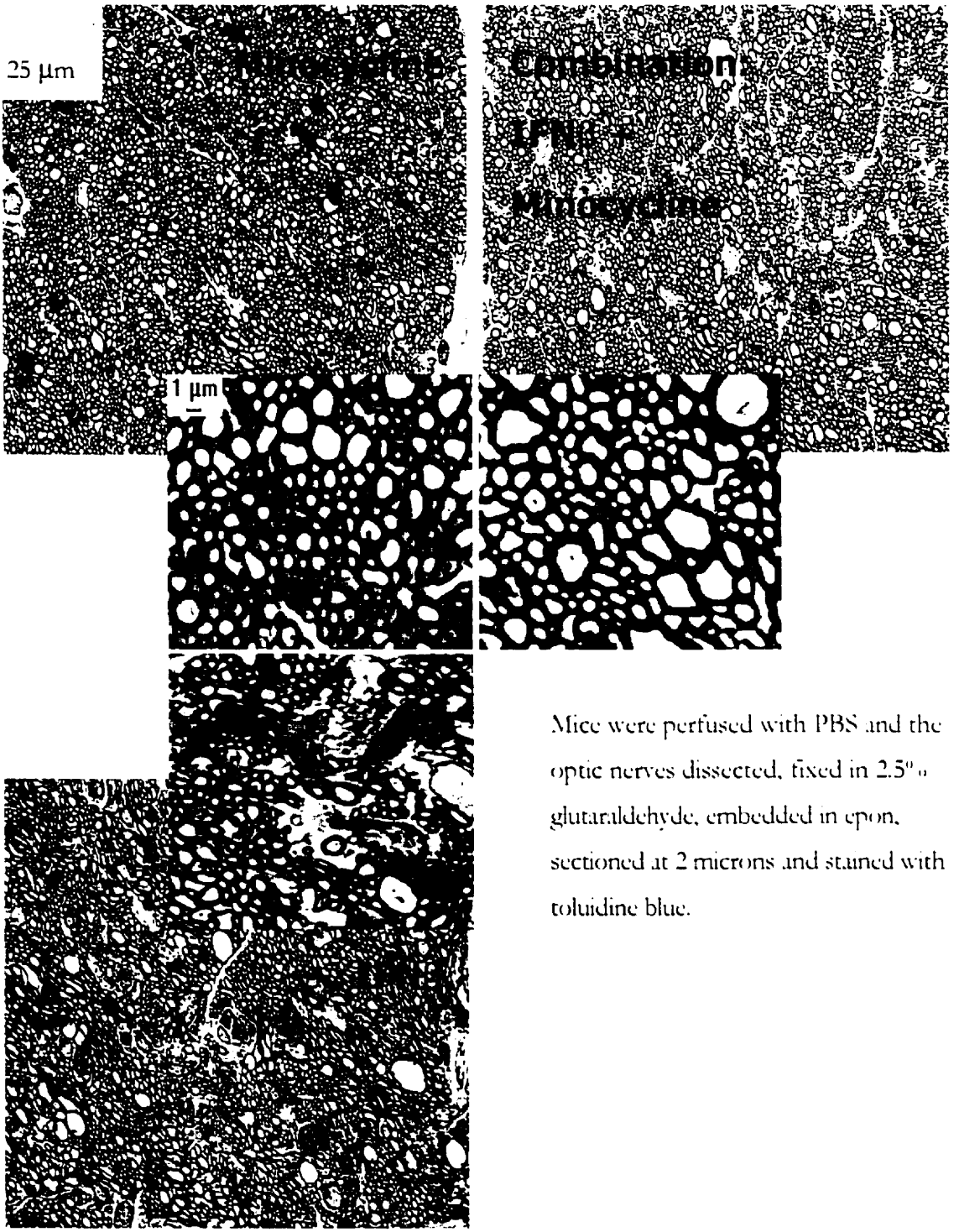
Mice were perfused with PBS and the sacral part of the spinal cord was dissected, fixed in 4% PFA over night and embedded in paraffin wax. Cross sections (6-8 microns) were stained with haematoxylin-eosin and Luxol fast blue.

Fig 3.3.7 MOG EAE mice have demyelination and gliosis



Mice were perfused at day 21 with PBS and the optic nerves were dissected and fixed in 2.5% glutaraldehyde over night, embedded in epon, sectioned at 2 microns and stained with toluidine blue. MOG EAE sections show demyelination and gliosis. **AX**: axon; **M**: myelin sheath; **black arrow**: demyelinated axon; **green arrow**: abnormal myelin sheath, evidence of demyelination.

Fig 3.3.8 Minocycline retards the appearance of demyelination



Mice were perfused with PBS and the optic nerves dissected, fixed in 2.5% glutaraldehyde, embedded in epon, sectioned at 2 microns and stained with toluidine blue.

CHAPTER 4

DISCUSSION

4.1 Overview

Multiple Sclerosis is an inflammatory disease characterized by the infiltration of large numbers of lymphocytes and monocytes into the CNS. It is believed that this infiltration leads to elevated levels of pro-inflammatory cytokines and an undesirable inflammatory response within the CNS. Therefore, arresting inflammatory cells from infiltrating the CNS could lead to the amelioration of MS. The entry of leukocytes into the CNS is dependent on several factors including the expression of MMPs that degrade the extracellular matrix proteins of the basal lamina in the BBB. Several lines of evidence implicate MMPs as being pathogenic factors in MS (reviewed in Yong et al, 1998b; Yong, 1999). First, MMPs are elevated in the CSF and brain of patients with MS. Second, serum MMP-9 levels are significantly elevated in MS patients compared to healthy controls; within the MS population, serum MMP-9 levels are higher during clinical relapse relative to periods of stability. In addition serum MMP-9 levels are correlated with the number of gadolinium enhanced lesions detected by MRI (Lee et al, 1999; Waubant et al, 1999). Third, inhibitors of MMP activity have efficacy in EAE. Fourth, young mice genetically deficient for MMP-9 are relatively resistant to EAE compared to age-matched controls (Dubois et al, 1999). Finally, one of the mechanisms by which IFNβ may act in the amelioration of MS is by inhibiting the production of MMP-9 since IFNβ treated MS patients show reduced serum MMP-9 levels (Trojano et al, 1999). This thesis tested the hypothesis that targeting MMPs could constitute an experimental approach to ameliorate CNS inflammation.

Our laboratory had previously demonstrated that the transmigration of T cells across a fibronectin barrier could be correlated with the expression of MMP-9, and that T cell traffic was inhibited by IFNβ in correspondence with the inhibition of MMP-9 production (Stuve

et al, 1996; 1997). While MMP-9 **production** by T cells was inhibited by IFNβ, MMP-9 **enzyme activity** was unaffected. Consequently, we hypothesized that the combination of IFNβ with a direct inhibitor of MMP-9 enzyme activity would be more effective in arresting leukocyte trafficking and that this combination could lead to a better therapeutic outcome in MS that either drug individually.

In considering inhibitors of MMP enzyme activity we chose minocycline because besides its MMP inhibitory capacity (Paemen et al, 1996) the drug was already in clinical use for other indications. Therefore, given that minocycline had already been approved by the FDA for human use, its potential employment as MS therapy would have been facilitated.

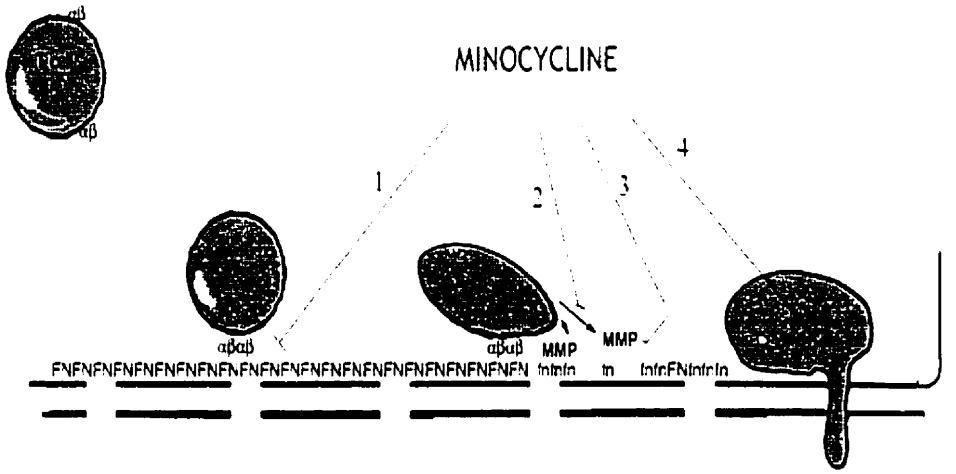
Firstly we have confirmed that minocycline has MMP inhibitory ability, and furthermore, that minocycline is able to decrease migration of T cells across a fibronectin barrier. The inhibitory effect on T cell transmigration is rapid (15 minutes) upon addition of minocycline and it is dose dependent. Moreover, it is not specific to any of the individual populations of lymphocytes tested (CD4+ T cells, CD8+ T cells, CD16+56 NK cells or CD25+ cells).

We have found that minocycline not only inhibited T cell **transmigration**, but that it also inhibited T cell **adhesion** onto fibronectin. The inhibitory action of minocycline could be overcome by the addition of increasing concentrations of Mg++, suggesting that the effect is related to Mg++. Other effects of minocycline on T cells are the inhibition of MMP-9 production at the level of both mRNA (demonstrated by RPA) and protein (demonstrated by western blot) (Fig 4.1.1).

These collective findings suggested minocycline to be a promising therapeutic approach for the treatment of MS. Moreover, because minocycline inhibited MMPs at both **production** and **activity** of MMPs, it was predicted that minocycline would offer better benefits than IFNβ as a therapy for EAE and possibly MS. Therefore we tested the efficacy of minocycline against IFNβ. MOG-EAE in C57BL/6 mice was used as a model for MS. Minocycline delayed the onset of EAE for 8 days compared to non-treated animals. Moreover, the average time for minocycline treated animals to become paralyzed (grade 4) was 8 days longer than non-treated or IFNβ treated EAE animals. Overall minocycline showed a greater efficacy in preventing EAE than IFNβ.

The following sections discuss in greater details my findings and their implications.

Fig 4.1.1 Minocycline inhibits T cell transmigration.



Minocycline has an inhibitory effect on (1) adhesion of T cells on fibronectin (FN, fn), on (2) MMP-9 expression and (3) MMP gelatinase activity with the resulting (4) inhibition of T cell transmigration.

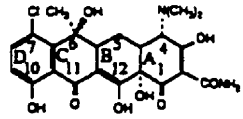
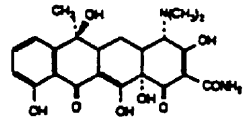
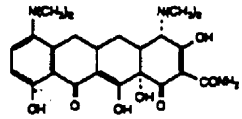
4.2 Minocycline and its effects on T cells in vitro

4.2.1 MINOCYCLINE INHIBITS T CELL MIGRATION IN VITRO

To determine the infiltrative capacity of lymphocytes across a proteinaceous barrier, the in vitro Boyden chamber assay was used. The Boyden chamber method evaluates the number of lymphocytes which, in accordance with the concentration gradient of migration factors, pass through a 3 μm millipore filter and migrate to the lower chamber. Because lymphocytes are floating cells (they do not adhere on plastic in vitro), they can be collected and easily counted by the use of a Coulter counter (Albini et al, 1987). It is noted that the method measures both chemotaxis and chemokinesis. As a model of the ECM barrier we used fibronectin since it is a component of the basal lamina of the BBB. In early MS and EAE lesions there is deposition of fibronectin and fibrinogen (Esiri and Morris, 1991). This deposition on endothelial cells is thought to enhance leukocyte adhesion (Languino et al, 1993). Similarly and as shown by my results, fibronectin coated chambers facilitate T cell transmigration in the Boyden system tested. Therefore, even though simplistic, fibronectin coated Boyden chambers seem to be a good model to study T cell transmigration, specifically when mechanisms associated with the basal lamina component of the BBB are of interest.

As shown by my results, minocycline attenuated adhesion and T cell transmigration through fibronectin. The inhibitory action of minocycline was overcome by the addition of Mg++. The family of tetracyclines has the ability to bind divalent ions with variable capacity. For example, calcium forms 2:1 metal-ion to tetracycline complex, while the magnesium complex can be formed at a 1:1 ratio. Formation of the calcium complex involves addition of one metal ion to the C-10, C-11 site with subsequent addition of a second metal ion at the C-12, C-1 site. The magnesium chelate occurs at the C-11, C-12 beta-diketone site (Fig 4.2.1) (Newman and Frank, 1976). Therefore, it is possible that minocycline could inhibit T cell adhesion and transmigration by chelating Mg++. The inhibitory action on granulocyte chemotaxis has also been related to the chelation property of minocycline, but in this case, specifically, of Ca++ ions (Sugita et al, 1995).

Fig. 4.2.1 Tetracyclines can bind to ions.

Chemical name	Generic name	Trade name	Year of discovery	Structure
7-chlorotetracycline	chlortetracycline	Aureomycin	1948	
Tetracycline	tetracycline	Achromycin	1953	
7-dimethylamino-6-demethyl-6-deoxy-tetracycline	minocycline	Minocin	1972	

Calcium can interact with C-10, C-11 site with subsequent addition of a second metal ion at the C-12, C-1 site. Magnesium interacts at the C-11, C-12 beta-diketone site (modified from Chopra et al, 1992).

The role of Mg++ in T cell transmigration is presumably at the level of adhesion; specifically, Mg++ is a required cofactor for β1 integrin activation. Firstly, fibronectin facilitates T cell migration. Secondly, the interaction of T cells with fibronectin is mediated by members of the β1 integrin family. This is specifically achieved in CD4+ T cells by α4- or α5- β1 heterodimer combinations. Third, Mg++ is required for T cell adhesion mediated by these integrins. Mg++ binds to the β1 chain as well as to the α4 and α5 chains inducing an activated conformational state that allows the receptor to bind fibronectin (Takamatsu et al, 1998; Masumoto, 1993).

MMPs have been implicated in the transmigration of T cells. TIMP-1 inhibits specifically the transmigration of T cells through the 3 micron fibronectin coated Boyden filter, which

suggests an MMP dependency (Uhm et al, 1999). Therefore minocycline could be also inhibiting the enzymatic activity of MMPs.

In 1983, Golub et al suggested that the inhibitory effect of minocycline on collagenolytic activity was probably due to its chelation of Ca++, since the inhibitory effect was diminished by the addition of $CaCl_2$. As their name implies it, MMPs are metalloproteinases, that is, they depend on Zn++ and Ca++. Both mechanisms are essential for the activity of MMPs. Zn++ acts as an intrinsic metal cation, being at the center of the catalytic site and required for the activation of MMPs, and Ca++ acts as an extrinsic one, needed as a cofactor to stabilize the tertiary structure of MMPs (Seltzer et al, 1977).

Other actions such as direct binding of minocycline to β1 integrin or MMPs cannot be discarded and these could also be involved in the inhibition of T cell transmigration. Minocycline has been shown to suppress collagenase activity in vivo for more than 19 weeks after the drug was discontinued (Golub et al, 1985). Greenwald et al (1987) have speculated that binding of minocycline to MMPs directly results in a loss of enzymatic activity and that the slow clearance of minocycline from such a complex provides the prolonged effect in vivo. In similar way, β1 integrins could have such a binding site for minocycline.

In an attempt to test the possibility that minocycline would bind to the β1 chain and change its configuration into a non-active state, cross linking experiments were performed where minocycline would be a possible competitor. Minocycline did not have any effect on the β1 signaling outcome suggesting that (1) the drug does not compete for the epitope recognized by the anti-β1 antibody utilized or (2) minocycline does not change the β1 chain into an inactive non-signaling state.

4.2.2 MINOCYCLINE HAS LONG TERM MECHANIMS OF ACTION ON T CELLS

The decrease of MMP-9 levels by minocycline may be explained by an effect on MMP-9 gene transcription and/or mRNA turnover. It should be noted that minocycline specifically affected mRNA levels of MMP-9, but not MT1-MMP or MMP-1. Similar specificity has been observed in the inhibitory action of minocycline on T cell cytokine production. In

human peripheral T cells, minocycline selectively suppressed TNFα and IFNγ production, but not IL-6, and it did so at the mRNA level (Kloppenburg et al, 1996a).

It has been described that minocycline penetrates leukocytes obtained from human peripheral blood. Agranulocytes absorb tetracyclines more actively than granulocytes (Kivman, 1984; Saivin et al, 1988). Once inside the cell minocycline could interfere with signal transduction cascades with the result of MMP-9 inhibition. For example, Pruzanski et al (1992) have shown that minocycline can inhibit phospholipase A2 enzymatic activity. Minocycline ingestion significantly enhances the rise in Ca++ influx by splenocytes from collagen immunized rats, when stimulated by Con A. Rising intracellular Ca++ is a vital second messenger for T cell activation. Sewell et al (1996) have postulated that by amplifying intracellular Ca++ during collagen II immunization and altering the normal signal transduction relationships between intracellular Ca++ and costimulatory events, minocycline may provide a tolerogenic state. This could explain the effect of minocycline on inducing nonresponsiveness or anergy in T lymphocytes, as determined by proliferation and IL-2 production to CD3 antigenic stimulation (Kloppenburg et al, 1995b).

4.3 Minocycline and its effects in vivo

4.3.1 Efficacy of minocycline in MOG EAE

Minocycline could delay the onset of MOG EAE for 8 days, nonetheless, after the onset of disease the progression was similar to that shown by non-treated EAE animals. Why is it that minocycline did not prevent completely the disease? It is possible that the explanation is related to the dose used and the severity of EAE induced. Minocycline was administered at 50 mg/kg for the first 7 days and subsequently, the dose was reduced to 25 mg/kg. It is possible that the reduction of the dose to half diminished the beneficial effects and thus the clinical outcome. Also it is noted that the majority of drug trials in EAE have involved the use of animals in grade 2 EAE; in this thesis, grade 4 EAE was the result.

Minocycline showed a greater efficacy than IFNβ in preventing MOG EAE. IFNβ has been shown to prevent or reverse the disease in various EAE models at a concentration of 10000 to 5000 U/animal (Yu et al, 1996). We showed that at a concentration of 7500 U/animal, IFNβ could prevent the onset of disease for 3 days. However, IFNβ did not alter the course of MOG EAE, since animals became paralyzed (grade 4) around the same time as non-treated EAE controls. At a concentration of 50 mg/kg administered for the first 7 days, minocycline had a better effect in preventing the onset of disease. Therefore, minocycline could be perceived as a better therapeutic approach for MOG EAE. Nonetheless, it cannot be concluded that minocycline has greater efficacy than IFNβ, because a higher dose of IFNβ could have shown better results. The effect of higher doses of IFNβ on prevention of MOG EAE remains to be explored.

4.3.2 MINOCYCLINE AND ITS MECHANISMS OF ACTION IN VIVO

The exact mechanisms by which minocycline might be acting in vivo are not known. Various levels of action are possible: periphery versus CNS; inhibition of immune response to induction versus T cell traffic into the CNS; inhibition of T cell activation versus inhibition of integrins versus MMPs versus inhibition of pro-inflammatory cytokines.

4.3.2.1 Minocycline as inhibitor of T cell transmigration in vivo

As shown in the in vitro experiments minocycline inhibits T cell transmigration at a concentration of 250 μg/ml. However, physiologically, levels of minocycline in the sera of patients treated with 100-200 mg of minocycline orally would not be expected to exceed 10 μg/ml. The peak serum concentration of minocycline is approximately 6 μg/ml when 200 mg of it is intravenously given to healthy adult male subjects (reviewed in Masumori et al, 1994). Thus, it may be concluded that the concentrations of minocycline used in this thesis for the in vitro experiments are beyond the physiologically relevant range. Whether studies of the effects of minocycline concentrations higher than those achievable in vivo in routine clinical practice are useful is a matter for discussion. It must be realized that an in vivo system, such as in a patient, is far more complex than an in vitro system, which is a controlled model. This implies that evaluation of the effects of high concentrations of minocycline in vitro provides an opportunity to help define the in vivo modulatory action of

a drug. Furthermore, minocycline has a high degree of lipid solubility that results in concentrations in tissue that exceed concentrations in serum. Consequently, lipid-soluble tetracyclines have been reported to accumulate in leukocytes (Saivin et al, 1988), which makes difficult to evaluate the efficacy of minocycline on the basis of concentrations in culture medium and serum.

Minocycline was shown to inhibit T cell adhesion and transmigration through mechanisms related to Mg++. In other words, Mg++ could be perceived as an inhibitor of minocycline on T cell migration in vitro. Therefore, it is valid to ask the question if minocycline, by its chelating property, would arrest T cell infiltration in vivo. Tetracyclines can be distributed in different complex species, proton and metal bound fractions. In combination with the protein bound fraction of the tetracyclines, the metal bound fraction represents more than 99% of these drugs in plasma, the extent of their free fraction commonly being less than 1%. The fraction of antibiotic not bound to proteins almost exclusively occurs as calcium and magnesium complexes (Berthon et al, 1983).

If the mechanism by which minocycline inhibits T cell adhesion and transmigration is by chelating magnesium and, if minocycline in plasma is mainly in the metallic form, then it would be logical to conclude that minocycline would not inhibit T cell transmigration into the CNS in MOG EAE. However, as shown in the histological sections, minocycline prevented MOG EAE onset by inhibiting mononuclear infiltrates into the CNS.

D-penicillamine, a protease inhibitor that prevents acute and abrogated chronic relapsing EAE, is also a chelator. The authors implied that this property could be the mechanism by which D-penicillamine directly inhibited MMPs contributing to the therapeutic effects shown by the drug in vivo (Norga et al, 1995). Moreover, the beneficial effects shown by minocycline in patients with rheumatoid arthritis (RA) have been associated with its ability to inhibit MMPs. MMPs are thought to contribute to the pathogenesis of RA by facilitating the inflammatory infiltrates into the joint and degrading the collagen in the cartilage (reviewed in Harris, 1990). In conclusion, it is possible that minocycline, by inhibiting β1 integrin and MMP activity in vivo, could prevent T cell infiltration and consequently MOG EAE.

Similar to the in vitro situation shown in this thesis, another additional mechanism of minocycline could be by inhibiting the expression of MMP-9 in T lymphocytes and consequently arrest T cell transmigration across the BBB. Moreover, a reduction in MMP-9 levels would be beneficial to the preservation of the BBB. Interestingly, it has been reported that doxycycline (50 μM) completely inhibits the phorbol-12-myristate-13-acetate (PMA)-mediated induction of MMP-8 and MMP-9 (Hanemaaijer et al, 1998) which could be an additional mechanism by which minocycline could preserve BBB integrity.

4.3.2.2 Minocycline as immunomodulator

In animals, tetracyclines have been shown to suppress the DTH response, the rejection of transplants and levels of serum immunoglobulin. Furthermore, in vitro tetracyclines inhibited the proliferative response of human peripheral blood mononuclear cells to mitogens (Potts et al, 1983).

Minocycline has been described as an immunosuppressor. Both minocycline and tetracycline suppress murine thymocyte co-mitogenesis induced by IL-1 (Ingham, 1990). In humans, T cells can be suppressed functionally by minocycline, resulting in a dose-dependent inhibition of T cell proliferation, decreased IL-2 responsiveness and reduction in production of IL-2, IFNγ, and TNFα (Kloppenburg et al, 1995b). Because a direct immunization model was used in this thesis, it is possible that minocycline acted as an immunosuppressor on the **induction phase** of the immune response. In order to measure the extent of the inhibitory action of minocycline on the transendothelial migration of T cells into the CNS, and on the course of chronic EAE, the use of an adoptive T cell transfer model is necessary. This experiment is contemplated as future directions of this project.

A reduction on IFNγ or TNFα levels is significant in MS. IFNγ and TNFα are pro-inflammatory cytokine with multiple functions, including the activation of cells of the monocyte lineage and the up-regulation of adhesion molecules on endothelial cells that regulate the entry of T cells into the CNS (reviewed in Hohlfeld, 1997; Yong et al, 1998a). In humans, IFNγ worsens the symptoms of MS. IFNγ is a potent promoter of MHC II expression on monocytes, microglia, endothelial cells and astrocytes; moreover, it promotes the differentiation of naïve CD4+ cells into Th1 cells, propagating inflammation. Cells isolated from the CSF during active disease expressed a Th1 pattern of cytokine production.

TNFα levels in CSF can be detected only during active disease and not inactive MS. MS relapses were preceded by increased IFNγ and TNFβ secretion by Con A stimulated mononuclear cells and were accompanied by increased IFNγ secreting cells in blood. PLP specific T cell clones generated from MS patients during clinical relapse secreted primary IFNγ and TNFα (reviewed in Hohlfeld, 1997; Yong et al, 1998a).

Minocycline have shown beneficial effects in patients with RA. It suppresses the laboratory parameters of disease activity, especially the acute-phase reactants (reviewed in Kloppenburg et al, 1995a). For example, in a study designed to analyze the anti-inflammatory effect of minocycline in rheumatoid arthritis, serum samples of 65 RA patients who completed a 26-week randomized double-blind trial of minocycline (100 mg twice a day) versus placebo were studied. Serum levels of IL-6 and rheumatoid factor (RF) decreased in the minocycline-treated group only. Minocycline significantly decreased serum IgM-RF, IgA-RF, total IgM and total IgA levels. In addition the ratio of IgM-RF/total IgM decreased in the minocycline-treated group (Kloppenburg et al, 1996b). Therefore, minocycline could act as immunosuppressor and as such be beneficial for MS.

4.3.2.3 Minocycline in the CNS

Minocycline (MW: 493.9 g/mol) has the ability to traverse the BBB. Indeed, because of their greater lipid solubility, minocycline and doxycycline are better distributed than other tetracyclines, to areas of the body such as eye, brain, cerebrospinal fluid, and prostate glands. This has made these drugs the choice of treatment in cases where these areas are infected. For example cerebral malaria is usually treated by intravenous administration of doxycycline or minocycline (Aronson et al, 1980).

Since it can enter the CNS, minocycline could act as an inhibitor of MMP-9 production on those T cells already in the CNS parenchyma as well as an inhibitor of MMP activity within the CNS. By inhibiting MMP activity, minocycline could contribute to the preservation of the myelin sheath, since MMPs are proteases able to degrade myelin into encephalitogenic components (Chandler et al, 1997; Opdenakker et al, 1994). The observation that minocycline affects MMP-9 also has consequences on the production of the oligodendrocyte-toxic cytokine, TNFα. The effect could be the prevention of conversion of pro-TNFα into its active form, which could be beneficial for EAE and MS (reviewed in

Yong, 1999). Minocycline might thus antagonize effects of cytokines which transcriptionally activate the MMP-9 gene (Johnatty et al, 1997). Because TNFα seems to be a disease promoting cytokine in MS and EAE, inhibition of this cytokine would be beneficial in MS (Selmaj, 1995; Raine, 1995).

Yrjanheikki et al (1998, 1999) have shown that doxycycline and minocycline inhibited inflammation and were neuroprotective against ischemic stroke, even when administered after the insult. Minocycline showed better efficacy than doxycycline. It increased the survival of CA1 pyramidal neurons; prevented completely the ischemia-induced activation of microglia; reduced mRNA induction of interleukin-1beta-converting enzyme in microglia; and, attenuated the expression of inducible nitric oxide synthase mRNA.

The inhibition of microglia activation could be of benefit in EAE and MS. Microglia have been considered as potential myelin antigen presenting cells being implicated in the initial phase of the pathogenic immune response in MS and EAE. Moreover, they release pro-inflammatory cytokines such as TNFα, TNFβ and IFNγ (Raine, 1994). Microglia are also responsible with monocyte/macrophages for the ingestion of myelin, probably contributing to demyelination. If minocycline acts by inhibiting microglial activation, the drug could have therapeutic efficacy not only for MS and ischemic stroke but for many CNS pathologies such as AIDS dementia, traumatic brain injury, Alzheimer's disease, experimental globoid cell dystrophy, and brain abscesses.

4.5 Minocycline as therapy for MS

The immunomodulatory and the anti-MMPs properties as well as the in vivo beneficial results shown in this thesis suggest minocycline as a potential therapy for MS. Because a progressive EAE was used for this study and since minocycline could delay the clinical manifestations, it is valid to rationalize that minocycline could ameliorate the course of the progressive form of MS.

In view of the fact that the relapses in MS are unpredictable, a continuous treatment with minocycline would have to be used to prevent relapses. The long term efficacy and safety of tetracyclines as disease modifying anti-inflammatory drugs is a concern in MS. For example, in a small trial of minocycline for RA, seven of ten patients treated with minocycline (maximal daily dose 400 mg) reported vestibular side effects and gastro-intestinal intolerance (reviewed in Kloppenburg et al, 1995a). Moreover, among acne treated patients with high doses (100-200 mg daily) and long exposure (6 months to 2 years) minocycline was shown to induce serum sickness, autoimmune induced hepatitis and systemic lupus erythematosus-like syndrome, and vasculitis, specially in women. In relation to the number of prescriptions, the number of serious adverse events of minocycline described is small and there is clinical and biochemical resolution after withdrawal of the drug (Gough et al, 1996; Bhat et al,1998; Akin et al, 1998; Elkayam et al, 1999). The fact that minocycline could induce autoimmune manifestations among women reflects the potential immunomodulatory effects of minocycline in humans.

In the MOG EAE trials shown in this thesis, two minocycline treated animals (of 20 animals in total) died before onset of EAE symptoms, suggesting a drug related death. The toxicity of minocycline could be related to the dose used and to the form of administration. In pigs and rabbits, the parental administration of tetracycline and oxytetracycline were rapidly adsorbed by lymphocytes and consumed by the epithelium of the kidney and cells of the liver parenchyma (Karput, 1976). It is possible that metabolites of tetracycline could be toxic for the liver and the kidney. Nonetheless, among the tetracyclines, minocycline and doxycycline can be given in full dosage with minimal risk to patients with renal impairment (Barza et al, 1977). Another means to utilize minocycline could be to administer the drug for short periods in MS subjects in combination with a drug already approved for MS use, such as IFNβ or copaxone (O'Dell et al, 1999).

The antibiotic capacity of minocycline could also be a disadvantage in its use for non-infectious diseases. Nonetheless, minocycline might be a lead compound for the development of more potent non-toxic MMP inhibitors. Indeed, the MMP inhibitory action of tetracyclines has become of such interest that tetracycline derivatives that have lost their antimicrobial properties altogether, but retain their anti-MMP activities, are being developed (Golub et al, 1992; Rifkin et al, 1994).

4.6 Conclusions

This thesis has been able to demonstrate that minocycline is able to decrease adhesion and migration through fibronectin of T cells in vitro. In vivo, minocycline delayed the onset of MOG EAE in C57/BL6 mice for 8 days compared to non-treated animals. Moreover, the average time for minocycline treated animals to become paralyzed was 8 days longer than non-treated or IFNβ treated EAE animals. Overall and at the concentrations used, minocycline showed a greater efficacy in preventing EAE than IFNβ. Mechanisms of action of minocycline involve MMP enzyme inhibitory activity as well as inhibition of MMP-9 production at the level of both mRNA and protein in T cells.

Because of these effects and because of additional immunomodulatory actions on the cell and humoral components of the immune system, we expect that minocycline will improve the prognosis of patients with MS by itself or in combination with IFNβ or copaxone.

4.7 Future directions

Future research can be expanded in various fields. Firstly, the mechanism of action of minocycline should be studied further. This can be achieved at two levels, cellular/molecular and physiological levels. At cellular/molecular levels it would be useful to know which cellular component minocycline affects. It is known that minocycline can enter leukocytes. Studies with doxycycline show that it distributes mainly to the mitochondria and not the nucleus. Similar studies could be done with minocycline in T cells in an attempt to investigate the effects on the internal cellular machinery. It is possible that minocycline could inhibit MMP-9 mRNA levels by interacting with signal transduction molecules or directly with the transcription or degradation of mRNA. There is specificity of action of minocycline on MMP-9 that should be further investigated. Since minocycline could have many beneficial effects as therapy for MS and other diseases, its activity on T cells as well as in monocytes should be pursued further. At the physiological level, it would be informative to analyze the

cytokines as well as MMPs expressed in the CNS and plasma of EAE animals in treatment with minocycline relative to non-treated controls.

MS patients after their diagnosis have already suffered from brain inflammation and have initiated a series of permanent autoimmune reactions which gradually or episodically damage their nervous system. Therefore chronic animal model systems and effective treatment regimens starting only after a first episode of brain inflammation are crucial to develop effective MS treatments. This requires the use of a relapsing model of EAE. There are various models used, for example PLP EAE in H-2^s mice or MOG EAE in H-2^{b7} mice or Lewis rats could be potential tools for future trials. Our data show encouraging results and there is a hope in obtaining similar beneficial effects in the RR-EAE model for the cure of the relapses.

Moreover, trials with minocycline in long term studies of animals would be useful to determine possible side effects. Also animal trials comparing the efficacy of minocycline against, or in combination with Copaxone or IFNβ, are worth investigating.

Finally, since minocycline is currently in use for the treatment of diseases other than MS, it may be worthwhile initiating clinical trials of minocycline in MS. In contrast to the drugs now available for MS, minocycline is an established, inexpensive and orally effective drug in humans.

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