Urinary neopterin: an inflammatory marker of disease activity in multiple sclerosis

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To my wife and best friend

Cheryl
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Abstract

Although inflammation probably plays a central role in the pathogenesis of multiple sclerosis (MS), suitable immunological markers of disease activity have remained elusive. Neopterin, an established pro-inflammatory marker of interferon-γ (IFNγ) induced macrophage activity, was investigated. In addition, the in vitro control of monocyte neopterin production by cytokines was studied. Urinary neopterin excretion was chosen over cerebrospinal fluid and serum neopterin, as it maximises neopterin’s sensitivity and allows for frequent monitoring. High performance liquid chromatography (HPLC) methods were set-up and validated to measure neopterin in urine and cell culture supernatants. Cell culture experiments using THP-1 cells (monocytic leukaemic cell line) and monocytes (purified by centrifugal elutriation) confirmed the stimulatory effects of pro-inflammatory cytokines (IFNγ, IL1, TNFα, GMCSF) and provided some new insights with regard to the “anti-inflammatory” cytokines (IL4, IL13, IL10, TGFβ) on neopterin production. New observations with regard to the mechanisms of action of IFNβ were also observed. Baseline studies on normal subjects confirmed that urinary neopterin excretion is subject to a diurnal rhythm. Cross-sectional and longitudinal studies in patients with MS demonstrated that urinary neopterin excretion is increased in all clinical subtypes of the disease. Increased urinary neopterin excretion occurs in association with clinical relapse and correlates with the appearance of new Gd-enhancing lesions on MRI. This thesis provides new insights into the immunology of monocyte/macrophage neopterin production and the immunopathogenesis of MS. It demonstrates the potential value of neopterin as an inflammatory marker of disease activity in MS. This work has resulted in two prospective long-term studies, involving patients with early relapsing and primary progressive MS, being set-up to investigate urinary neopterin excretion as a surrogate marker of disease progression.
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Chapter 1 - Introduction

1.1 Background

Until recently no therapy, without unacceptable side effects, had been shown to significantly affect the natural history of multiple sclerosis (MS). However the advent of interferon beta (The IFNB Multiple Sclerosis Study group, 1993; Jacobs et al., 1996; Ebers et al., 1997) and copolymer-1 or glitarmir acetate therapy (Johnson et al., 1998) in patients with early relapsing remitting MS and recently interferon-beta therapy in patients with secondary progressive MS (Kappos et al., 1998) has given patients with MS new hope with regard to the treatment of this devastating disease. Currently most new products in development, or clinical trials, for the treatment of MS act as anti-inflammatory agents (National Multiple Sclerosis Society, 1996). This is based on the rationale that MS is primarily an inflammatory disease of the CNS, and by controlling or stopping the inflammation the disease progression will be halted. The cost of these agents are often very high and with the introduction of world-wide reforms to reduce health-care expenditure, ever increasing pressure is being placed on clinicians to make rational decisions regarding these therapies. This task is made much harder by the clinical heterogeneity and lack of practical surrogate markers of inflammation and disease activity in patients with MS (Whitaker et al., 1995a). It is against this backdrop that the research presented in this thesis was performed. The primary aim of this work was to investigate neopterin, a product of activated macrophages as a potential surrogate marker of disease activity in MS.
1.2 Aims of the introduction

The aims of the introduction are to review the following:

the current theories on the pathogenesis of MS;

the clinical aspects of MS relevant to the monitoring of disease activity;

MRI and immunological markers of disease activity in MS;

the role of the macrophage and microglia in MS;

macrophage pterin and nitric oxide (•N=O) metabolism;

the potential role of pterins and •N=O in the pathogenesis of MS;

and to propose a testable hypothesis.

1.3 Introduction

MS is a demyelinating disease of the central nervous system that is characterised pathologically by inflammation, demyelination and variable degrees of axonal loss and gliosis (Allen, 1991). Although unproven MS is considered an organ-specific autoimmune disorder mediated by autoreactive T-cells, autoantibodies, or both, to various antigens (Calder et al., 1989; Hafler and Weiner, 1989; Martin et al., 1992; ffrench-Constant, 1994; Martin and McFarland, 1995; Giovannoni and Hartung, 1996). It occurs more commonly in females (Duquette and Girard, 1993), and Caucasians (Wynn et al., 1989), on a genetic background associated with specific MHC haplotypes (DR15/DQ6), and possibly other genetic loci (Oksenberg et al., 1993; Compston et al., 1995; Sawcer et al., 1996; Ebers et al. 1996; Haines et al. 1996). Epidemiological studies support an environmental agent(s) acting in early childhood as possible aetiological factor(s) (Wynn et al., 1989; Martyn, 1991; Poser, 1992; Kurtzke, 1993). This is then followed by a variable latency period (Wolfson, 1995) before the disease manifests clinically, usually in the third and fourth decades (McAlpine and Compston,
1952; Confavreux et al., 1980, Weinshenker et al., 1989). The pathogenesis of MS, based on current hypotheses, is summarised in Figures 1.1-1.5.

1.4 The pathogenesis of multiple sclerosis

Figure 1.1. The pathogenesis of multiple sclerosis: a hereditary predisposition combined with an environmental trigger establishes or maintains pathological auto-reactive T-cells. After a long latency period (10-20 years), these autoreactive T-cells are activated, possibly by a systemic trigger such as a viral infection or exposure to a superantigen. Once activated these T-cells selectively cross the blood brain barrier and on re-exposure to their auto-antigen initiate a cell mediated (Th1 T-cell) inflammatory reaction. The resultant inflammatory cascade causes demyelination and axonal loss, which in turn releases sequestered central nervous system antigens, which are hypothesised to initiate further episodes of auto-immune induced inflammation, by a process of inter- or intra-molecular determinant spreading. The inflammatory cascade and demyelination may also be triggered by a local neurotropic viral infection.

MBP = myelin basic protein, PLP = proteolipid protein, MOG = myelin oligodendrocte associated glycoprotein
Figure 1.2 Cellular mechanisms of acute inflammation in MS: activated CD4⁺ Th-cells cross the blood-brain barrier through a process involving the interaction of their cell surface adhesion molecules with those expressed on the central nervous system endothelium. Once within the perivascular space these cells are activated by professional antigen presenting cells (probably macrophages or microglia) to proliferate and produce cytokines. The profile of T-cell cytokines produced depends on the co-stimulatory signals received during activation. Th1-like cytokines (IL2, IFNγ and TNFα/β) initiate a classical cell mediated inflammatory cascade which activates macrophages, microglia, astrocytes and endothelial cells. This results in further cytokine production and recruitment of inflammatory cells by the up-regulation of endothelial cell adhesion-molecule expression and by the production of chemoattractants (e.g., chemokines MIP-1α/β). The inflammatory cascade also produces a host of noxious substances (oxygen and nitrogen free radicals, proteases, eicosanoids and complement), which in combination with auto-antibodies and cytokines (particularly TNFα) cause oligodendrocyte, axonal and neuronal damage. Immunomodulatory cytokines (IL4, IL10 and TGFβ) produced by suppressor and Th2 T-cells are important in down-regulating and controlling the resultant inflammation.
Figure 1.3. Antigen presentation and T-cell activation in MS: antigen recognition occurs via the trimolecular complex and requires additional co-stimulatory signals. T-cell activation results in proliferation and cytokine production. The trimolecular complex consists of the HLA, TCR and CD3 molecules. The important co-stimulatory signals required include interactions between HLA-MHC I and II molecules and their respective CD8 and CD4 molecules, CD40 and its ligand (CD40-ligand), and the CTLA/CD28-B7-1/B7-2 pair interaction. These latter co-stimulatory signals and the presence of specific cytokine, govern the type of T-helper response. Other accessory molecule interactions, including the LFA3/CD2 and LFA1/ICAM-1 pairs, facilitate positioning of the T-cell and APC. Cytokine signaling also helps governs the type of Th response. IL12 for example polarises the T-cell response towards a Th1 phenotype.

Figure 1.4. **Inflammatory cell trafficking and endothelial activation in MS:** proinflammatory cytokines IL1, TNFα/β, and IFNγ induce increased endothelial expression of E-selectin, ICAM-1, and VCAM-1. By binding to sialylated ligands, the selectins mediate attachment of leukocytes to the blood vessel wall by weak labile interactions, which allows the leukocytes to roll in the direction of the blood flow, i.e. rolling and capture. Rolling allows activation of leukocyte integrin molecules, e.g. LFA-1 and VLA-4, which causes a conformational change in the integrin (activation). This in turn allows the integrins to bind to their respective ligands (ICAM-1 and VCAM-1), which attaches the cells firmly. The cells then flatten and migrate across the endothelium (flattening). Extravasation occurs either through the endothelium or between cell junctions and requires the ICAM-1/LFA-1 interaction, as well as other adhesion molecules and the secretion of metalloproteases to negotiate the extracellular matrix.

Figure 1.5. Demyelination and phagocytosis: astrocytes and macrophages produce mutually stimulating cytokines (IL1 and TNFα). These and other proinflammatory T-cell cytokines up-regulate the production of numerous noxious substances which induce oligodendrocyte toxicity. These substances include TNFα/β, free oxygen and nitrogen radicals, complement, proteases and eicosanoids. Auto-antibodies, particularly to surface myelin antigens like myelin oligodendrocyte glycoprotein (MOG), appear crucial in the development of demyelination. In addition to myelin damage, programmed cell death or apoptosis of the oligodendrocyte may occur as a result of oxidative stress and death signaling induced by TNFα acting on its P55 receptor.

Antibodies and complement assist Fc receptor-mediated phagocytosis, by opsonisation.

Phagocytosis also occurs via the macrophage scavenger and LDL receptors.

IL - interleukin, LDL - low density lipoprotein, N0 - nitric oxide, NO2 - nitrogen dioxide ion, O2 - superoxide ion, OH - hydroxyl ion, ONOO - peroxynitrite, TNF - tumour necrosis factor.
1.5 The clinical aspects of MS relevant to the monitoring of disease activity

1.5.1 Diagnosis

Currently the diagnosis of MS is based on a clinical history and examination, supported by electrophysiological, CSF and MRI investigations (Poser et al., 1983; Paty et al., 1991). In addition, other diseases such as sarcoidosis, which could possibly masquerade as MS, should be excluded. The diagnostic criteria for MS set out by Poser et al. (1983) (Appendix I), are designed for research purposes with high specificity, and exclude groups of patients with asymptomatic disease or with clinically isolated syndromes compatible with demyelination. Recognition of these patients, who could possibly have “biological MS”, and predicting who will subsequently develop clinical MS, has become more important with the advent of disease-modifying therapy that may be beneficial when administered early in the course of the disease. An important question relates to whether these patients have biological markers of disease activity.

1.5.2 Clinical course and classification

MS is a clinically heterogeneous disorder with an unpredictable time-course and natural history (McAlpine and Compston, 1952; Confavreux et al., 1980, Weinshenker et al., 1989). It typically starts off with a relapsing-remitting course, characterised by relapses from which patients usually recover with minimal disability and periods of remission. After approximately 5 to 7 years the majority of patients with a relapsing course go on to develop progressive disease. This is characterised by a gradual, usually persistent, and irreversible deterioration in neurological function over an extended period of time. Patients also acquire disability by the failure to recovery fully from clinical relapses. The clinical decision whether or not to classify a patient as having progressive disease is often very difficult, especially in patients who are having frequent relapses with
incomplete recovery, or in patients who appear to have developed a progressive course but improve after the empirical administration of high-dose intravenous corticosteroids. Patients who develop progressive disease after an initial relapsing remitting course are classified as having secondary-progressive MS (Lublin and Reingold, 1996), and should possibly be further classified into 2 groups on the basis of whether or not they are having superimposed relapses (Kidd et al., 1996). Approximately 5-15% of patients have a progressive course from the outset without relapses and are classified as having primary-progressive disease (Table 1a, Lublin and Reingold, 1996). Finally, a rare group of patients have a progressive course from the outset with superimposed relapses and are classified as progressive relapsing (Lublin and Reingold, 1996).

1.5.3 Disease activity

Table 1a

<table>
<thead>
<tr>
<th>Clinical classification of patients with MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapsing-remitting (RR)</td>
</tr>
<tr>
<td>Secondary-progressive (SP)</td>
</tr>
<tr>
<td>2.1 with superimposed relapses</td>
</tr>
<tr>
<td>2.2 without superimposed relapses</td>
</tr>
<tr>
<td>3. Primary-progressive (PP)</td>
</tr>
<tr>
<td>4. Progressive-relapsing (PR)</td>
</tr>
</tbody>
</table>

Relapse rate and the acquisition of functional disability are currently the primary measures used to assess disease activity in MS. The clinical relapse rate was until recently considered a relatively weak predictor of the future development of disability (Weinshenker et al., 1986a). However recent longitudinal data on a large cohort of patients with MS followed for periods of up to 25 years in London Ontario,
demonstrates that early relapse rate is predictive of future disability (Weinshenker and Ebers, personal communication). Reasons for the failure of the earlier studies to detect a correlation between relapse rate and disability include poorly reactive disability scales that tend to be weighted in favour of the motor system, and the short periods of follow-up.

1.5.4 Disease Dynamics

Baseline lesion loads and longitudinal monitoring of MRI activity have provided radiological support for the clinical classification of MS (Table 1b). Patients with a relapsing course have a greater lesion load than patients with primary progressive disease (Thompson et al., 1991; Thompson et al., 1992; Kidd et al., 1996). Within the relapsing group the lesion load is greatest in the patients who have entered the progressive phase of the illness (secondary progressive). Patients with a benign course also have significantly fewer lesions (Thompson et al., 1992; Kidd et al., 1994). MRI activity in longitudinal studies, defined as either new lesion formation or enlargement of old lesions with or without gadolinium(Gd)-enhancement (Miller et al., 1991; Miller et al., 1996), correlates with a relapsing course and is highest in patients with relapsing secondary progressive disease, intermediate in patients with early relapsing remitting disease and low or absent in patients with a non-relapsing progressive course. It has become apparent from these natural history studies that MS can be separated into two groups, those with a relapsing course and/or evidence of MRI activity, and patients with a non-relapsing progressive course with little or no evidence of MRI activity. The observation that disease progression occurs in both these groups begs the question of what is responsible for progression and whether or not it is related to inflammatory activity.
Several criticisms have been leveled at these natural history studies. Firstly, claims that these studies are technically flawed as they only measured disease activity in the brain and not the spinal cord have been dispelled by two studies showing that the disease dynamics of MS in the spinal cord, although less frequent, are similar to that in the brain (Thorpe et al., 1996; Kidd et al., 1996). An important difference noted by Thorpe et al (1996) in their study was that 6 out of 19 enhancing or new non-enhancing lesions of spinal cord (32%) were symptomatic compared with 1 out of 166 (<1%) such lesions in the brain. This difference can be explained by anatomical factors and is probably unrelated to the dynamics of the disease. A second criticism is that the threshold and sensitivity of standard MRI imaging protocols is too low to detect focal activity in non-relapsing progressive patients. In an attempt to increase the threshold and sensitivity of Gd-enhanced MRI, Filippi et al. (1995a) using triple-dose Gd with delayed imaging in 10 patients with primary progressive MS, increased the sensitivity from 4 enhancing lesions in 2 patients to 14 enhancing lesions in 6 patients. Other investigators however, have not been able to reproduce this data (Silver et al., 1997) suggesting clinical heterogeneity in patient selection. Another strategy to improve the sensitivity of MRI has been the use of Gd-enhanced magnetisation transfer imaging which improves the detection of active lesions (Mheta et al., 1995; Silver et al.,1997). A third possibility is that the inflammatory process is diffuse rather than focal in non-relapsing progressive patients and is therefore undetectable by standard MRI imaging techniques. This has been suggested by studies documenting abnormalities in T1 and T2 measurements (Lacomis et al., 1986; Larsson et al., 1988; Miller et al., 1989), as well as abnormal MR spectroscopy findings (Husted et al., 1994; Hiehle et al., 1994; Fillipi et al., 1995c; Loevner et al., 1995) in the normal-appearing white matter (NAWM) of patients with MS. These studies demonstrate that the NAWM is abnormal in patients with MS, a fact
appreciated pathologically by the finding of microscopic foci of inflammation and
myelin damage in white matter that appears macroscopically normal (Allen, 1991;
Newcombe et al., 1991).

Although definitive pathological studies on the different subtypes of MS are lacking, a
post-mortem study in patients with end-stage disease showed that the inflammatory
activity in patients with primary progressive disease was significantly less than that
found in patients with secondary progressive disease (Revesz et al. 1994). Despite the
lack of immunological data to substantiate this clinicopathological dichotomy, the
evidence suggests that disease activity in non-relapsing progressive patients is different
from that in patients with a relapsing course.

Table 1b. Disease dynamics of MS on MRI.

<table>
<thead>
<tr>
<th>Clinical Classification</th>
<th>Baseline Lesion Load</th>
<th>MRI Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapsing Remitting</td>
<td>intermediate</td>
<td>moderate to high</td>
</tr>
<tr>
<td>Secondary Progressive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with superimposed relapses</td>
<td>high</td>
<td>high to very high</td>
</tr>
<tr>
<td>without superimposed relapses</td>
<td>high</td>
<td>low or absent</td>
</tr>
<tr>
<td>Primary Progressive</td>
<td>low</td>
<td>low or absent</td>
</tr>
</tbody>
</table>

Disease Severity (relapsing onset)

<table>
<thead>
<tr>
<th></th>
<th>Baseline Lesion Load</th>
<th>MRI Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>low to moderate</td>
<td>low</td>
</tr>
<tr>
<td>Non-benign</td>
<td>moderate to high</td>
<td>moderate to high</td>
</tr>
</tbody>
</table>

1.5.5 Disease Triggers

In MS, Gd-enhanced MRI studies demonstrate that disease activity occurs in bursts
(McFarland et al., 1992; Thorpe et al., 1996), which suggests that a systemic trigger (at
least early in the relapsing phase of the disease) may be responsible for disease
activation and clinical relapses. IFNγ is one of the well established triggers (Panitch et
al., 1987), and likely to be one of the mechanisms by which viral infections induce
disease activity, as up to a third of all clinical relapses occur in relation to viral
infections (Sibley et al., 1985; Panitch et al., 1994). Whether other triggers of IFNγ
production, such as vaccinations or skin tests for cell mediated immunity, are able to
induce disease activity has not been fully established. The converse occurs in
pregnancy, a state of relative immunosuppression, in which disease activity is
suppressed and the long-term course of the disease appears to be favourably affected
(Runmarker et al., 1995). However post-partum, the risk of relapse appears to be
increased (Ghezzi and Caputo, 1981, Poser and Poser, 1983). Epidemiological studies
have implicated an infectious agent as a possible cause of MS. It is plausible that a viral
infection of cells within the CNS may initiate the events that lead to focal
demyelination. Recently human herpes virus 6 (HHV-6) (Challoner et al., 1995) and an
MS associated retrovirus (Perron et al. 1997; Garson et al. 1998) has been added to the
long list of potential viral aetiologies or triggers (Cook et al., 1995).

1.5.6 Disease Severity

The clinical severity of MS varies widely, with some patients having a rapidly
progressive course leading to significant disability or death in a relatively short time,
and others who remain fully functional 15 years after disease onset. These two extremes
have been arbitrarily defined as malignant and benign disease respectively (Lublin and
Reingold, 1996). The principal factor underlying these definitions is the rate at which
patients become disabled. Although numerous scales have been developed or are in
development to measure disability, Kurtzke’s expanded disability status scale based on
functional scales (EDSS, Appendix II) (Kurtzke, 1980) is the most widely used and is
currently the scale of choice for clinical trials (Noseworthy, 1994). Major weaknesses in
the EDSS relate to its bias in favour of the motor system, its poor assessment of
cognitive function, the high intra- and inter-rater variability (Noseworthy, 1994) and its
non-linearity (Weinshenker, 1994). In a population of MS patients, EDSS scores are not
normally distributed but are bimodal, with patients clustered with scores less than 3.5
and greater than 5.5 (Weinshenker, 1994). This occurs as a result of the more rapid
progression through the scales 4 and 5; patients spend an average of 3 to 4 years at
EDSS 6 and EDSS 7 compared to 1 year each at EDSS 4 and EDSS 5 (Weinshenker,
1991). In an attempt to make the scale linear, it has been proposed to treat a change in
EDSS of 0.5 when the baseline EDSS is greater than 5.5 equivalent to a change of 1
when the baseline EDSS is less than 5.5 (Ellison, et al 1994). The EDSS is also subject
to diurnal fluctuations due to the effects of temperature and fatigue, which result in
intermittent neurological dysfunction. In addition, clinical relapses can dramatically
though transiently affect the EDSS. These factors have to be taken into account when
using the EDSS as the numerator in the assessment of the rate of disease progression.
Therefore, before a patient can be confidently classified as having progressed, the
change in EDSS should be maintained for a period of at least 3 months (Ellison, et al
1994). The previously used Poser index (Poser et al., 1982) illustrates these points. It
simply divides the change in EDSS by the time period it took for the change to occur.
As the EDSS is an ordinal scale, the bimodal distribution would falsely elevate the
progression index when changes in the EDSS occurred between 3.0 and 6.0 and lower
the index when changes in EDSS occurred above 6.0. In addition the index does not
make adequate provision for the reversible effects of a clinical relapse.
1.6 MRI and immunological markers of disease activity in MS

1.6.1 MRI markers of disease activity

Longitudinal MRI studies have demonstrated a strong association between MRI activity and acute relapse (Grossman et al., 1986; Miller et al., 1988; Thorpe et al., 1996), as well as a very dynamic disease process in which at least 5 to 10 "asymptomatic" MRI lesions are detected for each clinical relapse (Smith et al., 1993; Thompson et al., 1992; Wiebe et al., 1992). This is an underestimate, as studies with more frequent scanning intervals (weekly or biweekly) have demonstrated that approximately 30% of lesions enhance for less than 4 weeks and would therefore be missed in monthly studies (Lai et al., 1996; Capra et al., 1992). In addition MR imaging techniques using delayed scanning, triple dose Gd (0.3 ml/kg), and magnetisation transfer detect 126% more lesions than conventional single dose Gd (0.1mL/kg) studies (Silver et al., 1997).

Therefore a more realistic estimate would be at least 20-30 asymptomatic lesions for every clinical relapse, and if MR had the ability to detect microscopic lesions (Newcombe et al., 1991) this ratio would be even higher. Reasons for this probably relate to the relatively infrequent involvement of clinically eloquent sites, such as the optic nerves, brain stem, cerebellum and spinal cord compared with the more commonly involved "asymptomatic" paraventricular and deep white matter areas of the cerebral hemispheres. Acute relapses (Nesbit et al., 1991) and new Gd-enhancing MRI lesions (Katz et al., 1993; Rodriguez et al., 1993; Brück et al., 1997), correlate pathologically with acute perivascular inflammation. For the case of Gd-enhanced MRI, evidence comes from the post-mortem of a patient with MS who died from an unrelated cause 10 days after having had a Gd-enhanced MRI study (Katz et al., 1993), and two series of cases in which patients underwent diagnostic brain biopsies of large Gd-enhancing hemispheric lesions that proved to be demyelinating in nature (Rodriguez et al., 1993;
Brück et al., 1997). Some of these latter cases, however, are not typical of MS and have features which are suggestive of acute disseminated encephalomyelitis. Additional support for the MRI/pathological correlation comes from several animal studies on chronic relapsing (Hawkins et al., 1990) and acute experimental allergic encephalomyelitis (Seeldrayers et al., 1996; Morrisey et al., 1996). Because of this and its perceived sensitivity, particularly Gd-enhanced T1 weighted MRI studies, MRI has become the most widely accepted surrogate marker of inflammation in MS (Whitaker et al., 1995; Miller et al., 1991; Miller et al., 1996). However standard MRI parameters that are used to monitor inflammatory activity (Smith et al., 1993; Fillipi et al., 1995c; Paty and Li., 1993) are weak predictors of the future development of disability. Proposed reasons for this are many. Firstly, the disability scales used in these studies are weighted in favour of the motor system, which is predominantly related to posterior fossa and spinal cord disease, whereas the MRI monitoring has been largely restricted to the supratentorial region. This is supported by a stronger correlation between neuropsychiatric abnormalities, which are related to higher cognitive or cortical function, and the brain lesion load in patients with MS (Rao et al., 1989). Secondly, the abnormal increase in tissue water content or the breakdown of the blood-brain-barrier detected by standard MRI imaging are too non-specific to address the pathological heterogeneity of MS lesions (Barnes et al., 1991).

Pathologically, axonal loss and probably demyelination appear to be the major determinants of disability in MS. With the possible exception of hypointense lesions on T1 weighted sequences (Van Walderveen et al., 1995; Truyen et al., 1996) the radiological changes detected using conventional MRI techniques correlate poorly with disability (Filippi et al., 1995c). Several new putative MR markers of axonal loss and/or
demyelination have been shown to correlate more closely with clinical disability than Gd-enhanced MRI. These include (1) cerebral atrophy (Losseff et al., 1996a), (2) spinal cord atrophy (Kidd et al., 1996; Losseff et al., 1996b), (3) low magnetisation transfer (MT) ratios on MT imaging (Gass et al., 1994) and (4) reduced levels of N-acetylaspartate (NAA) on MR proton spectroscopy (Davie et al., 1994; Arnold et al., 1994). The mechanisms responsible for axonal loss and disability may be unrelated to, or only weakly associated with, inflammatory activity and hence the modest correlations.

1.6.2 Immunological markers of disease activity

Although MS is well characterised as an inflammatory disease, there are currently no well-established immunological markers of disease activity (Whitaker et al., 1995). Candidates investigated include cytokines, changes in cell surface markers, acute phase proteins, soluble adhesion molecules, chemokines, free immunoglobulin light chains, myelin breakdown products, neuronal and glial proteins, and markers of macrophage activity (Table 1c).

1.6.2.1 Cytokines

The proinflammatory Th1-like cytokines, IFNγ, IL2, and TNFα and β all appear pivotal in the inflammatory reactions of MS (Martin and McFarland, 1995). Prior to clinical relapses mitogen-stimulated peripheral blood cells of MS patients produce increased quantities of IFN-γ and TNFα (Beck et al. 1988; Lu et al., 1993; Rieckmann et al., 1995b; Navikas et al., 1996a). Proinflammatory cytokines, as well as their mRNA, have been demonstrated in inflamed MS plaques (Woodroffe et al., 1993; Raine et al., 1994), and increased levels of IL2 (Adachi et al., 1989; Gallo et al., 1989; Peter et al., 1991; Sharief et al., 1993), IL1 (Hauser et al., 1990; Peter et al., 1991; Tsukada et al., 1991a), IL6 (Hauser et al., 1990; Maimone et al., 1991a) and TNFα (Franciotta et al., 1989;
Hauser et al., 1990; Maimone et al., 1991a; Peter et al., 1991; Sharief et al., 1991;
Tsukada et al., 1991a; Perrella et al., 1993) have been found by some investigators in
the CSF and/or serum of patients with MS. In addition elevated levels of soluble IL2
receptors (Adachi et al., 1989; Gallo et al., 1989; Peter et al., 1991; Sharief et al., 1993)
and TNFα receptors (Tsukada et al., 1993; Rieckmann et al., 1994; Matsuda et al.,
1994; Hartung et al., 1995a) have been demonstrated in patients with MS.

IL12 is a pleiotropic cytokine that is assuming a more of central role in the proposed
immunopathogenesis of MS (Navikas and Link, 1996b). IL12 is produced by activated
monocytes/macrophages and promotes the development of a Th1 phenotype in activated
CD4+ T-cells. IL12 expression occurs in MS lesions (De Simone et al., 1995;
Windhagen et al., 1996). Increased levels are detected in the CSF of MS patients
(Drulovic et al., 1997) and raised serum IL12 levels have been reported in patients with
chronic progressive MS (Nicoletti et al., 1996). Activated T-cells from MS patients
express increased levels of CD40 ligand, which is responsible for inducing increased
IL12 production in mononuclear cells of patients with progressive MS (Balashov et al.,
1997). As the increased IL12 production appears to be limited to patients with
progressive disease (Balashov et al., 1997) the authors have suggested that a polarized
Th1-like T-cell response via IL12 production, is responsible for progressive disease.

Data is emerging with regard to the putative role of the anti-inflammatory cytokines,
IL4, IL10, IL13 and TGFβ in MS (Navikas and Link, 1996b). Increased levels of IL10
and TGFβ mRNA expression in mononuclear cells from the peripheral blood and CSF
are associated with the recovery phase of a relapse, periods of remission and a less
aggressive disease course in MS (Link et al., 1994; Rieckmann et al., 1995b;
Söderström et al., 1995). There is also evidence that these inhibitory or anti-inflammatory cytokines favourably modify the course of EAE and, by inference, MS (Kennedy et al., 1992; Khoury et al., 1992; for review see Hohlfeld, 1997).

Despite the obvious involvement of cytokines in the immunopathogenesis of MS, none of the current methods used for monitoring their levels has proved clinically useful. Reasons for this are multiple. Firstly, cytokines are produced locally which makes access to the site of their production in MS difficult. Secondly, they are produced in relatively small amounts to act mainly as paracrine and autocrine signals. Thirdly, as they are potent biological agents, numerous mechanisms exist to limit their activity, such as soluble receptors, natural neutralising antibodies, adsorption by serum proteins such as alpha-2-macroglobulin and proteolysis, all of which reduce their levels and chance of detection. Fourthly, the time-course of pro-inflammatory cytokines production in MS may be short, with a window too narrow to detect raised levels. To overcome these difficulties ex vivo cytokine production and techniques allowing one to determine cytokine production on the cellular level have been developed. Stimulated peripheral blood mononuclear cell TNFα, lymphotoxin and IL1 production appears to correlate with clinical disease activity (Chofflon et al., 1992; Chofflon et al., 1997; Glabinski et al. 1995), however, in other studies using a similar techniques, TNFα production does not correlate with Gd-enhanced MRI activity (Llewellyn-Smith et al., 1997). In situ mRNA hybridisation (Navikas et al., 1995; Matusevicius et al., 1996; Navikas et al., 1996a) and ELISPOT assays (Sun et al., 1991; Söderstrom et al., 1994) allow one to quantify cytokine production of individual cells from the CSF or peripheral blood. MS disease activity is associated with an up-regulation of IFNγ, TNFα, lymphotoxin, IL4, IL6 and IL12, and down-regulation of TGFβ and IL10. Cells
expressing TNFα and lymphotoxin are preferentially upregulated during relapses and those expressing TGFβ and IL-10 in remissions (Navikas and Link, 1996b). Finally, semi-quantitative, whole blood and CSF mononuclear cell, reverse transcriptase polymerase chain reaction cytokine-specific mRNA assays have also been employed (Rieckmann et al., 1995a). An increase in blood mononuclear cell TNFα and lymphotoxin mRNA levels appears to precede clinical and MRI disease activity whereas TGFβ and IL10 mRNA levels decline (Rieckmann et al., 1995b). In general the various cytokine assays demonstrate quite marked intra- and inter-patient variability, are labour intensive and are unsuitable for frequent monitoring. They have therefore not gained widespread support.

1.6.2.2 Cellular Changes

MS patients have significantly greater numbers of activated T-cells (high affinity IL2 receptor +ve) (Hafer et al., 1985; Selmaï et al., 1986; Bellamy et al., 1985), and fewer circulating CD8+ lymphocytes (Compston, 1983; Hauser et al., 1983; Bach et al., 1985; Reder et al., 1984; Paty et al., 1983; Hughes et al., 1986), more specifically CD8+CD28-suppressor T-cells (Crucian et al., 1995) in their circulation than normal controls. The decrease in CD8+ T-cells appears to correlate with acute relapse (Hauser et al., 1983; Bach et al., 1985; Reder et al., 1984) and the progressive phase of the disease (Paty et al., 1983; Hughes et al., 1986). Similarly, there have also been reports of fewer circulating NK-cells (CD56+) in patients with active relapsing and progressive MS (Munechaur et al., 1995). It has also been demonstrated from cell surface studies that circulating monocytes are activated in patients with MS compared to normal controls (Zaffaroni et al., 1992; Tsukada et al., 1994). CSF cell counts, although non-specific, are elevated in patients with MS and relate to disease activity (Freedman and Merritt, 1950; Tourtellotte, 1985). Unfortunately these peripheral blood and CSF cellular changes have
proved too insensitive to be suitable for monitoring disease activity in individual patients with MS.

1.6.2.3 T-cell Apoptosis

Apoptosis of antigen-specific T-cells in the lesions of EAE has been identified as an effective mechanism in stopping neural inflammation (Gold et al., 1997). Programmed cell death of infiltrating T-cells might be due to enhanced corticosteroid production, the presence (TNFα, IFNγ) or absence (IL2) of certain cytokines, lack of availability of co-stimulatory molecules (Ford et al., 1996), antigen induction, or due to Fas/APO 1 (CD95) expression on T-cells which mediates apoptosis (Dowling et al., 1996; Ichikawa et al., 1996; Bauer et al., 1995b). The proportion of apoptotic mononuclear cells in the CSF is lower in MS than in non-inflammatory neurological controls (Ciusani et al., 1998) and an increased CSF (Ciusani et al., 1998) and serum level (Zipp et al., 1998) of soluble CD95 (APO-1/Fas) is found in patients with relapsing remitting multiple sclerosis. Increased soluble CD95 correlated, albeit weakly, with an increase in EDSS over at least a 12-month period (Zipp et al., 1998).

1.6.2.4 Acute phase proteins

Inflammatory activity in MS is predominantly asymptomatic and is generally not associated with overt signs of systemic inflammation (Matthews, 1991a). Despite this an acute phase response due to the endocrine effects of proinflammatory cytokines on hepatic protein synthesis has been associated with clinical relapses and with Gd-enhanced MRI activity in the form of elevated levels of positive acute phase proteins such as C-reactive protein (CRP) (Dowling and Cook, 1976; Giovannoni et al., 1996a).

1.6.2.5 Adhesion molecules

Adhesion molecule expression is greatly up-regulated in MS (Canella and Raine, 1995), allowing recruitment of circulating leukocytes and functioning as accessory molecules
in antigen presentation (Gorski, 1994). Soluble forms of these molecules are produced by proteolytic cleavage or alternate RNA splicing and released from the cell (Gearing and Newman, 1993). The soluble forms probably function as natural inhibitors of cell adhesion and have immunomodulatory roles. Increased levels of soluble E-selectin (Giovannoni et al., 1996a; Hartung et al., 1995a; Dore-Duffy et al., 1995; Tsukada et al., 1995), L-selectin (Hartung et al., 1995a), ICAM-1 (Sharief et al., 1993; Tsukada et al., 1993; Hartung et al., 1995a, Rieckmann et al., 1993), ICAM-3 (Martin et al., 1995) and VCAM-1 (Matsuda et al., 1995; Hartung et al., 1995; Dore-Duffy et al., 1995) have been found in CSF and/or serum of patients with MS. Levels of adhesion molecules appear to correlate with clinical and MRI markers of disease activity but have not proved useful as surrogate markers of disease progression. By blocking adhesion molecule interactions, recruitment of effector cells into the nervous system and their interactions can be reduced, preventing or controlling the disease process - an approach which has proved partially successful in EAE (Yednock et al., 1992; Archelos et al., 1993) and has prompted clinical trials of anti-adhesion molecule therapy in patients with MS (Anti-VLA4, Antegren™ - Athena Neuroscience, personal communication Prof. DH Miller). Other adhesion molecules and related proteins studied in MS include β2-microglobulin (Bjerrum et al., 1988, Us et al., 1989; Ott et al., 1993), soluble-HLA molecules (Alvarez Cermeno et al., 1992), soluble CD4 (Tsukada et al., 1991b) and soluble CD8 (Maimone and Reder, 1991; Tsukada et al., 1991; Carrieri et al., 1992) which have all been shown to be elevated, with the exception of CD4, in cross-sectional studies in patients with MS. In general adhesion molecules show promise as inflammatory markers, as they occur distal to cytokines in the inflammatory cascade, are produced in greater quantities, are more stable and possibly have a longer time-course of production.
1.6.2.6 Chemokines

C-X-C chemokines (IL8, melanoma growth-stimulatory activity, platelet factor 4, and beta-thromboglobulin) are mainly responsible for attracting neutrophils and are unlikely to play a major role in MS. C-C chemokines are involved, as they are responsible for macrophage and T lymphocyte recruitment, and include macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemotactic protein-1, RANTES, and lymphotaxin. They play an important role in EAE (Karpus et al., 1995; Godiska, et al., 1995). Levels of MIP-1α are increased in the CSF of patients with MS (Miyagishi et al., 1995). Increased MIP-1α, MIP-1β and RANTES mRNA expression is found in active MS lesions (Hvas et al., 1997; Simpson et al., 1998). No data are yet available on the use of chemokines as inflammatory markers in MS.

1.6.2.7 B-Cell activity

The intrathecal oligoclonal plasma cell response which occurs in MS is useful diagnostically but has little if any role to play in monitoring disease activity (Giovannoni and Thompson, 1996). However immunoglobulin light chain synthesis, which occurs in excess to that of the heavy chains, has been used as a marker of B-cell activity. CSF (McLean et al., 1989; Sindic et al., 1991; Constantinescu et al., 1994) and urinary (Mehta et al., 1991) free light chains have both been used as markers of disease activity, but have not gained widespread clinical support.

1.6.2.8 Glial and neuronal markers

One strategy has attempted to quantitate myelin breakdown by measuring myelin basic protein-like material (MBPLM) in the urine of patients with MS (Whitaker et al, 1994). Although levels of urinary MBPLM are elevated in patients with MS, and are possibly higher in progressive patients, they correlate poorly with disease activity (Whitaker et al., 1995b). The neuronal marker neurone-specific enolase (NSE) and the astrocyte
marker S100 are clinically useful in certain types of CNS tumours and in conditions associated with gross neuronal death but have been disappointing in MS (Cunningham et al., 1994; Lamers et al., 1995). Another neuronal marker, neurofilament protein, which is a structural protein and a putative marker of axonal damage, appears more promising. Increased CSF neurofilament levels are found in patients with MS and appear to correlate with disability and relapse (Lycke et al., 1998). Gliosis, one of the pathological hallmarks of MS (Allen, 1991), occurs as a result of astrocyte proliferation. CSF glial fibrillary acidic protein (GFAP) an astrocytic marker, increases progressively with MS disease duration (Rosengren et al., 1995). Both neurofilament protein and GFAP need to be further investigated as a possible surrogate markers of disease progression in MS.

1.6.2.9 Monocyte/macrophage markers

Although monocytes and macrophages are a major source of cytokines, some inflammatory markers are relatively specific to macrophages. Neopterin is a well-established macrophage-specific marker of cell mediated inflammation in numerous autoimmune and infectious diseases, as well as in malignancy and transplant rejection. Several cross-sectional studies have demonstrated elevated levels of neopterin in the CSF (Fierz et al., 1987; Fredrickson et al., 1987a and 1987b; Ott et al., 1993; Shaw et al., 1995) and serum (Ott et al., 1993) of patients with MS. No studies have looked at its longitudinal role as a potential surrogate marker of disease activity in MS. Other potential macrophage markers include the tryptophan metabolites kynurenic and quinolinic acid, and the nitrogen oxides nitrate and nitrite. Activated macrophages upregulate their expression of 2,3-dioxygenase, an enzyme responsible for initiating catabolism of tryptophan to form quinolinic acid. Levels of quinolinic acid are raised in inflammatory diseases of the central nervous system, notably HIV-1 encephalopathy
(Heyes et al., 1991; Brouwers et al., 1993), poliomyelitis (Heyes et al., 1993), experimental allergic encephalomyelitis (EAE) (Flanagan et al., 1995) and possibly in patients with MS (Reinhard JF, Glaxo Wellcome Research). Similarly, inducible nitric oxide (\( \bullet N=O \)) synthase is upregulated in both CNS macrophages (Bagasara et al., 1995; DeGroot et al. 1997) and peripheral blood monocytes of MS patients (Lopez-Moratalla et al., 1997). Increased levels of the \( \bullet N=O \) metabolites nitrate and nitrite are found in the CSF (Johnson et al., 1995; Giovannoni, 1998) and serum of patients with MS (Giovannoni et al., 1997b). Ex vivo peripheral blood mononuclear cells from MS patients produce increased quantities of \( \bullet N=O \) metabolites which correlate with Gd-enhanced MRI activity (Sarchielli et al., 1997). Whether tryptophan and \( \bullet N=O \) metabolites are useful as macrophage-specific surrogate markers in MS requires performance of additional studies.
<table>
<thead>
<tr>
<th>Inflammatory Marker</th>
<th>Clinical Relapse</th>
<th>Disease Progression</th>
<th>MRI Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
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<tr>
<td>IL1</td>
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<tr>
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<td>-</td>
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<tr>
<td><strong>Cellular Changes</strong></td>
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<td>CSF cell counts</td>
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<td>CD8+</td>
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<td>NK Cells</td>
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<td><strong>Acute Phase Proteins</strong></td>
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<td>CRP</td>
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<td><strong>Adhesion Molecules</strong></td>
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<td>sICAM-1</td>
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<td>sICAM-3</td>
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<td>sVCAM-1</td>
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<td>sE-selectin</td>
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<td>sL-selectin</td>
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<td><strong>Chemokines</strong></td>
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<td>MIP-1α</td>
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<td><strong>B-cell Markers</strong></td>
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<td>Free Light Chains</td>
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<td>MBPLM</td>
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<td>GFAP</td>
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<td><strong>Others</strong></td>
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<td>Soluble CD95 (Fas/APO-1)</td>
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<td><strong>Macrophage</strong></td>
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<tr>
<td>Neopterin</td>
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<td>Tryptophan metabolites</td>
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<td>Nitric oxide metabolites</td>
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<td>(nitrate and nitrite)</td>
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CD = cluster differentiation, CRP = C reactive protein, CSF = cerebrospinal fluid, GFAP = glial fibrillary acidic protein, HLA = human leukocyte antigen, ICAM = intercellular adhesion molecule, IFN = interferon, IL = interleukin, MBLM = myelin basic protein like material, MIP = macrophage inhibitory protein, NF = neurofilament, NK = natural killer, NSE = neuron specific enolase, TNF = tumour necrosis factor, TGF = transforming growth factor, VCAM = vascular cell adhesion molecule.
1.7 The role of the macrophage and microglia in MS

1.7.1 Background

Although MS is hypothesised to be an antigen-specific T-cell mediated autoimmune disease, macrophages and CNS microglia are important effector cells of the inflammatory process and play a central role in the pathogenesis of MS (Dijkstra et al., 1992; Martin and McFarland, 1995). Microglia are the resident CNS macrophages, derived from bone marrow progenitors, which enter the CNS during embryonic development (Jordan et al., 1988). They differ morphologically from macrophages, having numerous dendritic extensions or spines (Sievers et al., 1994; Giulian et al., 1995), and electrically, having a distinctive pattern of membrane currents (Schmidtmayer et al., 1994; Eder et al., 1995). These properties appear to result from a trophic effect supplied by astrocytes, as blood- and spleen-derived peripheral monocytes develop the morphological (Sievers et al., 1994) and electrical characteristics (Schmidtmayer et al., 1994) of microglia when co-cultured with astrocytes or exposed to astrocyte-conditioned media. Once microglia are activated they gradually lose their dendritic processes and transform into typical phagocytic macrophages with similar functional properties (Ulvestad et al., 1994a). In the brains of patients with MS, macrophage activation is seen early in acute lesions (Esiri and Reading, 1987; Adams and Poston, 1990; Allen, 1991; Li et al., 1993) and their differentiation can be correlated with demyelinating activity (Brück et al., 1995). As a result of myelin phagocytosis, they form large oil red O positive foamy cells during active demyelination (Allen, 1991; Li et al., 1993). The phagocytosis of myelin occurs via clathrin-coated pits (Epstein et al., 1983), and is assumed to be mediated by Fc receptors (Ulvestad et al., 1994b). In vitro the Fc, LDL, and scavenger receptors have been shown to play a part in myelin uptake, which is augmented by opsonisation with anti-myelin
antibodies (Smith, 1993; Williams et al., 1994; Moseley and Cuzner, 1996). CSF from patients with MS contains anti-myelin antibodies (Sellebjerg et al., 1994; Warren et al., 1994; Link et al., 1990, Olsson et al., 1990) which could augment myelin phagocytosis as has been shown in EAE (Sommer et al., 1992; Smith, 1993). In addition to phagocytosis (Epstein et al., 1983) CNS microglia/macrophages are hypothesised to be responsible for antigen presentation (Hart and Fabry, 1995), the production of myelinotoxic and neurotoxic factors (Hartung et al., 1995), secretion of cytokines that augment the inflammatory cascade (Dijkstra et al., 1992, Merrill, 1992; Martin and McFarland, 1995) and assisting in the process of remyelination, presumably via the production of neurotrophic factors (Cuzner et al., 1994). The importance of macrophages in nervous system inflammation has been elegantly demonstrated in the adoptive transfer acute experimental allergic encephalitis (EAE) and neuritis (EAN) models, in which prior depletion of the macrophages and microglial population with liposomal-delivered toxin (dichlormethylene diphosphonate) not only prevented induction of the disease but suppressed the development of inflammation (Jung et al., 1993; Huitinga et al. 1996).

1.7.2 Macrophage pterin and •N=O metabolism

Macrophages are involved in all stages of the immune response, but their role in cell mediated immunity is particularly important in MS. Their recruitment, activation and effector functions are, at least initially, under the control of T-cell cytokines (Nacy and Meltzer, 1991). IFNγ, a major proinflammatory T-cell cytokine, is a potent activator of macrophages, which results in the production of neopterin (Huber et al., 1984; Nathan, 1986; Henderson et al., 1991) due to up-regulation of the enzyme GTP-cyclohydrolase I (Werner et al., 1990). This enzyme catalyses the rate-limiting step in the pathway producing tetrahydrobiopterin - an essential co-factor for the •N=O synthases (NOS)
which synthesise •N=O from the amino acid L-arginine (Fig.1.6) (Tayeh and Marletta, 1989; Kwon et al., 1989). Three distinct isoforms of •N=O synthase (NOS) have been described. Types I and III are constitutively expressed (cNOS) as neuronal and endothelial forms respectively and have also been demonstrated in other cells including pancreatic beta cells, monocytes, mast cells and platelets (Moncada and Higgs 1993; Knowles and Moncada, 1994). Type II or inducible NOS (iNOS) is induced by specific cytokines in macrophages (Tayeh and Marletta, 1989), hepatocytes (Green et al., 1990), fibroblasts (Lavnikova and Laskin, 1995), endothelial cells (Kilbourn, et al., 1990), pancreatic beta cells (Southern et al., 1990), chondrocytes (Stadler et al., 1991) astrocytes (Simmons and Murphy, 1992), and oligodendrocytes (Merrill et al., 1997).

The cNOS is calcium- and calmodulin-dependent and requires flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), nicotinamide dinucleotide phosphate (NADP) and the essential co-factor tetrahydrobiopterin (Knowles and Moncada, 1994). The inducible form shares similar characteristics except it is not calcium- and calmodulin-dependent and produces larger quantities of •N=O compared with its constitutive counterpart (Knowles and Moncada, 1994).

•N=O has several diverse physiological functions. Endothelial production is essential for the control of vasodilator tone and therefore blood pressure (Moncada and Higgs 1993). It acts as a neurotransmitter in the central and peripheral nervous systems where it has diverse actions including the formation of memory (Shibuki and Okada, 1991, Schuman and Madison, 1991), behavioural control (Nelson et al., 1995), neurogenic vasodilation, and the regulation of gastrointestinal, respiratory, and genitourinary tract functions (Moncada and Higgs, 1993). •N=O is also a product of immunological reactions and plays an important role in nonspecific immunity (Langrehr et al., 1993).
Activated murine macrophages produce $\cdot N=G$ which is responsible for cytotoxicity against tumour cells (Lorsbach et al., 1993) and the eradication of intracellular infections (Wei et al., 1995). The experimental data on $\cdot N=G$ production by human macrophage is inconclusive (Denis, 1994; Dugas et al., 1995). Evidence suggests that human macrophages produce $\cdot N=G$, but not in response to all the stimuli which have been shown to induce $\cdot N=G$ production in murine macrophages. For example, $\cdot N=G$ synthesis has been shown to occur in response to specific tumour cell lines but not to non-specific immunostimulants such as LPS, INF-$\gamma$ and TNF-$\alpha$ (Zembala et al., 1994). These immunostimulants do however result in induction of iNOS mRNA (Reiling et al., 1994), suggesting that the activity of iNOS is controlled either at a post-transcriptional or enzymatic level. There is clearly an interspecies difference with murine macrophages being able to produce large quantities in response to several stimuli whereas human macrophages produce much smaller quantities and then only in response to certain specific stimuli (Dugas et al., 1995). Both the murine (Lyons et al., 1992; Xie et al., 1992; Lowenstein et al., 1992) and human (Chartrain et al., 1994) iNOS genes have been sequenced and cloned with 80% and 60% homology in the coding and promoter regions respectively. It therefore seems likely that significant differences in the control of the murine and human iNOS systems exist.
Figure 1.6. The \( \text{\textit{NO synthase reaction, which is tetrahydrobiopterin-dependent}} \).

One of the hypothesised differences between the murine and the human macrophage system of \( \text{\textit{NO}} \) production relates to the availability of tetrahydrobiopterin. Unlike the murine pathway, human cells of the monocyte lineage have a partial block in the tetrahydrobiopterin synthetic pathway (Schoedon et al., 1987) due to a reduction in the activity of the 6-pyruvoyl-tetrahydropterin synthase (Werner et al., 1990). This results in reduced tetrahydrobiopterin synthesis and the shunting of 7,8-dihydroneopterin triphosphate into the production of 7,8-dihydroneopterin and neopterin (Fig. 1.7). The reduced tetrahydrobiopterin synthesis does not appear to limit \( \text{\textit{NO}} \) activity, as bypassing the block with the distal precursor sepiapterin does not result in an increase in \( \text{\textit{NO}} \) production (Weinberg et al., 1995). The significance of this neopterin shunt is currently unknown, but it has been proposed to give human and primates some phylogenetic advantage. One theory is that 7,8-dihydroneopterin acts as an intracellular reducing agent, which is capable of scavenging free radicals, in particular those related...
to •N=O and superoxide radicals, protecting the macrophage from self-induced •N=O damage. In vitro 7,8-dihydroneopterin is a potent anti-oxidant dramatically decreasing the oxidation of low density lipoprotein (LDL) and linoleate by copper ions (Cu^{2+}) or peroxyl radicals (Gieseg et al., 1995). 7,8-dihydroneopterin appears to functions by scavenging lipid peroxyl radicals (Gieseg et al., 1995). On the other hand Weiss et al. (1992) claim that neopterin has a role as a modulator of extracellular oxidants. They demonstrated that neopterin, in an alkaline medium (pH 7.5), augmented the bactericidal activity mediated by reactive oxygen and chloride species. In rat vascular smooth muscle cells neopterin is capable of inducing iNOS gene expression (Schobersberger et al., 1995). •N=O release and the nuclear translocation of the transcription factor nuclear factor-kappa B (NF-kB). It does this by modulating the intracellular redox state of the cell (Hoffmann et al., 1996). In another study 7,8-dihydroneopterin was found to augment TNFα induced apoptosis (Baier-Bitterlich et al., 1995), either due to enhanced formation of reactive oxygen intermediates or its ability to modulate the intracellular redox potential, which would affect NF-κB signalling. Neopterin has also been shown to be capable of reducing ischaemic neuronal damage in the gerbil (Arai et al., 1994). Whether it attenuates the ischaemic neuronal damage by scavenging free radicals or by inhibiting their generation is unknown.
GTP-cyclohydrolase I, is strongly induced by INFγ (Schoedon et al., 1986; Werner et al., 1990; Schott et al., 1993) and augmented by IL1 (Kasai et al., 1995), TNFα (Werner et al., 1991) and LPS (Hattori and Gross, 1993). These latter pro-inflammatory agents appear to have a small or moderate effect on neopterin production in the absence of INFγ (Werner-Felamayer, et al. 1993). Whether this occurs at the genetic level still needs to be elucidated as the promoter region of GTP-cyclohydrolase appears to lack an IFNγ-response element (Witter et al., 1996). It is of particular interest that in other cells of the body where tetrahydrobiopterin is required as a co-factor for aromatic amino acid decarboxylases and neurotransmitter synthesis (Ichinose et al., 1994), no block occurs in the tetrahydrobiopterin synthetic pathway. This discrepancy is not due to the presence

![Biochemistry of neopterin and 5,6,7,8-tetrahydrobiopterin synthesis.](image)

*Figure 1.7. Biochemistry of neopterin and 5,6,7,8-tetrahydrobiopterin synthesis.*

of separate GTP-cyclohydrolase I genes, as only a single functional copy has been mapped to chromosome 14q22.1-q22.2 (Ichinose et al., 1994).

GTP-cyclohydrolase I is under feedback inhibition by tetrahydrobiopterin the end-product of the metabolic pathway (Fig. 1.7) (Harada et al., 1993). 7,8-Dihydroneopterin and neopterin, end-products of the pterin shunt in macrophages, have no effect on GTP-cyclohydrolase I activity. This results in massive and relatively uninhibited synthesis of neopterin by activated macrophages. In vitro the addition of sepiapterin, a tetrahydrobiopterin precursor, to the culture medium dramatically decreases the production of neopterin by macrophages due to the feedback inhibition of tetrahydrobiopterin on GTP-cyclohydrolase I (Shen et al., 1988; Schoedon et al., 1986; Harada et al., 1993).

Whatever their function, the reduced and oxidised forms of free neopterin represent a by-product of monocyte tetrahydrobiopterin synthesis and are therefore an indirect measure of IFNγ production.

Neopterin, dihydroneopterin and tetrahydrobiopterin belong to a ubiquitous group of compounds, the pteridines, which have a bicyclic nitrogenous ring system (pyrazino-(2,3-d)-pyrimidine) and are formed from the fusion of a pyrazine with a pyrimidine (Fig. 1.7) (Wachter et al., 1992). Neopterin is formed from the oxidation of its reduced form 7,8-dihydroneopterin, and the term total neopterin refers to the sum of both the reduced and oxidised forms. It is found in all body fluids and is excreted in the urine (Wachter et al., 1992). The urinary concentration of neopterin is greater than two orders of magnitude higher than that of plasma and CSF (±500X). In humans continuous
production of neopterin occurs at low levels, which is greatly up-regulated by disease states characterised by immunological activation of the cell-mediated immune system (Huber et al., 1984; Wachter et al., 1992; Fuchs et al., 1992).

1.8 The potential role of pterins and nitric oxide in the pathogenesis of MS

In MS damage to oligodendrocytes and neurones has been hypothesised to occur as a result of non-specific mediators of inflammation (Fig.1.5) such as reactive oxygen species (Kim and Kim 1991; Noble et al., 1994), nitrogen free radicals (Merrill et al., 1993; Mitrovic et al., 1994; Mackenzie-Graham et al., 1994; Brosnan et al., 1994; Mitrovic et al., 1995), proteases (Richards and Cuzner, 1978; Sato et al., 1984b; Banik, 1992), pro-inflammatory cytokines (Selmaj and Raine, 1988; Selmaj et al., 1991; Zajicek et al., 1992; Chao et al., 1995), eicosanoids (Neu et al., 1992), and antibody/complement-mediated membrane attack (Compston and Scolding, 1991; Zajicek et al., 1992).

•N=O has been implicated in the immunopathogenesis of MS (Parkinson et al., 1997; Giovannoni et al., 1998b). Elevated levels of •N=O metabolites have been found in the cerebrospinal fluid (Johnson et al., 1995; Giovannoni, 1998) and serum of patients with MS (Giovannoni et al., 1997b), and pathological studies have demonstrated increased astrocyte iNOS activity (Bö et al., 1994), iNOS (DeGroot et al., 1997), as well as increased expression of iNOS mRNA in cells with a monocytic phenotype (Bagasara et al., 1995), in the brains of patients dying with MS.

The exact role that •N=O plays in MS is unknown but it has been shown in vitro to be cytotoxic to both oligodendrocytes (Merrill et al., 1993; Mitrovic et al., 1994; Mackenzie-Graham et al., 1994; Brosnan et al., 1994; Mitrovic et al., 1995) and
neurones (Boje et al., 1992; Chao et al., 1992; Mitrovic et al., 1994; Bolanos et al., 1995; Chao et al., 1996). Oligodendrocytes are also more vulnerable to •N=O induced cytotoxicity than are astrocytes and microglia (Mitrovic et al., 1994), and it has therefore been proposed that •N=O may act as one of the mediators of demyelination (Brosnan et al., 1994).

•N=O reacts with the superoxide radical (O$_2^-$) to form the peroxynitrite (ONOO$^-$), a potent oxidising agent capable of inducing many of the cytotoxic effects ascribed to •N=O (Beckman et al., 1990). The mechanisms of •N=O and peroxynitrite-mediated cytotoxicity are multiple: disruption of cellular function by lipid peroxidation (Beckman et al., 1990); depletion of intracellular energy stores due to inhibition of the mitochondrial electron transport chain (Bolanos, et al., 1995); consumption of intracellular reducing agents (Barker et al., 1996); damage to DNA (Inoue and Kawanishi, 1995) and inactivation of key intracellular enzymes such as aconitase (Hausladen and Fridovich, 1994), glyceraldehyde-3-phosphate dehydrogenase (Mateo et al., 1995) and ribonucleotide reductase (Roy et al., 1995).

Patients with MS have an increased level of lipid peroxidation, which is greater during periods of exacerbation (Hunter et al., 1985; Toshniwal and Zarling, 1992; Naidoo and Knapp, 1992). As a result of lipid peroxidation, the brains of patients with MS have significantly lower levels of polyunsaturated fatty acids than do controls (Baker et al., 1963). This may explain the apparent therapeutic response of the essential fatty acid linoleic acid in patients with MS (Dworkin et al., 1984). Linoleic acid has many therapeutic actions, one of which is to protect cell membranes from lipid peroxidation.
Oligodendrocyte death in MS is characterised by DNA fragmentation, which suggests apoptosis or programmed cell death (Ozawa et al., 1994). Possible mechanisms for apoptosis include oxidative stress (Ferrari et al., 1995; Bonfoco et al., 1995), depletion of intracellular energy stores (Richter et al., 1996), and a cytokine-induced death signal (D'Souza et al., 1995; Wilt et al., 1995). In vitro, IFNγ (Vartanian et al., 1995) and TNFα (D'Souza et al., 1995; Wilt et al., 1995) induce apoptotic cell death of oligodendrocytes. TNFα induces apoptosis through activation of its p55 or "death" receptor (Wilt et al., 1995). Oxygen and nitrogen free radicals can sensitize cells to cytokine-induced apoptosis by affecting redox-sensitive intracellular signalling (e.g. NFκB) (Dröge et al., 1994).

It therefore seems probable that free radicals such as •N=O, and its derivative peroxynitrite, play a role as non-specific mediators of CNS inflammation. In EAE •N=O appears to play a role in both acute and chronic models of the disease (Koprowski et al., 1993; Lin et al., 1993; Okuda et al., 1995; van Dam et al., 1995). Hooper et al. (1995) using an in vivo •N=O spin trapping technique in adoptive transfer Lewis rat EAE have demonstrated that large amounts of •N=O (20-30µM) are produced in the spinal cord 4 to 5 days after T cell transfer, which correlates with the time of clinical paralysis. An early study showed that an iNOS inhibitor, aminoguanidine, ameliorated MBP-specific T cell adoptive transfer EAE in SJL/J mice (Cross et al., 1994). These results with high-dose aminoguanidine have been confirmed in both MBP-peptide induced and T cell adoptive transfer EAE in PLJ/SJL mice (Parkinson et al., 1997). However other studies in the Lewis rat have shown that iNOS inhibitors result in a worsening of the disease course (Zielasek et al., 1995; Ruuls et al., 1996). Inducible NOS double knock-out mice are less resistant to the induction of EAE, and develop a
protracted non-remitting course compared to the acute monophasic illness in wild-type mice (Fenyk-Melody et al., 1998). Iron chelating agents such as desferrioxamine (Bowern et al., 1984) which decrease lipid peroxidation, and antioxidants such as n-acetylcysteine (Lehman et al., 1994) and vitamin E (Kryzhanovskii et al., 1984) ameliorate the severity of EAE presumably by preventing the actions of •N=O and peroxynitrite.

1.9 Conclusions

MS is a clinically heterogeneous disease. Clinical relapses, unlike progressive disease, correlate strongly with putative markers of acute CNS inflammation. The pathogenesis of axonal loss, which causes progressive disease, remains speculative. Production of the free radical •N=O, by inducible •N=O synthase, may be an important mediator of axonal loss. Tetrahydrobiopterin is an essential co-factor for the •N=O synthases. Nitric oxide metabolism is therefore closely linked to pterin metabolism. The signals inducing •N=O production also result in the excess production of neopterin by cells derived from monocytic precursors. Neopterin thus provides a handle by which to monitor, in vivo, the inflammatory activity of these cells and the metabolic pathways in diseases such as MS.

1.10 Primary Hypotheses

Increased neopterin production in patients with multiple sclerosis will correlate with clinical and MRI markers of disease activity.
Chapter 2 - Laboratory methods

2.1 Introduction

Neopterin is measured either by reversed phase high performance liquid chromatography (HPLC) with fluorescence detection (Niederwieser et al. 1982; Hyland et al. 1985) or by competitive immunoassay (Wachter et al. 1992). The main disadvantages of the commercially available immunoassays are the costs and, in the case of radioimmunoassays (RIA) (IMMУtest®, Henning Berlin; Neopterin ¹²⁵I RIA Kit, Incstar), the inconvenience of handling radioactive material. More recently this latter problem has been solved by a competitively priced enzyme linked immunosorbent assay (ELISA) becoming available (Immuchem™, ICN Biomedicals). A possible disadvantage of immunoassays in general relates to the pterin-specificity of the antibodies, which are unable to detect dihydronopterin (Wachter et al., 1992). Advantages with immunoassays however, include ease and speed of analysis, no need for sample preparation, and the requirement for minimal laboratory equipment. HPLC on the other hand requires expensive hardware, regular maintenance, sample preparation and is unsuitable for the rapid processing of large numbers of samples. One advantage that HPLC has is that it is possible to simultaneously measure creatinine using a second UV-detector. Creatinine is produced largely in muscle by irreversible non-enzymatic dehydration of creatine phosphate. The 24-hour urinary excretion of creatinine is remarkably constant from day to day, and is proportional to muscle mass (Rodwell, 1990). This makes it a suitable internal reference for assessing how concentrated a urine sample is. Creatinine makes it possible to make allowances for the variable concentration of urine (Hartman et al., 1994).
2.2 HPLC for determining levels of neopterin and creatinine in urine

2.2.1 Method

Reagent grade water, prepared by a water purification system (Milli-RO 10 and Milli-Q deioniser, Millipore™), was used for all HPLC applications. Urine samples were prepared by diluting them 1 in 10 with the 15 mmol/L potassium phosphate buffer (Sorensen's Buffer, Appendix III), pH 6.4, containing 5.4 mmol/L ethylenediaminetetra-acetic acid (EDTA, BDH prod. no. 10093) to dissolve urinary sediments (Fuchs et al. 1992). As the UV-detector saturated at creatinine concentrations greater than 2.7 mmol/L, specimens with concentrations greater than 2.5 mmol/L required further dilution and repeat analysis.

The method used in these studies was based on that of Niederwieser et al. (1982) and Fuchs et al. (1992). All HPLC methods were validated using either a 25cm ODS2 Anasil 80 column (Anachem, prod. no. IG80504252), or a 25cm 5μ ODS Techsphere column (HPLC Technology, cat. no. CTS-03525). A column heater (Jones Chromatography) was used to keep the column at a constant temperature of 40°C. A protective 5μ ODS Techsphere pre-column (HPLC Technology, cat. No. GTS-E03501) was also used. After each run (maximum 65 samples) the column was cleaned using a water methanol gradient that took approximately 2 hours to complete (Appendix IV).

Sample elution was performed using the 15 mmol potassium phosphate buffer (Sörensen’s Buffer, Appendix III), pH 6.4, containing 5% methanol (HPLC grade, Hipersolv™ BDH pro. no. 15250), with a flow rate of 1mL per minute (Kontron 420 HPLC pump). Two patients with MS, one on hormone replacement therapy and
multivitamins and the other on the oral contraceptive pill, had an unknown peak which interfered with the neopterin peak. Re-analysis of their samples with the same potassium phosphate buffer, but at pH 7.2 containing 10% methanol resolved the neopterin peak. During a chromatographic run the buffer was continuously degassed using helium (BOC). The buffer was recycled and reused for up to 5 runs (200-300 samples). Neopterin detection was by native fluorescence using an excitation wavelength of 353nm and an emission wavelength of 438nm. Initially a LS50 luminescence spectrometer (Perkin Elmer) and later a HPLC-dedicated CMA 280 fluorescence detector (Biotech Instruments) were used. Creatinine was measured using a Kontron 735LC UV detector with an absorption wavelength of 235nm. The fluorescence and UV detectors were connected in series, which allows the determination of neopterin and creatinine within the same chromatographic run. This has the advantage that any errors due to sample processing or variable injection volume, which would affect both neopterin and creatinine concentrations simultaneously, would have little effect on the ratio. A fixed volume 20μL sample loop was used. Methods were validated with a manual injection technique, using a 100μL glass syringe, and a Rheodyne™ series 25 sample injector. This was later replaced by a Kontron 360 autosampler. Data was initially collected using a double channel chart recorder (RE 571, Recorder Labs), with manual measurement of the peak heights (Fig. 2.1). Later the process was automated with a commercial 2-channel integration package (Integrator Pack ver. 3.92, Kontron), run on a Compaq™ 386 personal computer.
2.2.2 Assay calibration and performance

2.2.2.1 Neopterin

Figure 2.1. Urine chromatograph: a typical example of a 2-channel HPLC urine chromatograph.

The assays were calibrated using D-neopterin (Schirks Laboratories, prod. no. 11'303) and L-biopterin (Schirks Laboratories, prod. no. 11'203). Stock neopterin and biopterin standards, prepared by Dr Simon Heales (Dept. Neurochemistry, Institute of Neurology), were used throughout these studies. They were dissolved in reagent grade water with the aid of sonication, aliquoted in 1ml vials and stored in a light proof container at -20°C. Working standards were prepared by diluting stock neopterin, biopterin and creatinine standards in 15 mmol Sörensen’s buffer, aliquoted into autosampler vials, and stored at -20°C. The lower limit of detection with the fluorescence detector set to maximum sensitivity with an injection volume of 20μL, was 50nM. The recovery of neopterin from 20 urine samples spiked with a known quantity
of D-neopterin (Schirks Laboratories, prod. no. 11’303) diluted in distilled water was 98.6% (range = 92-102%). The intra-batch coefficient of variation for 20 samples assayed in duplicate was 9.8%, and the inter-batch coefficient of variation for 2 samples assayed in 20 separate runs was 13.4%. Serial dilutions of patient samples and calibrant demonstrated parallel curves.

2.2.2.2 Creatinine

The HPLC creatinine assay was calibrated using a set of commercially available standards (Sigma, 925-11), which were combined with the neopterin and biopterin stock standards as above. The creatinine assay’s lower limit of detection was not assessed as all urine samples tested had appreciable levels of creatinine and sensitivity was never a limiting factor. Recovery of creatinine (Sigma, prod. no. C-4255) from 20 spiked urine samples was 95.6% (range 87-106%). The intra- and inter-batch coefficients of variation were 7.6% and 11.2% respectively. Serial dilutions of urine samples and calibrant, below 2.5mmol/L, demonstrated parallel curves.

2.2.2.3 Urinary neopterin:creatinine ratio \( (\text{neopt:creat}_{\text{urine}}) \)

When the results of the neopterin and creatinine assays are combined in the form of the ratio, the reproducibility of the test is much improved compared to either assay on its own, with intra- and inter-assay coefficients of variation falling to 3.9% and 7.6% respectively. This improvement may result from the elimination of dilution errors occurring in sample preparation, which would affect individually both the neopterin and creatinine measurement but would not effect the ratio.

2.2.3 Measurement of neopterin versus total neopterin \( (\text{dihydroneopterin + neopterin}) \)
Dihydroneopterin, the reduced form of neopterin, is non-fluorescent. To be included within the total neopterin it has to be oxidised to neopterin before its measurement (Fuchs et al., 1989; Ruiz-Vázquez et al., 1991). Unlike neopterin, dihydroneopterin is unstable and undergoes a pH dependent spontaneous degradation into dihydroxanthopterin, xanthopterin and pterin a process that occurs even during storage at -20°C (Wachter et al., 1992). To prevent this loss of total neopterin, dihydroneopterin should be immediately oxidised to neopterin (Wachter et al., 1992). This however is impractical for clinical samples. Furthermore, since there is a close correlation between levels of neopterin and dihydroneopterin (Fuchs et al., 1989), there is little advantage to measuring total neopterin. Additionally acid oxidation dramatically reduces levels of creatinine (data not shown), which means there would have needed to be a separate assay for the determination of creatinine. In view of theses problems and, in keeping

![Figure 2.2. Freeze-thaw cycles: the effect of multiple freeze-thaw cycles on neopt:creat. urine in the urine samples of 3 healthy volunteers. (n = 3, p = 0.04 two-tailed paired Student's T-Test / error bars = ±1 s.d.)](image)
with current recommendations (Fuchs et al., 1992), it was decided to measure only neopterin and not total neopterin in the urine specimens during the clinical studies.

2.3 Specimen Preparation - collection and storage

Urine specimens were collected in either 30mL plastic universal containers (Bibby Sterilin, cat. No. 128B) or small 1.5mL polypropylene tubes (Sarstedt, prod. No. 72/609.001). The patients and staff responsible for collecting urine samples were instructed to protect them from unnecessary exposure to light by wrapping them in aluminium foil or dark paper, or placing them in a light proof container. Specimens were then frozen and stored at -20°C until processed. Specimens underwent two, and occasionally three, freeze thaw cycles prior to analysis.

2.4 Effect of freeze-thaw cycles on the neopt:creat_{urine}

An experiment to test for the effects of repeated freezing and thawing showed a small but significant effect on the neopt:creat_{urine} (Fig. 2.2), with a decrease in the neopt:creat_{urine} of approximately 10% with more than 1 freeze-thaw cycle.

2.5 Effect of light exposure on the neopt:creat_{urine}

As neopterin is sensitive to degradation by UV-light (Fuchs et al., 1992), exposure of samples to direct sunlight was avoided by processing them in a protected area of the laboratory. In an experiment performed to assess the effect of light exposure on urine samples stored in polypropylene tubes within the laboratory, aliquots of urine from 3 normal volunteers were exposed to continuous ambient light (fluorescent tube lighting) for up to 48 hours. The results show that the neopt:creat_{urine} is relatively stable for periods of up to 2 hours, but decreases progressively thereafter (Fig.2.3).
2.6 Twenty four-hour urinary neopterin excretion vs. early morning spot levels

One advantage of measuring the urinary concentration of substances such as neopterin, which are readily excreted by the kidneys is that the total daily production can be determined using a 24-hour urine collection. The impracticalities and unreliability of 24-hour urine collections, however, offset this advantage. Before embarking on a study to measure 24-hour neopterin production, a pilot study was performed to see whether spot urine specimens were as effective as 24-hour collections in assessing neopterin production.
2.6.1 Method

Four normal volunteers were recruited to undertake six 24-hour urine collections, at weekly intervals. Subjects were provided with large (4.5L) lightproof amber polypropylene bottles (Nalgene, cat. No. 2204-0010) and were instructed on how to collect a 24-hour specimen. They were also provided with appropriate urinals (Henleys Medical Supplies) and funnels (Nalgene, cat. No. 4260-0040) to minimise spillage. An additional, but separate, 5 mL spot specimen of urine was collected at the end of each 24 hour period for comparison. All specimens were brought to the laboratory on completion of the collection.

Figure 2.4. 24-hour urine: 24-hour neopt:creatinine vs. spot neopt:creatinine.
2.6.2 Results

The characteristics of the subjects, their mean levels of neopterin and creatinine excreted per 24 hours, both corrected for weight, and the ratio of the minimum to maximum levels of excretion are presented in Table 2a. Although individual urine samples are not independent of each other, the neopt:creat_{urine} of the 24 hour urine specimen correlated significantly with the neopt:creat_{urine} of the spot urine specimens collected on completion of the 24 hour collection (Fig. 2.4, n = 24, r = 0.75, p<0.001). No correlation was found between 24 hour neopterin excretion and the neopt:creat_{urine} of the spot specimen (Fig. 2.5, n = 24, r = -0.29, p = n.s.).

![Figure 2.5. 24-hour urine: 24-hour urinary neopterin excretion vs. spot neopt:creat_{urine}.](image-url)
Table 2a. Neopterin and creatinine excretion data for 4 subjects undergoing six 24-hour urine collections.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age (yrs.)</th>
<th>Weight (kg)</th>
<th>Mean Urine Volume (L)</th>
<th>Mean Neopterin Excretion μmol/24hrs</th>
<th>Mean Creatinine Excretion g/24hrs *</th>
<th>Mean Neopterin Excretion nmol/kg/24hrs</th>
<th>Mean Creatinine Excretion mg/kg/24hrs #</th>
<th>Min:Max Neopterin Excretion Ratio</th>
<th>Min:Max Creatinine Excretion Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK</td>
<td>M</td>
<td>42</td>
<td>76</td>
<td>2.000 (1.170-2.695)</td>
<td>2.48 (1.71-3.05)</td>
<td>1.65 (1.33-1.96)</td>
<td>32.7 (22.5-40.1)</td>
<td>21.7 (17.5-25.8)</td>
<td>56%</td>
<td>68%</td>
</tr>
<tr>
<td>GG</td>
<td>M</td>
<td>29</td>
<td>86</td>
<td>1.505 (1.010-1.740)</td>
<td>2.16 (1.42-2.62)</td>
<td>2.13 (1.98-2.23)</td>
<td>25.1 (16.6-30.5)</td>
<td>24.8 (23.1-25.9)</td>
<td>54%</td>
<td>89%</td>
</tr>
<tr>
<td>JT</td>
<td>F</td>
<td>43</td>
<td>50</td>
<td>1.830 (1.380-2.365)</td>
<td>1.22 (0.69-1.63)</td>
<td>0.70 (0.51-0.90)</td>
<td>24.4 (13.8-32.3)</td>
<td>13.6 (9.9-18.4)</td>
<td>42%</td>
<td>53%</td>
</tr>
<tr>
<td>CG</td>
<td>F</td>
<td>29</td>
<td>64</td>
<td>2.050 (1.690-2.775)</td>
<td>1.86 (1.38-2.47)</td>
<td>1.52 (1.27-1.81)</td>
<td>29.0 (21.6-38.6)</td>
<td>23.8 (19.8-28.3)</td>
<td>56%</td>
<td>70%</td>
</tr>
</tbody>
</table>

* Normal Values: Males = 1.0-2.0 g/24hrs, Females = 0.8-1.8 g/24hrs (Laposata, 1992).

# Normal Values: Males = 20-26 mg/kg/24hrs, Females = 14-22 mg/kg/24hrs (Laposata, 1992).

^ Normal Values > 85%. A value of less than 86% implies inaccurate 24 hour urine collections (Orth, 1995).
2.6.3 Conclusion

The fact that the neopt:creat:urine of the spot specimen correlates with the neopt:creat:urine of the 24-hour urine specimen (Fig. 2.4), but not total neopterin excretion (Fig. 2.5) suggests that the 24 hour urine samples were not collected reliably. This is borne out by the wide variation between the minimum and maximum creatinine excretion (Table 2a). In 3 out of 4 subjects this difference is less than 85%, a value above which is recommended to ensure accuracy of the 24 hour urine collections (Orth, 1995). The decision was therefore made not to use 24-hour urine collections to determine neopterin production, but to rather use spot specimens.

2.7 Affect of collection time on urinary neopterin excretion - diurnal rhythm

A previous report on 5 healthy young male subjects, demonstrated that neopterin production, and hence urinary neopterin excretion follows a diurnal pattern with maximal production between 2h00 and 6h00 (Auzéby et al. 1988). This study used total urine production over six 4-hour periods during the day to define the diurnal variation. Therefore a study was performed to assess the effect of diurnal variation on spot urinary neopterin levels, and to study the time dependent variability of neopterin excretion in individuals.

2.7.1 Methods

Six healthy volunteers, with no symptomatic infections, were asked to collect spot urine specimens over a period of 2 to 3 days (40 to 60 hours). Samples were collected as outlined above and the neopt:creat:urine was measured on each sample and related to the time of collection. Each subject’s serial neopt:creat:urine were analysed and plotted against time. The neopt:creat:urine has been expressed as a percentage of the maximum
level measured for each individual subject during the serial collection period. This was done to correct for inter-individual variability in neopt:creatinine levels and allow between-subject comparisons. The day was then divided into 5 time periods so as to separate the urine samples into specific time epochs.

2.7.2 Results

Table 2b summarises the demographic and neopt:creatinine data of six subjects, Fig. 2.6 is a box and whisker scatter plot of the values, and Fig. 2.7 a time course plot of the serial neopt:creatinine for each individual. The females subjects have higher mean neopt:creatinine levels (189.2 µmol/mol (SD 12.2)) than their male counterparts (126.2 µmol/mol (SD 5.4), p = 0.001). These data also demonstrate significant daily variation in neopt:creatinine with an average difference between the daily minimum and

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>No. Samples</th>
<th>Mean µmol/mol</th>
<th>SD µmol/mol</th>
<th>Min µmol/mol</th>
<th>Max µmol/mol</th>
<th>Max:Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG</td>
<td>32</td>
<td>F</td>
<td>9</td>
<td>188</td>
<td>49</td>
<td>128</td>
<td>272</td>
<td>2.1</td>
</tr>
<tr>
<td>CYG</td>
<td>29</td>
<td>F</td>
<td>20</td>
<td>202</td>
<td>26</td>
<td>156</td>
<td>266</td>
<td>1.7</td>
</tr>
<tr>
<td>JR</td>
<td>26</td>
<td>F</td>
<td>12</td>
<td>178</td>
<td>36</td>
<td>133</td>
<td>238</td>
<td>1.8</td>
</tr>
<tr>
<td>GG</td>
<td>29</td>
<td>M</td>
<td>16</td>
<td>122</td>
<td>23</td>
<td>92</td>
<td>165</td>
<td>1.8</td>
</tr>
<tr>
<td>GK</td>
<td>43</td>
<td>M</td>
<td>17</td>
<td>125</td>
<td>20</td>
<td>98</td>
<td>170</td>
<td>1.7</td>
</tr>
<tr>
<td>MR</td>
<td>31</td>
<td>M</td>
<td>18</td>
<td>132</td>
<td>42</td>
<td>71</td>
<td>200</td>
<td>2.8</td>
</tr>
</tbody>
</table>
maximum of 2.0 (range=1.7-2.8). Data on diurnal variation is summarised in Table 2c and plotted as a time course in Fig. 2.8. Although there is a large variation in neopt:creat.urine levels within the defined time epochs, there is a trend to higher neopt:creat.urine between 0h00 and 8h00, and significantly lower levels between 12h00 and 16h00 (p=0.03).

### Table 2c. Summary of diurnal neopt:creat.urine data

<table>
<thead>
<tr>
<th>Time of Day</th>
<th>No. Samples</th>
<th>Mean (%</th>
<th>SD (%)</th>
<th>SEM (%)</th>
<th>Range (%)</th>
<th>T-test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0h00 - 8h00 or awakening</td>
<td>7</td>
<td>75.7</td>
<td>21.8</td>
<td>8.2</td>
<td>(47 - 100)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>8h00 or awakening - 12h00</td>
<td>5</td>
<td>78.7</td>
<td>10.6</td>
<td>4.7</td>
<td>(69 - 95)</td>
<td>n.s</td>
<td></td>
</tr>
<tr>
<td>12h00 - 16h00</td>
<td>8</td>
<td>69.4</td>
<td>20.8</td>
<td>7.4</td>
<td>(35 - 100)</td>
<td>n.s</td>
<td></td>
</tr>
<tr>
<td>16h00 - 20h00</td>
<td>12</td>
<td>60.3</td>
<td>12.0</td>
<td>3.5</td>
<td>(40 - 81)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>20h00 - 24h00</td>
<td>11</td>
<td>75.3</td>
<td>16.8</td>
<td>5.1</td>
<td>(50 - 100)</td>
<td>n.s</td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h00 - 32h00 or awakening</td>
<td>9</td>
<td>77.6</td>
<td>13.8</td>
<td>4.6</td>
<td>(58 - 93)</td>
<td>n.s</td>
<td></td>
</tr>
<tr>
<td>32h00 or awakening - 36h00</td>
<td>7</td>
<td>71.2</td>
<td>13.3</td>
<td>5</td>
<td>(47 - 89)</td>
<td>n.s</td>
<td></td>
</tr>
<tr>
<td>36h00 - 40h00</td>
<td>7</td>
<td>73.9</td>
<td>11.6</td>
<td>4.4</td>
<td>(62 - 96)</td>
<td>n.s</td>
<td></td>
</tr>
<tr>
<td>40h00 - 44h00</td>
<td>9</td>
<td>66.4</td>
<td>11</td>
<td>3.7</td>
<td>(53 - 83)</td>
<td>n.s</td>
<td></td>
</tr>
<tr>
<td>44h00 - 48h00</td>
<td>8</td>
<td>76.4</td>
<td>12.1</td>
<td>4.3</td>
<td>(61 - 100)</td>
<td>n.s</td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48h00 - 56h00 or awakening</td>
<td>6</td>
<td>84.3</td>
<td>8.5</td>
<td>3.5</td>
<td>(76 - 100)</td>
<td>n.s</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.6. Diurnal variation: combined box and whisker scatter plot of the serial neopt:creat.urine of 6 normal volunteers collected over a period of 3 days. The box represents the 25th-75th quartile divided by the median, the whiskers the range, and the adjacent scatter plot the individual values.

Figure 2.7. Diurnal Variation: time course plots of serial UNCR collected over 3 days in 6 normal individuals. Data is summarised in Table 2b and combined in Fig. 2.8.
2.7.3 Conclusion

These results confirm that a diurnal variation in urinary neopterin excretion exists, with the highest excretion occurring between 0h00 and 8h00, and the lowest between 12h00 and 16h00. This diurnal variation is also responsible for large fluctuations in neopt:creatinine levels observed during the course of the day. To minimise this effect and to maximise neopt:creatinine levels for clinical studies it was decided to collect early morning spot urine specimens.

2.8 Normal Values

2.8.1 Methods

To establish a normal range for neopt:creatinine 40 adult subjects (20 females and 20 males, mean age = 36.4 ± S.D. 10.6 years [range = 20 - 57]) were recruited from the
general population and asked to collect an early morning spot urine specimen. The specimen was protected from light and frozen as soon as possible. No subject had any underlying medical problems and had to be free of any symptoms of infection on the day of collection.

**Figure 2.9. Normal control data: urinary neopterin, creatinine and neopt:creatinine in 20 normal female (●) and 20 normal male (■) subjects.**

### 2.8.2 Results

The neopt:creatinine of female control subjects was significantly greater (1.2 times) than their male counterparts (females = 129 ± S.D. 43 µmol/mol vs. males = 105 ± S.D. 37 µmol/mol, p = 0.04, Fig. 2.9). This was due to a significantly lower urinary creatinine concentration (0.75 times lower) in female subjects compared to males (females = 9.1 ± S.D. 3.8 mmol/L vs. males = 12.2 ± S.D. 3.3 mmol/L, p < 0.01, Fig. 2.9). Urinary neopterin levels did not differ between female and male subjects.
(females = 1.23 ± S.D. 0.75 mmol/L vs. males = 1.34 ± S.D. 0.68 mmol/L, p = n.s.,
Fig. 2.9).

2.8.3 Conclusion

The neopt:creατ.urine is significantly higher in females due to their lower muscle mass and hence lower urinary creatinine excretion. The factor of 1.2 obtained in these control subjects corresponds exactly to that already published in the literature (Fuchs et al., 1992). For the purposes of the clinical studies in the remainder of this thesis the upper limit of normal was set at the mean plus 2.5 S.D., i.e. = 200 µmol/mol for males.

To allow for direct comparisons between groups of subjects with varying proportions of male and female subjects, all female neopt:creατ.urine results were divided by the empirical factor of 1.2 obtained from these data.

2.9 Urinary Tract Infections – determination of urinary nitrite

2.9.1 Background

Patients with MS often have bladder dysfunction, leading to poor bladder emptying with an increased residual urine volume. Bacterial colonisation and infection of the lower urinary tract often complicates this. Common bacterial pathogens catalyse the reduction of urinary nitrate to nitrite. Finding an increased urinary nitrite concentration is a useful screening test for detecting bacterial colonisation of the urinary tract (Hurlbut & Littenberg, 1991; Lachs et al., 1992). In routine clinical practice a dipstick nitrite assay is commonly employed (e.g. N-multistix®, Ames). To check for possible bacterial colonisation of the urinary tract in these studies urine specimens were screened using a simple nitrite assay adapted for screening large numbers of samples. Increased urinary nitrite levels lack diagnostic sensitivity
however, as not all bacteria are nitrate reducing. Urinary nitrate and nitrite are also derived from dietary intake (Egberts and Soederhuizen, 1996), enteric bacterial metabolism (Mitchell et al, 1916; Tannebaum et al., 1978; Kurzer and Calloway, 1981) and the end-products of endogenous $\cdot N=O$ metabolism (Stuehr and Marletta, 1985; Beckman et al., 1990). The majority of urinary nitric oxides are in the form of nitrate. Nitrite can be measured using a simple colormetric reaction (Griess Reaction) (Vogal, 1959). Other methods for measuring nitrite include reverse phase (Green et al, 1982; Zangerle et al., 1995) and ion exchange high performance liquid chromatography (HPLC) (Michigami et al., 1989), gas chromatography with mass spectrometry (Tsikas et al., 1994), a biochemical fluorescence technique (Misko et al., 1993) and capillary electrophoresis (Leone et al., 1994; Meulemans and Delsenne, 1994).

2.9.2 Aim

To establish the value of raised urinary nitrite, as measured by the Griess reaction, in predicting the presence of UTI in patients with culture positive urinary tract infections.

2.9.3 Method

Urine samples sent for diagnostic microscopy and culture were prospectively collected and frozen by Mr K. Miles in the Department of Medical Microbiology, National Hospital for Neurology and Neurosurgery. Urinary nitrite was measured, using the Griess reaction (Vogal, 1959). Seventy-eight urine samples with a bacterial colony forming unit (CFU) count of greater than $100 \times 10^5$/mL were studied. Urinary
nitrite was also measured in 65 healthy volunteers recruited from the general public without symptoms of urinary tract infection.

2.9.3.1 Griess reaction

The Griess reaction is a simple colorimetric reaction between nitrite, sulfanilamide and N-(1-naphthyl) ethylenediamine to produce a pink/magenta azo product with a maximum absorbance at 543nm (Fig. 2.10).

\[
\text{NO}_2^- \quad \text{Nitrite} \quad + \quad \text{Sulfanilamide} \quad \xrightarrow{H^+} \quad \text{Azo product} \quad (\lambda_{\text{max}} = 543\text{nm})
\]

Figure 2.10. Griess reaction: a summary of the biochemical steps involved in the determination of nitrite using the Griess reaction.

2.9.3.2 Reagents and storage

Stock nitrite calibration standards (concentration range = 1000 µM) were prepared by diluting sodium nitrite (Sigma, product number S2252) in reagent grade water and storing them at -20°C. Griess reagent 1, 1% w/v sulfanilamide (Sigma, product number S9251) in 5% w/v concentrated phosphoric acid and Griess reagent 2, 0.1% w/v N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma, product number N9125) in water were stable for several months at 4°C in light proof containers.
2.9.3.3 Procedure

The assay was performed in a standard flat-bottomed 96-well polystyrene microtitre plate (e.g. ICN Biomedicals, prod. no. 76-307-05). A sodium nitrite standard curve, doubling-dilutions from 100μM down to 1.5μM, or urine samples diluted 1 in 200 with PBS were prepared. A volume of 50μL/well was used and the assay was blanked against PBS. Equivalent volumes (50μL) of Griess reagent 1 and 2 were added to the reaction mixture, and incubated at room temperature for approximately 10 minutes. The plate was then read using an ELISA plate reader (Anthos 2000) at an absorbance wavelength of 540nm.

2.9.3.4 Assay Performance

The standard curve and a doubling dilution of urine sample obtained from a normal control subject showed parallelism. The mean nitrite recovery after spiking 12 urine samples with a fixed quantities of sodium nitrite was 99.6% (range = 92-109%). The precision data is summarised in Table 2.d. The within-run precision was determined by measuring the coefficient of variation for 40 urine samples done in duplicate and the between batch variation by 4 samples, 2 with a high and 2 with a low nitrite concentration, measured in 6 different runs. As set out above the functional range of

<table>
<thead>
<tr>
<th>Concentration Range</th>
<th>Intra-assay CV</th>
<th>Inter-assay CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 - 10 μM</td>
<td>3.6%</td>
<td>3.8%</td>
</tr>
<tr>
<td>40 - 50 μM</td>
<td>3.9%</td>
<td>4.1%</td>
</tr>
<tr>
<td>2.5 - 50 μM</td>
<td>3.7%</td>
<td>-</td>
</tr>
</tbody>
</table>
this assay is 1.5-100μM, which means all urine samples have detectable levels of nitrite.

2.9.4 Results

Patients with greater than $100 \times 10^5$ bacterial CFU/mL had significantly higher urinary nitrite concentrations than normal control subjects (median urinary nitrite [25th-75th%] = 2937 [1002-4494] μmol/L vs. 463 [345-671] μmol/L, p<0.001, Fig. 2.11). Fifty-two out of 78 patients (67%) compared to only 2 out of 65 normal control subjects (3%) had urinary nitrite levels above 1375 μmol/L, i.e. greater than 2.5 standard deviations above the mean of the normal control subjects. Using 2.5 standard deviations above the mean of the normal control subjects as a cut-off, the positive

![Image of Figure 2.11]

**Figure 2.11. Urinary nitrite: natural logarithm of the urinary nitrite level in normal control subjects (●) and patients with urinary tract infections (■). The box represents the 25th-75th quartile divided by the median, the whiskers the range, and the adjacent scatter plot the individual values. The horizontal dotted line represents the mean + 2.5 S.D. of the normal control subjects.**
predictive value of a raised urinary nitrite level is 96% and the negative predictive value of a normal urinary nitrite level 71%.

2.9.5 Conclusion

Urinary nitrites can be reliably measured on frozen urine samples using the Griess reaction. Finding raised urinary nitrites levels in frozen urine specimens is a reliable indicator of bacterial colonisation of the urinary tract, with a positive predictive value of over 95%. However, the negative predictive value of urinary nitrites is poor and results in approximately a third of culture positive cases being missed. This finding is in concordance with published results using urinary dipstick nitrite assays (Hurlbut & Littenberg, 1991; Lachs et al., 1992). Although not ideal, the urinary nitrite level provides a simple screen for possible bacterial colonisation of the urinary tract.

2.10 HPLC for the detection of neopterin in tissue culture supernatants

2.10.1 Method

All tissue culture supernatants were treated by acid oxidation to convert dihydroneopterin to neopterin and to simultaneously deproteinise samples by acid precipitation (Ruiz-Vázquez et al., 1991). All supernatants were treated immediately after harvesting to prevent degradation of dihydroneopterin. As with the urine samples, precautions were taken to prevent unnecessary exposure of cell culture supernatants to UV-light. To 300μL of harvested tissue culture supernatant was added 25μL of 60% perchloric acid (HClO₄, Analar® BDH prod. no. 10176) and 25μL of 3% iodine solution (I₂/KI, Convol® BDH prod. no. 18008). Samples were vortexed
and incubated in the dark for 60 minutes. Following this 150μL of 2% ascorbic acid and 400μL 1M K₂HPO₄ (HiPerSolv® BDH prod.no. 15319) in reagent grade water, were added to reduce any excess iodine and to neutralise the excess perchloric acid respectively. The samples were then re-vortexed and incubated for a further 30 minutes and finally centrifuged at 14 000g for 10 minutes (Eppendorf micro-centrifuge). The supernatants were then removed for analysis or storage at -20°C. An alternative method using activated manganese dioxide powder (MnO₂, Sigma M1656) (Niederwieser et al., 1982) as the oxidising agent gave similar results to the iodine oxidation technique (data not shown). The iodine oxidation method was preferred, as it is simpler and much easier to perform than the MnO₂ powder technique.

HPLC was performed as for urine samples except that a 100μL sample loop was required and the mobile phase was not re-circulated because of the significant contamination that occurs during a run of treated samples. In tissue culture experiments the quantities of neopterin in the supernatants vary widely, and samples with high levels of neopterin required dilution and/or adjustment of the detector sensitivity to rectify this. Cell culture supernatants were “dirtier” than urine samples and because of the larger injection volumes shortened the life of both the HPLC pre-columns and columns significantly (approximately 400-600 injections/column).

2.10.1.1 Assay Performance

The lower limit of detection with the fluorescence detector set to maximum sensitivity with an injection volume of 100μL, was 1.25nM. This was the absolute lower limit, which after taking the dilutional effects of sample preparation into account, translated
to a level of 2.5-5nmol/L. The recovery of neopterin from 20 samples (RPMI tissue culture fluid with 5% foetal calf serum (FCS)) spiked with variable quantities (6.25 - 100nM) of D-neopterin (Sigma, N3386) diluted in distilled water was 87% (81-102%). The intra-batch coefficient of variation for 20 samples done in duplicate is 9.8%, and the inter-batch coefficient of variation for 2 samples assayed in 9 runs is 13.4%. A dilution curve demonstrated parallelism.

2.10.2 Effect of Protein on Neopterin Recovery

![Graph showing the effect of varying concentrations of bovine serum albumin on the recovery of neopterin after acid oxidation and deproteinisation.](image)

**Figure 2.12. Protein effects: effect of varying concentrations of bovine serum albumin on the recovery of neopterin after acid oxidation and deproteinisation. (n = 3, error bars = s.d., ** p <= 0.05)**

As the recovery of neopterin from cell culture fluid containing protein was incomplete, an experiment was designed to see whether the concentration of protein affected the recovery of neopterin.

2.10.2.1 Method

Samples of PBS containing 4 μmol/L D-neopterin (Shircks Lab., prod. no. 11’303), 2μM of 7,8-dihydro-d-neopterin (Shircks Lab., prod. no. 11’306), and various
concentrations of bovine serum albumin (BSA, Sigma, prod. no. A2153) were
prepared. Triplicate samples were then treated as above and the recovery calculated.

2.10.2.2 Results

The results are summarised in Table 2e and Fig. 2.12.

Table 2e Effects of protein concentration on neopterin recovery

<table>
<thead>
<tr>
<th>BSA g/L</th>
<th>Mean neopterin μM (n = 3)</th>
<th>S.D.</th>
<th>Mean Recovery % (n = 3)</th>
<th>T-test (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No acid oxidation</td>
<td>3.6</td>
<td>0.2</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>Acid Oxidation</td>
<td>0</td>
<td>8.7</td>
<td>0.3</td>
<td>109%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7.8</td>
<td>0.2</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.4</td>
<td>0.2</td>
<td>105%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.8</td>
<td>0.2</td>
<td>110%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.1</td>
<td>0.7</td>
<td>102%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.6</td>
<td>0.4</td>
<td>83%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.4</td>
<td>0.4</td>
<td>79%</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>6.4</td>
<td>1.0</td>
<td>79%</td>
</tr>
</tbody>
</table>

2.10.2.3 Conclusions

Acid oxidation and deproteinisation of samples with high concentrations of protein
(>4g/L) significantly reduced the recovery of neopterin and dihydroneopterin. This
has important implications for measuring neopterin in biological samples with varying
concentrations of protein. This however does not affect the interpretation of cell
culture experiments in which control samples, with similar concentrations of protein,
were used for comparison.
Chapter 3 - Cytokine control of neopterin production by human monocytes

3.1 Background

Cells of the human monocyte lineage produce neopterin in response to interferons (IFN) type I (α and β) (Henderson et al., 1991; Huber et al., 1984) and type II (γ) (Huber et al., 1984). However, IFNγ is more potent (Huber et al., 1984) and appears to be permissive in allowing other pro-inflammatory cytokines, such as IL1 (Kasai et al., 1995) and TNFα (Werner et al., 1991), to augment neopterin production. Bacterial lipopolysaccharide (LPS) also stimulates neopterin production and greatly augments the production of neopterin induced by IFNγ (Hattori and Gross, 1993). Phorbol esters, such as phorbol myristate acetate (PMA), also induce monocyte neopterin production (Seidl et al., 1986). They do not, however, require the permissive action of interferons as they activate protein kinase C, a signaling pathway distal to the cytokine receptors. Apart from the molecules listed above the effects of other cytokines on monocyte neopterin production, such as GMCSF, IL4, IL6, IL10, IL13 and TGFβ, have been poorly studied and are largely unknown. One study, which assessed the effect of IL4 on monocyte neopterin production found that it reduced unstimulated monocyte neopterin production but had little effect on LPS- and IFNγ- induced neopterin production (Moutabarrik et al. 1992).
3.2 Aims

The primary aim of the cell culture work presented in this chapter is to define the effects of the cytokines GMCSF, IL4, IL6, IL10, IL13 and TGFβ, as well as their interactions with pro-inflammatory cytokines, on the production of neopterin by monocytic cells of human origin. In addition the effects of immunoglobulin Fc-receptor stimulation, Fc-receptor mediated phagocytosis and non-Fc-mediated myelin phagocytosis on monocyte neopterin production were also studied.

3.3 Methods

3.3.1 Cells

All cell culture experiments were performed on either the monocytic THP1 cell line or monocytes.

3.3.2 THP1 cells

THP1 cells were obtained from the European collection of cell cultures (ECACC no. 88081201). THP1 cells are a CD14+ promonocytic leukaemic cell line, which have been extensively studied with regard to tetrahydrobiopterin and neopterin metabolism. They are known to produce neopterin in response to IFNγ stimulation (Werner et al., 1990; Werner-Felmayer et al., 1990). Stocks of the cells were preserved in 10% DMSO and 90% FCS and stored at -80°C. An experimental working stock of the cell line was maintained in RPMI with L-glutamine (Biowhitaker, Cat. no 5MB0102), containing 5% heat inactivated FCS (FCS, Sigma, Cat. no. F-7524), 100U/mL penicillin and 100μg/mL streptomycin (Biowhitaker, Cat. no 17-602E), at 37°C in the presence of 5%
CO\textsubscript{2}. The cells were fed 2-3 times/week as required and split weekly. For these experiments THP1 cells were used in their phase of logarithmic growth.

3.3.3 Monocytes

Enriched human peripheral blood mononuclear cells were isolated from the citrated buffy coats of normal human blood donors (London Blood Transfusion Service), by sucrose density gradient centrifugation and centrifugal elutriation.

3.3.3.1 Monocyte preparation

3.3.4.2 Density gradient centrifugation

In a 200mL tissue culture flask (Falcon) fresh buffy coats (less than 24 hours old) with an average volume of 40-60mL, were diluted to a final volume of 100mL with Hanks balanced salt solution (HBSS) containing no Ca\textsuperscript{2+} or Mg\textsuperscript{2+} (Biowhitaker, Cat.no 10-543F). Twenty five mL of the diluted buffy coat was then layered on to 20mL of Lymphoprep in each of four 50mL conical centrifuge tubes. To maximize cell recovery, the tissue culture flask was rinsed once with 20mL of the HBSS and a further 5mL added to each centrifuge tube. The tubes were then centrifuged at 2100g for 25 minutes at 10°C. After centrifugation the peripheral blood mononuclear cells (PBMC) were gently removed from the surface of the ficol by pipette, washed 3 times in HBSS, resuspended in 50mL of RPMI containing 0.5% FCS, 100U/mL penicillin G and 100μg/mL of streptomycin and placed on ice ready for elutriation.

3.3.4.3 Centrifugal elutriation

Counterflow centrifugal elutriation was performed using the laboratory’s standard protocol on a JE-MC elutriator system (Beckmann Instruments Inc., Palo Alto,CA). A strict protocol to avoid contamination was followed. After use, the JE-MC elutriator system was washed in sterile water and the centrifuge chamber autoclaved at 130°C for
60 minutes. The tubing system and bubble chamber were primed for storage with 70% ethanol. The ethanol was only removed prior to assembling the system. After assembly, the system was flushed with approximately 400mL of a 1% solution of E-Toxo-Clean (Sigma, Cat. No. 210-3) to limit and hopefully prevent LPS contamination. The system was then rinsed with double distilled sterile water to wash out any residual E-Toxo-Clean solution. The system was then primed with RPMI (containing 0.5% FCS,00U/mL penicillin G and 100µg/mL of streptomycin). Prior to starting elutriation the system was checked to ensure that it was free from air-bubbles and leaks. The pump was then calibrated manually to ensure an accurate flow rate. With the centrifuge running at a constant rate (2500rpm) cells were pumped into the elutriation chamber at an initial flow rate of 12 mL/minute. The rate was increased in a stepwise fashion (1mL/min increments) and at each flow rate fractions of fluid containing elutriated cells were collected. The 6 monocyte-rich fractions collected at flow rates from 18 to 23 mL/min were then pooled and resuspended in RPMI with L-glutamine (Biowhitaker, Cat.no 5MB0102), 10% bovine foetal calf serum (FCS, Sigma, Cat. no. F-7524), 100 U/mL penicillin and 100µg/mL streptomycin (Biowhitaker, Cat. no 17-602E).

3.3.4.4 Monocyte Recovery and Purity

The number of elutriated cells recovered from each buffy coat varied. In these experiments the mean was $68 \times 10^6$ (range = $34 - 108 \times 10^6$). The purity of the elutriated monocytes was assessed using fluorescence labeled anti-CD14 mAb staining (Becton Dickinson, Mountain View, CA View) and cytofluorometric analysis (FACScanV, Beckton Dickinson) according to standard laboratory protocols. The FACS analysis for these studies was kindly performed by Ms P Green. The proportion of isolated monocytes (CD14+ cells) in the enriched monocyte population varied: in these experiments the mean was 76% (range = 54 - 86%).
3.3.4 Cell Viability and Counting

Cell viability was assessed using trypan blue dye exclusion using a manual counting technique (Freshney, 1992). In all experiments it was greater than 95%.

3.3.5 Experimental design

All experiments were performed in either 96-well or 48-well tissue culture plates (Falcon). A final cell density of $1 \times 10^6$ cells/mL was used in a volume of 250 μL of culture fluid per well in the 96-well plates and 500μL per well in the 48-well plates. THP1 cells and monocytes are cultured in RPMI containing 5% or 10% FCS respectively, 100U/mL of penicillin G and 100μg/mL of streptomycin. Experiments confirming previously published observations were done at least twice. In the case of new observations all experiments were repeated at least 3 times to ensure reproducible results. Within each experiment the experimental conditions were tested in triplicate to allow appropriate statistical comparisons. All reagents were filtered using either a 0.2 or 0.45 μM membrane filters (Sartorius, Minisart® Cat. No. SM16534) to remove any particulate matter. Unless otherwise stated all reagents used in experiments were LPS free. LPS screening was done using a commercial amebocyte lysate assay (BioWhittaker, Cat. no. 50-648U). This assay was performed routinely by Ms P Green on all new reagents used in the laboratory. All the cytokines and commercial reagents used in these cell culture experiments are listed in Table 3a.
3.3.6 Sample preparation

Cell culture supernatants were harvested from individual wells after spinning the plates at 1500g for five minutes. Using the methods described in Chapter 2, acid oxidation and deproteinisation of specimens was done immediately after the supernatants were harvested. The remaining supernatants were frozen and stored at -20°C.

3.3.7 Assays

Neopterin was measured using HPLC as described in the Methods (Chapter 2). IL10 and TNFα were measured by Dr S Parry and Ms P Green respectively, using ELISAs developed at the Kennedy Institute of Rheumatology.
Table 3a. Reagents used in the cell culture experiments.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>RHu*</td>
<td>Bender Wien</td>
</tr>
<tr>
<td>IFNβ-1a</td>
<td>RHu</td>
<td>Serono Pharmaceuticals</td>
</tr>
<tr>
<td>TNFα</td>
<td>Rhu</td>
<td>Centre of Molecular and Macromolecular Studies</td>
</tr>
<tr>
<td>IL1α</td>
<td>RHu</td>
<td>Hoffman La-Roche</td>
</tr>
<tr>
<td>IL4</td>
<td>RHu</td>
<td>Sandoz</td>
</tr>
<tr>
<td>IL10</td>
<td>RHu</td>
<td>Schering-Plough</td>
</tr>
<tr>
<td>IL13</td>
<td>RHu</td>
<td>DNAX</td>
</tr>
<tr>
<td>GMCSF</td>
<td>RHu</td>
<td>Behringwerke AG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutralising anti-TNFα Ab</td>
<td>Centocor</td>
<td></td>
</tr>
<tr>
<td>IL1 receptor antagonist</td>
<td>R&amp;D Europe</td>
<td></td>
</tr>
<tr>
<td>Neutralising anti-IL10 Ab</td>
<td>Schering-Plough</td>
<td></td>
</tr>
<tr>
<td>Neutralising anti-TGF Ab</td>
<td>R&amp;D Europe</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Sepiapterin</td>
<td></td>
<td>Schirks Laboratories</td>
</tr>
</tbody>
</table>

RHu = recombinant human,

3.3.8 Statistics

Statistical significance was determined using a paired two-tailed Student’s t-test. A p-value of less than 0.05 was considered significant.
3.3.9 Cell survival and viability

Under experimental conditions cell viability, using trypan blue dye exclusion, drops off significantly with time for both THP1 cells and monocytes. This is a particular problem after 72 hours (Fig. 3.1). Cell death occurs sooner and to a much greater extent in monocytes than in THP1 cells. The poor viability of monocytes can be improved by culturing the cells in the presence of GMCSF, which probably affects neopterin production (see later).

![Graph showing cell viability over time](image)

**Figure 3.1. Cell Viability:** the time-course of cell viability, as assessed by trypan blue dye exclusion, of THP1 cells and monocytes in culture under standard experimental conditions. (Error bars = s.d., *P < 0.01, **P < 0.001 - unpaired two-tailed T-test, p values calculated relative to time = 0 hours).
3.3.10 Time-course of neopterin production

In time-course experiments IFNγ-stimulated THP 1 cells and monocytes produce detectable levels of neopterin from approximately 18 hours which peak at 72 hours (Fig. 3.2). The plateau in neopterin production after 72 hours is possibly due to a decrease in cell viability (see Fig. 3.1) or as a result of a drop-off in activity of IFNγ. Due to the poor viability of THP1 cells and monocytes beyond 72 hours, all experiments had to be performed within the maximum incubation period of 72 hours. Where possible neopterin production was assessed at 72 hours corresponding to the peak in its production. However when the experimental protocol required a period of preincubation prior to IFNγ stimulation, neopterin production was assessed at 48 hours post-stimulation.

![Figure 3.2. Time-course: the time-course of IFNγ-induced (100 U/mL) neopterin production in THP1 cells.](image)
3.4 Interferon-gamma (IFNγ)

3.4.1 Background immunology of IFNγ

IFNγ is a multifunctional cytokine produced by Th1 CD4+ lymphocytes, γδ T cells, CD8+ T lymphocytes and natural killer cells. It has documented antiviral, antiprotozoal and immunomodulatory activities (Locksley and Scott, 1991; Billiau and Dijkmans, 1990). The immunomodulatory activities of IFNγ are diverse. It is a potent inducer of macrophage effector functions, upregulating MHC class I and II, as well as Fc receptor expression and increasing the efficiency of macrophage-mediated intracellular killing (Farrar and Schreiber, 1993). IFNγ increases macrophage production of IL1, platelet activating factor, hydrogen peroxide and neopterin. It protects monocytes from cell-mediated lysis and activates monocytes to a cytotoxic state by downregulating TGFβ receptor expression and upregulating the expression of the IL2Rγ subunit (Billiau and Dijkmans, 1990; Sen and Lengyel, 1992; Gusella et al., 1993; Bulut et al., 1993; Espinoza-Delgado, 1994; Bosco et al., 1994). IFNγ has been reported to induce its own expression; IFNγ produced locally as a result of inflammation results in the induction of IFNγ mRNA at distant sites (Halloran et al., 1992). IFNγ also acts on endothelial cells upregulating ICAM-1, but not E-selectin or VCAM-1, expression (Thornhill et al., 1992). IFNγ also affects B cell function enhancing IgG1 and IgG3 secretion (Billiau, 1996). IFNγ activity is mediated by the induction of numerous proteins, some of which are specific to IFNγ (Weil et al., 1983; Harris et al., 1992, Billiau, 1996) and include the enzymes GTP cyclohydrolase I (Werner et al., 1990), iNOS (Tayeh and Marletta, 1989) and indoleamine 2,3-dioxygenase (Sen and Lengyel, 1992).
The IFN\(\gamma\) receptor is separate from the type I interferon (IFN\(\alpha\) and IFN\(\beta\)) receptors. It is composed of two separate transmembrane subunits; one located on chromosome 6 (Rashidbaigi et al., 1986; Pfizenmaier et al., 1988; Aguet et al., 1988) and the other on chromosome 21. The latter subunit is required for IFN\(\gamma\) signaling (Hemmi et al., 1994; Soh et al., 1994) and requires two protein tyrosine kinases, JAK1 and JAK2 (Muller et al., 1993; Watling et al., 1993).

### 3.4.2 Results - IFN\(\gamma\)

In general, unstimulated THP1 cells and monocytes do not produce detectable levels of neopterin (Fig. 3.3). However, monocytes isolated from occasional donors spontaneously produce detectable quantities of neopterin (Figs. 3.23, 3.27 and 3.28). These monocytes are presumably preactivated; possibly by endogenous IFN\(\gamma\) production as a consequence of sub-clinical viral infection, or by uncontrolled effects due to the in vitro handling and preparation of the monocytes. IFN\(\gamma\) produces a reproducible dose-responsive increase in neopterin production by both THP1 cells and monocytes (Fig. 3.3). In the absence of other exogenous co-stimulatory signals, such as

![Figure 3.3. IFN\(\gamma\): 72-hour dose response curve of IFN\(\gamma\)-induced neopterin production by THP1 cells and monocytes. (Error bars = s.d.)](image)
TNFα, IL1 and LPS (see later), 10U/mL of IFNγ is the minimal concentration of IFNγ that reproducibly induces detectable levels of neopterin production (>2.5nmol/L).

### 3.4.3 Summary - IFNγ

IFNγ is a major and potent stimulus for the induction of monocyte neopterin production.

### 3.5 Sepiapterin

#### 3.5.1 Background biochemistry of sepiapterin

It has been established in THP1 cells that GTP-cyclohydrolase I activity is inhibited by feedback control of the end-product tetrahydrobiopterin (Fig. 1.7) (Harada et al., 1993).

To confirm this and to demonstrate that THP1 cells and monocytes behave in a similar fashion the effect of tetrahydrobiopterin on IFNγ-induced neopterin production was explored. Sepiapterin is a stable intermediate in the tetrahydrobiopterin synthetic

![Figure 3.4. Sepiapterin: the effect of varying concentrations of sepiapterin on 72 hour IFNγ-induced (100U/mL) neopterin production in THP1 cells and monocytes. (Error bars = s.d., **p<0.001 - unpaired two-tailed T-test).](image)
pathway distal to the functional block in tetrahydrobiopterin synthesis (Fig. 1.7). It is converted to tetrahydrobiopterin by sepiapterin reductase. Sepiapterin is therefore a convenient substrate for bypassing the block in tetrahydrobiopterin synthesis and overcomes the poor stability of tetrahydrobiopterin.

3.5.2 Results

THP1 cells and monocytes were stimulated with IFNγ 100 U/mL in the presence of variable concentrations of sepiapterin. In both THP1 cells and monocytes, sepiapterin inhibited neopterin production in a concentration-dependent fashion (Fig. 3.4).

![Figure 3.5. Sepiapterin: the effect of sepiapterin (50μM) on 72-hour IFNγ-induced monocyte neopterin production. (Error bars = s.d., *p=0.03, **p<0.001 - unpaired two-tailed T-test).](image)

When THP1 cells and monocytes were stimulated with incremental concentrations of IFNγ (10-1000 U/mL) in the presence of a fixed concentrations of sepiapterin (50μM), they behaved in a similar fashion. Sepiapterin reduces neopterin production at all concentrations of IFNγ and inhibits neopterin production completely at an IFNγ
concentration of 10 U/mL. The results from a monocyte experiment are presented in Fig. 3.5.

3.5.3 Summary – sepiapterin

Sepiapterin decreases IFNγ-induced neopterin production in THP1 cells and monocytes.

3.6 Interleukin 1 (IL1) and tumour necrosis factor alpha (TNFα)

3.6.1 Background immunology of IL1

IL1 represents two separate but structurally related proteins, IL1α and IL1β, that are approximately 25% homologous (Oppenheim et al., 1986). They are the products of two separate genes and recognise the same cell surface receptors. IL1 is produced by many cells including monocytes (Mallardo, et al., 1994), macrophages (Foss, et al., 1994) and glial cells (oligodendrocytes, astrocytes and microglia) (Bergsteinsdottir, et al., 1991; da Cunha, et al., 1993). IL1α and β are proteins secreted as 31kDa precursors and cleaved by a cysteine protease IL1 converting enzyme (ICE) (Ceretti, et al., 1992; Thornberry, et al., 1992). There are two IL1 receptors; IL1 receptor type 1 (IL1-RI) and type II (IL1-RII). Only IL1-RI is capable of transducing a signal following IL1 binding and all IL1’s biological effects are produced via this receptor (Sims, et al., 1993; Stylianou, et al., 1992). IL1-RII acts as a decoy receptor and precursor for the soluble IL1-R (sIL1-R), which antagonises and modulates IL1 activity by binding to IL1 on the cell surface without resulting in signal transduction. The sIL1-R binds free IL1 preventing its activity (Sims et al., 1993). IL1 receptor antagonist (IL1-RA) is a naturally occurring inhibitor of IL1. IL1-RA is produced by monocytes, macrophages, neutrophils and fibroblasts (Arend, 1993). IL1-RA is upregulated by IL1, IL4, IL13,
IL6, IFNγ, GMCSF and TGFβ (Arend, 1993). IL1 production generally occurs as a consequence as of inflammation and is induced by IFNγ, TNFα/β, LPS and IL1 itself (Dinarello, 1992). It is also upregulated during bone formation, the menstrual cycle and in response to nervous system stimulation. IL1 possesses a wide range of biological activities. It induces prostaglandin synthesis, the hepatic acute phase response, production of collagenases, calcium and cartilage resorption in bones. CNS effects include fever (endogenous pyrogen effect), induction of sleep and the release of corticotrophin-releasing factor and adrenocorticotrophin. IL1 plays an important role immunologically. It acts on monocyte/macrophages inducing its own synthesis as well as the production of TNFα and IL6 (Navarro, et al., 1989; Lorre, et al., 1994). It further induces IL2, IL4 and GMSCF production, as well as the expression of IL2 receptors in activated T-cells. IL1 induces B-cell proliferation and maturation and increases immunoglobulin synthesis. IL1 synergises with other cytokines in activating NK cells.

IL1 has been implicated in the immunopathogenesis of CNS inflammation and in the immunopathogenesis of MS (see Chapter 1 - Introduction). IL1 increases the severity of both actively induced and adoptively transferred EAE (Mannie et al., 1987; Jacobs et al., 1991). The administration of sIL1-R and IL1-RA suppresses EAE (Jacobs et al., 1991; Martin and Near, 1995).

3.6.2 Background immunology of tumour necrosis factor (TNF)

TNFα and TNFβ (lymphotoxins) are closely related proteins with significant sequence homology, which bind to the same cell surface receptors. They produce a wide and similar range of biological activities but the regulation, expression and processing of the two cytokines are different (Vilcek and Lee, 1991). TNFα is produced by neutrophils,
activated lymphocytes, macrophages, NK cells, LAK cells, astrocytes, endothelial cells, smooth muscle cells and some transformed cells; TNFβ is produced by lymphocytes (Vilcek and Lee; 1991). TNFβ has a typical signal peptide sequence and is processed and secreted in a manner similar to other secretory proteins (Nedwin et al., 1985). TNFα has a longer precursor sequence with hydrophilic and phobic domains and occurs as a membrane bound or soluble form (Kriegler et al., 1988; Perez et al., 1990). The membrane bound form is found on the surface of monocytes and macrophages, where it serves as a reservoir for release of soluble TNFα, has cytotoxic activity and is involved in intercellular communication (Kriegler et al., 1988; Perez et al., 1990; Aversa et al., 1993; Macchia et al., 1993). There are two TNF receptors, TNF-RI (p55) and TNF-RII (p75), with almost all cells expressing one form or other. The intracellular domains of the two receptors differ and are responsible for different signals (Banner et al., 1993). Truncated soluble forms of the two receptors, which are found in biological fluids, can neutralise the activity of TNF (Vassalli et al., 1992). The soluble receptors may act as natural inhibitors of TNF activity, although at low concentrations they can stabilise and augment some TNF activities (Aderka et al., 1992).

TNF is implicated in the immunopathogenesis of CNS inflammation and multiple sclerosis (see Chapter 1 - Introduction). Inhibitors of TNF activity, which include neutralising monoclonal antibodies and soluble TNF receptors have shown positive effects in EAE (Ruddle et al., 1990; Baker et al., 1994; Selmaj et al., 1995; Klinkert et al., 1997). Other inhibitors of TNF activity such as thalidomide (Goihman-Yahr et al., 1972; Goihman-Yahr et al., 1974) and the phosphodiesterase inhibitors, pentoxifylline (Okuda et al., 1996; Nataf et al., 1993) and rolipram (Genain et al., 1995; Sommer et al., 1995; Sommer et al., 1997) have also shown positive effects in modulating EAE.
However in one study, TNFα knockout mice were more susceptible to MOG-induced EAE and the administration of TNFα reduced the severity of the disease implying that TNFα has an anti-inflammatory role (Liu et al. 1998). In an open labeled trial in two patients with MS, a human murine chimeric neutralising anti-TNFα monoclonal antibody (CA2, Centocor) increased the number of active lesions on MR (van Oosten et al. 1996) and a trial of soluble dimeric TNF-R55 coupled to the Fc portion of human IgG1 (Roche) was shown to increase disease activity (unpublished). It appears that TNFα may be both pro- and anti-inflammatory in CNS inflammation.
3.6.3 Results

3.6.3.1 Exogenous IL1β and TNFα

THP1 cells and monocytes only produced small quantities of neopterin in response to IL1 and TNFα and then only at relatively high concentrations (Fig. 3.6 and Fig. 3.7). However in the presence of IFNγ, both IL1 and TNFα greatly augmented neopterin production (Fig. 3.6 and Fig. 3.7).

In THP1 cells, the synergism of TNFα and IL1 with IFNγ could be demonstrated at a concentration of IFNγ (0.1 U/mL), two orders of magnitude lower than the usual concentration required (10 U/mL) to induce neopterin production in the absence of TNFα or IL1 (Fig. 3.8).

![Graph showing neopterin production](image)

**Fig. 3.6. IL1β: the effect of IL1β on 72-hour IFNγ-induced neopterin production in monocytes. (Error bars = s.d., **p<0.008).**
Fig. 3.7. TNFα: the effect of TNFα on 72-hour IFNγ-induced neopterin production in monocytes. (Error bars = s.d., **p<0.001).

Figure 3.8. IL1β and TNFα: the effect of IL1β and TNFα at variable concentrations of IFNγ on 72-hour neopterin production in monocytes. (Error bars = s.d., *p<0.05, **p<0.008).
3.6.3.2 Endogenous IL1 and TNFα

To see if endogenously produced TNFα and IL1, which are both induced by IFNγ albeit in small quantities, are responsible for some of the IFNγ-induced monocyte production of neopterin via an autocrine mechanism, monocytes were cultured in the presence of 10μg/mL of neutralising anti-human TNFα (CA2, Centocor) and 10μg/mL of recombinant ILRA (Fig. 3.9). TNFα and IL1 autocrine stimulation varied according to the degree of IFNγ stimulation. TNFα and IL1 inhibition reduced IFNγ-induced neopterin production by 19%, 26% and 42% at 100U/mL, 500U/mL and 1000U/mL of IFNγ respectively (Fig. 3.9).

3.6.4 Summary – IL1β and TNFα

IL1β and TNFα have little effect on their own but significantly augment IFNγ-induced neopterin production in both THP-1 cells and monocytes.

![Graph](image)

**Figure 3.9. IL1β and TNFα: the effect of inhibiting TNFα and IL1 activity with 10μg/mL of neutralising anti-TNFα (CA2, Centocor) and 10μg/mL of IL1-RA, on 72-hour IFNγ-induced neopterin production in monocytes.** (Error bars = s.d., *p=0.03, **p=0.006).
3.7 Granulocyte macrophage colony stimulating factor (GMCSF)

3.7.1 Background immunology of GMCSF

GMCSF stimulates the proliferation, maturation and function of haematopoetic cells. It is produced by a wide variety of cell types which include T cells, B cells, macrophages, mast cells, endothelial cells and fibroblasts (Moore, 1991). GMCSF stimulates neutrophil, monocyte/macrophage and eosinophil colony formation. It is responsible for inducing proliferation and differentiation of progenitors. On mature haematopoetic cells, GMCSF enhances the function of differentiated cells and prolongs survival in vitro (Robin et al., 1991; Dimri et al., 1994). In addition, GMCSF induces the synthesis and release of a number of cytokines, including IL1, IL8 and TNFα from monocytes (Sisson and Dinarello, 1988; Takahashi et al., 1993). GMCSF is a pleiotropic cytokine produced in inflammatory reactions. Increased CSF levels of GMCSF have been described in patients with RR MS during clinical relapse (Perrella et al., 1993).

3.7.2 Results

THPl cells and macrophages fail to produce neopterin in response to GMCSF. However, GMCSF did augment the production of IFNγ-induced neopterin production in monocytes (Fig. 3.10). However, this was only significant at high concentrations of GMCSF (100ng/mL). This does not occur in THP1 cells (data not shown). This is probably not due to a direct stimulatory effect of GMCSF on monocytes, but rather as a result of indirect effects due to improved survival of monocytes (greater than 75% viability at 72 hours in the presence of GMCSF, compared to less than 50% viability in the absence of GMCSF, data not shown). In addition GMCSF induces monocyte IL1
and TNFα production (Sisson and Dinarello, 1988), which would also augment neopterin production.

![Graph showing the effect of GMCSF on 72-hour IFNγ-induced neopterin production in monocytes.](image)

**Figure 3.10. GMCSF: the effect of GMCSF on 72-hour IFNγ-induced neopterin production in monocytes.** (Error bars = s.d., *p*<0.02).

### 3.7.3 Summary – GMCSF

GMCSF does not induce monocyte neopterin production. However IFNγ-induced neopterin production is augmented.
3.8 Interleukin 6 (IL6)

3.8.1 Background Immunology

IL6 is a multifunctional cytokine produced by numerous cells including T-cells, B-cells, monocytes/macrophages, astrocytes and endothelial cells. IL6 production is upregulated by mitogens, antigenic stimulation, LPS, IL1, IL2, IFNγ, TNF, PDGF and viral infection (van Snick, 1990). IL4 and IL13 inhibit IL6 production by monocytes but augment IL6 production by human embryonal microglial cells (Sebire et al., 1996). IL6 binds to a high affinity receptor complex consisting of a 80 kDa IL6-binding receptor protein (IL6-R) (Yamasaki et al., 1988; Sugita et al., 1990) and a 130 kDa signal transduction protein (gp130) (Hibi et al., 1990; Saito et al., 1992). A naturally occurring soluble form of IL6-R, which binds IL6 and mediates signaling through the interaction with gp130, is found in biological fluids. A soluble form of gp130 is also present in the serum of normal individuals and is capable of inhibiting gp130 mediated cytokine activities. IL6 has effects on B cell differentiation and antibody production, on cytotoxic T-cell differentiation, on T-cell activation, growth and differentiation and on the induction of IL2 and IL2-R production in T-cells. IL6 synergises with IL3 in promoting haemopoeisis and synergises with IL11 and LIF in the induction of the hepatic acute phase response (Bauman et al., 1984). Other biological activities attributed to IL6 include the differentiation and survival of neuronal cells (Satoh et al., 1988; Hama et al., 1989), the activation of osteoclasts and possible anti-viral activity. Increased CSF levels of IL6 have been found in patients with active MS (Hauser et al., 1990; Maimone et al., 1991) and patients with relapsing remitting MS demonstrate an acute phase response which correlates with clinical and MR markers of inflammatory disease activity (Giovannoni et al., 1996a).
3.8.2 Results

IL6 did not induce neopterin production in monocytes and THP 1 cells and had no effect on IFNγ-induced neopterin production (Fig.3.11).

3.8.3 Summary – IL6

IL6 did not induce, or augment IFNγ-induced, neopterin production.

Figure 3.11. IL6: the effect of IL6 on 72-hour IFNγ-induced neopterin production in monocytes. (Error bars = s.d., n.s. = not significant).
3.9 Bacterial Lipopolysaccharide (LPS)

3.9.1 Background immunology of LPS

Bacterial LPS or endotoxins are the cell wall components of gram negative bacteria. LPS are potent stimuli which activate monocytes and macrophages. LPS binds to a serum protein LPS-binding protein, forming the LPS/LPS-binding protein complex, which in turn binds to the CD14 receptor on monocytes and macrophages (Kielian and Blecha, 1995; Schletter et al., 1995). CD14 is a glycosylphosphatidylinositol (GPI) anchored cell surface receptor that is unable to transduce an intracellular signal directly. However, once the LPS/LPS-binding protein complex binds to CD14, CD14 is capable of interacting with other cell surface molecules that then transduce an activating signal (Schletter et al., 1995). LPS also activates monocytes via a CD14 independent process that has been postulated to occur through the intracellular second messenger ceramide (Wright and Kolesnick, 1995). LPS activation of monocytes and macrophages involves numerous protein kinases (protein tyrosine kinases, mitogen-activated protein kinases, protein kinase C, protein kinase A, ceramide-activated protein kinase) and G-proteins (Sweet and Hume, 1996). Numerous nuclear transcription factors (rel, C/EBP, Ets, Egr, fos and jun) are implicated in the activation of LPS-inducible gene expression (Sweet and Hume, 1996). Although LPS has not been directly implicated in the immunopathogenesis of demyelinating diseases it provides a good control stimulus for investigating monocyte activation.
3.9.2 Results - LPS

![Graph showing neopterin production](image)

**Figure 3.12. LPS: 72-hour LPS-induced neopterin production in the presence and absence of in human monocytes.** (Error bars = s.d., *p=0.01,**p<0.001).

LPS is able to induce moderate levels of neopterin production in both THP1 cells and monocytes and greatly augments IFNγ-induced neopterin production (Fig. 3.12). The effects of LPS are partially mediated by TNFα and IL1. Neutralising TNFα and IL1 activity with neutralizing anti-human TNFα antibodies (CA2) and IL1RA respectively, reduces LPS-induced neopterin production by approximately 50% (Fig. 3.13).

After prolonged culture a batch of THP1 cells became CD14 negative (FACS staining) and failed to produce neopterin in response to LPS, whilst maintaining IFNγ responsiveness (data not shown).
Figure 3.13. LPS: effect of inhibiting IL1 and TNFα activity with IL1RA (10μg/mL) and neutralising anti-human TNFα (10μg/mL, CA2) on 72-hour LPS and LPS plus IFNγ-induced neopterin production in human monocytes. (Error bars = s.d., * p = 0.03, ** p = 0.02).

3.9.3 Summary - LPS

1. These data confirm that LPS is capable of inducing and augmenting IFNγ-induced, neopterin production in monocytes.

2. LPS mediates part of its effects via IL1 and TNFα.

3. CD14 is important for LPS-induced neopterin production.
3.10 Anti-inflammatory cytokines – interleukin-10 (IL10) and transforming growth factor beta (TGFβ)

3.10.1 Background immunology of IL10

IL10 is secreted by T cells (particularly Th2 cells), macrophages and other immune cells (Moore et al., 1993). IL10 is a pleiotropic cytokine with multiple in vitro effects on different cell types. IL10 inhibits the production of a number of cytokines, especially IFNγ by Th1 cells responding to antigen in the presence of antigen presenting cells (Powrie et al., 1993). IL10 inhibits monocyte/macrophage-dependent, antigen-stimulated cytokine synthesis by human peripheral blood mononuclear cells (PBMC) and NK cells (Yssel et al., 1991) as well as monocyte/macrophage dependent antigen-specific T cell proliferation (de Waal et al., 1991). IL10 is also capable of inducing a long-term antigen-specific state of unresponsiveness in human CD4+ T-cells (Groux et al., 1996). IL10 is a potent modulator of monocyte/macrophage function, downregulating the cell-mediated response and suppressing the production of prostaglandin E2 and numerous pro-inflammatory cytokines, including TNFα, IL1 and IL6. IL10 also enhances the release of soluble TNF receptor, inhibits the expression of ICAM-1 and B-7 (Leeuwenberg et al., 1994; Willems et al., 1994) and downregulates MHC class II expression (de Waal et al., 1991). IL10 suppresses the synthesis of superoxide anion and reactive oxygen intermediates (Niro et al., 1992). IL10 is also capable of facilitating macrophage/monocyte function (including upregulation of both IL1-RA and FcγR1) and an increase in antibody-dependent cellular cytotoxicity (Moore et al., 1993). IL10 has effects on B cells; inducing IgA synthesis, selecting for the
secretion of IgG1 and IgG3 subtypes and enhancing B cell survival. IL10 also has documented effects on endothelial cells, mast cells and granulocytes.

IL10 prevents EAE in Lewis rats (Rott et al., 1994). In SJL mice relapses could be prevented by IL10 (Crisi et al., 1995). However in another study IL10 had no effect on, or even caused worsening of, chronic relapsing EAE (Cannella et al., 1996). Preliminary evidence suggests that IL10 has an important role to play in the immunopathogenesis of MS (see Chapter 1 - Introduction).

3.10.2 Background immunology of TGFβ

TGFβ is a pleiotropic group of growth factors, consisting in man of three isoforms (TGFβ1, TGFβ2 and TGFβ3) (Sporn and Roberts, 1992). In most in vitro assays the biological activity of the three isoforms is similar. However, they differ in their potencies and some biological activities. All the isoforms are secreted in an inactive latent form which requires extracellular proteolytic cleavage to become active (Barr, 1991). There are three TGFβ receptors. The type III receptor, which exists as a membrane bound and soluble form, binds TGFβ but does not transmit a signal (Cheifetz et al., 1989; Andres et al., 1989). The formation of an oligomeric complex consisting of receptor type I and II is required for signalling (Wrana et al., 1992). TGFβ receptors are widely expressed and are found on most cell types. A TGFβ effect depends on the cell type and the conditions of growth. TGFβ tends to be stimulatory for cells of mesenchymal origin and inhibitory for cells of epithelial or neuroectodermal origin. TGFβ mediates a wide variety of biological processes related not only to inflammation but also to development, tissue repair and tumourgenesis (Stavnezer, 1995). Potent and varied effects of treatment with TGFβ have been reported for B cells, T helper and
cytotoxic T lymphocytes, thymocytes, NK cells, lymphokine activated killer cells, monocyte/macrophages and other haemopoietic cells (Sporn and Roberts, 1992; Hooper, 1991). The most commonly reported effects of TGFβ on immune cells are inhibitory (Sporn and Roberts, 1992; Hooper, 1991). A number of studies have demonstrated a favorable effect of TGFβ in EAE (Schluesener and Lider, 1989; Johns et al., 1991; Kuruvilla et al., 1991; Racke et al., 1991; Stevens et al., 1994; Fabry et al., 1995). However, in a one reported study the administration of a neutralising antibody to TGFβ1 enhanced the severity of EAE (Johns and Sriram, 1993). A favorable role for TGFβ in the immunopathogenesis of MS has been proposed (see Chapter 1 - Introduction).

![Figure 3.14. IL10: the effect of IL10 on 72-hour IFNγ-induced neopterin production in monocytes.](Error bars = s.d., * p=0.03,**p=0.009).
3.10.3 Results - IL10 and TGFβ2

To prevent significant loss of TGFβ2 activity due to matrix protein binding, dilutions of TGFβ2 are done in gas sterilized (ethylene dioxide) siliconised (Sigmacote®, Sigma - Cat. No. SL-2) microcentrifuge tubes (Eppendorf).

THP1 cells did not respond consistently to IL10 and TGFβ2. Therefore all experiments using these two cytokines were performed on elutriated monocytes only. IL10 (Fig. 3.14) and TGFβ2 (Fig. 3.15) do not induce neopterin production in monocytes. They do however inhibit IFNγ-induced neopterin production, which is dose-responsive (Fig. 3.14 and Fig. 3.15). IL10 (10ng/mL) and TGFβ2 (10ng/mL) are able to suppress the production of neopterin induced at more physiological concentrations of IFNγ, i.e. 10U/mL (Fig. 3.16). Incubating monocytes with IL10 or TGFβ2 for variable periods of time in relation to IFNγ-stimulation demonstrates that the inhibitory effects of IL10

![Graph](image)

**Figure 3.15. TGFβ: The effect of TGFβ2 on 72-hour IFNγ-induced neopterin production in monocytes.** *(Error bars = s.d., *p=0.02,**p=0.007).*
(Fig. 3.17) and TGFβ2 (Fig. 3.18) occurs up to 4 hours after IFNγ stimulation.

**Figure 3.16. IL10 and TGFβ: the effect of IL10 and TGFβ2 on 72-hour neopterin production in monocytes stimulated with various concentrations of IFNγ.** (Error bars = s.d., *p <

**Figure 3.17. IL10: the effect of IL10 (10ng/mL), administered at various times in relation to IFNγ(100U/mL) stimulation, on 48-hour neopterin production in monocytes.** (Error bars = s.d., *p <0.04, **p< 0.003).
Figure 3.18. TGFβ: the effect of TGFβ2 (10ng/mL), administered at various times in relation to IFNγ (100U/mL) stimulation, on 48-hour neopterin production in monocytes.

(Error bars = s.d., *p < 0.03, **p < 0.003).

3.10.4 Summary - IL10 and TGFβ2

IL10 and TGFβ2 inhibit IFNγ-induced neopterin production by human monocytes.
3.11 Interleukin 4 and 13

3.11.1 Background immunology

3.11.1.1 IL4

IL4 is a pleiotropic cytokine with multiple effects on the immune system. CD4+ Th0 and Th2 cells, fetal thymocytes, CD8+ T cells, mast cells and basophils produce IL4 (Keegan and Pierce, 1994). IL4 activity is mediated via a specific high affinity cell surface receptor complex. The functional receptor complex consists of a ligand binding subunit (IL4-R) and a common γ chain. Dimerization of these components is required for signalling. IL4-R is also an essential component of the IL13 receptor complex, explaining the similar biological actions of IL4 and IL13 (Zurawski and de Vries, 1994). IL4 is thought of as an anti-inflammatory cytokine with numerous immunomodulatory functions on a variety of cells, including T cells, B cells, monocytes, neutrophils, haemopoietic progenitors, fibroblasts, endothelial cells and epithelial cells. IL4 is an important modulator of the differentiation of precursor T helper cells into Th2-like cells which mediate humoral immunity by assisting in antibody production. IL4 suppresses cytokine production by Th1 cells, downregulating cell mediated immune responses. IL4 increases B cell viability, cell size and upregulates surface expression of CD23, CD40, IgM, BB1/B7, IL4R and MHC class II antigen, which results in enhancement of the antigen-presenting capacity of B cells. IL4 is an important regulator of immunoglobulin isotype switching, inducing the production of IgE and IgG4 (Paul et al., 1991). IL4 exerts a predominantly anti-inflammatory effect on macrophages. It upregulates the expression of class II MHC antigens, LFA-1 and CD23 whilst downregulating the expression of Fc receptors (FcγRI, FcγRII, FcγRIII). IL4 inhibits monocyte IL1, IL6, IL8 and TNFα production, but stimulates ILRA
expression (Paul et al., 1991). IL4 inhibits macrophage superoxide production and the release of collagenase. IL4 also has effects on neutrophils, haematological progenitors, endothelial cells, fibroblasts and epithelial cells.

Clinical signs of adoptive transfer EAE in mice, could be suppressed by the administration of IL4, despite significant numbers of CNS-infiltrating inflammatory cells (Racke et al., 1994). IL4 is abundantly expressed in MS lesions (Woodroofe et al., 1993) and is hypothesised to have an immunomodulatory role in the immunopathogenesis of MS (see Chapter 1 - Introduction).

3.11.1.2 IL13

IL13 shares many of the properties of IL4. It has approximately 30% amino acid sequence homology with IL4 and exhibits IL4-like activity on monocytes/macrophages and B cells (Minty et al., 1993; McKenzie et al., 1993; Zurawski and de Vries, 1994). IL13 is produced by activated Th0, Th1-like, Th2-like and CD8+ T cells (McKenzie et al., 1993). IL13 receptor shares the IL4-Rα chain component and is activated by both IL4 and IL13. IL13 is however unable to bind and activate the IL4 receptor (Callard et al., 1996). IL13 is a potent regulator of inflammatory and immune responses. Like IL4, it upregulates the monocyte/macrophage expression of CD23 and MHC class I and II antigens and downregulates the expression of Fcγ receptors and inhibits antibody-dependent cytotoxicity. IL13 inhibits the expression of proinflammatory cytokines IL1, IL6, IL8, IL12, TNFα and the chemokines MIP-1 and MCP, but enhances the production of IL1RA. Unlike IL4, IL13 has no effects on human T cells due to the absence of the IL13 ligand binding receptor subunit (Callard et al., 1996). In Lewis rats, EAE could be suppressed by injection of hamster ovary cells transfected with human
IL13 (Cash et al., 1994). Whether or not IL13 plays a role in the immunopathogenesis of MS has yet to be determined.

3.11.2 Results

3.11.2.1 Effect of IL4 or IL13 on IFNγ-stimulated neopterin production

Figure 3.19. IL4: the effect of IL4 on 72-hour IFNγ-induced neopterin production in THP1 cells. (Error bars = s.d., *p<0.006, **p<0.001).
**Figure 3.20. IL4**: the effect of IL4 on 72-hour IFN-γ-induced neopterin production in monocytes. (Error bars = s.d., *p=0.04).

**Figure 3.21. IL13**: the effect of IL13 on 72-hour IFN-γ-induced neopterin production in monocytes. (Error bars = s.d., *p=0.03, **p=0.007).
Figure 3.22. IL4: the effect of IL4 (10ng/mL), administered at various times in relation to IFNγ (100U/mL) stimulation, on 48-hour neopterin production in monocytes. (Error bars = s.d., *p=0.05, **p<0.001).

Figure 3.23. IL13: the effect of IL13 (10ng/mL), administered at various times in relation to IFNγ (100U/mL) stimulation, on 48-hour neopterin production in monocytes. (Error bars = s.d., **p<0.001).
IL4 significantly suppressed neopterin production, in a dose-dependent fashion, in THP1 cells (Fig. 3.19). IL13 was not tested in THP-1 cells. IL4 (Fig. 3.20) and IL13 (Fig. 3.21) did not induce appreciable quantities of neopterin production in monocytes. However, compared to unstimulated monocytes, IL4 (10ng/mL) occasionally resulted in a small (<15nmol/L) but significant increase in monocyte neopterin production secondary to the induction of TNFα synthesis (see later, Fig. 3.24). The effect of IL4 and IL13 on IFNγ-stimulated monocyte neopterin production is complex. When the IL4 or IL13 was added simultaneously with IFNγ to elutriated monocytes, the effect on neopterin production was not what was expected from the results of IL4 treated THP1 cells (Fig. 3.19). IL4 and IL13 had minor and inconsistent effects on neopterin production, which depended on the concentration of IL4 or IL13 used. In some experiments low concentrations of IL4 and IL13 (5-10ng/mL) had a small inhibitory effect on neopterin production, with a mean reduction of -11% (range = -37% to +19%, n=6) in neopterin production. In comparison, high concentrations of IL4 and IL13 (100ng/mL) tended to cause a small increase in neopterin production with a mean increase of +4% (range = -28% to +30%, n=6). The latter increase was only statistically significant in one out of six experiments (Fig. 3.20). In comparison to the effects of the other cytokines TNFα, IL1β, TGFβ2 and IL10 these changes were inconsistent and relatively minor.
3.11.2.2 Preincubation of monocytes with IL4 or IL13 augments neopterin production.

Preincubating monocytes with more physiological concentrations of IL4 or IL13 (10ng/mL) for 24 hours prior to IFNγ-stimulation resulted in a significant augmentation of neopterin production (Fig. 3.22 and 3.23). With a period of preincubation of less than 6 hours this augmented neopterin production did not occur. Consistent with their common receptor and mechanism of action on monocytes (Callard et al., 1996), the effects of IL4 and IL13 on monocyte neopterin production were identical. Further experiments were therefore limited to the effects of IL4 alone. Augmented IFNγ-induced monocyte neopterin production due to IL4-preincubation was seen at all concentrations of IFNγ (Fig. 3.24). Occasionally IL4 was able to stimulate small (<15nmol/L) but significant quantities of neopterin production in the absence of exogenous IFNγ-stimulation (Fig. 3.24).
3.11.2.3 The augmentation of IFNγ-induced neopterin production by IL4 can be partially reversed by neutralising TNFα and IL1RA activity

Preincubating monocytes with IL4 for 20 hours has been shown to induce TNFα production (D’Andrea et al., 1995). Therefore to test the hypothesis that IL4 (and by inference IL13) augment neopterin production via the induction of TNFα and possibly IL1, experiments were repeated in the presence and absence of neutralising anti-human TNFα (10μg/mL) and IL1-RA (10μg/L) (Fig. 3.25). The experiments demonstrated that the augmentation could be partially reversed by neutralising the activity of TNFα and IL1 (Fig. 3.25). However this only partially reversed the effect of IL4 (Fig. 3.25).

**Figure 3.25 IL4: the effect of neutralising TNFα activity on IL4 augmented (10ng/mL) 48-hour neopterin production in IFNγ stimulated monocytes.** *(Error bars = s.d., *p < 0.02, **p < 0.005)*
Low levels of immunoreactive TNFα could not be detected in the monocyte cell culture supernatants 24 hours after IFNγ stimulation (100U/mL) (Fig. 3.26). However, monocytes pre-incubated for 24 hours with IL4 or IL13 (10ng/mL) prior to IFNγ-stimulation, produced small quantities of TNFα (Fig. 3.26). These levels were an order of magnitude lower than the quantity of TNFα induced by 10μg/mL of LPS (mean = 649pg/mL (range = 609-704, n = 3), which were included in all experiments as a positive control. TNFα levels were not detected in supernatants harvested after 36 hours (data not shown).
3.11.3 Discussion

The effects of IL4 and IL13 on monocyte neopterin production are complex and affected by the ability of IL4 and IL13 to enhance the production of TNFα (D'Andrea et al., 1995). IL4 has previously been shown to decrease spontaneous neopterin production in human monocytes but to have no effect on LPS- or IFNγ-stimulated neopterin production (Moutabarrik et al., 1992). In the study by Moutabarrik et al’s (1992) the monocytes were purified by selective adherence, which results in significant activation of monocytes (Shaw et al., 1990; Sporn et al., 1990). Therefore the effects of IL4 on unstimulated monocyte neopterin production could not be assessed in their experiments. On the other hand unstimulated monocytes, purified by elutriation using LPS-free conditions, generally did not produce detectable levels of neopterin (>2.5nmol/L). However, in one isolated experiment IL4-treated monocytes in the absence of exogenous IFNγ induced a significant, albeit small (<15nmol/L), quantity of neopterin compared to untreated monocytes. In the same experiment neutralising TNFα and IL1 activity abrogated this effect. These monocytes may have been pre-activated in vivo so that the small quantities of IL4-induced TNFα synthesis may have been sufficient to induce neopterin production. On balance, however, IL4 and IL13 do not have an effect on IFNγ-stimulated monocyte neopterin production unless there is a 24 hour preincubation period prior to IFNγ-stimulation, in which case neopterin production is greatly augmented. This effect appears to be, at least partially, mediated by the ability of IL4/13 and to induce TNFα production (D'Andrea et al., 1995). Whether this effect is directly due to IL4/13 or possible via the action of a second mediator is unknown and is currently under active investigation. One possible mediator is soluble CD23 (sCD23) or FcεRII, which is induced by IL4 (Lee et al. 1993) and augments monocyte TNFα production (Armant et al., 1994). Soluble CD23 is probably responsible for IL4-
mediated induction of iNOS in human macrophages (Paul-Eugene et al., 1995; Dugas et al., 1995). Whether monocyte/macrophage GTP-cyclohydrolase I expression and activity is controlled in a similar fashion to iNOS is currently being investigated.

3.11.4 Summary IL4 and IL13

IL4 and IL13 have little direct effect on human monocyte neopterin production. However, preincubating human monocytes with IL4/13 for 24 hours greatly augments IFNγ-induced neopterin production.
3.12 Phagocytosis and Fc-receptor (FcR) stimulation

3.12.1 Background Immunology

Phagocytosis is a highly specialised active process. It involves the attachment of particulate matter via specific receptors to a local segment of the macrophage plasma membrane, followed by engulfment through the sequential interaction of surface ligands around the circumference of the particle (zipper mechanism). Phagocytosis is temperature- and energy-dependent and is sensitive to agents which disrupt the activity of actin filaments (e.g. cytochalasin). Although numerous receptors are involved in the process of phagocytosis, Fc and complement (CR3, C1qR) receptors are involved in the phagocytosis of opsonised particles. Unlike FcR phagocytosis, which initiates a cytotoxic response and release of inflammatory mediators, CR3 ligation effects engulfment of a target without triggering a respiratory burst or release of arachidonate products (Gordan, et al., 1992). FcRs can be classified as serving either an effector or transport function. Those involved in effector functions include FcRs for IgG (FcγRI, II and III), IgE (FcεRI and II) and IgA (FcαRI) and those responsible for transport of immunoglobulin across epithelial surfaces include poly IgA receptor and the neonatal IgG transporter. The effector class of FcRs are potent triggers of cellular activation (Ravetch, 1997), although an inhibitory role for FcγRII on B-cells has been described (Amigorena et al., 1992; Muta et al., 1994). The Fcγ-FcγR interaction in monocyte/macrophages has been shown to initiate the release of TNFα (Debets et al., 1988; Debets et al., 1990; Polat et al., 1993; Kindt et al., 1993), IL1β (Kindt et al., 1993; Remvig et al., 1990), IL6 (Krutmann et al., 1990) and IL4 (Ben-sasson et al.,
1990), as well as the production of reactive oxygen intermediates, prostaglandins and leukotrienes (Nitta and Suzuki, 1982; Rouzer et al., 1980).

In MS, phagocytosis of myelin debris by macrophages results in the formation of large oil-red positive, lipid laden, foamy cells (Allen, 1991). In vitro the phagocytosis of myelin occurs via Fc, complement, scavenger and LDL receptors (Mosley and Cuzner, 1996). The binding of immunoglobulins to myelin and the resultant complement activation appear to be important early events in the formation of new lesions in MS (Zajicek and Compston, 1995). In EAE anti-myelin antibodies and complement activation appear critical in causing demyelination (Linnington et al., 1989; Piddlesden et al., 1991).

The following studies explore the effects of Fc-receptor stimulation and phagocytosis on neopterin production by monocytes in vitro.

3.12.2 Methods

3.12.2.1 Fc-R Stimulation

Fc-receptor stimulation was achieved by pre-coating wells of a 48-well microtitre plate with 200μL of 20μg/mL OX12 (ECACC, Cat. No. 88051301, mouse IgG2a anti-rat kappa chain) in HBSS for 24 hours, at 4°C. The wells were then washed with culture medium to remove non-adherent immunoglobulins. THP1 cells and monocytes were then incubated under standard conditions.

3.12.2.2 Phagocytosis via Fc-Receptors

Anti-D IgG opsonised autologous erythrocytes were used as a substrate according to a previously published methods (Gebran et al., 1992).
3.12.2.3 Opsonisation of erythrocytes

Autologous erythrocytes from Rhesus positive (Rh*) blood packs were used. A small aliquot of erythrocytes from the red cell fragment of the density gradient centrifugation was used. The erythrocytes were washed three times in HBSS solution, resuspended in RPMI, counted manually and a suspension containing $4 \times 10^8$ erythrocytes per mL prepared. One mL of the erythrocyte suspension was incubated for 2 hours with 5μg/mL of human IgG antiD (Weak anti-D, Bio Products Laboratory, Prod. No. HJG3). After three washes the erythrocytes were resuspended to $1 \times 10^7$ per mL in cell culture medium. All the FCS serum in the cell culture media was decomplemented by heat inactivation (56°C for 30 minutes). Sensitization of the erythrocytes was tested by agglutination using a drop of rabbit anti-human IgG (Polyspecific anti-human Globulin Reagent, LSEZ Diagnostics/National Blood Transfusion Service).

3.12.2.4 Phagocytosis of erythrocytes

Elutriated monocytes were exposed to $1 \times 10^6$ IgG-sensitized and non-sensitized erythrocytes. Phagocytosis was confirmed by observing rosette formation due to the IgG-sensitized erythrocytes sticking to the surface of the monocytes and noting a change in monocyte morphology (enlargement with coarse granularity of cytoplasm). Phagocytosis was semi-quantified by measuring the peroxidase activity of haemoglobin (Hb) in non-phagocytosed erythrocytes. To measure the level of extracellular Hb due to erythrocyte lysis, the cell culture plates were centrifuged at 2000 rpm for 5 min before 100μL of the supernatants were removed for analysis. The cell pellets were then resuspended using a pipette and 100μL transferred to micro-centrifuge tubes. The non-phagocytosed erythrocytes were lysed by the addition of 900μL of a 0.2% NaCl
solution for 3 minutes. The preparations were then centrifuged at 5000 rpm for 5 minutes and the supernatants removed for analysis.

3.12.2.5 Colorimetric assay for detection of Haemoglobin (Hb)

A stock solution was prepared by dissolving 100mg of 2,7-diaminofluorene (Sigma Cat. No. D9251) in 10mL of 90% glacial acetic acid (BDH, Prod. No. 10001) in water with vigorous vortexing at room temperature. One mL of the 2,7-diaminofluorene solution and 100μL of hydrogen peroxide were added to 10mL of 0.2M Tris-HCl buffer (Appendix V) containing 6M urea (Sigma Cat. No., U0631). A 100μL of this solution was mixed with 100μL of the cell culture supernatant or erythrocyte cell lysis extract. The reaction mixture was incubated for 5 minutes and the green reaction product measured 620nm using an ELISA plate reader (Anthos-2000). The kinetics of IgG-

![Figure 3.27. Erythrocyte phagocytosis: kinetics of erythrocyte phagocytosis in THP1 cells (O = unsensitised / • = IgG-sensitised) and monocytes (□ = unsensitised / ■ = IgG-sensitised). The absorbance of light at 620nm due to 2,7-diaminofluorene is a measure of the peroxidase activity of unphagocytosed haemoglobin (Hb).]
sensitized erythrocyte phagocytosis in THP1 cells and monocytes are presented in Fig. 3.27.

3.12.2.6 Myelin phagocytosis

Dr Stephan Peuchen (Dept. Neurochemistry, Institute of Neurology), (using a standard method) kindly prepared a myelin extract from normal human brain (Blomstrand and Hamberger, 1969). Normal brain tissue was kindly provided by Prof. F Scaravilli (Dept. Pathology, Institute of Neurology) and Dr Jia Newcombe (MS Society Brain Bank, Institute of Neurology). Using the limulus amoebocyte lysate assay (Biowhittaker, cat. no. 50-648U), the myelin extract was found to contain 0.027 endotoxin units per mL (~27pg/mL). In the myelin phagocytosis experiments the myelin extract was diluted 1:10 making the final concentration of LPS 2.7pg/mL. All myelin phagocytosis experiments were performed in the presence of polymyxin B (10μg/mL), which neutralises LPS-activity (Coyne et al., 1994). The low levels of LPS contamination in the myelin preparation were therefore unlikely to be a confounding factor. Myelin phagocytosis was not quantified, but was easily observed by a change in cell morphology (enlargement and cytoplasmic vacuolation).

3.12.3 Results

Fc-R stimulation, Fc-mediated erythrocyte phagocytosis (IgG sensitized) and non-Fc-mediated myelin phagocytosis do not induce neopterin production in monocytes (Figures 3.28 and 3.29). However, Fc-R stimulation (Fig. 3.28) and Fc-mediated phagocytosis of IgG-sensitized erythrocytes (Fig. 3.29) significantly augmented IFNγ-mediated neopterin production in monocytes. Non-Fc mediated myelin phagocytosis in
monocytes tended to augment IFNγ-mediated neopterin production but this was not significant (Fig. 3.29).

**Figure 3.28. Fc-Receptor: the effect of Fc-Receptor stimulation on 72-hour neopterin production in IFNγ-stimulated monocytes.** (Error bars = s.d., *p = 0.04, **p < 0.01).

**Figure 3.29. Fc-Receptor mediated phagocytosis: the effect of Fc-R mediated (IgG sensitised erythrocytes) and non-Fc-R mediated phagocytosis (human myelin) on 72-hour neopterin production in IFNγ-stimulated monocytes.** (Error bars = s.d., *p < 0.05, **p = 0.002, relative to control).
3.12.4 Discussion

Stimulation of monocytes via Fc-R’s, Fc-R-mediated phagocytosis and non-Fc-R-mediated myelin phagocytosis did not induce neopterin production. However Fc-R stimulation, with or without phagocytosis, was found to augment IFNγ-induced neopterin production. This is not surprising as Fc-R stimulation activates macrophages leading to the production of the pro-inflammatory cytokines, TNFα (Debets et al., 1988; Debets et al., 1990; Polat et al., 1993; Kindt et al., 1993) and IL1β (Kindt et al., 1993; Remvig et al., 1990), both of which augment IFNγ-induced neopterin production. Non-Fc-R-mediated myelin phagocytosis did not augment IFNγ-induced neopterin production.

3.12.5 Summary Fc-Receptor Stimulation

Fc-R stimulation and Fc-R-mediated phagocytosis, but not non-Fc-R-mediated myelin phagocytosis, augmented IFNγ-induced neopterin production.
3.13 Interferon beta (IFNβ) (type I interferons)

3.13.1 Background immunology of type I interferons

Type I interferons are a family of molecules which includes more than 20 different proteins. The interferons were initially classified by their source, but current nomenclature is determined by the sequence analysis of their genes (Ackrill et al., 1991). There are four varieties of type I IFNs (α, β, ω and τ), of which IFNα and IFNβ are the best studied. IFNα and β form a cluster of genes on chromosome 9. IFNα is a family of closely related proteins, with at least 18 non-allelic genes, four of which are pseudogenes. There is only a single IFNβ gene. The type I IFNs all bind and compete for a specific cell surface receptor. The receptor consists of two subunits, one of which is essential for binding and the other for signal transduction, requiring two cytoplasmic protein tyrosine kinases (tyk 2 and JAK1) (Muller et al., 1993; Watling et al., 1993). Low levels of IFNs are detected in human tissues in the absence of a specific inducer, but tend to be upregulated in response to viral, bacterial, mycoplasmal and parasitic infections (Billiau, 1996). Double-stranded RNA is a potent inducer and is thought to be one of the IFN-inducing factors produced during virus replication (Fields et al., 1967). IFNα and β synthesis is regulated at both transcriptional and post-transcriptional levels (Taylor and Grossberg, 1990; Kerr and Stark, 1991; Darnell et al., 1994). The biological effects of type I IFNs are numerous and include antiviral, antiproliferative, antitumour and immunomodulatory effects (Weinstock-Guttman et al., 1995). Type I IFNs increase suppressor (Noronha et al., 1990; Noronha et al., 1992) and cytotoxic cell function (Borden et al., 1990; Schiller et al., 1990), reduce T cell activation and T cell IFNγ production (Panitch et al., 1987; Rudick et al., 1993), increase MHC class I expression
and inhibit IFNγ induction of MHC class II antigens (Ling et al., 1985; Inaba et al., 1986; Barna et al., 1989).

Although there is no direct evidence that type I interferons play a role in MS they do have a therapeutic benefit. With recombinant human IFNβ there is approximately a 30% reduction in the relapse rate (IFNB Multiple Sclerosis Study Group et al., 1993; IFNB Multiple Sclerosis Study Group, 1995; Jacobs et al., 1996), a significant reduction in MRI activity (Paty et al., 1993; IFNB Multiple Sclerosis Study Group, 1995; Stone et al., 1995; Jacobs et al., 1996) and a delay in time to sustained clinical progression (Jacobs et al., 1996). Similarly recombinant IFNα also seems to reduce relapse rate and MRI activity (Durelli et al., 1994; Jacobs and Johnson, 1994). Type I interferons are also effective in EAE (Abreu, 1985; Brod et al., 1995; Brod and Khan, 1996b; Yu et al., 1996).
3.13.2 Results

3.13.2.1 IFNβ1a induced neopterin production

Figure 3.30. IFNβ: the effect of IFNβ-1a on 72-hour neopterin production by THP-1 cells in the presence and absence of IFNγ stimulation. (Error bars = s.d., ** p < 0.001).

Figure 3.31. IFNβ: the effect of IFNβ-1a on 72-hour neopterin production by monocytes in the presence and absence of IFNγ stimulation. (Error bars = s.d., * p = 0.05, ** p < 0.008).
In the THP1 cells IFNβ1a induced and synergised in a dose-dependent manner with IFNγ to further upregulate, neopterin production (Fig. 3.30). In contrast using monocytes, IFNβ1a whilst inducing neopterin production at low concentrations, did not induce it in a concentration-dependent manner (Fig. 3.31). Furthermore, monocyte IFNγ-induced neopterin production was inhibited by IFNβ1a in a dose-dependent fashion (Fig. 3.31).

3.13.2.2 TGFβ and IL10 effects

To further explore the mechanism whereby IFNβ1a inhibited IFNγ-induced neopterin production in monocytes, experiments were repeated in the presence of neutralising anti-IL10 (rat monoclonal IgG2a – DNAX, ATTC Cat. No. HB10487) or TGFβ (R&D Systems, Cat. No. AB-100-NA) antibodies, using previously determined effective concentrations of 10 and 5 μg/mL respectively. The addition of neutralising anti IL10 antibodies, but not anti TGFβ antibodies, to IFNγ stimulated monocytes reversed the inhibition of neopterin production due to IFNβ1a (0 – 5000u/mL) (Fig. 3.32). Similarly the inhibitory effect of IFNβ1a at high concentrations on its own induction of neopterin production in monocytes was partially reversed by the addition of neutralising anti-IL10 antibodies, but not anti-TGFβ antibodies (Fig. 3.33). Antibodies OX20 (ECACC, Cat. No. 88070801, rat IgG1 anti-mouse kappa chain) and OX14 (ECACC, Cat. No. 87112401, mouse IgG2a anti-rat kappa chain), used as control antibodies for the neutralising anti-IL10 and anti-TGFβ experiments had no effect on neopterin production.
Figure 3.32. IFNβ: neopterin production in monocytes stimulated with IFNγ (100U/mL) and cultured in the presence of IFNβ1a (0-5000U/mL), and either neutralising anti-TGF-β (5µg/mL) or anti-IL10 (10µg/mL) antibodies for 72 hours. The neutralising anti-TGFβ (5µg/mL) or anti-IL10 (10ng/mL) antibodies, had no effect on neopterin production in the absence of IFNβ1a (data not shown).

Figure 3.33. IFNβ: The effect of neutralising anti-IL10 antibodies on IFNβ-1a-induced 72-hour neopterin production in elutriated monocytes. (Error bars = s.d., * p < 0.04, ** p < 0.001).
3.13.2.3 IL10 levels

Despite the effects of neutralising anti-IL10 antibody, IL10 (>40pg/mL) was not detected in the culture supernatants of IFNβ1a (all concentrations) treated monocytes or THP1 cells. Whereas the same monocytes, cultured in the presence of LPS (100ng/mL), but not the THP1 cells, produced significant quantities of IL10 (mean = 142 pg/mL (range = 98-207)).

3.13.3 Discussion

IFNβ-1a induces neopterin production in THP1 cells, in a manner, which is both concentration-dependent and synergistic with IFNγ. Interestingly, IFNβ-1a-induced neopterin production in monocytes was not dose-responsive and produced a bell-shaped curve with peak neopterin production occurring at approximately 500U/mL. In addition IFNβ-1a unexpectedly inhibits IFNγ-induced neopterin production in monocytes. Neutralising the activity of IL10 partially reversed these effects. Thus in the presence of neutralising anti-IL10 antibodies IFNβ-1a induced a response in monocytes, similar to that found in THP1 cells. Although there are reports of IL10 production in THP1 cells (Takeshita et al., 1995; Li et al., 1997), the THP1 cells used in these experiments did not produce IL10. Reasons for this are unknown and may be due to an acquired deficit in IL10 production in the THP1 cell line used in these experiments. This data strongly suggests that IFNβ-1a induces IL10 production in monocytes despite no detectable soluble IL10 (>40pg/mL) in these experiments. Reasons for this may relate to the sensitivity of the ELISA used (lower limit of detection = 40 pg/mL), or the possibility that the IL10 activity was due to a membrane-bound isoform rather than soluble protein (Flemming et al., 1996). Indeed, in a recent publication it has been shown that the inclusion of a neutralising anti-IL10 monoclonal antibody markedly increased monocyte
TNFα production in response to fixed T cells even though secreted IL10 was undetectable (Parry et al., 1997). This supports the argument that the effect of IL10 is due to a membrane bound rather than a soluble form. Augmented LPS-induced IL10 production by monocytes is observed when the monocytes are co-stimulated with IFNβ-1a, but no soluble IL10 production is detected when IFNβ1a is used in isolation (unpublished observations, Dr Sarah Parry). Other groups have reported increased monocyte IFNβ-induced IL10 mRNA expression and IL10 production in vitro (Shakir et al., 1994; Porrini et al., 1994; Rudick et al., 1996a). In addition, 12 and 24 hour serum levels of IL10 are increased in normal volunteers after the administration of IFNβ–1a (Rudick et al., 1996a). IFNβ–1a (Avonex®, Biogen) treatment also significantly increases CSF IL10 levels in patients with MS (Rudick et al., 1996b). These data suggest IFNβ is capable of inducing IL10 production in human monocytes.

3.13.4 Summary IFNβ

IFNβ–1a is able to induce neopterin production in human monocytes but antagonises the effects of IFNγ on neopterin production. This antagonism is mediated by IL10.
Table 3b. Summary of the control of neopterin production in THP1 cells and monocytes.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>THP1 Cells</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IFNγ</strong></td>
<td>+3 ↑</td>
<td>+3 ↑</td>
</tr>
<tr>
<td>No additives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additives + IFNγ</td>
<td>+1 ↑</td>
<td>+1 ↑</td>
</tr>
<tr>
<td>TNFα</td>
<td>+1 ↑</td>
<td>+1 ↑</td>
</tr>
<tr>
<td>IL1b</td>
<td>+1 ↑</td>
<td>+1 ↑</td>
</tr>
<tr>
<td>GMCSF</td>
<td>-</td>
<td>+2 ↑</td>
</tr>
<tr>
<td>IL6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LPS</td>
<td>+1 ↑</td>
<td>+2 ↑</td>
</tr>
<tr>
<td>Fc-receptor stimulation</td>
<td>-</td>
<td>+2 ↑</td>
</tr>
<tr>
<td>Phagocytosis (Fc-receptor)</td>
<td>-</td>
<td>+2 ↑</td>
</tr>
<tr>
<td>Phagocytosis (Myelin)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFNβ</td>
<td>+2 ↑</td>
<td>+2 ↑</td>
</tr>
<tr>
<td>IL4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL13</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>IL10</td>
<td>-</td>
<td>? -1 ↓</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>-</td>
<td>-2 ↓</td>
</tr>
<tr>
<td>Sepiapterin</td>
<td>-</td>
<td>-2 ↓</td>
</tr>
</tbody>
</table>

* Concentration-dependent, # Time-dependent
Chapter 4 - Urinary neopterin excretion as a marker of disease activity in multiple sclerosis: a cross-sectional study.

4.1 Background

To assess the potential of neopterin as an immunological marker of disease activity in patients with MS, a cross-sectional study was performed to see if urinary neopterin excretion is elevated in patients with MS. The results from this study prompted the more informative longitudinal study presented in Chapter 5. This study was also designed to see if urinary neopterin excretion differed between the clinical subtypes of MS, and whether there was any correlation with clinical and MRI markers of inflammation, i.e. clinical relapse and Gd-enhancement on MRI. Patients with demyelinating disease (clinically isolated syndromes compatible with demyelination and clinically definite MS) undergoing MRI were compared to a large group of healthy control subjects, as well as patients with either HIV-1 infection, active rheumatoid arthritis, or posterior uveitis. Patients with HIV-1 infection and active rheumatoid arthritis were chosen as inflammatory controls, as increased neopterin production has been well documented in both of these conditions. Patients with posterior uveitis were also selected as an inflammatory control group as they have active inflammation within the confines of the central nervous system that can be documented clinically without the need for invasive investigations.

4.2 Hypotheses

Patients with MS have increased urinary neopterin excretion which correlates with clinical and MRI markers of disease activity.
4.3 Methods

4.3.1 Patients and Subjects

The study group consisted of 58 normal subjects, 106 patients with clinically or laboratory supported definite MS (Poser et al., 1983; Appendix I), 23 patients with a clinically isolated syndrome compatible with demyelination, 19 patients with acquired immune deficiency syndrome (AIDS) due to HIV-1 infection, 35 patients with rheumatoid arthritis and 20 patients with posterior uveitis. All patients with demyelinating diseases were recruited from the National Hospital for Neurology and Neurosurgery, London. Their disability was rated using Kurtzke’s EDSS (Kurtzke, 1983; Appendix II). Patients with clinically isolated syndromes were not examined acutely, but 3 to 12 weeks after their initial presentation. Patients with MS were classified as having either benign, relapsing remitting, secondary progressive, or a primary progressive course using established definitions (Lublin and Reingold, 1996). The time when patients with MS were studied varied and included 12 patients during a clinical relapse. Clinical relapse was defined using standard criteria (Poser et al., 1983). Sixty six out of 106 patients with MS (62%) and all 23 patients with clinically isolated syndromes (100%) underwent a T1-weighted Gd-enhanced MRI study of the brain, using a standardised protocol to assess disease activity (Miller et al., 1996). A neuroradiologist, who counted the number of Gd-enhancing lesions per study, assessed all the MRI studies. Normal control subjects were healthy volunteers, with no overt medical problems, recruited from the general population. The HIV-1 infected patients were all inpatients of the University College London Hospitals’ HIV/AIDS unit. All had a CD4+ T cell counts performed in the routine haematology laboratory. Patients with rheumatoid arthritis, fulfilling the American Rheumatism Association diagnostic criteria (Arnett et al., 1987), were recruited from the Charing Cross Hospital, London. All
patients with rheumatoid arthritis underwent a clinical examination and had their
disease activity assessed using a joint count (Felson et al., 1993). Patients with posterior
uveitis were recruited from the ophthalmology outpatient department at St Thomas’
Hospital, London. Active posterior uveitis was defined symptomatically by the presence
of floaters or clinically using fundoscopy and/or fluorescein angiography to show
inflammatory activity within the posterior chamber of the eye.

Urine specimens were collected at various times during the day, protected from light,
coded and frozen at -20°C as soon as possible. The urinary nitrite level was used as a
screen for bacterial colonisation of the urinary tract in patients with MS (see Chapter 2).
Patients with evidence of bacterial colonisation were not included in this study.

4.3.2 Neopterin and creatinine Assays

Urine neopterin and creatinine were measured by HPLC as described in Chapter 2. All
samples were assayed without knowledge of the clinical status of the patients.

4.3.3 Data transformation

The neopt:creat.urine female subjects were divided by the factor of 1.2, to correct for a
lower creatinine excretion (see Chapter 2), thereby allowing direct comparisons
between male and female subjects.

4.3.4 Statistics

Levene’s test of homogeneity of variances was used to test whether variables were
normally distributed. Normally distributed continuous variables were compared using a
one-way analysis of variance (ANOVA). If the ANOVA was significant, individual
groups were compared with each other using a post-hoc Tukey’s honestly significant
difference test. This test delineates the inter-group differences responsible for the
significant ANOVA. The majority of variables could be normalised using a simple logarithmic transformation, when this was not possible, groups were compared using the non-parametric Kruskal-Wallis one-way analysis of variance. Other non-parametric data was compared using the Yates corrected Chi-squared test or, if an expected value was less than 5, the Fisher exact test. Normalised continuous data were correlated using simple linear regression and a two-tailed Pearson’s test. A p value of <0.05 was considered statistically significant.

Table 4a - Clinical classification and diagnoses of patients by group.

<table>
<thead>
<tr>
<th>Clinical Groups</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Demyelinating Disease (n=129)</td>
<td></td>
</tr>
<tr>
<td>Clinically isolated syndrome compatible with demyelination</td>
<td>23</td>
</tr>
<tr>
<td>Optic neuritis</td>
<td>16</td>
</tr>
<tr>
<td>Brainstem syndrome</td>
<td>6</td>
</tr>
<tr>
<td>Transverse myelitis</td>
<td>1</td>
</tr>
<tr>
<td>Benign MS</td>
<td>12</td>
</tr>
<tr>
<td>Relapsing remitting MS</td>
<td>34</td>
</tr>
<tr>
<td>Secondary progressive MS</td>
<td>29</td>
</tr>
<tr>
<td>Primary progressive MS</td>
<td>31</td>
</tr>
<tr>
<td>2. HIV-1 infection (n=19)</td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>10</td>
</tr>
<tr>
<td>Pneumocystis carinii</td>
<td>6</td>
</tr>
<tr>
<td>Acute bacterial pneumonia</td>
<td>4</td>
</tr>
<tr>
<td>Cerebral Infection</td>
<td></td>
</tr>
<tr>
<td>Cerebral toxoplasmosis</td>
<td>2</td>
</tr>
<tr>
<td>CMV encephalitis</td>
<td>1</td>
</tr>
<tr>
<td>Progressive multifocal leukoencephalopathy</td>
<td>1</td>
</tr>
<tr>
<td>Varicella Zoster acute retinal necrosis</td>
<td>1</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
</tr>
<tr>
<td>Idiopathic thrombocytopenic purpura</td>
<td>1</td>
</tr>
<tr>
<td>Kaposi’s sarcoma (skin)</td>
<td>1</td>
</tr>
<tr>
<td>Stevens Johnson Syndrome</td>
<td>1</td>
</tr>
<tr>
<td>Tuberculous osteomyelitis</td>
<td>1</td>
</tr>
<tr>
<td>3. Rheumatoid arthritis (n=35)</td>
<td></td>
</tr>
<tr>
<td>4. Posterior uveitis (n=20)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1. Group comparison (see Table 4b): combined scatter plus box and whisker plots of neopt:creat.uro. The box represents the 25th-75th quartile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2SDs above the mean of the normal control subjects).

4.4 Results

4.4.1 Clinical Features

The clinical classifications and diagnoses of the patients are presented in Table 4a. The demographic and urinary neopterin data are given in Table 4b. Significant differences were noted in the sex ratios and mean ages of the groups (Table 4b). The majority of patients with HIV-1 infection were male compared to the other groups in which females predominated (p<0.001, Table 4b). Patients with rheumatoid arthritis were significantly older than those in the other groups (p<0.001, Table 4b). After correcting for the lower urinary creatinine excretion in females, all the male and female subjects participating in the study had similar neopt:creat.uro. levels (median neopt:creat.uro. males = 165
μmol/mol [25th-75th% = 109-322] vs. females = 169 μmol/mol [107-301], p = n.s.). No correlation was found between age and neopt:crea:urine levels in this study (n = 261, Pearson’s correlation coefficient = 0.05, p = n.s.). Similarly when limiting these analyses to the normal control subjects no correlations were found (data not shown).

The neopt:crea:urine ratios were significantly elevated in patients with demyelinating disease, HIV-1 infection and rheumatoid arthritis compared to normal control subjects (p<0.001, Table 4b and Fig. 4.1). The neopt:crea:urine was significantly greater in patients with HIV-1 infection and rheumatoid arthritis compared to patients with demyelinating disease (p < 0.009, Table 4b and Fig.4.1). Only 38% of subjects with demyelinating disease and 35% of patients with posterior uveitis had elevated neopt:crea:urine compared to 95% of HIV and 86% of rheumatoid arthritis patients (p < 0.001, Table 4b).

4.4.2 Demyelinating Diseases

In patients with demyelinating disease, significant differences were noted in the sex ratios and mean ages of the clinical subgroups (Table 4c). More patients with clinically isolated syndromes and primary progressive MS were male compared to the other subgroups (p<0.001, Table 4c). Patients with benign, secondary progressive and primary progressive MS were older than patients with clinically isolated syndromes and relapsing remitting MS (p<0.001, Table 4c). There were no differences in the neopt:crea:urine ratios between the demyelinating disease subgroups (Table 4c, fig.4.2).

After excluding patients with clinically isolated syndromes (not MS by definition) and primary progressive MS (who characteristically have little MRI activity), no correlation was found between the neopt:crea:urine ratios and Gd-enhancing MRI activity. Patients
with Gd-enhancing lesions on MRI had a slight trend towards having a higher
neopt:creatinine than patients without Gd-enhancement on MRI (median neopt:creatinine
= 256 μmol/mol [25th-75th% = 105-551] vs. 151 μmol/mol [108-523], p = n.s.).

After excluding patients who by definition could not have a clinical relapse, i.e. patients
with clinically isolated syndromes and primary progressive MS, patients sampled during
a clinical relapse had similar neopt:creatinine to those MS patients sampled in remission
(median neopt:creatinine = 121 μmol/mol [25th-75th% = 97-275] vs. 169 μmol/mol [117-
416], p = n.s.). Urine samples were collected a median of 8 days (range = 1 – 18) after
the clinical onset of symptoms. The mean neopt:creatinine of the 6 patients in whom the
specimen was collected less than 8 days after the clinical onset of the relapse was
significantly higher compared to the 6 patients in whom the specimen was collected
greater than 8 days after the onset of the clinical relapse (mean neopt:creatinine = 359
μmol/mol [range = 118-684] vs. 97 μmol/mol [77-124], p = 0.003). In addition the
natural logarithm of the neopt:creatinine correlated negatively with the time in days after
the clinical onset of the relapse when the urine specimen was collected (n = 12, r = - 0.7,
p = 0.01; Fig. 4.3). There was no correlation between disability, as measured by
Kurtzke’s EDSS (Kurtzke, 1983) and the neopt:creatinine (Spearman’s rank correlation
coefficient = 0.01, p = n.s.).
**Figure 4.2.** Demyelinating diseases (see Table 4c): combined scatter plus box and whisker plots of neopt:creat. urine. The box represents the 25<sup>th</sup>-75<sup>th</sup> quartile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2SDs above the mean of the normal control subjects).

**Figure 4.3.** Neopt:creat. urine vs. time post onset of clinical relapse: a linear regression of the natural logarithm of the neopt:creat. urine and the time in days post onset of the clinical relapse. The solid line represents the regression line and the dotted horizontal line the upper limit of normal for the neopt:creat. urine.
Table 4b - Demographic data and the neopt:creat-urine by group.

<table>
<thead>
<tr>
<th></th>
<th>Normal Controls (NC)</th>
<th>Demyelinating Diseases (DD)</th>
<th>HIV-1 Infection (HIV)</th>
<th>Rheumatoid Arthritis (RA)</th>
<th>Posterior Uveitis (PU)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>58</td>
<td>129</td>
<td>19</td>
<td>35</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>22:36</td>
<td>59:70</td>
<td>18:1</td>
<td>9:36</td>
<td>10:10</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Mean age yrs (SD)</td>
<td>37.1 (11.0)</td>
<td>39.5 (9.9)</td>
<td>34.6 (7.7)</td>
<td>55.1 (11.7)</td>
<td>42.4 (12.1)</td>
<td>p &lt; 0.001a</td>
</tr>
<tr>
<td>Median neopt:creat-urine µmol/mol (25th-75th %tile)</td>
<td>99 (76-151)</td>
<td>163 (119-266)</td>
<td>972 (653-1456)</td>
<td>389 (257-623)</td>
<td>135 (99-213)</td>
<td>p &lt; 0.001b</td>
</tr>
<tr>
<td>No. of subjects with elevated neopt:creat-urine µmol/mol</td>
<td>2/58 (3%)</td>
<td>49/129 (38%)</td>
<td>18/19 (95%)</td>
<td>30/35 (86%)</td>
<td>7/20 (35%)</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

Post-hoc Tukey’s honestly significant difference test

*a* HIV < NC/DD/RA/PU, p < 0.001.

*b* NC < DD/HIV/RA, p < 0.001; DD/PU < HIV/RA, p < 0.001; HIV > RA, p = 0.01.
Table 4c – Clinical and neopt:creatinine urine data of patients with demyelinating diseases.

<table>
<thead>
<tr>
<th></th>
<th>Clinically Isolated Syndromes (CIS)</th>
<th>Benign MS (B)</th>
<th>Relapsing Remitting MS (RR)</th>
<th>Secondary Progressive MS (SP)</th>
<th>Primary Progressive MS (PP)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>23</td>
<td>12</td>
<td>34</td>
<td>29</td>
<td>31</td>
<td>-</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>14:9 (61%)</td>
<td>5:7 (42%)</td>
<td>9:25 (26%)</td>
<td>9:20 (31%)</td>
<td>22:9 (71%)</td>
<td>p = 0.02</td>
</tr>
<tr>
<td>Mean age yrs. (SD)</td>
<td>32.4 (6.1)</td>
<td>49.5 (9.4)</td>
<td>33.7 (7.8)</td>
<td>40.1 (7.0)</td>
<td>46.7 (8.5)</td>
<td>p &lt; 0.001(^a)</td>
</tr>
<tr>
<td>Median Kurtzke's EDSS (range)</td>
<td>1.0 (1.0-8.0)</td>
<td>2.0 (1.0-3.0)</td>
<td>3.5 (0.0-6.5)</td>
<td>6.5 (5.5-8.0)</td>
<td>7.0 (3.5-8.5)</td>
<td>p &lt; 0.001(^b)</td>
</tr>
<tr>
<td>No. of subjects in clinical relapse</td>
<td>-</td>
<td>1/12 (8%)</td>
<td>5/34 (15%)</td>
<td>6/29 (21%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>No. of subjects with active MRI</td>
<td>2/23 (9%)</td>
<td>1/9 (11%)</td>
<td>10/20 (50%)</td>
<td>8/14 (57%)</td>
<td>2/23 (9%)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Median neopterin:creatinine urine µmol/mol (25th-75th %tile)</td>
<td>147 (124-266)</td>
<td>126 (108-342)</td>
<td>161 (117-266)</td>
<td>186 (118-454)</td>
<td>165 (130-253)</td>
<td>n.s.</td>
</tr>
<tr>
<td>No. of subjects with elevated neopterin:creatinine urine</td>
<td>10/23 (43%)</td>
<td>3/12 (25%)</td>
<td>13/34 (38%)</td>
<td>11/29 (38%)</td>
<td>12/31 (39%)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Post-hoc Tukey's honestly significant difference test
\(^a\) CIS/RR < B/SP/PP, p < 0.01; B/PP > CIS/RR/SP, p < 0.02.
\(^b\) CIS/B < RR/SP/PP, p < 0.001; RR < SP/PP, p < 0.001.
4.4.3 HIV-1 Infection

In the 19 patients with HIV-1 infection, there was a correlation between the CD4+ T-cell count and the neopt:creat. urine (Pearson’s correlation coefficient = -0.59, p < 0.01; Fig. 4.4). The neopt:creat. urine of the 11 HIV-1 infected patients with a CD4 T-cell count less than 100 / μL (normal CD4 T-cell count = 350 - 2200 / μL) was significantly higher than the 8 patients with a CD4 T-cell count greater than 100 (median neopt:creat. urine = 1271 μmol/mol [25th-75th% = 804-2106] vs. 532 μmol/mol [240-1064], p = 0.03).

Figure 4.4. Neopt:creat. urine vs. CD4+ T-cell count: a linear regression of the natural logarithm of the neopt:creat. urine and the CD4+ T-cell count in 19 patients with HIV-1 infection. The dotted line represents the regression line.

4.4.4 Rheumatoid Arthritis

Patients with rheumatoid arthritis had a median active joint count of 14 [range = 8-48]. No correlation was found between the active joint count and the neopt:creat. urine (Pearson’s correlation coefficient = 0.1, p = n.s.). Patients with an active joint count greater than 14 had a similar neopt:creat. urine to patients with a joint count less than or
equal to 14 (median neopt:creat._urine = 417 μmol/mol [25\(^{th}\) - 75\(^{th}\)% = 260-627] vs. 384 μmol/mol [253-496], p = n.s.).

4.4.5 Posterior Uveitis

Using an unpaired T-test, patients with posterior uveitis had a significantly higher urinary neopterin excretion compared to normal control subjects (median neopt:creat._urine = 135 μmol/mol [25\(^{th}\) - 75\(^{th}\)% = 98-214] vs. 99 μmol/mol [76-281], p = 0.01). Similarly, the group with posterior uveitis had a significantly higher proportion of patients with an elevated neopt:creat._urine than normal controls (7/20 vs. 2/56, p < 0.001). Seven out 20 patients (35%) with posterior uveitis had active disease defined symptomatically by the presence of floaters or clinically using fundoscopy and/or fluorescein angiography. Patients with active disease had a slight trend towards higher neopt:creat._urine than patients without active disease (median neopt:creat._urine = 300 μmol/mol [25\(^{th}\) - 75\(^{th}\)% = 96-395] vs. 117 μmol/mol [99-215], p = 0.08). Sixteen out of 20 (80%) patients with posterior uveitis were on oral immunosuppressive agents at the time of the urine collection; 15 patients were on oral prednisone (mean daily dose = 14 mg (range = 4 – 40)), 3 patients were on azathioprine (2 patients on 100 mg/day, and one patient on 150 mg/day), and one patient was on cyclosporin A (230 mg/day). The neopt:creat._urine of the 4 patients not on immunosuppressive agents was similar to the 16 patients on immunosuppression (median neopt:creat._urine = 112 μmol/mol [25\(^{th}\) - 75\(^{th}\)% = 95-300] vs. 143 μmol/mol [98-414], p = n.s.)
Figure 4.5. Urinary neopterin vs. creatinine: a linear regression of the natural logarithm of the urinary neopterin and creatinine concentrations in all 261 subjects participating in the study (left hand graph) and in the 155 subjects with normal neopt:creat.urine (< 200 μmol/mol, right hand graph). The dotted lines represent the regression lines.

4.4.6 Urinary neopterin vs. urinary creatinine

The urinary concentrations of neopterin and creatinine correlated (n = 261, Pearson’s correlation coefficient = 0.29, p < 0.01, Fig. 4.5). When the analysis was limited to the subjects with a normal neopt:creat.urine (< 200 μmol/mol) the correlation was further improved (n = 155, Pearson’s correlation coefficient = 0.92, p < 0.001, Fig. 4.5).
4.4.7 Creatinine vs. disability in patients with multiple sclerosis

In patients with multiple sclerosis the absolute concentration of urinary creatinine (\(\text{creat}_{\text{urine}}\)) was higher in patients with progressive disease compared to patients with relapsing disease (median \(\text{creat}_{\text{urine}}\) = 189 mol/L [25\(^{th}\)-75\(^{th}\)% = 53-271] vs. 98 mol/L [25\(^{th}\)-75\(^{th}\)% = 30-157], \(p = 0.006\)). The \(\text{creat}_{\text{urine}}\) correlated weakly with the EDSS (Spearman’s rank correlation coefficient = 0.27, \(p = 0.001\)). MS patients with and EDSS of less than 3.5 had a significantly lower \(\text{creat}_{\text{urine}}\) than patients with and EDSS of 3.5 to 6.0 or patients with an EDSS greater than 6.0 (median \(\text{creat}_{\text{urine}}\) = 84 mol/L [25\(^{th}\)-75\(^{th}\)% = 45-152] vs. 172 mol/L [25\(^{th}\)-75\(^{th}\)% = 69-266] vs. 152 mol/L [25\(^{th}\)-75\(^{th}\)% = 28-271], \(p = 0.03\), Fig. 4.6)

**Figure 4.6. Urine creatinine by EDSS in patients with MS:** combined scatter plus box and whisker plots of \(\text{creat}_{\text{urine}}\). The box represents the 25\(^{th}\)-75\(^{th}\) quartile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived.
4.5 Discussion

The primary aim of this study was to see whether urinary neopterin excretion had any potential as a surrogate inflammatory marker in MS. Urine neopterin was chosen over blood or CSF because of the high fractional excretion of neopterin (Fuchs et al., 1992) and the advantages for collection, which urine has over other body fluids in an intensive longitudinal study requiring frequent monitoring (see Chapter 5). This cross-sectional study demonstrated that a proportion of patients with CNS demyelination have increased urinary neopterin excretion, as well as patients with HIV-1 infection, rheumatoid arthritis and posterior uveitis. Possible confounding factors in the cross-sectional study relate to differences in the age and sex ratios of the patient groups, as a result of the rheumatoid arthritis patients being older and the striking male predominance in the patients with HIV-1 infection. These differences probably had little effect on the studies outcome as the lower creatinine excretion in females was corrected for and the increased urinary neopterin excretion associated with increasing age (Fuchs et al., 1992) typically occurs in subjects over the age of 65 years, which accounted for only 3% (8/261) of this study’s patient population. In addition, the neopt:creatinine of the male and female subjects in this study were similar and no correlation was found between the neopt:creatinine and age.

All clinical subtypes of demyelinating diseases, except patients with benign relapsing disease, showed elevated urinary neopterin excretion. No correlation was found between either clinical relapse or Gd-enhanced MRI activity and the urinary neopterin excretion. However during clinical relapse there was a clear temporal profile with the neopt:creatinine being higher in patients sampled within the first week after the onset of symptoms compared to patients whom were sampled greater than a week after the
onset of symptoms. This suggests that there is an inflammatory timetable to a clinical relapse with a greater quantity of pro-inflammatory markers being produced within the first week after the relapse. When trying to correlate inflammatory markers with clinical disease activity the timing of the specimen collection in relation to the onset of the clinical relapse should be taken into account.

In addition the temporal profile of urinary neopterin excretion in relation to Gd-enhanced MRI activity in MS has not been defined. MRI activity tends to occur in clusters (Smith et al., 1993), with lesions enhancing for variable periods (range from one to ten weeks) (Lai et al., 1996). Whether the inflammatory timetables of the individual lesions are synchronous or asynchronous is unknown, therefore the timing of the specimen collection in relation to the time-course of the individual lesions or clusters of lesions is critical in determining the relationship between inflammatory markers and MRI activity. A cross-sectional study would have to be sufficiently large to provide enough time points across the life cycle of various lesions to answer to this question. A small detailed MRI study is presented in the Chapter 6, which attempts to answer this question. In this context it is worth noting that neopterin excretion tended to be higher in patients with Gd-enhancing MRI activity. A larger cross-sectional study may be required to demonstrate a significant relationship between neopterin excretion and MR activity. Another caveat to be kept in mind is that the raised levels of neopterin excretion in patients with primary progressive disease are not associated with Gd-enhancing MR activity as these patients have little if any MR activity (Thompson et al., 1991; Kidd et al., 1996).
The patients with clinically isolated syndromes are a particularly interesting group as approximately two thirds of them will go on to develop clinically definite MS within 5 years (Morrissey et al., 1993). Finding increased urinary neopterin excretion in some patients with clinically isolated syndromes suggests the presence of ongoing inflammation possibly indicating “sub-clinical” MS. An interesting question will be whether or not the presence of elevated levels of inflammatory markers, after the acute presentation of an isolated syndrome, predict the future development of clinically definite MS. This is currently being investigated in a prospective study.

Patients with HIV-1 infection and rheumatoid arthritis had significantly higher levels of neopterin excretion compared to patients with demyelinating diseases; implying that the inflammatory activity is greater in these two systemic diseases compared to multiple sclerosis. In the HIV-1 infected patients the CD4+ T lymphocyte count did correlate with neopterin excretion. This supports previous reports that neopterin is useful as an inflammatory, as well as a surrogate, marker of disease activity in HIV-1 infection (Fuchs et al., 1988; Melmed et al., 1989). In the patients with rheumatoid arthritis, raised urinary neopterin excretion confirms observations from an earlier study (Reibnegger et al., 1986), but failed to demonstrate a correlation with disease activity. The lack of correlation between these inflammatory markers and clinical disease activity may relate to fact that all the patients with rheumatoid arthritis had active disease, resulting in a selection bias due to a skewed population of patients.

The majority of patients with posterior uveitis, despite being on oral immunosuppressive agents, still had a mildly elevated neopt:crea:urine compared to normal control subjects. This was not statistically apparent using ANOVA and a post-
hoc honestly significant difference test, but became apparent with an unpaired T-test. These results confirm a previous study which included several of the same patients but not the control subjects participating in the present study (Palmer et al., 1995). Patients with clinically active posterior uveitis tended to have higher levels than those with inactive disease, suggesting that neopt::creatinine may be a suitable surrogate marker of inflammation in this disease. Raised neopt::creatinine in idiopathic posterior uveitis is interesting for several reasons. Firstly, the retina is anatomically located within the CNS and has the added advantage of being accessible for direct observation of disease activity. Finding raised urinary neopterin excretion in patients with posterior uveitis, particularly active posterior uveitis, implies that increased CNS derived neopterin can be detected in the urine. Secondly, the inflammatory volume in posterior uveitis is relatively small. Therefore the raised urinary neopterin excretion in posterior uveitis implies that neopt::creatinine is a very sensitive inflammatory marker, a prerequisite for monitoring other diseases with relatively small inflammatory volumes such as MS. Thirdly, idiopathic posterior uveitis is aetiologically related to MS, since a proportion of patients with idiopathic posterior uveitis will go on to develop MS (Graham et al., 1989) and patients with MS frequently have perivenous sheathing of the retinal veins (Lightman et al., 1987) which pathologically resembles the perivascular inflammatory cuffing seen within the CNS (Kerrison et al., 1994). It is conceivable that some of the patients with posterior uveitis had asymptomatic active MS. This could have been determined using Gd-enhanced MRI, but unfortunately this was not performed in this study.

The urinary creatinine concentration was higher in MS patients with progressive disease compared to patients with relapsing disease and correlated very weakly with
the EDSS ($r^2 = 0.07$). This observation has been noted previously in a study on urinary myelin basic protein-like material (MBPLM) in a subgroup of patients participating in a interferon-$\beta$-1b trial (Whitaker et al., 1995b). In this study levels of urinary creatinine were found to be a major confounding variable, with increased levels correlating with both clinical and MRI parameters of disease progression and accounting for some of the positive correlations between urinary MBPLM and disease progression. The reasons for the association between disability and creat.$_{\text{urine}}$ are unknown but may relate to voluntary dehydration MS patients with bladder dysfunction impose on themselves, to control troublesome urinary frequency (Matthews, 1991). This tends to affect patients who are more disabled, unless permanently catheterised, since urinary complaints are more common in patients with increased disability.

4.6 Conclusion

In a cross-sectional study, patients with all clinical subtypes of MS except benign disease had increased urinary neopterin excretion, however it did not correlate with clinical relapse or Gd-enhanced MRI activity. Raised neopterin levels in relation to a clinical relapse appear to be higher in the first week after the onset of clinical symptoms compared to later time points.

5.1 Background

Following on from the results of the cross-sectional study presented in Chapter 4, an intensive longitudinal study was performed to assess the potential of urinary neopterin excretion as a marker of disease activity in MS. The primary aim of this was to see whether differences between clinical subtypes of patients with MS existed and to correlate urinary neopterin excretion with clinical disease activity. Patients with MS were compared to healthy control subjects.

5.2 Hypotheses

Increased urinary neopterin excretion correlates with clinical relapse and is elevated in patients with relapsing remitting and secondary progressive disease compared to patients with primary progressive disease.

5.3 Methods

5.3.1 Patients and Subjects

Fourteen normal control subjects and 31 patients with clinically definite MS (Poser et al., 1983; Appendix I) were asked to collect consecutive daily urine specimens for periods from 2 to 12 weeks (Table 5a). Normal control subjects were recruited as volunteers from laboratory staff and the general population, with the aim of obtaining equal numbers of male and female subjects of similar age to the patients with MS. They were only included if they had no underlying medical problems. All patients were recruited from the National Hospital for Neurology and Neurosurgery, London. The
Study was approved by the Hospital’s Ethics Committee and written informed consent was obtained from all participants (see Appendix VI). Subjects were instructed to collect daily early morning urine specimens as outlined in Chapter 2. All specimens from individual subjects were brought to the laboratory at the end of the study period. In addition to collecting urine, all subjects had to keep a detailed daily diary throughout the study period (see Appendix VII). The diary used a fixed format documenting any infections, intercurrent medical illnesses, use of corticosteroids or immunosuppressive agents and comments on changes in neurological function. At the end of the study each patient underwent a structured interview to discuss the significance of any entries in the diary and also had a neurological examination so that physical disability could be rated according to Kurtzke's EDSS (Kurtzke, 1983; Appendix II). Patients were classified into as either primary progressive, relapsing remitting or, secondary progressive using established criteria (Lublin and Reingold, 1996). The presence of excess urinary nitrites was used as a screen to test for either bacterial colonisation or infection of the lower urinary tract (see Chapter 2).

5.3.2 Neopterin and Creatinine Assays

Urinary neopterin and creatinine were measured by HPLC as described in Chapter 2. All samples were assayed without the knowledge of the clinical status of the patients.

5.3.3 Data transformation

The neopt:creat.urine in females was divided by the correction factor of 1.2 as highlighted in Chapter 2. As infection can cause an increase in neopterin production (Fuchs et al., 1992), which may precede the onset of symptoms by 24 to 48 hours, all measurements on days of documented infection including the two days prior to the symptomatic phase of the infection were excluded from the analysis. Due to the nature and design of this
A longitudinal study, infective episodes were not documented objectively, and subjects were given the benefit of the doubt with regard to their own assessment and interpretation of infection. The neopt:creatinine were then processed to generate several variables: (1) The mean of all ratios for an individual was used as an overall indicator of neopterin production. (2) The intra-individual variance of neopt:creatinine was used to uncover fluctuations in day to day neopterin production that would not necessarily be associated with well defined peaks in the serial levels or with an elevation in the mean above normal. (3) Peaks in the neopt:creatinine time curve were identified and counted by calculating the second order derivative of the plot. This derivation was checked visually and if any peaks were missed by the algorithm, they were manually flagged. A peak had to be of at least 2 days duration to be considered significant. Other variables analysed were related to the specific pattern of elevated neopterin excretion and included (4) the proportion of days in which the neopt:creatinine were above normal (greater than 200 μmol/mol) or (5) whether these elevated levels were associated with definite peaks or merely reflected an elevated baseline.

5.3.4 Statistics

Levene’s test of homogeneity of variances was used to test whether variables were normally distributed. Normally distributed continuous variables were compared using a one-way analysis of variance (ANOVA). If the ANOVA was significant, individual groups were compared with each other using a post-hoc Tukey’s honestly significant difference test. This test delineates the inter-group differences responsible for the significant ANOVA. The majority of variables could be normalised using a simple logarithmic transformation and when this was not possible, groups were compared using the non-parametric Kruskal-Wallis one-way analysis of variance. Other non-parametric data were compared using the Yates corrected Chi-squared test or the Fisher exact test
was used when an expected value was less than 5. Normalised continuous data were correlated using simple linear regression and a two-tailed Pearson’s test. A p value of <0.05 was considered statistically significant. Data involving serial measurements were computed as subject means using all values available for that particular subject, after the exclusion of those measurements related to infection.

5.4 Results

5.4.1 Clinical Features

The clinical data are presented in Table 5a. Ten patients had primary progressive MS, 10 relapsing remitting and 11 secondary progressive disease. There was a significant difference in the sex ratios of the 4 groups (p=0.03, Table 5a). The mean age, calculated on the final day of urine collection for each study participant, differed between groups with the primary progressive patients being significantly older than the other subjects (p = 0.001, Table 5a). Similarly, the mean age of disease onset of the primary progressive patients was significantly older than the relapsing remitting and secondary progressive patients (p = 0.006, Table 5a), which is in accordance with previously published observations (Confavreux et al., 1980). As expected the disease duration and level of disability, was significantly greater in the progressive MS groups (p = 0.004 and p < 0.001 respectively, Table 5b). Nine clinical relapses occurred in 9 patients during the study period; 5 in the relapsing and 4 in the secondary progressive groups. Two of these relapses were treated with high dose intravenous corticosteroids (1g methylprednisolone intravenously for 3 days). In addition to these relapses, 2 patients in the secondary progressive group received intravenous methylprednisolone, one patient 3 weeks prior to commencing the study and another patient during the study period. A single patient in the primary progressive group was on low dose oral prednisone (10-25mg/day)
throughout the study period. No other patients received any other immunosuppressive or immunomodulatory therapy. Twenty-nine infections occurred in 21 of the 45 subjects during the study. These all involved the upper respiratory tract except for 2 urinary tract infections, and an episode each of gingivitis, herpes labialis and a superficial skin infection. When the number of infections occurring in each group was normalised for the duration of the study period no significant differences were noted in the incidence of infection between the study groups, although the MS patients tended to have fewer infections (Table 5a). Asymptomatic bacterial colonisation of the lower urinary tract was detected in 4 subjects, all of whom were using intermittent self-catheterisation.
Table 5a. Demographic and clinical data.

<table>
<thead>
<tr>
<th></th>
<th>Normal Controls</th>
<th>Primary Progressive</th>
<th>Relapsing Remitting</th>
<th>Secondary Progressive</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>14</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>7:7</td>
<td>8:2</td>
<td>2:8</td>
<td>3:8</td>
<td>0.03</td>
</tr>
<tr>
<td>Age</td>
<td>35.1 (SD 9.4)</td>
<td>47.5 (SD 9.5)</td>
<td>31.8 (SD 8.0)</td>
<td>37.7 (SD 6.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>Age at disease onset</td>
<td>37.5 (SD 10.2)</td>
<td>27.0 (SD 6.9)</td>
<td>26.4 (SD 6.7)</td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>Disease duration</td>
<td>9.9 (SD 4.0)</td>
<td>4.7 (SD 2.9)</td>
<td>11.3 (SD 5.3)</td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>Median EDSS*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(range)</td>
<td>6.5 (4.5 - 7.5)</td>
<td>2.0 (0 - 3.5)</td>
<td>6.0 (6.0 - 8.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. relapses</td>
<td>0</td>
<td>5</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. infections / month</td>
<td>0.6 (SD 0.8)</td>
<td>0.2 (SD 0.3)</td>
<td>0.3 (SD 0.4)</td>
<td>0.2 (SD 0.3)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

*EDSS = Kurtzke's Expanded Disability Status Scale (Kurtzke, 1983; Appendix II).
Figure 5.1. Mean neopt:creatinine (see Table 5b): combined box plus whisker and scatter plots of the natural logarithm (Loge) of the mean neopt:creatinine for normal control subjects and patients with primary progressive, relapsing remitting and secondary progressive MS. These measurements excluded all neopt:creatinine associated with symptomatic infections. The box represents the 25th-75th quartile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived. The horizontal dashed line is the upper limit of normal.
Figure 5.2. Variance neopt:creat.urine (see Table 5b): combined box plus whisker and scatter plots of the natural logarithm (Loge) of the variance of the serial neopt:creat.urine for normal control subjects and patients with primary progressive, relapsing remitting and secondary progressive MS. These measurements excluded all neopt:creat.urine associated with symptomatic infections. The box represents the 25th-75th quartile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived. The horizontal dashed line is the upper limit of normal.
Figure 5.3. Peaks per month (see Table 5b): combined box plus whisker and scatter plots of the number of peaks per month in the serial neopt:creatinine time-course plots unrelated to infection for normal control subjects and patients with primary progressive, relapsing remitting and secondary progressive MS. These measurements excluded all neopt:creatinine associated with symptomatic infections. The box represents the 25th-75th quartile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived.
Figure 5.4. Proportion of days greater than normal (see Table 5b): combined box plus whisker and scatter plots of the percentage of days with neopt:creatinine greater than normal for normal control subjects and patients with primary progressive, relapsing remitting and secondary progressive MS. These measurements excluded all neopt:creatinine associated with symptomatic infections. The box represents the 25th-75th quartile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived.
5.4.2 Neopt:crea\textsubscript{urine} Results

Urine specimens were collected daily for approximately 4 weeks in normal control subjects and for 7 to 9 weeks in patients with MS (Table 5b), with a minimum collection period of 14 days. The number of days excluded due to infections in all groups was similar (Table 5b). After measurements related to symptomatic infections were excluded, the overall mean neopt:crea\textsubscript{urine} was significantly higher in patients with MS than normal controls (p < 0.001, Table 5b and Fig. 5.1). To analyse the pattern of this observed difference the other variables relating to the individual neopt:crea\textsubscript{urine} time-course plots described above were analysed. Firstly, the natural logarithm (Log\textsubscript{e}) of the variance of the mean neopt:crea\textsubscript{urine} for each subject was found to be greater in patients than normal control subjects (p<0.001, Table 5b and Fig.5.2). This indicates greater day to day fluctuations in neopterin excretion, or increased variability of the baseline of the neopt:crea\textsubscript{urine} time-course plot. Secondly, the proportion of days as a percentage on which the neopt:crea\textsubscript{urine} was found to be greater than normal (defined as the mean plus two and half times the standard deviations of the neopt:crea\textsubscript{urine} for normal control subjects, i.e. > 200 \(\mu\)mol/mol) was significantly elevated in all patient groups (p<0.001, Table 5b and Fig. 5.3). By using a cut-off, as having at least 50% of the serial neopt:crea\textsubscript{urine} measurements above the upper limit of normal, 3/10 primary progressive, 2/10 relapsing remitting and 5/11 secondary progressive had elevated baselines, with only the secondary progressive group significantly different from normal control subjects (p=0.009, Table 5b).

In 3/14 of the control subjects 3 small peaks were found in the neopt:crea\textsubscript{urine} time curve, which could not be attributed to a symptomatic infection in a total of 14
months (424 days) of serial urine testing. This compared to 143 peaks in 59 months (1759 days) of serial urine testing, which were found in all except 2 of the primary progressive patients (Table 5b, p < 0.001). When expressed as a rate, the normal control subjects had significantly fewer peaks unrelated to infection than the MS patient groups (p<0.001, Table 5b and Fig. 5.3).

Although the secondary progressive patients tended to have higher mean neopt:creat-urine, greater variability and elevations in their baselines with more peaks than the primary progressive and relapsing remitting patients, this did not reach statistical significance (Table 5b and Figs. 5.1-5.4).
Table 5b. Neopt:creat_urine data.

<table>
<thead>
<tr>
<th></th>
<th>Normal Controls</th>
<th>Primary Progressive</th>
<th>Relapsing Remitting</th>
<th>Secondary Progressive</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average no. of collection days per subject</td>
<td>30 (SD 16)</td>
<td>64 (SD 29)</td>
<td>49 (SD 33)</td>
<td>57 (SD 33)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Average no. of days excluded per subject due to recorded infection</td>
<td>2.9 (SD 3.9)</td>
<td>1.6 (SD 2.1)</td>
<td>3.7 (SD 5.2)</td>
<td>4.5 (SD 7.0)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Mean neopt:creat_urine (median μmol/mmol (interquartile range))</td>
<td>134 (97 - 152)</td>
<td>187 (135 - 231)</td>
<td>187 (165 - 277)</td>
<td>218 (164 - 517)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mean Loge variance neopt:creat_urine</td>
<td>6.5 (SD 1.0)</td>
<td>8.2 (SD 1.8)</td>
<td>9.2 (SD 1.6)</td>
<td>10.3 (SD 3.2)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>% Days with neopt:creat_urine greater than normal (median (interquartile range))</td>
<td>0% (0-6)</td>
<td>16% (6-62)</td>
<td>28% (21-36)</td>
<td>49% (14-86)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>No. subjects with an elevated baseline</td>
<td>0 / 14</td>
<td>3 / 10</td>
<td>2 / 10</td>
<td>5 / 11</td>
<td>0.05#</td>
</tr>
<tr>
<td>No. subjects with peaks in serial neopt:creat_urine unrelated to infection</td>
<td>3 / 14</td>
<td>8 / 10</td>
<td>10 / 10</td>
<td>11 / 11</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mean no. peaks in serial neopt:creat_urine per subject / month</td>
<td>0.2 (SD 0.6)</td>
<td>2.1 (SD 1.8)</td>
<td>3.0 (SD 1.7)</td>
<td>3.3 (SD 2.3)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

* All comparisons of normal controls with MS patients groups are significant (p< 0.01).
# Comparison of secondary progressive patients with controls is significant (p=0.009).
5.4.3 Relapses

Five of the 9 relapses involved the sensory pathways, 2 the brainstem, and one each the cerebellum and optic nerve. All patients who had relapses had elevated mean neopt:creat.urine. The median maximum neopt:creat.urine measured during a relapse was 559μmol/mol (range 218 - 13329μmol/mol). The median neopt:creat.urine of the symptomatic days of the relapses was higher than the median levels of days not associated with the relapse in the same patients (215μmol/mol (interquartile range (179-358) vs. 160μmol/mol (interquartile range (152-189), p = 0.02, Fig. 5.5). All patients also had increased variability in their baseline neopt:creat.urine during the relapse. The median natural logarithm of the variance of the neopt:creat.urine during the symptomatic days of the relapse was 9.7 (range = 7.6 - 16.4), which although greater than the variability during other periods of the study as well as greater than variability of the patients without clinical relapse, did not reach statistical significance. Five relapses were temporally associated with peaks in the baseline, one of which was the highest detected in the study (Fig 5.12, patient SP-3). These peaks preceded the onset of symptoms in 4 out of 5 cases by a variable period of time (range 1 to 14 days). In one case however the onset of symptoms preceded the neopterin peak by 5 days. Of the 4 relapses not associated with a clear peak in the neopt:creat.urine, 3 had elevated baselines and one had a single elevated neopt:creat.urine measurement during the relapse. This latter relapse involved a recurrence of symptoms related to a previous brainstem episode with vertigo, dysequilibrium and facial myokymia.
Figure 5.5. Relapse: combined box plus whisker and scatter plots of the natural logarithm ($\log_{e}$) of the mean neopt:creat.urine for 9 patients with MS. The open circles (O) represent the mean neopt:creat.urine for the days prior to the onset of the clinical relapse and the open squares (□) the mean neopt:creat.urine during symptomatic phase of the clinical relapse. These measurements excluded all neopt:creat.urine associated with symptomatic infections. The box represents the 25th-75th quartile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived. Solid lines link the two points related to each individual patient. The horizontal dashed line represents the upper limit of normal.
5.4.4 Corticosteroids

A brainstem relapse associated with a modest increase in the neopt:creat. urine (maximum neopt:creat. urine = 291 μmol/mol) was treated with high dose intravenous corticosteroids (1g methylprednisolone intravenously for 3 days). The neopt:creat. urine normalised to levels below 100 μmol/mol for a 6 day period, from day 3 of the infusion, but then increased to pre-treatment levels for the remainder of the study. The second patient who received intravenous corticosteroids for a sensory spinal cord relapse associated with a positive Lhermitte's sign, had a dramatic fall in her neopt:creat. urine, from 1467 μmol/mol to 168 μmol/mol on day 2 of the methylprednisolone infusion. This response was maintained for a further 7 days before the neopt:creat. urine increased again to levels above 300 μmol/mol coinciding with an upper respiratory tract infection. The patient with secondary progressive MS who received intravenous corticosteroids 3 weeks prior to entry into the study, had a persistently elevated neopt:creat. urine from day 1 of the study and therefore no effects of the corticosteroid treatment on his neopt:creat. urine could be inferred. The other secondary progressive patient, who had a severe cerebellar tremor but no evidence of a clinical relapse, received intravenous methylprednisolone when the baseline neopt:creat. urine were normal; no effects were noted. Finally, the one patient with primary progressive MS who was on low dose oral prednisone had significantly elevated neopt:creat. urine throughout the study.
5.4.5 Infections

During the study 10 infections were recorded by 8 control subjects, and 19 by 13 patients with MS. To see if infection precipitated relapse or an increase in urinary neopterin excretion, the “at risk period” related to these infectious episodes was analysed. This is defined as the 6 week period, starting a week prior to and ending 5 weeks after the onset of the infection (Sibley et al., 1985). As this period includes the episode of infection, a known stimulus for neopterin production, the mean and variance of the neopt:creat._urine could not be used. The proportion of days associated with an elevated neopt:creat._urine in relation to the onset of the infection was used instead. Not all subjects had a complete set of data points for each infectious episode and in 2 cases this period had to be modified as a result of a second infection occurring prior to the end of the first “at risk period”. In these cases the first “at risk period” was terminated the day prior to the onset of the second infection and the next “at risk period” was started the following day. No data point was used more than once in this analysis. Eight out of 13 patients had clearly defined neopt:creat._urine peaks following documented infections. This inflammatory activity tended to persist following the infection for variable periods of time of up to 6 weeks. In comparison, normal control subjects had a brisk return to baseline, usually by day 10, from the elevated levels associated with the infectious episode (Fig. 5.2). Of note 3 of the 9 relapses were temporally associated with infections, becoming symptomatic in the “at risk period” on days 23, 31, and 33 post-infection. Two such examples are given in Fig. 5.3, as patients SP-3 and SP-4. Of the 4 subjects with asymptomatic bacterial colonisation of the lower urinary tract, one had a normal baseline with 2 small peaks, 2 had elevated baselines with increased variability and one had a normal baseline with intermittent activity that was associated with a clinical relapse.
Figure 5.6. Infection: longitudinal serial scatter plot of the proportion of days with a neopt:creat urine greater than normal in relation to the onset of a symptomatic infection, for 10 infections in 8 normal control subjects and 19 infections in 13 patients with MS. The shaded area represents the approximate period related to the infectious episode. Day 0 represents the day of onset of symptoms related to the infection.
5.4.6 Individual Profiles and Frequency of Testing

As daily monitoring is impractical for long term follow-up, it was compared to alternate daily and weekly urine sampling frequencies (Table 5c). No differences are observed between these different sampling frequencies with regard to the cross-sectional data analysis, except that the degree of variability in the baseline is reduced with fewer samples. However, the detailed time-course and peak analysis becomes more difficult with smaller numbers of samples as some peaks are short lived and may be missed by infrequent sampling. Daily serial individual neopt:creat.urine profiles of 3 control subjects and 10 patients with brief case histories are shown in Fig.5.3. They illustrate the different patterns of elevated neopt:creat.urine found in patients with MS, and demonstrate the effects of clinical relapses and infections on the neopt:creat.urine time-course plot.
Table 5c. Comparison of the neopt:creat.urine data for daily, alternate day and weekly sampling.

<table>
<thead>
<tr>
<th></th>
<th>Normal Controls</th>
<th>Primary Progressive</th>
<th>Relapsing Remitting</th>
<th>Secondary Progressive</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean neopt:creat.urine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(median µmol/mmol (interquartile range))</td>
<td>daily 134 (97 - 152)</td>
<td>187 (135 - 231)</td>
<td>187 (165 - 277)</td>
<td>218 (164 - 517)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>alternate daily 127 (98 - 151)</td>
<td>188 (135 - 231)</td>
<td>190 (155 - 246)</td>
<td>247 (162 - 468)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>weekly 126 (95 - 140)</td>
<td>184 (130 - 240)</td>
<td>184 (180 - 340)</td>
<td>237 (145 - 444)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Mean Loge variance neopt:creat.urine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>daily 6.5 (SD 1.0)</td>
<td>8.2 (SD 1.8)</td>
<td>9.2 (SD 1.6)</td>
<td>10.3 (SD 3.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>alternate daily 6.4 (SD 1.1)</td>
<td>8.3 (SD 2.0)</td>
<td>8.9 (SD 1.5)</td>
<td>10.2 (SD 3.3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>weekly 4.9 (SD 2.7)</td>
<td>7.6 (SD 1.6)</td>
<td>8.5 (SD 3.1)</td>
<td>9.5 (SD 3.5)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Case Histories:

Figures 5.7-5.12. Longitudinal profiles of serial neoP:creatinuria for 3 normal subjects and 10 patients with multiple sclerosis, for periods of 6 and 12 weeks respectively. The dotted line represents the upper limit of normal for the neoP:creatinuria (200 μmol/mol), the dark bars episodes of symptomatic infection, and the open squares, clinical relapses. The episodes of infection and clinical

Figure 5.7. Healthy control subjects: NC-1: A healthy 30 year male, with a flat baseline, limited variability and no peaks. NC-2: A normal 30 year old female with an episode of herpes labialis clearly associated with a well formed peak. NC-3: A normal 33 year old female with a neoP:creatinuria peak related to a mild upper respiratory tract infection, which was followed by a second peak that could not be attributed to a symptomatic infection. Only 3 such peaks occurred in 14 months of serial testing in the normal control subjects and probably represent episodes of subclinical viral infection.
**Figure 5.8. Primary progressive MS:** **PP-1:** A 50 year old male with primary progressive MS of 12 years duration and an Kurtzke's Expanded Disability Status Scale (EDSS) of 7. He demonstrates a slight increase in variability in the baseline with some peaks unrelated to infection. **PP-2:** A 41 year old female with primary progressive MS of 8 years duration, an EDSS of 6.5, and a persistently elevated neopt:creatinine throughout the study period. She had progressed from 4.5 to 6.5 on the EDSS, in the 15 months preceding this study.
Figure 5.9. Primary progressive MS: PP-3: A 43 year old male with primary progressive MS of 7 years duration, an EDSS of 4.5, and a normal neopt:creat. urine time-course plot. His disability had not progressed in the preceding 3 years. PP-4: A 46 year old male with primary progressive MS of 20 years duration and an EDSS of 7.5. His disability had also not progressed in the preceding 3 years.
Figure 5.10. Relapsing remitting MS: **RR-1**: A 27 year old female with relapsing remitting (RR) MS of 2.5 years duration and an EDSS of 1.5, demonstrates intermittent increases in neopterin excretion with a large peak occurring in the “at risk period” of a symptomatic infection. **RR-2**: A 29 year female with relapsing remitting MS of 6 years duration, an EDSS score of 3.5, and an intermittent elevation in neopterin excretion with a variable baseline and numerous peaks.
Figure 5.11. Secondary progressive MS: SP-1: A 39 year old female with secondary progressive MS of 8 years duration, and an EDSS of 6.0. Her plot demonstrates a persistently elevated baseline, increased variability and clear peaks occurring in the at risk periods post infection. She did not develop any new neurological symptoms during these periods but did complain of increased fatigue. She was one of the more rapidly progressive patients moving from an EDSS of 4.5 to 6.0 in the 12 months preceding this study. SP-2: A 40 year old female with secondary progressive MS of 10 years duration and a EDSS of 6.0. She had the highest elevation in neopterin excretion throughout the study period. Although she did not have any neurological symptoms that could be classified as a relapse, she complained of numerous intermittent symptoms, marked day to day fluctuations in activities of daily living and severe fatigue. Her disability, however had not changed in the preceding 12 months.
Figure 5.12. Secondary progressive MS: SP-3: A 35 year female with SP MS of 16 years duration and an EDSS of 6.0. She had a persistently elevated baseline with numerous peaks and a symptomatic spinal cord relapse, with sensory symptoms and an associated Lhermitte’s sign. Her relapse and increased neopterin excretion was probably precipitated by the preceding viral upper respiratory tract infection. SP-4: A 46 year old female with SP MS of 21 years duration, an EDSS of 6.5, and a peak in her neopt:creat:urine associated with a mild episode of optic neuritis.

5.5 Discussion

Patient groups could not be age and sex matched as primary progressive MS is commoner in males, and has an older age of disease onset (Confavreux et al., 1980.; Compston et al., 1991; Thompson et al.; 1997). The differences between the ages and sex ratios of the groups could however have possible confounding effects. Firstly, neopt:creat:urine is approximately 20% higher in subjects over the age of 65 (Fuchs et al., 1992), but as only one subject in this study was over 65 this would be unlikely to
affect the results. Secondly, the higher \text{neopt:creatinine} which occurs in females was corrected for and no differences were found between male and female control subjects after this correction. As patients and normal control subjects were not randomly selected nor age and sex matched, other unidentified confounding variables may have been present.

Neopterin excretion is clearly raised in the majority of the patients studied longitudinally, particularly in those with secondary progressive MS who tended to have the highest levels, followed by the patients with relapsing remitting and primary progressive disease. This inflammatory hierarchy between the different patient subgroups corresponds approximately to the one found using Gd-enhanced T1-weighted MRI (Thompson et al., 1991; Thompson et al., 1992). In addition, clinical activity in the form of a relapse was without exception associated with some elevation in neopterin excretion, albeit modest in some cases. Neopterin excretion tended to be higher during clinical relapse, implying that one is more likely to detect inflammation using \text{neopt:creatinine} during a relapse than when patients are in clinical remission. This has been demonstrated by MRI studies which show that the majority of patients in relapse (80%), compared to approximately 25% of patients in remission, have detectable Gd-enhancement (Thompson et al., 1991; Thompson et al., 1992; Thorpe et al., 1996). Due to the small number of relapses which occurred during this study, it was not possible to assess the association between the level of neopterin excretion and the severity of clinical relapse, or whether increases in neopterin excretion can predict the onset of a clinical relapse. An important thing to consider is that the clinical severity of a relapse is not necessarily related to the size of the offending lesion, or to its inflammatory mass, but rather to whether it is located in a clinically eloquent site.
Therefore the level of neopterin excretion, and for that matter other inflammatory markers, may bear no relation to the severity of a clinical relapse.

Asymptomatic elevations in neopterin excretion in the form of a persistently elevated baseline with or without superimposed peaks, or a normal baseline with intermittent peaks was common in patients with MS, supporting the MRI data showing that much of the disease activity in MS is asymptomatic (Miller et al., 1988; Koopmans et al., 1989; Willoughby et al., 1989; Capra et al., 1992). Two primary progressive cases who had been clinically stable for several years had normal neopterin excretion, suggesting that there was no inflammatory component to their disease process during the study period. Finding elevated levels of neopterin excretion in some patients with primary progressive MS, indicates that ongoing inflammation is occurring in these patients and supports findings from a pathological study demonstrating low grade inflammation in this subgroup of patients (Revesz et al., 1994). The fact that inflammatory activity can be detected by measuring urinary neopterin is very important, as neopterin may provide a means of monitoring inflammation in these progressive patients who characteristically lack MRI activity (Thompson et al., 1991; Kidd et al., 1996).

Although only a few patients received high dose intravenous corticosteroids, it appears that this form of treatment suppresses the production of neopterin for a short period of time. This is similar to the transient suppression of Gd-enhancement (< 1 week) seen after high dose intravenous methylprednisolone (Miller et al., 1992), and to that which occurs in renal and bone marrow transplant rejection, where the administration of high dose methylprednisolone exerts a dramatic suppression of
neopterin production (Wachter et al., 1992). However, the transplant scenario, in which neopterin excretion is used to monitor immunosuppressive therapy, is confounded by the persistent use of other immunomodulatory drugs, which if successful, cause a sustained suppression of neopterin excretion.

Since infections, particularly viral, result in increased IFNγ and neopterin production (Wachter et al., 1992), could the elevated neopt:crea:t urine be due to subclinical or low grade chronic infections? This is unlikely for several reasons (unless MS is caused by an unidentified viral infection). Firstly, in normal controls only 3 peaks in 14 months of daily urine testing could not be accounted for by a symptomatic infection, and secondly the number of subclinical infections would have had to have been very frequent (2 -3 per month). Thirdly, patients were instructed to document all ailments however minor and were always given the benefit of the doubt with regard to their interpretation of an infection. Fourthly, after excluding urinary tract infections, there is no evidence that patients with MS are more predisposed to infection than normal controls. On the contrary, patients with MS have been documented to have significantly fewer viral infections than healthy subjects (Sibley et al., 1985), a trend noted in this study (Table 5a). Finally, neopterin levels are higher in the CSF than in the serum of patients with MS, a finding opposite to that in non-inflammatory controls (Shaw et al., 1995). This indicates that neopterin is synthesised intrathecally which would be incompatible with a non-CNS infection causing the increased neopterin production. Taken together, I feel that the increase in urinary neopterin excretion is due to inflammatory activity within the CNS.
The observation that infection is associated with clinical relapse in patients with MS has been known for several years (Sibley et al., 1985; Andersen et al., 1991; Panitch, 1994). This longitudinal study confirms this and demonstrates that this effect is not limited to symptomatic relapses. A third of the relapses in this study occurred in the "at risk period" and 62% of subjects had a persistent increase in neopterin excretion post infection for a period much longer than expected (Fig. 5.2). From these data it appears that infections are a potent stimulus for causing prolonged elevations in neopterin excretion beyond the time period expected for an uncomplicated infection. This elevation in neopterin production, which occurs for up to and possibly beyond 6 weeks post infection, is presumably due to an increase or precipitation of both symptomatic and asymptomatic disease activity. As this was not seen in the normal control subjects it suggests a phenomenon related to MS disease activity. How infections precipitate disease activity is unknown, but it may result from the systemic induction of IFNγ. This data also suggests that the "at risk period" may be longer than that defined in the literature (Sibley et al., 1985).

This study demonstrates the informative nature of daily longitudinal data on individual patients with MS. A comparative analysis demonstrates that alternate day and weekly sampling is sufficient to detect inter-group differences, but a sampling frequency of less than this would miss some of the short lived, intermittent inflammatory activity.

5.6 Conclusion

When measured longitudinally urinary neopterin excretion is increased in the majority of patients with MS. Levels are raised in primary progressive, relapsing remitting and secondary progressive subtypes. However, no differences were noted between these
clinical subtypes. Clinical relapse is associated with increased urinary neopterin excretion and intravenous corticosteroids appears to suppresses the neopt:creat.urine for several days. Infections increase the neopt:creat.urine in patients with MS and the increase persists for several weeks, which can be interpreted as further evidence for infection being a precipitant of disease activity in MS.
Chapter 6 - The sensitivity and time-course of urinary neopterin excretion in response to a known immunological stimulus.

6.1 Background

In the longitudinal study presented in Chapter 5, increased urinary neopterin excretion was noted in response to symptomatic infections and in patients with MS during asymptomatic periods, suggesting that neopterin is a suitable marker for monitoring sub-clinical disease activity. MS is only rarely associated with a large systemic inflammatory response, which is more typical in systemic inflammatory disorders such as rheumatoid arthritis or infection. It is rare for MS patients to develop pyrexia, anorexia, cachexia or a marked acute phase response due to their disease. Possible reasons for this relate to the small inflammatory volumes present in MS, as well as the anti-inflammatory micro-environment which is present in the CNS (Cserr and Knopf, 1992). Any useful inflammatory marker would therefore have to be sensitive enough to detect relatively small amounts of inflammation. Results from the cross-sectional study presented in Chapter 4 suggest that urinary neopterin excretion is such a marker. Raised urinary neopterin levels, albeit mild, were found in patients with posterior uveitis, a disease of the CNS which is not only characterised by focal inflammation in a relatively small volume of tissue but it is also tolerated very poorly, affecting sight early on and usually requiring prompt immunosuppressive therapy. To study further the sensitivity of neopterin as an inflammatory marker in adults, serial urinary neopterin excretion was measured in a group of subjects undergoing various vaccinations or Mantoux testing with tuberculin purified protein derivative (PPD). The advantage of using vaccination or antigen inoculation as an immunological stimulus is that it provides a clearly defined
time point from which to study the time-course of increased neopterin production in vivo.

6.2 Hypothesis

Vaccination or Mantoux skin testing would result in an increase in urinary neopterin excretion with a characteristic time-course.

6.3 Methods

6.3.1 Subjects

Fifty eight healthy adults who were either receiving a vaccination or undergoing a Mantoux skin test with tuberculin PPD were included in the study. Informed consent was obtained from all subjects (Appendix VIII). They were divided into two groups depending on whether the predominant immunological stimulus was cell mediated or humoral. Forty five subjects undergoing Mantoux skin testing with tuberculin PPD (Evans Medical) and one subject who had a live rubella vaccine (Evans Medical) constituted the cell mediated immune (CMI) group. Twelve subjects undergoing vaccination with non-live vaccines were included in the humoral immune group. These latter vaccinations included 5 influenza (Fluvirin®, Evans Medical), 2 hepatitis B (Engerix B®, SmithKline Beecham), 2 typhoid (Typhim Vi®, Pasteur Mérieux), 1 hepatitis A (Havrix®, SmithKline Beecham) and 2 tetanus toxoid (Wellcome). A disproportionately large number of patients undergoing Mantoux skin tests were recruited to allow a comparison between subjects with positive or negative skin reactions. Subjects were instructed to collect daily urine specimens starting 2 days prior to and finishing 10 to 12 days after the vaccination or skin test. Early morning urine specimens were collected according to the methods outlined in Chapter 2. Patients were
also instructed to keep a daily diary documenting and describing any local or systemic side-effects of the vaccination as well as any infections contracted during the study period (Appendix IX).

6.3.2 Neopterin and Creatinine Assays

Neopterin and creatinine were measured by HPLC and the neopt:creat.urine values in female subjects were corrected as outlined in Chapter 2.

6.3.3 Data transformation

A baseline threshold was established for each subject by taking the mean of the neopt:creat.urine from day 2 pre- to day 3 post-vaccination inclusive. Subjects were only considered to have a significant increase in their neopterin excretion if at least one neopt:creat.urine after day 3 was more than double their calculated baseline threshold. The time-course plot was then generated for each group by plotting the mean daily corrected neopt:creat.urine against time. Peaks were identified and only considered significant if the height was at least double that of the baseline.

6.3.4 Statistics

Data was compared using the Student’s T, Kruskal-Wallis, Chi-squared and Fisher’s Exact tests as appropriate. A p-value of less than 0.05 was considered significant.

6.4 Results

The mean ages of the cell mediated and humoral immune groups were 36.6 ± 11.6 years and 42.0 ± 13.3 years respectively. Twenty six out of 46 subjects (57%) in the CMI and 10/12 (83%) in the humoral immune group were females. These differences are not significant.
Figure 6.1. Peak neopt:creat.urine post vaccination: scatter and box and whisker plot of the mean baseline neopt:creat.urine (day 2 pre to day 3 post-vaccination □ / ○) and maximum peak UNCR (days 4 to 10 post-vaccination ■ / ●) in 46 subjects receiving vaccinations known to stimulate cell mediated immunity (cell mediated group □ / ■) and 12 subjects receiving vaccines known to stimulate humoral immunity (humoral group ○ / ●). The boxes represent the 25th to 75th percentiles divided by the median and the whiskers the maximum and minimum values. The horizontal dotted line represents the neopt:creat.urine upper limit of normal (200μmol/mol).
Post-vaccination, most subjects had an increase in their neopt:creat.urine (Fig. 6.1). The cell mediated group had a median average increase of 206% (interquartile range = 150-338%) in their peak neopt:creat.urine from a median baseline of 138μmol/mol (interquartile range = 94-209) to a median peak of 232μmol/mol (interquartile range = 170-338). The humoral group had a median average increase of 165% (interquartile range = 142-412%) from a median baseline of 124μmol/mol (interquartile range = 92-182) to a median peak of 227μmol/mol (interquartile range = 140-404). Only 24/46 (52%) subjects in the CMI group and 5/12 (42%) subjects in the humoral immune group had clear peaks in their neopt:creat.urine (Fig. 6.1). In the CMI group the maximum levels were all detected from day 5 to 10 post skin test, with the mode on day 5. By contrast the 5 subjects in the humoral immune group all developed their peaks on day 7 or 8. Three of the latter subjects received vaccinations against influenza, one against typhoid and one received tetanus toxoid vaccine.

Eighteen out of 45 subjects (40%) receiving Mantoux skin tests had had a previous BCG vaccination and no patient had a previous history of tuberculosis. Eight out of 18 subjects (44%) who had previously had a BCG vaccination developed clearly defined peaks in response to PPD, compared with 15/27 subjects (56%) who had not had a previous BCG vaccination (p = n.s.). The former patients tended to have a lower median neopt:creat.urine peak than the latter patients (179μmol/mol (interquartile range 160-288), vs. 248μmol/mol(interquartile range 184-348), p = 0.07). All Mantoux skin tests were read at 48 hours and considered positive if the diameters in two planes were greater than 5mm. Nineteen skin tests were negative (42%) and 26 positive (58%).
Figure 6.2. Cell mediated group: mean serial urinary neopt:creat.urine of 46 subjects in the cell mediated (☐ / ■). All vaccinations or skin tests were administered on day 0.

Data is presented as the mean ± standard error of the mean. The horizontal dotted line represents the neopt:creat.urine upper limit of normal (200µmol/mol).
Figure 6.3. *Humoral group*: mean serial urinary neopt:creat. urine of 12 subjects in the humoral group (○ / ●). All vaccinations or skin tests were administered on day 0. Data is presented as the mean ± standard error of the mean. The horizontal dotted line represents the neopt:creatinine upper limit of normal (200μmol/mol).
Eleven out of 19 (58%) subjects with a negative Mantoux skin test, compared with only 10/26 (38%) subjects with a positive Mantoux skin test, had a peak in their neopt:creatinine as defined above (p = n.s.). The subjects with negative Mantoux skin tests tended to have higher median peak neopt:creatinine levels (257μmol/mol (interquartile range 170-360)), than subjects with positive Mantoux skin tests (198μmol/mol (interquartile range 165-288), p = n.s.).

No association was found between the local or systemic side-effects of vaccination and raised neopterin levels. Thirty one patients documented local reactions of pain, swelling or erythema due to vaccination. Their average median peak neopt:creatinine from day 5 to 10 was slightly lower than patients without local reactions, but did not reach statistical significance (205μmol/mol (interquartile range 163-313), vs. 256μmol/mol (interquartile range 173-354), p = n.s.). Similarly 31 subjects who complained of mild systemic symptoms such as fever, myalgias, headache and tiredness due to the vaccination, tended to have an average median peak neopt:creatinine lower than the 27 subjects without systemic reactions (201μmol/mol (interquartile range 165-313), vs. 258μmol/mol (interquartile range 169-360), p = n.s.).

The average time-course plots for the CMI and humoral immune groups are presented in Figs. 6.2 and 6.3 respectively. In the CMI group a clear peak occurred between days 4 and 10, with the maximum height on day 5. The mean area under the graph from days 4 to 9 post-vaccination was significantly greater than the area under the graph from day 2 pre- to day 3 post-vaccination (p = 0.009). In the humoral immune group the post-vaccination peak is noted on day 8, with the area under the curve from days 4 to 9 being
statistically greater than the area under the curve graph from day 2 pre- to day 3 post-vaccination (p = 0.04).

6.5 Discussion

In contrast to Wildgrube et al (1985), who were unable to demonstrate increased neopterin production in response to various skin tests in 25 subjects, we have shown that neopterin is sensitive enough in adult subjects to detect the CMI response to low dose PPD as well as to vaccines known to stimulate a humoral response. Fuchs et al. (1990) have demonstrated previously that the vaccination of 4 children with live measles/mumps vaccine induced a marked and brisk increase in neopterin excretion which peaked between day 8 and day 11. This time-course was similar to the 5 subjects in the humoral group but differed from subjects in the cell mediated group, who tended to have an earlier peak between day 4 and day 8. This difference in time-courses is probably related to the fact that the immune response in the 4 paediatric cases and subjects in our humoral group was a primary response to a new set of antigens. This would therefore be delayed compared with a secondary response to a previously recognised set of antigens, like PPD in the CMI group. Children have higher baseline neopt:creat:urine than adults (Fuchs et al., 1992). The peak neopt:creat:urine (1000-2000 μmol neopterin/mol creatinine) found by Fuchs et al. (1990) in their 4 paediatric subjects are higher than those in our CMI group (±350 μmol neopterin/mol creatinine). This may reflect the baseline differences between children and adults.

Suprisingly, 3 subjects receiving influenza vaccine, 1 a typhoid and 1 a tetanus toxoid booster had an increase in their neopt:creat:urine. The primary aim of these vaccines is to induce a good humoral IgG response, which depends on the Th2 cytokine IL-4. This
provides evidence that neopterin is not simply a marker of the Th1 or IFN\(\gamma\) inflammatory activity.

A striking and very interesting observation is the elevated level of neopterin production in subjects who have negative skin reactions to PPD. These subjects are able to generate a systemic response which is unaccompanied by a positive skin reaction. These patients may have low or absent circulating memory T-cells to PPD and when exposed to the antigen they mount a more vigorous primary immune response. In contrast the patients with positive skin reactions have a good pool of circulating memory T-cells and are able to localise the immune reaction to the site of inoculation as a secondary immunological response. Against this suggestion is the similar time-course of neopterin production in patients without and with a positive skin reaction, as one would expect the neopterin production of a primary immunological response to be delayed relative to a secondary response. Another possibility is that PPD contains sufficient quantities of mycobacterial heat shock proteins, which can activate large numbers of T-cells non-specifically as superantigens (Pfeffer et al. 1992), resulting in systemic IFN-\(\gamma\) production.

The time-course of peak neopterin excretion in response to PPD is compatible with the time-course of local IFN-\(\gamma\) production. Using immunohistochemistry of PPD-induced skin lesions, the maximum local production of IFN-\(\gamma\) was detected at 48 hours (Chu et al. 1992) compared with day 5 for neopterin production in this study. This 72 hour delay in peak neopterin production from that of local IFN-\(\gamma\) production is equivalent to that found in vitro, using stimulated monocytes or THP-1 cells (see Chapter 3).
These results show that urinary neopterin excretion, although non-specific, is a very sensitive inflammatory marker. Monitoring urinary neopterin excretion is therefore capable of detecting minor degrees of inflammation of the order of magnitude associated with vaccinations or immunological skin tests.

6.6 Conclusion

Vaccination or Mantoux skin testing result in an increase in urinary neopterin excretion with a characteristic time-course.
Chapter 7 - Immunological time-course of gadolinium enhancing MRI lesions in patients with multiple sclerosis.

7.1 Background

Studies correlating MRI activity and immunological markers of inflammation often give disparate, inconsistent and in some circumstances conflicting results (Giovannoni et al., 1998a). Reasons cited for the poor correlation between MRI activity and immunological parameters are numerous and include anatomical, temporal and methodological factors, all of which are poorly elucidated (see Chapter 1 – Introduction). In serial MRI studies individual lesions enhance with Gd for variable periods of time (McDonald 1993), with some lesions shrinking in volume and intensity of enhancement or even disappearing, whilst other lesions are appearing or enlarging. This implies that the inflammatory timetables of individual lesions are probably not dependent on each other and are possibly asynchronous. This has implications for interpreting levels of inflammatory markers, such as neopterin, which may be produced within a discrete time frame in the life cycle of an active lesion. If neopterin is only produced in the initial few days of a Gd-enhancing lesion, then its levels should only be correlated with newly enhancing lesions (i.e. those enhancing for less than 8 days duration). Support for this concept comes from a large serial study on 47 patients with relapsing remitting and secondary progressive MS undergoing monthly Gd-enhanced MRI, where serum soluble intercellular adhesion molecule-1 (sICAM-1) levels associated with inactive MR studies were significantly lower than the levels associated only with persistently enhancing lesions (enhancing for at least a month), or newly enhancing lesions (enhancing less than a month) (Giovannoni et al., 1997a). To study further the immunological time-
course of Gd-enhancement urinary neopterin was measured in patients with active MS undergoing weekly MRI.

7.2 Hypothesis

Urinary neopterin excretion correlates with the presence of new Gd-enhancing lesions on MRI.

7.3 Methods

7.3.1 Patients

Patients with clinically definite MS (Poser et al., 1983; Appendix I) who had had a clinical relapse in the last 6 months were screened for possible recruitment into the study. All patients underwent a standard Gd-enhanced T1-weighted MRI study. Only patients with actively enhancing lesions were recruited into the study to undergo weekly Gd-enhanced MRI studies for a period of 12 weeks. Disability was rated using Kurtzke’s EDSS (Kurtzke 1983; Appendix II) at the beginning and end of the study. A spot urine specimen was collected at the time of the study. All the patients were recruited from the National Hospital for Neurology and Neurosurgery, London. The study had Ethics Committee approval and written informed consent was obtained from all the patients.

7.3.2 MRI

Brain MRI was performed according to our standardised protocol (Miller et al., 1996). All studies were done on a General Electric 1.5 Tesla Signa system and were examined by a neuroradiologist (Dr C Good, National Hospital for Neurology and Neurosurgery)
who was unaware of the patients’ clinical details. For the purposes of this study lesions were defined as active if Gd-enhancement was noted on a T1-weighted sequence 10 minutes after the administration of 0.1 mmol/kg of gadolinium-diethylenetriamine penta-acetic acid (Schering). The number of Gd-enhancing lesions per study was counted. Care was taken to ensure that those lesions appearing on two slices were only counted once. Viewing each patient’s studies as a series, each lesion’s duration of enhancement could be assessed. All MRI studies, except the initial study, were classified as either having new Gd-enhancing lesions (those enhancing for less than 8 days duration), or as having no new Gd-enhancing lesions. This latter category includes MRI studies with either no enhancement or persistent enhancement present on at least two consecutive scans (i.e. enhancing for greater than 7 days duration).

7.3.3 Urine neopterin and creatinine assays

Urine and serum samples were protected from light, coded, frozen and stored at -20°C. All samples were assayed without knowledge of the patients’ MRI results. Neopterin and creatinine were measured by HPLC and the neopt:creat:urine values in female subjects were corrected as outlined in Chapter 2.

7.3.4 Data transformation and statistics

Neopt:creat:urine were first transformed using the natural logarithm. Mean levels for each patient were then compared using a paired T-Test. A p value of <0.05 was considered statistically significant.
Table 7a – Demographic and baseline clinical data

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>35</td>
<td>38</td>
<td>40</td>
<td>22</td>
<td>24</td>
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<tr>
<td>Sex</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Disease Course*</td>
<td>SP</td>
<td>RR</td>
<td>SP</td>
<td>RR</td>
<td>RR</td>
</tr>
<tr>
<td>EDSS</td>
<td>7.0</td>
<td>6.0</td>
<td>7.0</td>
<td>2.5</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* SP = secondary progressive, RR = relapsing remitting (Lublin and Reingold, 1996).

7.4 Results

Only 5 out of 17 patients (29%) who had Gd-enhancing lesions on their initial MRI study were recruited into the study. Preliminary MRI data on 3 of these patients has been published previously (Lai et al., 1996). The clinical characteristics and demographic data of these 5 patients are presented in Table I. The MRI data is presented in Table II.

Three patients had relapses during the study: patient 2 suffered a mild sensory relapse involving the left arm, patient 3 had an episode of optic neuritis and in patient 5 a brain stem event with diplopia occurred. Patient 1 received a course of corticosteroids (1g methyl-prednisolone intravenously for 3 days) 3 weeks prior to starting the study because of progressive deterioration in clinical course. Patient 5 also received a course of intravenous corticosteroids midway through the study for his brain stem relapse.

Sixty-four MRI studies were performed, 4 patients each had 13 MRI studies and one patient had 12 studies. A total of 71 individual enhancing lesions were noted (Fig. 7.1),
averaging 4.3 (SD2.9) Gd-enhancing lesions per MRI study. Fifty-three of the lesions could be classified as new, i.e. not present on the preceding study, giving an average of 1.1 (SD1.3) new Gd-enhancing lesions per MRI study. The life cycle of the Gd-enhancement in 34 individual lesions could be traced from beginning to end (Fig. 7.1).

Figure 7.1. Gd-enhancing time-course plot: The time-course (duration of Gd-enhancement) of all 71 Gd-enhancing lesions detected in 5 patients with multiple sclerosis over a 13 week period. For the purpose of clarity the cessation of Gd-enhancement is presented as occurring mid-week.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. MRI studies</td>
<td>13</td>
<td>12</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>64</td>
</tr>
<tr>
<td>No. MRI studies with Gd-enhancing lesions (%)</td>
<td>13 (100%)</td>
<td>12 (100%)</td>
<td>12 (92%)</td>
<td>4 (31%)</td>
<td>13 (100%)</td>
<td>54 (84%)</td>
</tr>
<tr>
<td>No. of individual Gd-enhancing lesions</td>
<td>18</td>
<td>20</td>
<td>12</td>
<td>6</td>
<td>15</td>
<td>71</td>
</tr>
<tr>
<td>Mean Gd-enhancing lesions / MRI study (SD)</td>
<td>5.5 (2.4)</td>
<td>6.8 (1.5)</td>
<td>3.3 (2.4)</td>
<td>0.6 (1)</td>
<td>5.2 (2.3)</td>
<td>4.3 (2.9)</td>
</tr>
<tr>
<td>No. of MRI studies with new Gd-enhancing lesions (%)*</td>
<td>8 (75%)</td>
<td>10 (91%)</td>
<td>7 (58%)</td>
<td>2 (17%)</td>
<td>6 (50%)</td>
<td>33 (56%)</td>
</tr>
<tr>
<td>No. of individual new Gd-enhancing lesions*</td>
<td>17</td>
<td>13</td>
<td>10</td>
<td>4</td>
<td>9</td>
<td>53</td>
</tr>
<tr>
<td>Mean new Gd-enhancing lesions / MRI study (SD)*</td>
<td>1.4 (1.4)</td>
<td>2 (1.5)</td>
<td>0.8 (0.9)</td>
<td>0.3 (0.8)</td>
<td>0.8 (1.1)</td>
<td>1.1 (1.3)</td>
</tr>
<tr>
<td>Mean duration of Gd-enhancement weeks (SD)**</td>
<td>3.7 (1.7)</td>
<td>4.0 (1.6)</td>
<td>3.3 (3.2)</td>
<td>1.0 (0)</td>
<td>4.3 (1.2)</td>
<td>3.5 (1.9)</td>
</tr>
</tbody>
</table>

* Excludes initial or baseline study, ** calculation excludes lesions enhancing on the first and last studies.
Figure 7.2. **Duration of Gd-enhancement**: a frequency histogram of the 34 lesions in which the life cycle of the enhancement could be traced from beginning to end. The mean duration of enhancement of these lesions is 3.5 (SD 1.9) weeks.

The mean duration of enhancement of these 34 lesions was 3.5 (SD 1.9) weeks (Fig. 7.2). To compare the sensitivity of weekly versus 4-weekly scanning, the first (week 0), fifth (week 4), ninth (week 8) and thirteenth (week 12) studies were compared with preceding weekly studies. Studies 9, 10 and 11 of patient 2 were excluded from the analysis, because a 12-week study was not performed. Performing weekly studies a total of 50 Gd-enhancing lesions were detected compared to 38 Gd-enhancing lesions in a 4-weekly protocol (paired T-test, p = 0.02). Monthly scanning would therefore have missed 12 out 50 (24%) of the new Gd-enhancing lesions. Thirty two out of 59 (54%) MRI studies had new Gd-enhancing lesions, i.e. enhancing lesions that were not present on the previous week’s study implying that the enhancement was new and present for less than 8 days. Although the individual MRI studies are not independent of each other, the results and scatter plots of the neopt:creat_uine
associated with those MRI studies demonstrating new Gd-enhancing lesions and those studies not associated with new Gd-enhancement are presented in Table 7c and Fig. 7.3 respectively. Using a paired T-test the mean neopt:creat-urine associated with new Gd-enhancement was significantly higher than the mean neopt:creat-urine not associated with new Gd-enhancement (Table 7c, p = 0.03). Three out of the 5 patients showed a clear increase in the neopt:creat-urine associated with new Gd-enhancing lesions (Fig. 7.3). The 2 patients who did not have an increase in the neopt:creat-urine in association with new Gd-enhancing lesions were the 2 cases who had received intravenous corticosteroids. These 2 cases did however have elevated neopt:creat-urine during the study period (Fig. 7.4, patients 1 and 5). The individual profiles of the weekly neopt:creat-urine in relation to new and total Gd-enhancing lesions are presented in Fig. 7.4.

Table 7c Neopt:creat-urine Data

<table>
<thead>
<tr>
<th>Immunological Parameter</th>
<th>Persistent or no Gd-enhancing lesions</th>
<th>New Gd-enhancing lesions</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All MRI Studies*</td>
<td>n = 24</td>
<td>n = 32</td>
<td>-</td>
</tr>
<tr>
<td>Median neopt:creat-urine</td>
<td>258 (167-424)</td>
<td>346 (209-718)</td>
<td>-</td>
</tr>
<tr>
<td>µmol/mol (25th-75th %tile)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number associated with a raised neopt:creat-urine</td>
<td>14 (58%)</td>
<td>24 (75%)</td>
<td>-</td>
</tr>
<tr>
<td>Mean values for each subject **</td>
<td>n = 5</td>
<td>n = 5</td>
<td></td>
</tr>
<tr>
<td>Mean geometric neopt:creat-urine</td>
<td>250 (132-492)</td>
<td>413 (207-521)</td>
<td>p = 0.03</td>
</tr>
</tbody>
</table>
Figure 7.3. Neopt:creat. urine data versus new Gd-enhancement: on the left hand side of each graph is a combined scatter and box-and-whisker plot of the natural logarithms of the neopt:creat. urine. The open circles (O, n = 24) represent values associated with MRI studies without new Gd-enhancing lesions and the filled circles (●, n = 32) those associated with the presence of new Gd-enhancing lesions. To the right hand side of each graph are the mean values of the respective markers for each patients’ MRI studies associated with no Gd-enhancing lesions (O) and with the presence of Gd-enhancing lesions (●), the mean of these 5 values and standard error of the mean (vertical error bars) are represented on either side of the individual values. The p-value is derived from a paired T-test comparing the respective mean values. The horizontal dotted lines represent the upper limit of normal for neopt:creat. urine.
Figure 7.4. Neopt:creat.urine vs. number of Gd-enhancing lesions: individual time-course plots for each patient. The filled histograms (■) represent the total number of Gd-enhancing lesions and the open bars (□) the number of new Gd-enhancing lesions (enhancing for less than 8 days) detected on each MRI study. The initial bar is hashed as some of the lesions may be newly enhancing. The horizontal dashed line represents the neopt:creat.urine upper limit of normal.
7.5 Discussion

Although this is a small study of only 5 patients with active MS it demonstrates that patients with MRI studies demonstrating new Gd-enhancing lesions (i.e. enhancing for less than 8 days duration) are associated with higher neopt:creatinine than when their MRI studies show either no or persistent Gd-enhancement (i.e. Gd-enhancement for greater than 7 days). Although Gd-enhancement has been correlated with acute perivascular inflammation in both biopsy (Rodriguez et al., 1993; Brück et al., 1997) and post-mortem (Katz et al., 1993) specimens, this studies findings suggest that the time-course of immunological events in active MS lesions may not be accurately inferred by simply noting the presence or absence of Gd-enhancement on MRI. Data from experimental allergic encephalomyelitis (EAE), a commonly used model of MS, supports this view. In EAE CNS inflammation is either monophasic (acute EAE) or polyphasic (chronic relapsing EAE), with episodes of CNS inflammation following a distinct time-course (Baker et al., 1990). The induction of the immune response, cell trafficking into the CNS, cytokine expression, production of inflammatory mediators and the development and recovery of paralysis are predictable. Furthermore, blood brain barrier breakdown, plasma protein extravasation and cell trafficking into the CNS, correlate with the development of weight loss prior to the onset of paralysis.

Specific cytokine profiles, which orchestrate the immunological response in EAE, are noted within the CNS. Prior to the onset and during clinical EAE there is an increased mRNA expression and production of the pro-inflammatory cytokines IL-2, interferon-\(\gamma\), IL-1, TNF-\(\alpha\) and IL-6, which abates during remission. IL-4, IL-10 and TGF-\(\beta\) putative anti-inflammatory cytokines are also observed within the CNS in both the acute and remitting phases. In a guinea pig model of chronic relapsing EAE, inflammatory lesions generally enhanced with Gd for 10-15 days, spanning both the
acute pro-inflammatory and recovery phases of the illness. It seems therefore, that Gd-enhancement of inflammatory CNS lesions, which indicates increased focal blood-brain-barrier water permeability, also detects lesions during their regressive phase.

Given that CNS inflammation in MS is generally multi-focal and multi-phasic, it is very difficult to develop immunological strategies to monitor the inflammatory events of individual lesions in vivo. Gd-enhanced T1-weighted MRI is one such strategy but unless it is done frequently, accurate ageing of the lesions is not possible. Other important issues relate to whether the immunological events occurring in simultaneously enhancing lesions are synchronous or asynchronous, and whether immunological signalling via cytokines occurs between such lesions.

Possible confounding factors not taken into account in this study relate to the sensitivity of Gd-enhanced MRI. Administering triple dose Gd, in conjunction with delayed scanning and the use of magnetisation transfer imaging, detects 126% more active lesions than using standard single dose protocol (Silver et al., 1997). However, this is unlikely to affect the findings of this study as improving the sensitivity of MRI rarely detects Gd-enhancing lesions in studies in which no Gd-enhancement was found using a standard single dose protocol (Silver et al., 1997). Whether or not improving the MRI sensitivity with triple dose Gd, delayed scanning, and MT imaging would alter the detection rate of new lesions by detecting them sooner is unknown. Spinal cord imaging also has the potential to increase the detection rate of active lesions by a further 5-10% (Thorpe et al., 1996; Kidd et al., 1996). However, missed spinal cord activity is unlikely to be significant as over 85% of Gd-enhancing
spinal cord lesions are associated with simultaneous activity in the brain (Thorpe et al., 1996; Kidd et al., 1996). At the level of individual lesions immunological events may not be temporally related and the immunopathogenesis may be heterogeneous. Numerous serial MR studies, including the current study, have clearly shown that individual lesions do not enhance for a uniform period of time (McDonald, 1993; Capra et al., 1992), and post-mortem histopathology also demonstrates lesions of varying age within the same subject (Brück et al., 1997). Individual MS lesions are therefore unlikely to be synchronised immunologically: one lesion could be producing pro-inflammatory immunological markers early in its life cycle whilst other lesions may not. This probably makes any strong correlation between immunological markers and Gd-enhancement an unrealistic expectation. Although less plausible, it is also possible that inflammatory mediators produced by one lesion may have an effect on the activity of other lesions via local (paracrine) or distant (endocrine) signalling. For example, a dominant lesion producing large amounts of anti-inflammatory cytokines might decrease pro-inflammatory activity in other active lesions.

In conclusion, in both cross-sectional studies and serial studies, in which there are wide time intervals between MRI studies, are unsuitable for demonstrating a relationship between Gd-enhanced MRI activity and immunological markers of disease activity as the age of Gd-enhancing lesions cannot be accurately dated. Pro-inflammatory markers such as neopterin, which are probably produced within the initial few days of the life cycle of an active lesion, only correlate with young newly enhancing lesions (enhancing for less than a week). This may explain the variable results of earlier studies attempting to correlate immunological markers of disease activity with Gd-enhancement on MRI (for review see Giovannoni et al., 1998a).
7.6 Conclusion

Urinary neopterin excretion correlates with the presence of new Gd-enhancing lesions on MRI, i.e. enhancing for less than 8 days. This suggests that individual MS lesions have asynchronous inflammatory timetables and that Gd-enhancement on MRI persists beyond the initial pro-inflammatory immunological phase of the lesions.
Chapter 8 - Conclusions

8.1 Background

The primary aim of this thesis was to establish whether urinary neopterin excretion could be used as an inflammatory marker of disease activity in patients with MS. An important secondary aim was to further elucidate the immunological significance of increased urinary neopterin excretion by studying its production in human monocytes. In the pursuit of these aims additional lessons have been learnt which provide a useful framework to investigate other immunological or biochemical markers of disease activity in patients with MS.

8.2 Neopterin as an inflammatory marker in MS

Urinary neopterin excretion is intermittently increased in patients with all clinical subtypes of MS. Neopterin production tended to be higher in patients with secondary progressive compared to patients with relapsing remitting disease. Increased levels were also noted in patients with a primary progressive course, whom characteristically have low levels of disease activity on MRI (Thompson et al., 1991; Thompson et al., 1992; Kidd et al., 1996). Raised neopterin excretion in patients with primary progressive disease provides further evidence of an inflammatory role in the pathogenesis of this disease subtype (Thompson et al., 1997).

Elevated levels of neop:crea:urine are also found in patients with clinically isolated syndromes compatible with demyelination; a large proportion of whom will go onto to develop MS in the future (O’Riordan, et al. 1998). Whether patients with clinically
isolated syndromes and persistently raised neopt:crea_{urine} are more likely to develop MS than patients with normal neopt:crea_{urine} is currently under investigation in a large natural history study at the Institute of Neurology.

Clinical relapse and Gd-enhanced MRI activity are associated with raised neopt:crea_{urine} however this is not an absolute finding as levels tend to be higher early on in the inflammatory life cycle of enhancing lesions, i.e. the first week. Raised neopt:crea_{urine} did not correlate with baseline disability and the length of follow-up was too short to determine whether patients with raised neopt:crea_{urine} are more likely to develop progressive disability. A longitudinal study is currently being performed to see if increased urinary neopterin excretion is associated with the development of clinical disability. Whether raised neopt:crea_{urine} levels correlate with putative MR markers of axonal loss and/or demyelination, like hypointense lesions on T1-weighted MRI (van Walderveen et al, 1995; Truyen et al. 1996) cerebral atrophy (Losseff et al. 1996a), spinal cord atrophy (Kidd et al, 1996; Losseff et al. 1996b), low magnetisation transfer (MT) ratios on MT imaging (Gass et al. 1994), and reduced levels of N-acetylaspartate (NAA) on MR proton spectroscopy (Arnold et al., 1990; Arnold et al., 1994; Davie et al. 1994) will also be answered in this study.

Urinary neopterin excretion was elevated in patients with posterior uveitis, despite a significant number of these cases receiving oral immunosuppressive agents. This finding is relevant to MS for several reasons; (1) a proportion of these patients will go onto to develop MS (Graham et al., 1989); (2) uveitis is analogous to MS in that the posterior uveal tract is anatomically part of the CNS (Cserr and Knopf 1992); (3) the size of the posterior uveal tract is relatively small confirming the sensitivity of urinary
neopterin in detecting small inflammatory volumes. In addition vaccination, a relatively minor immunological stimulus, is capable of increasing urinary neopterin excretion demonstrating its sensitivity as an inflammatory marker.

Confirming numerous previous studies urinary neopterin excretion was shown to be elevated in patients with RA and HIV-1 infection, which was significantly higher compared to patients with demyelinating diseases. This is not surprising, as both RA and HIV-1 infection are associated with a large systemic inflammatory response and a large volume of inflammatory tissue.

8.3 The immunological significance of increase neopterin production in MS

The cell culture experiments confirmed that the interferons, particularly IFNγ (Huber et al., 1984), are essential for the induction of neopterin production in monocytes. Other stimuli (TNFα, IL1, GMCSF, LPS, Fc-receptor stimulation), which can be broadly classified as monocyte/macrophage activators, clearly augment IFNγ-induced neopterin production and lower the absolute requirements of IFNγ to induce significant neopterin production. The inhibitory cytokines IL10 and TGFβ suppress IFNγ-induced neopterin production, but the role of IL4 and IL13 is more complex. Pre-incubating monocytes with IL4/IL13 for 24 hours greatly augments IFNγ-induced monocyte neopterin production. Without the appropriate pre-incubation period IL4/IL13 has little or no effect on neopterin production.

Although neopterin is a well-established inflammatory marker in the fields of infectious diseases, autoimmunity, transplantation and oncology (Fuchs et al., 1992), the current
dogma that neopterin is a specific marker of a Th1 T-cell mediated immunological reactions may not necessarily be correct. Although IFNγ is the primary immunological stimulus for monocyte neopterin production pre-incubation with IL4, the prototypical Th2 cytokine, augmented neopterin production. Therefore neopterin cannot simply be viewed as a Th1 marker and is possibly influenced in vivo by IL4 and IL13. This may explain some of the conflicting views on the significance of raised levels of neopterin production in patients with HIV-1 infection (Fuchs et al., 1995). In progressive HIV-1 infection the reduction in the CD4+ T cell count is associated with a progressive increase in neopterin production (Strathdee et al., 1996), “implying” greater IFNγ production. However, a central theme in the current theories on the immunopathogenesis of HIV-1 infection is an immunological shift from a Th1 (IFNγ) to a Th2 (IL4) driven response with disease progression (Clerici and Shearer, 1994). If neopterin production was simply a Th-1 marker its levels should decrease. However the observation that IL4 greatly augments IFNγ-induced neopterin production may help explain why neopterin levels are massively elevated in end-stage HIV-1 infection (Planella et al., 1998), a state dominated by Th2 immunological environment (Clerici and Shearer, 1994). This has implications for interpreting the significance of raised neopterin levels in patients with MS, a disease considered to be mediated by a predominantly Th1 T-cell response (Martin and McFarland, 1995). Raised neopterin excretion in patients with MS, may imply the presence of both a Th1 and Th2 cytokine response.

8.4 A possible mechanism of action of interferon-beta in MS

The clinical efficacy of human recombinant IFNβ in MS is based on empirical observations, with the mechanism/s of action unknown. Suggestions include functional
antagonism of the IFNγ-induced macrophage activation (Garotta et al., 1986), decreased antigen presentation (Jiang et al., 1995) via down regulation of MHC class II receptors (Kato et al., 1989; Soilu et al., 1995; Huynh et al., 1995), increased NK cell function (Fujimiya et al., 1995), improved non-specific suppressor cell activity (Noronha et al., 1990), decreased T-cell metalloproteinase production (Stuve et al., 1996; Leppert et al., 1996), anti-viral activity (Billiau, 1987), and by decreasing TNFα (Brod et al., 1996) and glial nitric oxide production (Hua et al., 1998). IFNβ’s ability to induce monocyte IL10 production is another possible mechanism of action.

IFNβ-1a is able to induce neopterin production in human monocytes, but at higher concentrations it antagonises the effects of IFNγ as well as its own effects on neopterin production. This antagonism appears to mediated by IL10. These effects are most marked at high concentrations of IFNβ-1a and may therefore have most relevance in the pharmacological use of IFNβ (The IFN-B Multiple Sclerosis Study Group, 1993; Jacobs et al., 1996; Kappos et al., 1998). These data support the findings of other groups who have also demonstrated IL10 induction in monocytes stimulated with IFNβ (Shakir et al., 1994; Porrini et al., 1994; Rudick et al., 1996a).

Further studies are required to see if these effects occur in vivo. The upregulation of IL10, possibly via the membrane form may be an important component of the pharmacological efficacy of IFNβ in MS.
8.5 Other lessons learnt – a case for urinary monitoring?

8.5.1 High fractional excretion

For substances like neopterin, which are excreted in the urine with a high fractional excretion, the urinary concentration is an order of magnitude higher than that found in CSF or blood (Bannister and Field, 1996). This makes it easier to detect elevated neopterin production despite relatively low levels in the CSF or plasma. A tea-strainer or sieve serves as a useful analogy for describing the utility of a high fractional excretion of a substance by the kidneys.

*Increasing the flow rate of tea (production) through a tea-strainer (kidneys) will only raise the fluid level in the tea-strainer by a small amount (plasma or CSF concentration), despite a large increase in the volume of tea passing through the strainer (urinary concentration).*

This helps to explain why urinary neopterin is such a sensitive inflammatory marker.

8.5.2 Ease of collection
Urine is easy to collect, allowing for frequent sampling, with little patient discomfort. Ethical considerations and patient compliance limit the frequency of invasive procedures such as lumbar puncture and venesection. This makes urine monitoring an attractive alternative to CSF and/or serum. Urine sampling can be performed by patients at home and does not require trained personnel or clinic/hospital attendance. In addition, most patients have access to -20°C home refrigeration which solves the problem of interim specimen storage.

*Figure 8.1. Urine as a natural integrator*: A 24-hour time-course plot of the average volume of urine produced per hour in a normal adult. The diurnal variation is due to a nocturnal increase in the production of antidiuretic hormone (ADH). An early morning spot specimen (07h00) captures the nocturnal excretion of substance over a prolonged period of time (shaded light grey area). This allows urine to act as a natural integrator providing information on the average excretion of a substance over a defined period (i.e. the area under the curve).
8.5.3 The bladder as a natural integrator

The bladder functions as a urinary reservoir, collecting and storing urine over a period of hours. Any substance measured in a spot urine specimen represents an average of its excretion over a particular period of time. A urinary level is therefore an integral of the production of a substance i.e. the area under the time curve (Fig. 8.1). This has particular advantages in relation to the diurnal variation of neopterin production and selecting the most suitable time point for urinary sampling.

8.5.4 Diurnal variation

Most biological processes are affected by diurnal rhythms, the immune system is no exception. For example IL6 levels may vary by as much as 350% over 24 hours (Arvidson et al., 1994). Similarly levels of urinary neopterin excretion vary by as much

\[ r^2 = 0.87 \]

\[
\begin{array}{c|c|c|c|c|c|c|c|c}
00h25 & 07h30 & 12h20 & 16h25 & 20h40 & 00h25 & \\
2h20 & 16h25 & 07h30 & 00h25 & 20h40 & \\
45 & 40 & 35 & 30 & 25 & 20 & 15 & 10 & 5 & 0
\end{array}
\]

Figure 8.2. Diurnal variation of CSF production: a 48-hour time-course plot of the average CSF flow, via the Sylvian aqueduct, as measured by MRI. The dotted line represents the best-fit polynomial demonstrating a clear diurnal variation in the rate of CSF production.
as 50% during 24 hour period (Auzéby et al., 1988, see Chapter 2). CSF flow is also subject to diurnal variation and aqueductal flow rates vary by as much as factor of 3.5, with minimum CSF production occurring around 18h00 (12±7 mL/hour) and a maximum at approximately 02h00 (42±2 mL/hour) (Nilsson et al., 1992; Fig. 8.2). As the CSF is a major outflow conduit for inflammatory markers produced in the central nervous system sampling during the period of maximal CSF flow seems appropriate. The first urine specimen of the day has the advantage of integrating events over this period of maximal CSF flow.

8.5.5 Anatomical factors

The lumbar subarachnoid space is a cul-de-sac. CSF obtained from lumbar punctures cannot provide accurate information on inflammatory events in the brain which occur distal to the CSF outflow foramina of the fourth ventricle, i.e. inflammation in relation to the surface of the brainstem, cranial nerves, and cerebral hemispheres. In addition, the extracellular space of intraparenchymal lesions may not necessarily communicate with the free CSF space (Jacobi et al., 1986). Neopterin measured in the blood and urine is not affected by such anatomical constraints and represent a summation of the brain- and systemic-derived pool. Whether plasma or urinary levels of a particular substance accurately reflect disease activity in the central nervous system is a moot point. Invasive human and/or animal studies combining CSF, blood and urine measurements would help to answer this question.
8.5.6 Spot urine samples vs. 24-hour urine collections

Although a 24-hour urine collection should, at least theoretically, provide a better index of the daily neopterin production than a spot specimen a comparison between the two procedures produced comparable results. The 24-hour urine collections were laborious, impractical, and unreliable. It is doubtful if it would be possible to ensure patient compliance for 24-hour urine collections especially if frequent sampling is planned. The first urine specimen of the day also has the advantage of capturing the overnight period during which the production of neopterin is maximal.

8.5.7 Frequency of sampling

The longitudinal study (Chapter 5) took advantage of these points and demonstrated that changes in urinary neopterin excretion occurs over a time-course of days rather than weeks in patients with MS. An example of a daily, weekly, biweekly and monthly neopt:crea:urine time-course profile of a patient with secondary progressive MS is presented in Fig. 8.3. The inflammatory profiles of biweekly or monthly sampling detect very little of the inflammatory activity compared to daily or weekly sampling. For practical purposes weekly sampling appears to be a reasonable compromise and is currently being used in a large natural history study.
Figure 8.3. Longitudinal neopt:creat-urine profiles: a daily, weekly, biweekly and monthly longitudinal profile of the serial urinary neopterin: creatinine ratios for a 35 year old female with secondary progressive multiple sclerosis. The profiles are derived from the same data. The dotted line represents the upper limit of normal for the urinary neopterin: creatinine ratios (200 μmol/mol).
8.5.8 Cross-sectional vs. longitudinal studies

The proportion of MS patients in the cross-sectional study who had an elevated neopt:creat:urine was significantly lower than the proportion of patients with an elevated mean neopt:creat:urine in the longitudinal study (49 out of 129 patients (38%) vs. 29 out of 31 patients (94%), p = 0.002). Cross-sectional studies are only useful in screening potential inflammatory markers in patients with MS, which then require detailed assessment in longitudinal studies.

8.5.9 Variable concentration of urine

A disadvantage of urinary measurements is the variable volume of urine produced over a 24-hour period (1.0-3.5 litre/24 hours; Laposata, 1992), which affects the neopterin concentration. Correcting for this using creatinine as an internal reference creates an additional problem related to differences in creatinine excretion between subjects, especially between male and female subjects.

Whitaker et al. have reported a positive correlation between urinary creatinine concentration and disability in patients with MS (Whitaker et al., 1995b). The cross-sectional study confirms their findings. The reasons for the correlation between disability and urinary creatinine levels are currently speculative. It is unlikely to be due to a change in muscle mass. MS patients with urinary symptoms are known to restrict their fluid intake to control troublesome urinary frequency (Matthews, 1991). Patients with bladder dysfunction tend to be more disabled the higher urinary creatinine concentrations may simply reflect voluntary dehydration.
8.5.10 Urinary tract infection

Asymptomatic bacterial colonisation of the urinary tract and infection is common in patients with MS. This is a particular problem in patients with bladder dysfunction who are unable to void completely. In addition some patients require intermittent self-catheterisation or rarely an indwelling catheter. These factors encourage bacterial growth and metabolism, which could affect substances excreted in the urine. In addition bacterial infections are associated with an increase in the production of neopterin (Fuchs et al., 1992) and other inflammatory markers which need to taken into account when interpreting results.

8.6 Conclusion

Neopterin production in macrophages is primarily induced by IFNγ and augmented by TNFα and IL1. IL10 and TGFβ inhibit IFNγ–induced neopterin production. IL4 and IL13 have little effect on neopterin production but can increase neopterin production by augmenting TNFα production. This effect is time-dependent and requires a period of preincubation. In conclusion urinary neopterin is a very sensitive and useful inflammatory marker in MS. Whether it proves to be a useful surrogate marker of disease progression awaits the outcome of a large natural history study currently being performed. Importantly, urinary neopterin excretion appears to be sensitive enough to detect relatively small volumes of inflammation of the order of magnitude of that occurring in MS and provides a very powerful immunological handle by which to study a dynamic disease process in vivo. It has numerous theoretical advantages over other blood and CSF markers of inflammation.
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V


W


X


Y


Z


## Appendix I

### Diagnostic Criteria for Multiple Sclerosis (Poser et al., 1983)

<table>
<thead>
<tr>
<th>Category</th>
<th>Attacks</th>
<th>Clinical Evidence</th>
<th>Paraclinical Evidence</th>
<th>CSF OCB/IgG*</th>
</tr>
</thead>
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<tr>
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<td>B. Laboratory-supported definite</td>
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<tr>
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<tr>
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<td>D. Laboratory-supported probable</td>
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<tr>
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* CSF OCB/IgG = cerebrospinal fluid oligoclonal bands or increased intrathecal IgG synthesis.

### Definitions

**Attack**: The occurrence of a symptom or symptoms of neurological dysfunction, with or without objective confirmation, lasting more than 24 hours constitutes an attack.

**Clinical Evidence**: Signs of neurological dysfunction demonstrable by neurological examination. Such neurological signs are acceptable even if no longer present, provided that they were elicited and recorded in the past by a competent examiner.

**Paraclinical Evidence**: The demonstration by means of various tests and procedures of the existence of a lesion of the central nervous system which has not produced signs of neurological dysfunction but which may or may not have caused symptoms in the past.
Appendix II

Rating neurologic impairment in multiple sclerosis (Kurtzke, 1983)

Functional Systems (FS)

Pyramidal Functions

0. Normal.
1. Abnormal signs without disability.
2. Minimal disability.
3. Mild or moderate paraparesis or hemiparesis; severe monoparesis.
4. Marked paraparesis or hemiparesis; moderate quadriparesis; or monoplegia.
5. Paraplegia, hemiplegia, or marked quadriparesis.
6. Quadriplegia.
V. Unknown.

Cerebellar Functions

0. Normal.
1. Abnormal signs without disability.
2. Mild ataxia.
3. Moderate truncal or limb ataxia.
4. Severe ataxia, all limbs.
5. Unable to perform coordinated movements due to ataxia.
V. Unknown.
X. Is used throughout after each number when weakness (grade 3 or more on pyramidal) interferes with testing.

Brain Stem Functions

0. Normal.
1. Signs only.
2. Moderate nystagmus or other mild disability.
3. Severe nystagmus, marked extraocular weakness or moderate disability of other cranial nerves.
4. Marked dysarthria or other marked disability.
5. Inability to swallow or speak.
V. Unknown.

Sensory Functions (revised 1982)

0. Normal.
1. Vibration or figure-writing decrease only, in one or two limbs.
2. Mild decrease in touch or pain or position sense, and/or moderate decrease in vibration in one or two limbs; or vibratory (c/s figure writing) decrease alone in three or four limbs.
3. Moderate decrease in touch or pain or position sense, and/or essentially lost vibration in one or two limbs; or mild decrease in touch or pain and/or moderate decrease in all proprioceptive tests in three or four limbs.
4. Marked decrease in touch or pain or loss of proprioception, alone or combined, in
one or two limbs; or moderate decrease in touch or pain and/or severe proprioceptive decrease in more than two limbs.
5. Loss (essentially) of sensation in one or two limbs; or moderate decrease in touch or pain and/or loss of proprioception for most of the body below the head.
6. Sensation essentially lost below the head.
V. Unknown.

**Bowel and Bladder Functions (revised 1982)**

0. Normal.
1. Mild urinary hesitancy, urgency, or retention.
2. Moderate hesitancy, urgency, retention of bowel or bladder, or rare urinary incontinence.
3. Frequent urinary incontinence.
4. In need of almost constant catheterization.
5. Loss of bladder function.
V. Unknown.

**Visual (or Optic) Functions**

0. Normal.
1. Scotoma with visual acuity (corrected) better than 20/30.
2. Worse eye with scotoma with maximal visual acuity (corrected) of 20/30 to 20/59.
3. Worse eye with large scotoma, or moderate decrease in fields, but with maximal visual acuity (corrected) of 20/60 to 20/99.
4. Worse eye with marked decrease of fields and maximal visual acuity (corrected) of 20/100 to 20/200; grade 3 plus maximal acuity of better eye of 20/60 or less.
5. Worse eye with maximal visual acuity (corrected) less than 20/200; grade 4 plus maximal acuity of better eye of 20/60 or less.
6. Grade 5 plus maximal visual acuity of better eye of 20/60 or less.
V. Unknown.
Is added to grades 0 to 6 for presence of temporal pallor.

**Cerebral (or Mental) Functions**

0. Normal.
1. Mood alteration only (Does not affect DSS score).
2. Mild decrease in mentation.
3. Moderate decrease in mentation.
5. Dementia or chronic brain syndrome—severe or incompetent.
V. Unknown.

**Other Functions.**

0. None.
1. Any other neurologic findings attributed to MS (specify).
V. Unknown.
Expanded Disability Status Scale (EDSS)

0.0 = Normal neurologic exam (all grade 0 in Functional Systems [FS]; Cerebral grade 1 acceptable).

1.0 = No disability, minimal signs in one FS (i.e. grade 1 excluding Cerebral grade 1).

1.5 = No disability minimal signs in more than one FS (more than one grade 1 excluding Cerebral grade 1).

2.0 = Minimal disability in one FS (one FS grade 2, others 0 or 1).

2.5 = Minimal disability in two FS (two FS grade 2, others 0 or 1).

3.0 = Moderate disability in one FS (one FS grade 3, others 0 or 1), or mild disability in three or four FS (three/four FS grade 2, others 0 or 1) though fully ambulatory.

3.5 = Fully ambulatory but with moderate disability in one FS (one grade 3) and one two FS grade 2; or two FS grade 3; or five FS grade 2 (others 0 or 1).

4.0 = Fully ambulatory without aid, self-sufficient, up and about some 12 hours a day despite relatively severe disability consisting of one FS grade 4 (others 0 or 1), or combinations of lesser grades exceeding limits of previous steps. Able to walk without aid or rest some 500 meters.

4.5 = Fully ambulatory without aid, up and about much of the day, able to work a full day, may otherwise have some limitation of full activity or require minimal assistance; characterized by relatively severe disability, usually consisting of one FS grade 4 (others 0 or 1) or combinations of lesser grades exceeding limits of previous steps. Able to walk without aid or rest for some 300 meters.

5.0 = Ambulatory without aid or rest for about 200 meters; disability severe enough to impair full daily activities (e.g. to work full day without special provisions). (Usual FS equivalents are one grade 5 alone, others 0 or 1; or combinations of lesser grades usually exceeding specifications for step 4.0).

5.5 = Ambulatory without aid or rest for about 100 meters; disability severe enough to preclude full daily activities. (Usual FS equivalents are one grade 5 alone, others 0 or 1; or combinations of lesser grades usually exceeding those for step 4.0.)

6.0 = Intermittent or unilateral constant assistance (cane, crutch, or brace) required to walk about 100 meters with or without resting. (Usual FS equivalents are combinations with more than two FS grade 3+).

6.5 = Constant bilateral assistance (canes, crutches, or braces) required to walk about 20 meters without resting. (Usual FS equivalents are combinations with more than two FS grade 3+).
7.0 = Unable to walk beyond about 5 meters even with aid, essentially restricted to wheelchair; wheels self in standard wheelchair and transfers alone; up and about in w/c some 12 hours a day. (Usual FS equivalents are combinations with more than one FS grade 4+; very rarely, pyramidal grade 5 alone).

7.5 = Unable to take more than a few steps; restricted to wheelchair; may need aid in transfer; wheels self but cannot carry on in standard wheelchair a full day; may require motorized wheelchair. (Usual FS equivalents are combinations with more than one FS grade 4+).

8.0 = Essentially restricted to bed or chair or perambulated in wheelchair, but may be out of bed itself much of the day; retains many self-care functions; generally has effective use of arms. (Usual FS equivalents are combinations, generally grade 4+ in several systems.)

8.5 = Essentially restricted to bed much of the day; has some effective use of arm(s); retains some self-care functions. (Usual FS equivalents are combinations, generally 4+ in several systems.)

9.0 = Helpless bed patient; can communicate and eat. (Usual FS equivalents are combinations, mostly grade 4+).

9.5 = Totally helpless bed patient; unable to communicate effectively or eat/swallow. (Usual FS equivalents are combinations, almost all grade 4+).

10.0 = Death due to MS.
Appendix III

Sørensen's / Phosphate Buffer at 18°C (Varley et al., 1980)

\[ pK_2 = 7.20 \quad \Delta \text{pH}^\circ C = -0.003 \quad I = 77 \text{ to } 191 \text{ (pH 5.8 to 7.9)} \]

Volume (ml) of 15 mmol/L monopotassium phosphate (KH$_2$PO$_4$, FW = 136.1, Sigma Cat. No. P-5379) to be diluted to 1 litre 15 mmol/L disodium phosphate (Na$_2$HPO$_4$, FW = 142.0, Sigma Cat. No. S-7907).

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Appendix IV

Water methanol gradient to clean HPLC column

Flow rate 1.5mL / minute.

Reagent grade water (Milli-RO and Milli Q systems, Millipore, Watford, UK) for 60 minutes.

Reagent grade water with 50% methanol (HPLC grade, Hipersolv™ BDH prod. No. 15250) for 30 minutes.

100% methanol for 30 minutes.
Appendix V

Tris-HCl (Gomori) Buffer (Varley et al., 1980)

\[ p\text{K} = 0.08 \ \Delta p\text{H}/^\circ C = -0.020 \ I = 44 \text{ to } 5 \ \text{(pH 7.2 to 9.0)} \]

Volume (ml) of 200 mmol/L hydrochloric acid (HCl, FW = 36.46, BDH Prod. No. 14157) to be added to 250 mL of 200 mmol/L tris[hydroxymethyl] aminomethane and diluted to 1 litre with water (C₄H₁₁NO₃, FW = 121.1, Trizma® Base Sigma Cat. No. T-1503).

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Appendix VI

Patient information letter for the daily urine neopterin study

Thank you for agreeing to participate and help in our research project.

In multiple sclerosis the macrophage, which is a white blood cell, is responsible for the demyelination that causes your nervous system to malfunction. A by-product of the macrophage's metabolism is a substance called NEOPTERIN, which is excreted in your urine. By measuring your daily urine neopterin levels, we may be able to tell how active your macrophages are over a period of time and see if correlates with the activity of your disease.

We will need you to collect a small specimen of urine each morning for a 3-month period. Enclosed is a packet with 90 small bottles, caps, and plastic pipettes to aid you in filling the bottles. The pipettes can be rinsed with water and re-used. We have enclosed stickers that need to be filled in so that the samples can be identified and dated. Neopterin is light and temperature-sensitive. It must be stored in the dark and frozen as soon as possible. The bottle caps have a seal so that they will not leak, but we still recommend that you keep them in a separate container or packet. Please remember to label the samples with the stickers provided before you freeze them.

As neopterin excretion is also increased by infections, please keep a note of any infections you may develop over the period of collection. This includes even minor infections like a common cold or a cold sore. A diary is enclosed for this purpose. It has a column for infections to be noted and space for a short comment to describe the infection. There is also a column for any neurological symptoms. These may be new or existing complaints that fluctuate from day to day; for example you may notice new or increased weakness in a limb, strange sensory symptoms, pain, spasticity, muscle twitching etc.

Please bring the urine samples and diary to your appointment. As discussed, this appointment will take approximately 30 minutes when you will be examined, your diary discussed and a blood sample taken.
Finally, all research done at the National Hospital for Neurology and Neurosurgery requires a signed consent form. If you understand the study and are willing to participate, please sign the form and return it in the self-addressed envelope. You are under no obligation to participate and may withdraw from the study at any time. If you do withdraw, it will not affect your usual treatment in any way.

If you have any queries, please contact me on (071) 837-3611 ext. 4212 during office hours.

Thank you for your time.

Yours sincerely

Dr Gavin Giovannoni (MBBCH)
Multiple Sclerosis Society Research Fellow
### APPENDIX VII - DAILY STUDY DIARY

**NAME:**

Are you taking any medications or vitamin supplements? (Please list)

- Evening Primrose Oil - 1000 mg daily

<table>
<thead>
<tr>
<th>DAY</th>
<th>DATE</th>
<th>INFECTION</th>
<th>COMMENT (Infection)</th>
<th>NEUROLOGICAL SYMPTOMS</th>
<th>COMMENT (Neurological)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20-1-95</td>
<td>—</td>
<td>Tingling under eye</td>
<td>Right leg —— Tingling, sensation, burning, hypersensitive to touch, burning eye.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21-1-95</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>22-1-95</td>
<td>—</td>
<td>—</td>
<td>Right leg —— Tingling sensation, hypersensitive to touch, burning eye.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>23-1-95</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>24-1-95</td>
<td>—</td>
<td>—</td>
<td>Right leg —— Tingling, sensation, burning, hypersensitive to touch, burning eye.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>25-1-95</td>
<td>—</td>
<td>—</td>
<td>Right leg —— Tingling, sensation, burning, hypersensitive to touch, burning eye.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>26-1-95</td>
<td>—</td>
<td>—</td>
<td>Right leg —— Tingling, sensation, burning, hypersensitive to touch, burning eye.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>27-1-95</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>30-1-95</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>29-1-95</td>
<td>Slight right side neck</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>30-1-95</td>
<td>Slight right side neck</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>31-1-95</td>
<td>Slight right side neck</td>
<td>—</td>
<td>Right leg —— Tingling, sensation, burning, hypersensitive to touch, burning eye.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1-2-95</td>
<td>—</td>
<td>—</td>
<td>Right leg —— Tingling, sensation, burning, hypersensitive to touch, burning eye.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>2-2-95</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
Appendix VIII

Patient information letter for the vaccine study

Thank you for agreeing to participate and help in our research project.

When an infection or another inflammatory process activates your immune system, your white cells produce a chemical substance called neopterin. This substance is a by-product of cellular metabolism and is released into your blood, once in the blood stream it is filtered by the kidneys and excreted in your urine. We are currently investigating urine neopterin excretion in patients with various inflammatory diseases to see if we can use neopterin to monitor the levels of inflammation in these diseases. We would like to see if we can detect increased neopterin production in response to skin tests or vaccinations, relatively minor inflammatory stimuli, and to see if increased neopterin production correlates with reactions to vaccination.

We will need you to collect a small specimen of urine each morning starting 3 days before and continuing until 10 days after your vaccination or skin test. Enclosed is a packet with 14 small bottles, caps, and plastic pipettes to aid you in filling the bottles. The pipettes can be rinsed with water and re-used. We have enclosed stickers that need to be filled in so that the samples can be identified and dated. Neopterin is light and temperature-sensitive. It must be stored in the dark and frozen as soon as possible. The bottle caps have a seal so that they will not leak, but we still recommend that you keep them in a separate container or packet. Please remember to label the samples with the stickers provided before you freeze them.

We would also like you to keep a diary, documenting any symptoms you may have as a reaction to the vaccine (e.g. fever, sore muscles, fatigue, headache, etc.), as well as any local reactions to the vaccine (e.g. swelling, pain, redness, etc.). As neopterin excretion is also increased by infections, please keep a note of any infections you may develop over the period of collection. This includes even minor infections like a common cold. A diary is enclosed for these purposes.
Finally, all research done at the National Hospital for Neurology and Neurosurgery requires a signed consent form. If you understand the study and are willing to participate, please sign the form. You are under no obligation to participate and may withdraw from the study at any time.

If you have any queries, please contact Sister Joan Bell on (071) 837-3611 ext. 3037 or Dr Gavin Giovannoni (071) 837-3611 ext. 4212 during office hours.

Thank you

Sr Joan Bell and Dr Gavin Giovannoni
Appendix IX

Vaccine Daily Diary

**Neopterin Vaccine Study**

**Date:** 7.2.95  
**Study Code:** A.1.

**Name:**  
**Date of Birth:**  
**Sex:** F

**Address:**

**Telephone No.:**

David Ferrier, PhD

**Current Medical Problems:**

Nil.

**Medications (including vitamins etc.):**

Nil.

**Type of Vaccination:**

- **Mantoux 4500:** 7.2.95
- **BCG 1984:**
  - 9.2.95 faint redness 9 x 5mm.
  - 10.2.95 more pronounced 12 x 10mm.

**Reason for Vaccination:**

Check TB status

**Site of Vaccination:**

- Forearm.
## Neopterin Vaccine Study

<table>
<thead>
<tr>
<th>Date</th>
<th>Symptoms</th>
<th>Vaccine Site</th>
<th>Infection</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-2-95</td>
<td>NIL</td>
<td>NORMAL</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>8-1-95</td>
<td>NIL</td>
<td>NORMAL</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>9-1-95</td>
<td>NIL</td>
<td>NORMAL</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>10-2-95</td>
<td>FATIGUE</td>
<td>SLIGHTLY RED</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>11-2-95</td>
<td>NIL</td>
<td>SLIGHTLY RED</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>12-2-95</td>
<td>NIL</td>
<td>RED</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>13-2-95</td>
<td>NIL</td>
<td>RED</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>14-2-95</td>
<td>NIL</td>
<td>SLIGHTLY RED</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>15-2-95</td>
<td>NIL</td>
<td>SLIGHTLY RED</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>16-2-95</td>
<td>NIL</td>
<td>SLIGHTLY RED</td>
<td>NIL</td>
<td></td>
</tr>
</tbody>
</table>

Please protect samples from light and freeze as soon as possible.

Thank you for your participation and help.

Sr. Joan Bell (071) 837-3611 ext. 3037.
Dr. Gavin Giovannoni (071) 837-3611 ext. 4212.
Appendix X

Publications


The immunopathogenesis of multiple sclerosis and Guillain–Barré syndrome
Gavin Giovannoni and Hans-Peter Hartung

This review covers current hypotheses regarding antigen specific disease induction, immune responses within the central and peripheral nervous systems, the nonspecific inflammatory cascade, and mechanisms responsible for demyelination, axonal loss, and disease progression in multiple sclerosis and Guillain–Barré syndrome. We also discuss the new evidence on the role of infection as an aetiological factor in multiple sclerosis and Guillain–Barré syndrome.

Department of Neuroimmunology, Institute of Neurology, Queen Square, London and the Department of Neurology, Julius Maximilians University, Würzburg, Germany

Corresponding author: Gavin Giovannoni, Department of Neuroimmunology, Institute of Neurology, Queen Square, London WC1N 3BG, UK


Abbreviations

CSF cerebrospinal fluid
EAE experimental allergic encephalomyelitis
GHD experimental allergic neuritis
GBS Guillain–Barré syndrome
HHV-6 human herpes virus 6
HLA human leukocyte antigen
ICAM intracellular adhesion molecule
IFN gamma interferon gamma
IL interleukin
MBP myelin basic protein
MHC major histocompatibility complex
MRI magnetic resonance imaging
MS multiple sclerosis
PLP proteolipid protein
TCR T-cell receptor
TGF- beta transforming growth factor beta
Th T-helper cell
TNF tumour necrosis factor
VCAM vascular cell adhesion molecule

Introduction

Multiple sclerosis (MS) and Guillain–Barré syndrome (GBS) remain the most common demyelinating diseases of the central and peripheral nervous systems, respectively. They are, although unproven, considered organ-specific autoimmune disorders, mediated by autoreactive T-cells, autoantibodies or both, to various antigens. MS occurs more commonly in Caucasians on a genetic background associated with specific major histocompatibility complex (MHC) haplotypes (DR1/DQ6), and possibly to other loci [1*]. Epidemiological studies support an environmental agent, acting in early childhood as a possible aetiological factor. The course of MS is highly variable and unpredictable. GBS is an acute monophasic disease with a more predictable and defined clinical outcome. It is commonly precipitated by infections with a particularly strong association with the gastrointestinal pathogen Campylobacter jejuni. The humoral immune response appears to be more important in GBS. In this disease, certain autoantibodies, short of inciting structural damage, also impact on peripheral nerve function. Figures 1 and 2 summarize the immunopathogenesis of MS and GBS.

Disease induction

Disease induction in MS, and at least in a proportion of patients with GBS, requires establishment and activation of autoreactive T-cells; current proposals are presented in Table 1. T-cell activation usually requires an antigen-specific signal transduced via the T-cell receptor (TCR)–CD3–CD8/CD4 complex interacting with MHC class I or II molecules, with additional costimulatory signals [Fig. 2(a)]. The costimulatory signals occur via the interaction of a group of accessory molecule ligand pairs, B7-1/CD28 and B7-2/cytotoxic T-lymphocyte antigen 4, which also governs the type of T-helper (Th) response induced: B7-1/CD28 interaction results in a Th-1 phenotype producing interferon gamma (IFNγ) and interleukin (IL)-2, whereas B7-2/cytotoxic T-lymphocyte antigen 4 interaction results in a Th-2 phenotype, producing predominantly an IL-4 response [5*]. Inhibiting the
Figure 1. The pathogenesis of multiple sclerosis and Guillain-Barré syndrome

(a) A hereditary predisposition combined with an environmental trigger establishes or maintains pathological autoreactive T-cells. After a long latency period (10-20 years), these autoreactive T-cells are activated, possibly by a systemic trigger like a viral infection or exposure to a superantigen. Once activated these T-cells selectively cross the blood-brain barrier and on re-exposure to their autoantigen initiate a cell-mediated (Th1) inflammatory reaction. The resultant inflammatory cascade causes demyelination and axonal loss, which in tum releases sequestered central nervous system antigens that are hypothesised to initiate further episodes of autoimmune-induced inflammation. The inflammatory cascade and demyelination may also be triggered by a local neurotropic viral infection. Recently human herpes virus 6 (HHV-6) expression has been noted in the oligodendrocytes and neurones of patients with multiple sclerosis (MS) [2*].

(b) The pathogenesis of Guillain-Barré syndrome (GBS). Unlike multiple sclerosis, there appears not to be a hereditary predisposition to acquiring GBS. However, a systemic trigger, usually in the form of an infection, is well established as an aetiological factor. The infecting organism results in a humoral and cellular immune response which because of molecular mimicry cross-reacts with peripheral myelin antigens. These autoreactive antibodies and T-cells cross the blood-nerve barrier and initiate an inflammatory reaction, which results in demyelination and possible axonal damage by a process not too dissimilar to that occurring in MS. Although GBS is a monophasic illness, it may be followed on rare occasions by a chronic relapsing illness. CNS, central nervous system; HLA, human leukocyte antigen.

B7-1/CD28 interaction prevents transfer of experimental allergic encephalomyelitis (EAE) and abrogates established disease through the activity of IL-4 [5**,6,7]. In brains of patients with MS, B7-1 was localized to activated microglia and infiltrating macrophages [8].

Cellular response CD4+ αβT-cells

Current evidence supports MS and, in some patients, GBS as diseases mediated by Th1/CD4+ αβT-cells [9,10**]. Increased numbers of activated αβT-cells to central- and peripheral-nervous system-specific antigens occur more often in MS and GBS patients, respectively.

The frequency of somatic mutations in autoreactive T-cells and T-cell clones is higher in MS and GBS patients than normal control individuals suggesting an in-vivo proliferative response and participation in disease pathogenesis [11,12]. Following earlier studies in EAE, some investigators have found restricted usage of TCRs to the immunodominant epitopes of myelin basic protein (MBP) and proteolipid protein (PLP) in MS patients [10**,13-17] however other studies, including studies on identical twins [18,19], have produced conflicting results. Indeed, it has become clear that even in acute EAE in the Lewis rat, TCR usage is broader than previously reported [20]. It therefore remains to be established whether a definitive
Figure 2. Cellular mechanisms of acute inflammation in multiple sclerosis

(a) Activated CD4+ T helper cells (Th) cross the blood-brain barrier through a process involving the interaction of their cell surface adhesion molecules with those expressed on the central nervous system endothelium. Once within the perivascular space, these cells are activated by professional antigen-presenting cells (probably macrophages or microglia) to proliferate and produce cytokines. The profile of T cell cytokines produced depends on the costimulatory signals received during activation. Th-1 like cytokines (interleukin (IL)-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-α) initiate a classical cell-mediated inflammatory cascade which activates macrophages, microglia, astrocytes and endothelial cells. This results in further cytokine production and recruitment of inflammatory cells by the upregulation of endothelial cell-adhesion molecule expression and by the production of chemokines. The inflammatory cascade also produces a host of noxious substances (oxygen and nitrogen free radicals, proteases, eicosanoids and complement), which in combination with autoantibodies and cytokines (particularly TNFα), cause oligodendrocyte damage and demyelination. Immunomodulatory cytokines [IL-4, IL-10 and transforming growth factor (TGF-β)] produced by suppressor and Th-2 T-cells are important in down-regulating and controlling the above inflammation. (b) Antigen presentation and T-cell activation. Antigen recognition occurs via the trimolecular complex and requires additional costimulatory signals. T-cell activation results in proliferation and cytokine production. The trimolecular complex consists of the human leukocyte antigen (HLA), T-cell receptor (TCR) and CD3 molecules. The important costimulatory signals required include interactions between HLA major histocompatibility complex (MHC) I and II molecules and their respective CD8 and CD4 molecules, and the cytokotic T-lymphocyte antigen/B7-1 or CD28/B7-2 pairs. These latter costimulatory signals and the presence of specific cytokines, govern the type of T helper response. Other accessory molecule interactions, including the lymphocyte function associated antigen (LFA3/CD2 and LFA1/ICAM-1 pairs, facilitate positioning of the T-cell and antigen presenting cell. Figure 2 is continued on page 168.
Apoptosis of antigen-specific T-cells, in the lesions of inflammation, is an effective mechanism in stopping neural cell death of these infiltrating cells identified as an effective mechanism in stopping neural cell death of these infiltrating cells. Impaired counterregulatory influence of αβ T-cells, they have a smaller CD4 receptor, interact with non-classical MHC molecules and do not necessarily require specific peptide complexed to these molecules for activation. αβTCR restriction with disease reinduction [27]. Purified CD8+ T-cells, activated in the autologous mixed lymphocyte reaction, suppress the proliferation of autologous T-cells through the release of IFNγ. Patients with chronic progressive MS appear to have a defect in this mechanism [28]. Impaired counterregulatory influence of CD8+ T-cells in MS might be related to the recently described reduction in density of MHC class I gene products on T-cells, B-cells, and macrophages [29, 30]. Summarizing, CD8+ T-cells, like their CD4 counterparts, have dual roles in the immunopathogenesis of MS.

Apoptosis of antigen-specific T-cells, in the lesions of EAE and experimental allergic neuritis (EAN), has been identified as an effective mechanism in stopping neuronal inflammation. Programmed cell death of these infiltrating T-cells might be the consequence of enhanced corticosteroid production, the presence or absence of cytokines, lack of availability of costimulatory molecules, or antigen induction [31–34].

**γδT-cells**

Although αβ T-cells predominate in MS lesions, significant numbers of T-cells expressing γδTCRs also exist. It is controversial whether their presence indicates early or long-standing disease. They are phylogenetically older than αβ T-cells, and are responsible for a more primitive immune response to gut-derived bacterial antigens as well as heat-shock proteins which are expressed in MS lesions. Compared with αβ T-cells they have a smaller TCR repertoire, interact with non-classical MHC molecules and do not necessarily require specific peptide complexed to these molecules for activation. γδTCR restriction with disease reinduction [27].
clonal expansion is observed in MS patients [35–37], implying a pathogenic role.

Natural killer cells
Some report fewer circulating natural killer cells in patients with active relapsing and progressive MS but their role in the immunopathogenesis is unknown [38].

B-cells
Oligoclonal plasma cell responses occur in MS and GBS. Complement-fixing anti-myelin antibodies are responsible for injury to the myelin sheath, possible damage to the oligodendrocyte and Schwann cell, Fc-receptor stimulation, chemotaxis and myelin opsonisation for phagocytosis. In GBS, antibodies to specific peripheral myelin antigens are believed to be central to the pathogenesis of the disease, and GBS responds to plasmapheresis. Antibodies to specific gangliosides are responsible for functional effects. Anti-GM1 antibodies, found in approximately 25% of GBS patients have been shown to cause conduction block in some studies [39*], and to affect voltage gated Na+ channel function in vitro [40*,41]. However, in the most recently published study on the effects of intraneural injection of GM1 antisera, no signs of electrophysiological disturbances or pathology were recordable despite deposition of immunoglobulin (G and M) at the nodes of Ranvier in rat tibial nerves [42]. Anti-GQ1b immunoglobulins in the Miller Fisher syndrome variant of GBS, interfere with neuromuscular transmission by blocking acetylcholine release [43*,44*]. It is quite possible in GBS that T-cells cooperate by opening the blood–nerve barrier to allow circulating autoantibodies access to myelin antigens which are then responsible for the nerve damage [45].

Macrophages and microglia
Macrophages and microglia play a central role in the pathogenesis of both MS and GBS. They are responsible for antigen presentation, production of myelinotoxic and neurotoxic factors, and phagocytosis, there is some in-vitro evidence that they may be responsible for assisting in the process of remyelination [46], presumably via the production of growth factors. In brains of patients with MS, macrophage differentiation can be correlated with demyelinating activity [47].

Antigen-presenting cells and the site of T-cell activation
Memory T-cells (CD45RO+) expressing high levels of adhesion molecules are selectively recruited into the nervous system [48]. If activated, they initiate an inflammatory response, in which non-activated cells migrate along with antibodies and possibly inflammatory mediators [49]. Although the site of antigen specific T-cell activation in MS is unknown, evidence from adoptively transferred EAE demonstrates that disease-inducing MBP-specific T-cells remain in the perivascular space [50,51]. This makes the perivascular macrophage the most likely antigen-presenting cells in MS. Other possible antigen-presenting cells include astrocytes, smooth-muscle cells or pericytes, endothelial cells, microglia and dendritic cells in deep cervical lymph nodes [52]. In the peripheral nervous system, endothelial macrophages are the most likely antigen-presenting cells, although based on in-vitro studies, Schwann cells may serve as facultative antigen presenters [53,54].

Endothelial cells
After activation, proliferating T-cells of the Th-1 phenotype produce proinflammatory cytokines. These result in activation of resident macrophages and microglia which then produce an array of proinflammatory monokines and chemokines. This results in astrocyte and endothelial activation, upregulation of adhesion molecules and blood–brain or nerve barrier breakdown (Fig. 2).

Adhesion molecules and cytokines
Adhesion molecules
Adhesion molecule expression is greatly upregulated in MS [55,56] and probably GBS, allowing recruitment of circulating leukocytes and functioning as accessory molecules in antigen presentation. Soluble forms of these molecules are produced by proteolytic cleavage or alternate RNA splicing and released from the cell. The soluble forms probably function as natural inhibitors of cell adhesion and have an immunomodulatory role. Soluble E-selectin and vascular cell adhesion molecule (VCAM)-1 stimulate angiogenesis [57*]. Increased levels of soluble E-selectin [58–60], L-selectin [60], intercellular adhesion molecule (ICAM)-1, ICAM-3 [61] and VCAM-1 [62] have been found in cerebrospinal fluid (CSF) and serum of patients with MS. These levels appear to correlate, albeit poorly, with clinical and magnetic resonance imaging (MRI) markers of disease activity (Table 2). Soluble E-selectin levels are also elevated in GBS [63,64]. By blocking adhesion molecule interactions, recruitment of effector cells into the nervous system can be reduced, preventing or controlling the disease process—an approach that has proved partially successful in EAE and EAN [65–69].

Chemokines
Chemokines are chemotaxtractant cytokines that are responsible for the selective recruitment of specific subsets of inflammatory cells. Based on the arrangement of cysteine residues, chemokines are divided into two groups. C-X-C chemokines (IL-8, melanoma growth-stimulatory activity, platelet factor 4, and β-thromboglobulin) are mainly responsible for attracting neutrophils and are therefore unlikely to play a major role in MS and GBS. C-C chemokines, however, are likely to be involved as they are responsible for macrophage and T-lymphocyte recruitment, and include macrophage inflammatory protein-1α and -1β, monocyte chemotactic protein-1, RANTES and lymphotaxin. They play an important role
Table 2. Recent correlations between immune markers and magnetic resonance imaging and clinical disease activity in patients with multiple sclerosis

<table>
<thead>
<tr>
<th>Immune marker</th>
<th>Gadolinium-enhanced MRI</th>
<th>Relapse</th>
<th>Remission</th>
<th>Disease progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines and soluble receptors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumour necrosis factor α (protein and mRNA)</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>soluble tumour necrosis factor-R</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>interferon γ (mRNA)</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>transforming growth factor (mRNA)</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>interleukin 4 (mRNA)</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Soluble adhesion molecules</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>sl-selectin</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>vascular cell adhesion molecule-1</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Acute phase proteins</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Data are emerging with regard to the putative role of the anti-inflammatory cytokines, IL-4, IL-10, IL-13 and transforming growth factor (TGFβ1) in MS and GBS. Increased levels of IL-10 and TGFβ1 mRNA expression in mononuclear cells from the peripheral blood and CSF are associated with the recovery phase of a relapse, periods of remission and a less aggressive disease course in MS [74,80,81]. In EAN, mRNA for TGFβ1 is upregulated to high levels in spinal ganglia and motor and sensory nerves with peak levels preceding the first signs of clinical recovery. Macrophages and a subpopulation of T-cells were identified as the cellular source of TGFβ1 [82]. There is other evidence that inhibitory cytokines favourably modify the course of EAE and EAN.

Autoantigens

Assuming that MS and GBS are primarily diseases of myelin, most work has concentrated on the myelin-
specific proteins as putative autoantigens. Central nervous system myelin proteins implicated on the basis of T-cell reactivity include MBP, PLP, myelin-associated glycoprotein, and lately myelin oligodendrocyte glycoprotein (MOG) [83,84]; other potential antigens include transaldolase, 2',3'-cyclic nucleotide 3'-phosphodiesterase and αβ-crystallin [10*,85*,86]. In addition, autoantibodies to MBP [87,88], PLP [87] and myelin oligodendrocyte glycoprotein [86] have been found in the CSF of MS patients. Despite reactivity to 'classical' myelin antigens, the presence of retinal perivascular inflammation in MS, an area devoid of myelin, and the absence of inflammation in the peripheral nervous system which contains high concentrations of some putative myelin antigens, cannot be explained.

In GBS the peripheral nervous-system myelin antigens include P0, P1, P2 and gangliosides [89*]. Recently, it has been shown that it is possible to raise T-cell lines to human P2 protein from the blood of patients with GBS, while no reactivity was detected towards P0 [90-92]. The regional distribution and function of glycolipids in different areas of the human nervous system may have a role in determining the pathophysiological features of the associated neuropathy. For example, GQ1b is enriched in human ocular motor nerves compared to other sites which may explain the exquisite vulnerability of these nerves to immune-mediated attack in the Miller-Fisher syndrome. Similarly, varied antigen distribution and expression may account for regional or fibre selective variants, e.g. GM1 in the myelin sheath is highly enriched in motor versus sensory nerves [54].

Access of circulating autoantibodies to the peripheral nervous system through a leaky blood–nerve barrier at specific anatomical sites (exit zones, motor terminals), the autoantibody fine specificities and the glycolipid membrane topography, may all be crucial factors that could explain in pathogenic terms the recently appreciated heterogeneity of GBS. Pathological studies of Chinese acute motor axonal neuropathy demonstrating penetration by macrophages of the outer basal lamina of the Schwann cell and invasion of the periaxonal space of the internode with deposition of activated complement components at the adaxonal Schwann cell plasma membrane raise the possibility that antibodies in these cases bind to an axolemmal antigen [93*,94**,95**], see Fig. 3.

Almost certainly other myelin and non-myelin autoantigens will be implicated in the pathogenesis of demyelinating diseases. Possible candidates in MS include heat-shock proteins, astrocytic antigens like the calcium-binding protein S-100, endothelial antigens, and possibly nuclear factors. Reactivity to so many antigens and peptides may arise from inter- and intramolecular determinant spreading, a process that occurs in EAE [96] and probably in MS [10**].

**Triggers**

**Systemic triggers**

In MS, gadolinium-enhanced MRI studies demonstrate that disease activity occurs in bursts. This suggests that a systemic trigger at least early in the disease is responsible for disease activation and clinical relapses. IFNγ is one of the well-established triggers [76], and likely to be one of the mechanisms by which viral infections induce disease activity, as up to a third of all clinical relapses occur in relation to viral infections [97]. Whether other triggers of IFNγ production, like vaccinations or skin tests for cell-mediated immunity, are able to induce disease activity, has not been fully established. The converse occurs in pregnancy, a state of relative immunosuppression, in which disease activity is suppressed and appears to favourably affect the long-term course of the disease [98]. However, in the postpartum period the risk of relapse is increased.

Antecedent infections occur in approximately 65% of patients with classical GBS, the most common caused by

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**Figure 3. Macrophage Infiltration in Guillain–Barré syndrome**

In acute motor axonal neuropathy (AMAN), macrophages (M0) penetrate the outer basal lamina of the Schwann cell and invade the periaxonal space of the internode; deposition of activated complement components on the adaxonal side of the Schwann cell plasma membrane raises the possibility that autoantibodies in these cases bind to an axolemmal antigen. In acute inflammatory demyelinating neuropathy (AIDP) or classical GBS, this process occurs at the nodal and paranodal areas suggesting a distinct and different set of autoantigens are involved.

- **Attack of M0 on axon**
- **Attack of M0 on myelin**

Axonal GBS

AMAN

AIDP

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the enteric pathogen *C. jejuni* [review: 99**,100**]. Additional microbial triggers include cytomegalovirus and Epstein–Barr virus, among others. Additional evidence has been furnished that at least in a proportion of patients with GBS and its variant, the Miller-Fisher syndrome, molecular mimicry of epitopes common to both glycolipids on peripheral nerve and lipopolysaccharide of GBS-associated *C. jejuni* serotypes causes these immune-mediated neuropathies [101–103].

**Local triggers**

Epidemiological studies over the years have implicated an infectious agent as the cause of MS [104]. It is plausible that a viral infection of cells within the central nervous system may initiate the events that lead to focal demyelination. Recently human herpes virus 6 (HHV-6) has been added to a long list of such triggers [2**]. Although the HHV-6 genome was found in the brains of a similar number of patients and control individuals (>70%), nuclear expression of HHV-6 protein was only demonstrated in oligodendrocytes of MS cases. This staining was more prominent in and around plaques, but was also seen in areas without inflammation. Neuronal cytoplasmic HHV-6 protein expression was seen in certain controls but was much more prominent in the grey matter, adjacent to plaques, in cases with MS. In contrast, HHV-6 DNA could only be detected in acellular CSF of a minority (four out of 36) of MS patients examined [105].

**Demyelination and axonal loss**

Damage to oligodendrocytes and Schwann cells occurs as a result of nonspecific mediators of inflammation, such as reactive oxygen and nitrogen free radicals, proteases, proinflammatory cytokines, and antibody or complement-mediated membrane attack. Oligodendrocytes and Schwann cells are sensitive to the damaging effects of free radicals, produced by activated macrophages and astrocytes [106]. They cause cellular damage by lipid peroxidation, depletion of intracellular energy stores because of inhibition of the mitochondrial electron transport chain [107,108], consumption of intracellular reducing agents, and damage to DNA. Both lipid peroxidation and nitric oxide metabolites [109] are increased in the CSF of MS patients. It appears that human macrophages are unable to produce significant quantities of nitric oxide, despite the expression of inducible nitric oxide synthase mRNA [110,111]. Astrocytes, however, demonstrate increased activity of inducible nitric oxide synthase in MS specimens [112]. Scavenging or inhibiting oxygen free-radical production has a positive effect on the clinical course of both EAE [113] and EAN, and beneficial effects have also been shown with inducible nitric oxide synthase inhibitors [114,115].

Oligodendrocyte death in MS is characterized by DNA fragmentation that suggests apoptosis or programmed cell death [116]. Possible mechanisms for apoptosis include oxidative stress, depletion of intracellular energy stores, and a cytokine-induced death signal. In vitro, IFNγ [117] and TNFα [118] induce apoptotic oligodendrocyte cell death, TNFα through activation of its p55 receptor. An established mechanism of oligodendrocyte and Schwann-cell damage is immunoglobulin-induced complement activation [119]. Decomplementation using cobra venom factor or limiting complement activation with soluble recombinant complement receptor 1, inhibits antibody-mediated demyelinating and damage to myelin [120–122]. Apparently, an inflammatory attack overcomes the normal regulatory mechanisms that downregulate complement activation on myelin [123].

In Lewis rats, when non-neural antigen (ovalbumin) specific CD4⁺ T-cells are targeted to the tibial nerve by prior intraneural injection of ovalbumin, and administered with a systemic demyelinating anti-galactocerebroside antibody, local conduction block and damage to myelin sheaths occur in the region of T-cell accumulation [124,125]. Therefore, breakdown of the blood–nerve barrier by activated non-neural specific T-cells, allows the development of nerve dysfunction and tissue damage in the presence of circulating antimyelin antibodies. These observations serve to revive the concept of demyelination as a bystander phenomenon.

Ultimately, the most important aspect affecting clinical prognosis in MS and GBS is the development of disability. Although it is tempting to equate levels of inflammation in MS with the ultimate development of disability, MRI studies have not borne this out. Firstly, non-relapsing progressive patients acquire disability with little evidence of active inflammation on MRI, and secondly the association of disability with MRI activity is weak. Inflammation is not all bad, and if MS is caused by an infection the inflammatory activity may be an appropriate response to contain the infection. Therefore the assumption that axonal loss is caused by inflammatory activity may not necessarily be correct.

The mechanisms that result in oligodendrocyte and Schwann cell injury are also damaging to axons, but as the nerve cell bodies are usually some distance from the site of inflammation they are able to survive. However, if destruction of myelin-producing cells is severe and prolonged, axonal involvement and subsequent nerve cell loss will occur. In GBS axonal involvement is associated with a poorer outcome, and is more likely to occur in association with preceding *C. jejuni* infection [100**]. In the acute motor-sensory axonal GBS variant, associated with *C. jejuni* infection, the immunological attack may be directed at the axon [94**,126**]. It has also been proposed that an increase in endoneurial pressure from breakdown of the blood–nerve barrier, reduces nerve blood flow, further damaging axons and Schwann cells by ischaemia [126].
Disease progression
Each MS lesion has some degree of axonal loss [127], which only manifests as disability if a certain threshold of axons are lost in a particular neuronal system [128]. This mechanism of how disability manifests does not explain the immunopathogenesis of disease progression. Some hypotheses follow (see Tables 3 and 4).

If demyelination in early MS is not a result of a direct immunological attack but rather the result of a bystander phenomenon, oligodendrocyte damage will not be severe and recovery will occur. However, as the disease progresses with concomitant spread of antigenic determinants, the immunological response becomes directed toward the oligodendrocyte, which is then unable to survive and progressive disease ensues.

If the immunological response becomes directed to some early myelin components, maturation of progenitors into oligodendrocytes may be prevented, e.g., sulfatide-reactive antibodies which inhibit oligodendrocyte progenitor differentiation have been described in MS patients [129].

If there is a limited population of oligodendrocyte progenitors, their gradual depletion limits recovery and progressive disease ultimately develops.

Quinolinic acid, a byproduct of IFNγ-induced macrophage metabolism of tryptophan, is elevated in the CSF of many inflammatory conditions. It is an N-methyl-D-aspartate receptor agonist with excitotoxic activity, and is hypothesized to be involved in neuronal cell death. Elevated levels are found in HIV encephalopathy (a disease with marked neuronal loss) and in the spinal cord of animals with EAE [130]. Quinolinic acid may be involved in neuronal loss in MS.

Gliosis, a pathological hallmark of MS, prevents axonal sprouting in vitro and may limit axonal recovery in vivo. CSF glial fibrillary acidic protein, an astrocytic marker, increases progressively with disease duration suggesting a relationship between gliosis and disease progression [131]. As gliosis occurs in areas of recurrent demyelination it may be an important factor inhibiting the recovery of damaged axons.

Table 3. Possible mechanisms in the development of progressive disease in multiple sclerosis patients

<table>
<thead>
<tr>
<th>Hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shift of primary immunological attack to oligodendrocyte, causing</td>
</tr>
<tr>
<td>oligodendrocyte cell death</td>
</tr>
<tr>
<td>Development of an immunological response to early myelin components</td>
</tr>
<tr>
<td>preventing maturation of oligodendrocyte progenitors</td>
</tr>
<tr>
<td>Depilation of oligodendrocyte progenitors</td>
</tr>
<tr>
<td>Excitotoxic neuronal damage, e.g., quinolinic acid</td>
</tr>
<tr>
<td>Excessive gliosis preventing regrowth of axons</td>
</tr>
<tr>
<td>Failure to re-establish immunological tolerance</td>
</tr>
<tr>
<td>Cytotoxic antineuronal T-cell response</td>
</tr>
<tr>
<td>Cytopathic effects of a neurotropic virus (e.g., human herpes virus 6)</td>
</tr>
</tbody>
</table>

Another possibility may be a failure of the immunological tolerance. Evidence suggests that relapsing–remitting MS follows cycles of immunological activation as a result of Th-1 response (relapse), which is then followed by a suppressor response that downregulates inflammation, re-establishes tolerance and encourages remyelination and repair (recovery and remission). Initially this may be controlled systemically, explaining the cycles of activity seen on MRI. However, if the systemic control breaks down the inflammatory processes desynchronize and result in continuous cycles of inflammation occurring at the level of individual lesions. This would then manifest clinically as progressive disease with superimposed relapses and high levels of MRI activity. The other side of the immunological spectrum would include the non-relapsing progressive cases. These would be unable to mount a suppressor response, have continuous low-grade inflammation with no obvious relapses or remissions and little MRI activity. An analogy would be the immunological spectrum seen in chronic infections, e.g., leprosy, tuberculosis and leishmaniasis, in which a link to certain MHC haplotypes accounts for the observed clinical and immunological spectrum.

Neurons may express MHC class I molecules [132**] making them a target for cytotoxic T-cells—a potential mechanism for clearing neurotropic viruses that could cause neuronal loss.

If a neurotropic virus causes MS, cytopathic effects from viral replication may be sufficient to cause progressive oligodendrocyte and neuronal loss, independent of inflammation.

Conclusion
Since the last review on the immunopathogenesis of demyelinating diseases, there has been further progress on the specific and nonspecific mechanisms of nervous system inflammation, thus providing new avenues of therapeutic intervention. The costimulatory molecules B7-1 and B7-2 have been shown to control the type of T-helper response in EAE. The manipulation of this interaction provides a new and novel approach to control T-cell-mediated autoimmune disease. The list of putative autoantigens in MS continues to grow with the demonstration of T-cell reactivity to the small heat-shock protein αB-crystallin. The significance of HHV-6 infection in MS remains speculative. In GBS, C. jejuni has firmly been established as the most common precipitating infection and is associated with a poorer clinical outcome. It has also been shown to be responsible for outbreaks of acute
motor axonal neuropathy with features suggestive of GBS [133*,134]. Molecular mimicry, i.e. existence of epitopes shared between glycolipid antigens of peripheral nerve and lipopolysaccharide constituents of C. jejuni may be crucial to the pathogenesis of GBS (demyelinating and axonal). Anti-GMI and GQ1b antibodies found in some cases of GBS affect ion channel function with specific electrophysiological and clinical effects.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
• Of outstanding interest
• Of special interest


Comprehensive and current literature review on genes and susceptibility to MS.

2 Chaffron PB, Smith KT, Parker JD, Macleod DL, Coulter SN, Rose TM, Challoner PB, Smith KT, Parker JD, Macleod DL, Coulter SN, Rose TM, et al: Comprehensive and current literature review on genes and susceptibility to MS.


These authors identified peptide motifs required for MHC class II binding and TCR recognition of the human- and mouse-specific MBP peptides. These could be relevant to inflammatory disease of the central nervous system. MBP(85-99) specific T-cell clones could be activated by mimicry peptides of herpes simplex. Epstein–Barr virus, adenovirus 12, influenza type A and Pseudomonas aeruginosa. Viral mimicry peptides were more effectively presented by MS-associated DR2 molecules. Activation of DR2-specific T cells by viral peptides would constitute a mechanism by which autoimmune responses are triggered in MS.


Very important findings documenting the different T-helper developmental pathways and the potential therapeutic implications from manipulating their development.


An excellent authoritative review with 384 references.


16 Kuchroo VK, Das MT, Brown JA, Das MR, Kuchroo VK, Das MT, Brown JA, Das MR, et al: Comprehensible and current literature review on genes and susceptibility to MS.


24 Kuchroo VK, Das MT, Brown JA, Das MR, Kuchroo VK, Das MT, Brown JA, Das MR, et al: Comprehensive and current literature review on genes and susceptibility to MS.


Immunopathogenesis of multiple sclerosis and Gullain–Barré syndrome Giovanni and Hartung


41 A demonstration of the functional effects of anti-GM1 antibodies on functions of peripheral markers in patients with MS.


Both papers (43,44) demonstrate the probable functional effects of anti-GM1 antibodies in the motor system, weakening the spectrum of auto-immune phenomena occurring at this site.


55 Important immunological finding of a functional role for soluble E-selectin and VCAM-1.


A review of the role of TNF in EAE and MS, as well as an editorial accompanying a paper on the antiinflammatory roleplay which suppresses the production of TNF and prevents EAE.


85 Van Noort JM, van Sechel AC, Bajramovic JJ, Ouagmiri ME, Polman CH, Sparkes RS, Breakefield XO: The expression of extracellular matrix molecules by astrocytes in inflammatory and degenerative diseases of the central nervous system.


91 Definite study. A prospective series of over 100 GBS patients, demonstrating a more severe course with a worse prognosis in patients with C. jejuni-related GBS.


131 Neumann H, Cavalie A, Jenne DE, Wekerle H: Induction of MHC class I antigens and zIgE therefore balle to attack by cytotoxic T lymphocytes. Transcription of MHC I genes occurred only in electrically silent neurons. This has important implications for the immunology of neurotropic viruses and neuronal transplantation. J Neuroimmunol 1995, 56:191-200.


Correlating immunological and magnetic resonance imaging markers of disease activity in multiple sclerosis

G Giovannoni, B Kieseier, H P Hartung

Abstract

Inflammation plays a central part in the pathogenesis of multiple sclerosis. However, current surrogate magnetic resonance (MR) and immunological markers of inflammation are weakly associated and correlate poorly with clinical progression. Reasons for this are multiple and probably relate to the non-specific changes and insensitivity of current MR techniques, disease dynamics, anatomical factors, and the temporal profile and poorly defined complexities of the inflammatory reaction in multiple sclerosis. This paper provides an overview of the principles involved in the monitoring of inflammation in multiple sclerosis, discusses possible reasons for the weak correlation between MR and immunological markers of inflammation, and briefly reviews the studies correlating these modalities. In addition, the predictive values of MRI and CSF oligoclonal immunoglobulin are compared in determining future progression to clinically definite multiple sclerosis in patients presenting with clinically isolated syndromes compatible with demyelination.

(J Neural Neurosurg Psychiatry 1998;64(Supplement 1):S31–S36)

Keywords: multiple sclerosis; magnetic resonance imaging; disease monitoring

In a meeting held under the auspices of the American Multiple Sclerosis Society, it was decided by a consensus opinion that the primary outcome measure of definitive therapeutic or phase III clinical trials in multiple sclerosis should remain clinical. The magnetic resonance imaging (MRI) indices of disease activity, such as new or enlarging lesion formation and gadolinium (Gd) enhancement, although more sensitive, should only be used as secondary outcome measures. The various immunological markers discussed were considered unsuitable as outcome measures as none satisfied the criteria for a surrogate marker of disease activity. The discrepancy between clinical, MRI, and immunological markers of disease activity in multiple sclerosis has important implications for their use in clinical practice, as well as our understanding of the disease dynamics in multiple sclerosis. The principles behind the immunological monitoring of disease activity are based on the model that multiple sclerosis is an organ specific autoimmune disease caused by autoreactive CD4+ T cells, which orchestrate a cell mediated immunological reaction. The resulting complex immunological cascade with T cell, B cell, macrophage and endothelial activation, and the induction of cytokines and inflammatory mediators, results in demyelination and axonal loss. By measuring or quantifying levels of inflammation, it should have been possible to predict the development of disability. Unfortunately this did not prove to be the case.

In multiple sclerosis, clinical relapse and MRI activity are associated pathologically with acute plaques showing perivascular inflammation and demyelination. Hence both relapse and MRI activity are currently used as surrogate markers of inflammation and are important secondary outcome measures in clinical trials. However, both the relapse rate and MRI activity correlate poorly with disability, suggesting that inflammatory activity may not necessarily be directly responsible for disease progression. Further support for this comes from patients with primary progressive multiple sclerosis, who by definition have no clinical relapses, little or no evidence of MRI activity, and less inflammation pathologically than patients with secondary progressive multiple sclerosis, but who continue to progress clinically. In addition, therapeutic trials with various anti-inflammatory and immunomodulatory agents (with the possible exception of interferon β-1a(IFNβ-1a)) have failed to show a convincing effect on disease progression. Immunological studies using various CSF, blood, serum, and urine markers have also found no consistent relation between inflammation and disease progression. However, some of these markers correlate with clinical relapse and Gd enhanced MRI activity. We review the literature concerned with correlating immunological indices with MRI markers of disease activity in multiple sclerosis, and present a clinically based model in an attempt to explain the findings.

The inflammatory cascade and the principles of immunological monitoring

Immunological monitoring is based on the principle that multiple sclerosis is an organ...
specific cell mediated autoimmune disease, orchestrated by antigen specific CD4+ Th1 cells (for reviews see Martin and McFarland1 and Giovannoni and Hartung14). Once activated, antigen specific T cells produce interleukin-2 (IL2) and express the high affinity IL2 receptor, IL2R. Both results in autocrine stimulation and clonal proliferation of the T cell population. In addition to IL2 activated T cells produce various cytokines which include IFNγ, tumour necrosis factor (TNF) α/β, and IL4. These cytokines result in endothelial, microglial, and astrocytic activation with the induction or upregulation of adhesion molecules, production of chemokines, and other proinflammatory cytokines. This results in breakdown of the blood-brain barrier and the non-selective recruitment of circulating lymphocytes and monocytes. Various non-specific inflammatory mediators which are mainly produced by activated microglia, macrophages, and astrocytes are implicated in oligodendrocyte toxicity and demyelination, as well as axonal and neuronal toxicity, the pathological substrates of irreversible disability. In addition to typical cell mediated inflammation, antigen specific B cell activation with isotype switching and affinity maturation results in a plasma cell response with oligoclonal immunoglobulin and light chain production. The release of cytokines into the systemic circulation results in an acute phase hepatic endocrine response as well as changes in the population of circulating lymphocytes. In addition to proinflammatory events there are inhibitory immunological responses that are hypothesised to down regulate and terminate acute inflammation associated with disease activity.

Various immunological markers, which include changes in circulating subsets of lymphocytes, cytokines, and their soluble receptors, adhesion molecules, chemokines, immunoglobulins and free light chains, macrophage activation markers, activated complement, myelin, and neuronal breakdown products, have been studied in multiple sclerosis. A detailed discussion of these markers and their clinical significance, unless correlated with MRI activity, is beyond the scope of this article.

Diagnosis
With the advent of disease modifying treatments it is becoming more important to diagnose multiple sclerosis sooner. Potential therapies may have to be started as early as possible, before the development of clinical disability, to have maximum effect. In about 90% of patients with multiple sclerosis, the initial event is an acute clinically isolated syndrome involving either the spinal cord, brainstem, or optic nerves. However, not all patients presenting with such syndromes go on to develop clinically definite multiple sclerosis. Much attention has been focused on the capabilities of MRI to predict who will go on to develop multiple sclerosis (for review see Miller15). Although no immunological index is specific for multiple sclerosis, the detection of local oligoclonal bands or IgG synthesis in the CSF is a helpful diagnostic aid. At presentation about 95% of cases with clinically definitive multiple sclerosis are oligoclonal band positive.16 Similarly at diagnosis, areas of increased signal on MRI which are also non-specific, are positive in 70%-100% of patients.17 Table 1 represents a summary of the studies in which the positive predictive values of both MRI and CSF oligoclonal bands were directly compared. Although the presence of CSF oligoclonal bands provides immunological rather than anatomical information, it seems to be equivalent or slightly superior to MRI in predicting future progression to clinically definite multiple sclerosis.

Disease dynamics
Multiple sclerosis is a heterogeneous disease which is associated with various clinical manifestations of disease activity. Most disease activity detected by MR is clinically asymptomatic. What determines whether a lesion is symptomatic or not depends primarily on the site of the lesion. Lesions involving eloquent sites such as the optic nerves, brain stem, and spinal cord are more often symptomatic, compared with lesions occurring in the paraventricular and deep white matter. Other factors that have not been well defined but intuitively play a part in determining whether a lesion is symptomatic or not, are the size of the lesion and whether it involves a previously compromised pathway. Serial monthly MR studies have shown that for every clinical relapse there are between five and 10 asymptomatic lesions.18 This is an underestimate, as studies with more frequent scanning intervals (weekly or biweekly) have shown that about 30% of lesions enhance for fewer than four weeks and would therefore be missed in monthly studies.19 In addition MRI techniques using delayed scanning, triple dose Gd (0.3 ml/kg), and magnetisation transfer detect 126% more

Table 1  Predictive value of CSF oligoclonal IgG bands (O CB) v MRI

<table>
<thead>
<tr>
<th>Patients</th>
<th>Duration of follow up (s)</th>
<th>MRI + ve n (%)</th>
<th>MRI - ve n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>18/22 (82)</td>
<td>12/22 (55)</td>
<td>24/26 (92)</td>
</tr>
<tr>
<td>Rellini et al.</td>
<td>83</td>
<td>37 (30)</td>
<td>25 (5)</td>
</tr>
<tr>
<td>Sharrack et al.</td>
<td>45</td>
<td>17/24 (71)</td>
<td>5/21 (24)</td>
</tr>
<tr>
<td>Nederbrant et al.</td>
<td>60</td>
<td>22/14 (37)</td>
<td>0/22 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>232</td>
<td>60/121 (50)</td>
<td>0/22 (0)</td>
</tr>
</tbody>
</table>
lesions than conventional single dose Gd (0.1 ml/kg) studies.5 Therefore a more realistic estimate would be at least 20–30 asymptomatic lesions for every clinical relapse, and if MR had the ability to detect microscopic lesions39 this ratio would be even higher. Most studies quantify Gd enhancement by simply noting its presence or absence, or by simply counting the number of Gd enhancing lesions per study (Table 2). Reickmann et al. have recently shown a correlation between the area of Gd enhancement and the sICAM-1, sVCAM-1 index, and the serum concentrations of these two soluble adhesion molecules.40 Unfortunately, no comparison between lesion volumes and a simple lesion count was presented. Improving the quantification of the “inflammatory burden” on MR, in terms of the true number or volume of active lesions, may improve the correlation between MR and immunological indices of inflammation.

Temporal effects

Studies in experimental allergic encephalomyelitis (EAE), and by inference in multiple sclerosis, have delineated a cell mediated inflammatory reaction with a defined inflammatory timetable. Initially antigen specific T cells enter the CNS.41 Presumably, on recognizing their autoantigen in the presence of the appropriate costimulatory molecules and cytokines, these T cells become activated, express the high affinity IL2 receptor, and produce IL2 and IFNγ. This results in T cell proliferation, microglial and endothelial cell activation, and the upregulation and induction of adhesion molecules, chemokines, and a cytokine cascade. This leads to disruption of the blood-brain barrier, the recruitment of circulating monocytes, T cells, and B cells, and the production of various non-specific inflammatory mediators. The resultant cascade results in oligodendrocyte, axonal, and neuronal toxicity. As part of this reaction, the anti-inflammatory or regulatory cascade counteracts the proinflammatory elements. If successful, inflammation is suppressed and recovery occurs (monophasic or relapsing EAE), otherwise continuous inflammation issues and the EAE becomes chronic.

In multiple sclerosis, evidence suggests that disease activity is temporally related. Firstly, MR activity in the form of single or multiple Gd enhancing lesions is more likely to be found in association with a clinical relapse than in periods of remission.42 Secondly, serial MR studies have shown cycles of disease activity with multiple new lesions forming in bursts.43 Thirdly, disease activity in the spinal cord rarely occurs in isolation and is strongly associated with simultaneous activity in the brain.44 The temporal clustering of lesions affects immunological monitoring. During a clinical relapse proinflammatory markers are more likely to be raised than during periods of clinical inactivity. Studies recruiting patients during relapse are more likely to find positive correlations than studies recruiting patients in periods of remission.

Natural history studies of multiple sclerosis have shown that individual lesions enhance with Gd for a variable period of time.45 Whereas some lesions are shrinking or disappearing other lesions are appearing or enlarging, implying that the inflammatory time courses of individual lesions are not necessarily dependent on each other, and are probably asynchronous. This has implications for interpreting levels of inflammatory markers which may be produced in a discrete time frame, in the life cycle of an active lesion. For example, if a particular marker is only produced in the initial few days of a Gd enhancing lesion, then its levels should only be correlated with newly enhancing lesions (< seven days). Support for this comes from a large serial study in which 47 patients with relapsing-remitting or secondary progressive multiple sclerosis underwent monthly scanning46: this study showed that serum sICAM-1 concentrations associated with inactive MR studies were significantly lower than the concentrations associated with only persistently enhancing lesions (enhancing for at least a month), or newly enhancing lesions (enhancing less than a month) (median sICAM-1 (interquartile range) = 200 (85–561) ng/ml v 349 (82–615) ng/ml v 497 ng/ml (108–667), p = 0.03). The temporal profile of Gd enhancing lesions should be considered when attempting to correlate inflammatory markers with Gd enhancement.

Anatomical factors

Multiple sclerosis has a predisposition for certain anatomical areas, such as the periventricular white matter, the external surfaces of the cervical spinal cord, thepons, and the optic chiasm and nerves.47 It has been shown in patients with single periventricular Gd enhancing lesions, that the CSF/serum index for sICAM-1 and sVCAM-1 is inversely related to the distance of the lesion from the lateral ventricle.48 These data imply that CSF concentrations are related to the distance of the lesion from the CSF pathways, a fact well established for CSF white cell counts in patients with brain abscesses.49 Anatomical factors need to be considered when interpreting immunological indices.

Other possible confounding factors: CSF dynamics and diurnal variation

For most proteins produced intrathecalely there is a rostrocaudal gradient with ventricular fluid having higher concentrations than lumbar CSF.50 This affects the absolute concentrations of proteins depending on which aliquot of CSF is used for analysis. Similarly, posture affects the lumbar CSF protein concentrations by its effect on CSF drainage.51 For example, patients who are kept supine with a severe clinical relapse will have higher CSF protein concentrations than patients who are mobile. Most biological functions are affected by a diurnal variation and the immunological system is no exception. For example, IL6 concentrations may vary by as
much as 350% during a day. Similarly levels of urinary neopterin excretion, a marker of IFNy-induced macrophage activity, can vary by as much as 50%. Unfortunately, most studies fail to adequately control for the rostrocaudal gradient and the postural affects on CSF protein concentration, as well as diurnal variation in inflammatory markers. CSF dynamics as well as the effects of diurnal variation may bias results and contribute to the poor correlation between immunological markers and MR activity.

Non-specific versus “specific” MR indices

The currently used MR indices of disease activity (new or enlarging lesions on T1, T2, and Gd-enhanced T1-weighted images) are non-specific changes due to an increase in water content within the brain or spinal cord or, in the case of Gd-enhancement, a breakdown of the blood-brain barrier. Changes in these indices provide no information on the pathological substrate of the lesions—that is, whether or not demyelination and axonal loss is occurring (destructive v non-destructive lesions), or the type and severity of the inflammation. It would therefore be naïve to think that the complex cascade of immunological reactions which occurs in multiple sclerosis lesions could be simply quantified by measuring alterations in water content, or by crudely calculating the breakdown of the blood-brain barrier. Several new putative MR markers of axonal loss or demyelination have been shown to correlate more closely with clinical disability than non-specific MR markers of inflammation. These putative markers include hypointense lesions on T1-weighted MRI, measures of cerebral atrophy, low magnetisation transfer ratios (MTRs) on MT imaging, and reduced concentrations of N-acetyl aspartate (NAA) on MR proton spectroscopy. Correlations between immunological markers and longitudinal changes in these “specific” MR derived indices are eagerly awaited.

MRI and immunological correlations

Table 2 summarises the studies correlating inflammatory markers with MR activity. Most studies, cross sectional and longitudinal, find a correlation between proinflammatory markers and MR activity. Although significant, these correlations do not always hold. Levels of these markers vary widely, and raised levels are not necessarily raised due to disease activity as most of these markers are non-specific. Some markers are more stable in body fluids (for instance, soluble adhesion molecules and soluble cytokine receptors) than others (for example, cytokines and nitric oxide). Many are produced locally, as autocrine and paracrine signals, with evanescent half lives in serum and CSF which may result in an underestimation of the rate of synthesis in situ when systemic product concentrations are relied on.

Conclusion

Improving the quantification of the “inflammatory burden” on MR, in terms of the true number or volume of active lesions, as well as defining the temporal and anatomical profile of these lesions, may improve the correlation between MR and immunological indices of inflammation. Effects due to dynamics of CSF flow and diurnal variation should be controlled to limit potential confounding factors. New, specific MR indices of axonal loss and demyelination may provide better indices than established non-specific MR markers of disease activity, for immunological correlations. Although numerous markers of inflammation have been studied in multiple sclerosis, they are non-specific, correlate weakly with MR activity, and do not reproducibly predict disease progression. Other inflammatory markers which correlate with clinical disease activity—for example, urinary neopterin excretion, sIL2 and soluble IL2 receptors, and newer markers such as T cell TNF receptor expression, serum nitric oxide metabolites (nitrate and nitrite), circulating monocyte inducible nitric oxide synthase expression and activity, and IL12

<table>
<thead>
<tr>
<th>Index</th>
<th>Cases (n)</th>
<th>Clinical subtypes</th>
<th>Type of study</th>
<th>Duration</th>
<th>Clinical activity</th>
<th>Clinical progression</th>
<th>MRI activity</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVCAM-1, sICAM-1, s-selectin, sL-selectin, sVLA-4</td>
<td>11</td>
<td>RR, SP, PP</td>
<td>Long</td>
<td>8:12</td>
<td>N S</td>
<td>N  S</td>
<td>- +</td>
<td>Calabrese et al.</td>
</tr>
<tr>
<td>SVCAM-1, sICAM-1</td>
<td>15</td>
<td>RR, SP, PP</td>
<td>Long</td>
<td>12:12</td>
<td>N  S</td>
<td>N  S</td>
<td>+ +</td>
<td>Rieckmann et al.</td>
</tr>
<tr>
<td>sICAM-1, sICAM-1</td>
<td>17</td>
<td>RR, SP, PP</td>
<td>Long</td>
<td>12:12</td>
<td>N  S</td>
<td>N  S</td>
<td>+ +</td>
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</tr>
<tr>
<td>sICAM-1, sICAM-1</td>
<td>28</td>
<td>RR, SP, PP</td>
<td>Long</td>
<td>12:12</td>
<td>N  S</td>
<td>N  S</td>
<td>+ +</td>
<td>Rieckmann et al.</td>
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<tr>
<td>sICAM-1, sICAM-1, sICAM-1</td>
<td>46</td>
<td>RR, SP, PP</td>
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<td>12:12</td>
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Correlating immunological and MRI markers of disease activity in multiple sclerosis


The potential role of nitric oxide in multiple sclerosis

G Giovannoni, SIR Heales, JM Land and EJ Thompson

Department of Neuroimmunology and Clinical Biochemistry, The National Hospital for Neurology and Neurosurgery, and the Neuroimmunology Unit, Institute of Neurology, Queen Square, London WC1N 3BG

Nitric oxide (NO) and its reactive derivative peroxynitrite (ONOO-) have been implicated in the pathogenesis of multiple sclerosis (MS). They are cytotoxic to oligodendrocytes and neurones in culture by inhibiting the mitochondrial respiratory chain (complexes III and IV) and inhibiting calcium/calmodulin-dependent ionomycin and calmodulin-dependent NOS (iNOS) activity is found in the cerebrospinal fluid (CSF) and serum of patients with MS. In addition inducible NO synthase (iNOS) activity is found in pathological specimens from patients with MS as NO and its reactive derivative peroxynitrite are cytotoxic to both oligodendrocytes and neurones. Therefore potential mediators of demyelination and axonal loss in MS. We will review the experimental evidence supporting NO as an inflammatory mediator in neurological diseases and discuss its role in experimental allergic encephalomyelitis (EAE) and (MS).

Nitric oxide (NO)

NO is an inorganic gas and free radical which mediates a variety of biological functions including neurotransmission, vasodilation, and cytotoxicity. NO is produced from the conversion of L-arginine to L-citrulline by the enzyme NO synthase (NOS). The NOS's are a family of related enzymes that are different gene products and include neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) isoforms. nNOS and eNOS are constitutively expressed and are calcium and calmodulin dependent. iNOS on the other hand is calcium and calmodulin independent and induced by the action of various proinflammatory cytokines.

Inducible NOS (iNOS)

Although the human and murine iNOS genes are 80% and 60% homologous in their coding and promoter regions respectively, their genetic regulation and possibly their cellular expression differ. Human iNOS is less induced by pro-inflammatory cytokines and bacterial lipopolysaccharides (LPS) than rodent iNOS, which produce large quantities of NO in response to these stimuli. Although iNOS and iNOS mRNA are demonstrable in human macrophages, there is conflicting evidence regarding the production of NO by human cells of the monocyte lineage. In contrast to the murine system in which IL4 inhibits the induction of iNOS as well as NO production, the current evidence suggests that IL4 stimulates the production of NO in human monocytes via the production of soluble FcεR1 or CD23. TGF-β inhibits human and murine NO expression. Fetal human astrocytes and possibly adult human astrocytes are capable of expressing iNOS and producing NO. Enriched human fetal astrocyte cultures produce NO when stimulated with IL-1β, which is augmented by co-stimulation with IFN-γ or TNF-α. In contrast human fetal microglia fail to produce significant amounts of nitrite in response to various stimuli. Although one group has demonstrated that rat oligodendrocytes are capable of iNOS expression and NO production, no data on human oligodendrocytes is available.

When human fetal mixed glial cultures are stimulated with cytokines, iNOS mRNA is expressed within...
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2 h and decreases to baseline by 2 days. iNOS protein appears within 24 h after stimulation and remains present for at least 3 days. The iNOS is responsible for a dramatic increase in •NO production and •NO-mediated events, such as the induction of cyclic guanosine monophosphate (cGMP), NADPH diaphorase activity, and nitrotyrosine production which occur 3 days after stimulation (a delay of 48 h from the time of the first expression of iNOS enzyme).

Interestingly pretreating rat astrocytes with IFNα/β, an effective strategy in early relapsing remitting MS,55–57 prevents subsequent stimulation of •NO release with IFNα/β. In addition IFNα/β, in a murine co-culture system using IFNα/LPS stimulated astrocytes, prevented •NO and peroxynitrite induced mitochondrial complex II/III and IV damage in neighbouring neurons.33 These data suggest that one of the mechanisms of IFNα/β’s activity is via the modulation iNOS activity.

•NO and cellular metabolism

Intracellularly, the mitochondrial respiratory chain is an important target for •NO.32,33 On exposure to •NO neuronal O2 consumption decreases due to a combination of reversible and irreversible inhibition of complexes II/III and IV.142 Mitochondrial damage has important implications for cell survival and the induction of cell death. •NO and peroxynitrite are also damaging to cellular function by causing lipid peroxidation,11 consumption of intracellular anti-oxidants,45 DNA damage,12 and inhibition of several key enzymes such as aconitase,13 glyceraldehyde-3-phosphate dehydrogenase14 and ribonucleotide reductase.46

•NO induced cell death

•NO and peroxynitrite are cytotoxic, causing cell death by necrosis or apoptosis depending on the level and duration of exposure.34,35 •NO induces mitochondrial permeability by disrupting the mitochondrial transmembrane potential which causes the generation of reactive oxygen species and liberates pro-apoptotic factors like cytochrome oxidase which induce nuclear apoptosis.44 In neurons this process is augmented by local or autocrine NMDA-induced excitotoxicity and prevented by NMDA antagonists.45,46 The pro-apoptotic effects of •NO can also be prevented by bcl-2 expression (an anti-apoptotic gene product)47 or anti-oxidants like reduced glutathione or N-acetylcysteine.51

•NO in EAE

In EAE •NO appears to play an important role in CNS inflammation, in both acute and chronic models of the disease.32–35 Hooper et al. using an in vivo •NO spin trapping technique in adoptive transfer Lewis rat EAE have demonstrated that large amounts of •NO (20–30 μM) are produced in the spinal cord 4–5 days after T cell transfer, which correlates with the time of clinical paralysis.48 An early study showed that an iNOS inhibitor, aminoguanidine, ameliorated MBP-specific T cell adoptive transfer EAE in SJL mice.59 These results with high-dose aminoguanidine have been confirmed in both MBP-peptide induced and T cell adoptive transfer EAE in PL/J/SJL mice.60 However other studies in the Lewis rat have shown that iNOS inhibitors result in a worsening of the disease course.59,60 INOS double knock-out mice are less resistant to the induction of EAE, and develop a protracted non-remitting course compared to the acute monophasic illness in wild-type mice.61 More targeted strategies using an iNOS induction inhibitor (tricyclo-decan-9-yl-xanthogenate), a •NO scavenger (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide), and a peroxynitrite scavenger (uric acid) all showed significant therapeutic benefit.62 Interestingly, peroxynitrite scavenging using high dose uric acid almost abrogated the manifestations of the clinical disease. This is strong evidence in favour of peroxynitrite as a major inflammatory mediator in EAE. Although the EAE data is conflicting it suggests that •NO and peroxynitrite play an important role in neurotoxicity due to inflammation but they also have an immunomodulatory role in the CNS inflammatory reactions.

How then does •NO favourably affect the course of autoimmune CNS inflammation? A possible mechanism may relate to •NO’s role in modulating the process of apoptosis.58 •NO plays a role in the elimination of antigen-specific autoreactive T-cells by apoptosis. Its production may therefore augment T-cell apoptosis and assist in the elimination of autoreactive T-cells in EAE.58–64 In the absence of •NO these autoreactive T-cells may persist, resulting in a lower threshold for the induction of autoimmune disease or maintenance of an ongoing autoimmune inflammatory reaction.

•NO in MS

Although •NO is hypothesised to play an important role in MS57–60 the current evidence is circumstantial and based mainly on data extrapolated from animal models or in vitro experiments. In cross-sectional studies on patients with MS increased CSF,7,17 serum* and urine (manuscript submitted) levels of the •NO metabolites nitrate and nitrite are found. In a longitudinal study in which we followed 25 patients with relapsing remitting and secondary progressive MS with serial MRI and clinical examinations over 18 months, serum nitrate and nitrite was significantly higher in patients with relapsing remitting compared to secondary progressive disease.65 Patients with either no or one relapse during the 18 months had significantly higher serum •NO metabolites than patients with two or more relapses. No correlation was found between the mean serum •NO metabolites and (1) Gd-enhanced MRI activity, (2) the development of cerebral atrophy (as measured by MRI) or (3) clinical progression as measured by a sustained increase in the EDSS over the 18-month follow-up period. The significance of the findings are uncertain as nitrate and nitrite are only a crude index of endogenous •NO production. As a large proportion of •NO is consumed by nitrosylation reactions in vivo40
and nitrates levels are affected by gastrointestinal bacterial production and dietary intake.\(^9\)\(^10\) Although the clinical findings are preliminary and need to be confirmed in larger studies, using more sensitive and specific measurements of the in vivo production of \(\cdot\text{NO}\), they raise interesting questions about the role of \(\cdot\text{NO}\) in the pathogenesis of MS.

Pathological studies support the clinical and animal studies and suggest that \(\cdot\text{NO}\) plays a role in the pathogenesis of MS. Patients dying with MS demonstrate increased astrocytic iNOS activity\(^9\) as well as increased levels of iNOS mRNA\(^11\) and iNOS\(^12\) localising to cells with a macrophage phenotype. In EAE both astrocytes and microglia express iNOS.\(^13\) Increased nitrotyrosine residues from peroxynitrite-induced protein nitrosylation are observed in MS patients supporting pathological effects of \(\cdot\text{NO}\) production in vivo.\(^14\) Increased protein nitrosylation is also observed in EAE.\(^15\)

Certain clinical manifestations in MS arise as a result of failure of action potentials to propagate along damaged axons, i.e. transient neurological symptoms and signs. Although demyelination contributes to the conduction block, clinical evidence suggests that inflammatory mediators may also contribute to this process.\(^16\) In a recent study \(\cdot\text{NO}\) donors were capable of causing reversible conduction block in demyelinated dorsal columns of the rat, but not in normally myelinated axons.\(^17\) As \(\cdot\text{NO}\) is a diffusible gas these results suggest a much wider potential role for \(\cdot\text{NO}\) in the symptomatic manifestations of MS.

Other possible sources of \(\cdot\text{NO}\) production with relevance to MS

Currently only three \(\cdot\text{NO}\) synthases are known to produce nitric oxide in mammals.\(^18\) If \(\cdot\text{NO}\) is generated by another source, the levels of \(\cdot\text{NO}\) metabolites would not necessarily reflect the activity of \(\cdot\text{NO}\) synthases. The enzymatic conversion of arginine to citrulline by peptidylarginine deiminase in myelin basic protein (MBP) may be another source of nitric oxide.\(^19\) Peptidylarginine deiminase converts a variable number of arginine residues in MBP to citrulline.\(^20\) Although the biochemistry of this reaction has not been fully delineated, it has been hypothesised to produce either an ammonium ion or \(\cdot\text{NO}\) as a by-product.\(^21\) Citrullination of MBP produces a less cationic or developmentally immature isoform of MBP, which is increased in the white matter of patients with MS, compared with normal control subjects and patients with other neurological diseases.\(^22\) If the citrullination of myelin produces \(\cdot\text{NO}\), elevated levels of nitrate and nitrite in patients with MS may be associated with myelin synthesis and a better prognosis.

Conclusion

Progress has been made in identifying \(\cdot\text{NO}\)'s role in the pathogenesis of inflammatory demyelinating diseases of the CNS. Whether or not \(\cdot\text{NO}\) is central to the pathogenesis of these diseases is unknown, and may depend on the outcome of clinical trials of iNOS antagonists, inhibitors of iNOS induction, or \(\cdot\text{NO}\) and peroxynitrite scavengers. The role of \(\cdot\text{NO}\) in inflammation appears to be complex, on the one hand mediating important cytotoxic reactions and on the other hand modulating immunological functions which in the setting of autoimmunity may be protective. It has been postulated that although \(\cdot\text{NO}\) formation may be deleterious in chronic demyelinating disease, early on in the disease process it might prove to be protective.\(^23\) Further studies in patients with MS, using more sensitive and specific techniques for measuring \(\cdot\text{NO}\) production in vivo are required to delineate the role that \(\cdot\text{NO}\) plays in the pathogenesis of the disease and to monitor the effects of emerging MS treatments.

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Bonfoco E et al. (1995) Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci USA* 92: 7162–7166.


Raised serum nitrate and nitrite levels in patients with multiple sclerosis

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Abstract

Nitric oxide and its highly reactive derivative peroxynitrite have been implicated as non-specific inflammatory mediators of neuronal and oligodendrocyte damage and death in multiple sclerosis. In a cross-sectional study we found levels of the nitric oxide metabolites nitrate and nitrite to be raised in the serum of patients with demyelinating disease (65.6 μM (SD 32.9)), acquired immune deficiency syndrome (57.9 μM (SD 34.9)) and inflammatory neurological disease (57.5 μM (SD 31.3)), compared with normal control subjects (32.8 μM (SD 12.2)) and patients with non inflammatory neurological disease (41.1 μM (SD 12.3), p < 0.001). Nitric oxide metabolites were raised in all clinical subtypes of multiple sclerosis, as well as in clinically isolated syndromes compatible with demyelination, and were not related to progressive disease or disability. This study provides further evidence for a role of nitric oxide in the immunopathogenesis of inflammatory diseases of the central nervous system, including multiple sclerosis. © 1997 Elsevier Science B.V.

Keywords: Serum; Nitrate; Nitrite; Nitric oxide; Multiple sclerosis; HIV-1; AIDS

1. Introduction

Serum nitrate and nitrite are considered to be the major end-products of the endogenous synthesis of nitric oxide (NO) from the guanidino nitrogen of L-arginine. Increases in the metabolic end-products of NO' production are associated with infections in humans due to the induction of the inducible form of NO' synthase (iNOS). NO' plays an important role in non-specific immunity, has cytotoxic effects and is hypothesised to cause significant tissue damage in inflammatory diseases. In vitro, NO' and its highly reactive derivative peroxynitrite (Beckman et al., 1990) are toxic to both oligodendrocytes (Merrill et al., 1993; Mackenzie-Graham et al., 1994; Mitrovic et al., 1994) and neurones (Boje et al., 1992; Bolaños et al., 1995). In vivo there is increasing evidence that NO' plays an important role in CNS inflammation. In experimental allergic encephalomyelitis (EAE), a putative animal model of multiple sclerosis (MS), the administration of the iNOS inhibitor aminoguanidine decreases the severity of the disease (Cross et al., 1994). Evidence of elevated levels of NO' metabolites have been found in the cerebrospinal fluid (CSF) of patients with MS (Johnson et al., 1995), and pathological specimens from patients dying with MS demonstrate increased astrocytic iNOS activity (Bö et al., 1994), as well as increased levels of iNOS mRNA co-localising to cells with a macrophage phenotype (Bagasra et al., 1995). To further study the role of NO' in the pathogenesis of MS, we measured serum levels of nitrate and nitrite in patients with demyelinating disease (clinically isolated syndromes and MS), and compared them to healthy controls, patients with other inflammatory and non-inflammatory neurological disease, and patients with HIV-1 infection. The HIV-1 group was chosen as a positive control as they have previously been shown to have elevated levels of serum nitrate and nitrite (Zangerle et al., 1995).
2. Method

2.1. Patients

Serum nitrate and nitrite were measured in 39 patients with clinically or laboratory supported definite MS (Poser et al., 1983), and in 16 patients with a clinically isolated syndrome compatible with the diagnosis of demyelination. For comparative purposes four control groups were studied: 22 normal subjects, 27 patients with acquired immune deficiency syndrome (AIDS) due to HIV-1 infection, 16 patients with other inflammatory neurological diseases and 14 patients with non-inflammatory neurological disease. All patients, excluding those with HIV-1 infection, were recruited from the National Hospital for Neurology and Neurosurgery, London. All patients with MS and clinically isolated syndrome were examined, and their disability rated using Kurtzke’s expanded disability status score (EDSS) (Kurtzke, 1983). Patients with a clinically isolated syndrome were examined and had blood taken between 3 weeks and 3 months after the clinical onset of their syndrome. All patients with relapsing MS were examined during a period of clinical remission. Patients with other neurological diseases were recruited prospectively from hospital inpatients, and were classified into inflammatory or non-inflammatory groups after review of their medical records. The HIV-1 infected group, were all being investigated as inpatients for AIDS related CNS complications in the HIV/AIDS inpatient unit of the University College London Hospitals. Normal controls were healthy volunteers recruited from laboratory staff and the general population.

2.2. Serum assay

Serum samples were separated as soon as possible, coded, frozen and stored at −20°C. All samples were assayed blind. Serum nitrate and nitrite were measured with a modified nitrate reductase and Griess reaction method (Hevel and Marletta, 1994). The assay is performed in a standard flat-bottomed 96-well polystyrene microtitre plate. Samples are first ultrafiltrated using 10 000 kDa molecular mass filters (Ultrafree-MC*, Millipore). Nitrate is then reduced to nitrite by the action of nitrate reductase (Boehringer Mannheim) in the presence of β-NADPH (Sigma). Excess β-NADPH is then consumed and the nitrite concentration measured by the addition of equal volumes of 1% sulfanilamide in 5% concentrated phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride. The Griess colour reaction forms a pink azo dye with an absorbance wavelength of 540 nm. The assay was standardised using various dilutions of sodium nitrate and nitrite (Sigma), had a lower limit of detection of 2.5 μM, a mean recovery of 95% (range = 86–113%), and an average intra-assay coefficient of variation across the functional range of the assay of 14%.

2.3. Statistics

Normally distributed continuous variables were compared using a one-way analysis of variance, and if not normally distributed by a Kruskal-Wallis one-way analysis of variance. Non-parametric data were compared using the Yates corrected Chi-squared test and, if an expected value was less than 5, the Fisher exact test. A p value of < 0.05 was considered statistically significant.

3. Results

The clinical classifications and diagnoses of the patients are presented in Table 1, and their demographic data and serum nitrate and nitrite levels in Table 2. Significant differences were noted in the sex ratios and ages of the groups (Table 2). Levels of serum nitrate and nitrite were

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</tr>
<tr>
<td></td>
<td>Basal ganglia degeneration</td>
</tr>
<tr>
<td></td>
<td>Cerebellar degeneration</td>
</tr>
<tr>
<td></td>
<td>Generalised dystonia</td>
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<tr>
<td></td>
<td>Supra-nuclear gaze palsy (Steele-Richardson syndrome)</td>
</tr>
<tr>
<td></td>
<td>Motor neuron disease</td>
</tr>
<tr>
<td></td>
<td>Primary generalised epilepsy</td>
</tr>
<tr>
<td></td>
<td>Benign intracranial hypertension</td>
</tr>
<tr>
<td></td>
<td>Normal pressure hydrocephalus</td>
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<td></td>
<td>Mitochondrial myopathy</td>
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<td></td>
<td>Toxic optic neuropathy</td>
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<tr>
<td></td>
<td>Compressive VII nerve palsy</td>
</tr>
<tr>
<td></td>
<td>Inflammatory neurological controls (n = 16)</td>
</tr>
<tr>
<td></td>
<td>Acute inflammatory demyelinating polyneuropathy</td>
</tr>
<tr>
<td></td>
<td>Neuro-sarcoidosis</td>
</tr>
<tr>
<td></td>
<td>Acute meningitis (meningococcal)</td>
</tr>
<tr>
<td></td>
<td>Tuberculous meningitis</td>
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<tr>
<td></td>
<td>Chronic meningitis</td>
</tr>
<tr>
<td></td>
<td>Neuro-Behcet’s disease</td>
</tr>
<tr>
<td></td>
<td>Mononeuritis multiplex</td>
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<tr>
<td></td>
<td>Brainstem encephalitis</td>
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</table>

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Table 2
Demographic data and the serum nitrate and nitrite levels by group

<table>
<thead>
<tr>
<th></th>
<th>Normal controls</th>
<th>Demyelinating disease</th>
<th>AIDS</th>
<th>Non-inflammatory neurological controls</th>
<th>Inflammatory neurological controls</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>22</td>
<td>55</td>
<td>27</td>
<td>14</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>11:11</td>
<td>23:32</td>
<td>24.3*</td>
<td>7.7</td>
<td>5.11</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>33.8 (SD 7.4)</td>
<td>39.7 (SD 9.4)</td>
<td>39.1 (SD 11.6)</td>
<td>47.8 (SD 17.8)*</td>
<td>47.4 (SD 16.5)*</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>Serum nitrate + nitrite (µM)</td>
<td>32.8 (SD 12.2)</td>
<td>65.6 (SD 32.9)*</td>
<td>57.9 (SD 34.9)*</td>
<td>41.1 (SD 12.3)</td>
<td>57.5 (SD 31.3)*</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

* Significant using analysis of variance.

Fig. 1. Combined scatter and box and whisker plots of serum nitrate and nitrite levels in normal controls (O, n = 22), patients with demyelinating disease (□, n = 55), AIDS (▲, n = 27), and other non-inflammatory (●, n = 14), and inflammatory neurological diseases (■, n = 16). The box represents the 25th-75th quartile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived. The horizontal dotted lines represents the upper limit of normal (2 SDs above the mean of the normal control subjects).

Table 3
Demographic and clinical data, and the serum nitrate and nitrite levels by multiple sclerosis sub-group

<table>
<thead>
<tr>
<th></th>
<th>Clinically isolated syndrome compatible with demyelination</th>
<th>Relapsing remitting MS</th>
<th>Secondary progressive MS</th>
<th>Primary progressive MS</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>16</td>
<td>21</td>
<td>10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>8:8</td>
<td>5:16</td>
<td>3:7</td>
<td>7:1*</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.2 (SD 4.1)</td>
<td>40.6 (SD 10.7)</td>
<td>40.6 (SD 8.8)</td>
<td>45.2 (SD 11.7)</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>Mean age of disease onset (years)</td>
<td>35 (SD 4.1)</td>
<td>25 (SD 6.2)</td>
<td>28 (SD 6)</td>
<td>37 (SD 8.8)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Median EDSS (range)</td>
<td>0 (0–3)</td>
<td>3.5 (1.0–6.0)*</td>
<td>7.0 (5.5–8.0)</td>
<td>7.0 (5.5–8.0)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Median serum nitrate + nitrite (µM) (interquartile range)</td>
<td>43.2 (36.5–71.7)</td>
<td>81.6 (51.2–103.6)</td>
<td>54.2 (43.7–84.7)</td>
<td>46.6 (39.6–93.35)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

* Significant using analysis of variance.
Fig. 2. Combined scatter and box and whisker plots of serum nitrate and nitrite in different sub-groups of patients with demyelinating disease; clinically isolated syndrome compatible with demyelination (■ n = 16), relapsing remitting (▲ n = 21), secondary progressive (● n = 10), and primary progressive (♦ n = 8) multiple sclerosis patients. The box represents the 25th–75th percentile divided horizontally by the median, the whiskers the range, and the adjacent scatter plot the individual values from which the box and whiskers are derived. The horizontal dotted lines represent the upper limit of normal (2 SDs above the mean of the normal control subjects).

significantly elevated in patients with demyelinating disease, AIDS and inflammatory neurological diseases compared to normal controls and patients with non-inflammatory neurological disease (p < 0.001, Table 2 and Fig. 1). Serum nitrate and nitrite levels did not differ between the patients with demyelinating disease, AIDS and inflammatory neurological diseases. Levels in patients with non-inflammatory neurological diseases tended to be higher than normal controls but did not reach statistical significance (p = 0.053).

The clinical data of the patients with demyelinating disease is presented in Table 3. There was an unequal sex distribution as the majority of primary progressive patients were male. As expected progressive patients were older with significantly greater disability than the patients with an isolated syndrome or relapsing remitting disease. The mean age of the relapsing patients was unusually high in this study because of the inclusion of 8 patients with a benign course, defined as a disease duration of greater than 10 years with an EDSS score of less than 3.5. No statistical differences in serum levels of nitrate and nitrite were noted between the different sub-groups of patients with demyelinating disease (Table 3 and Fig. 2). After excluding patients with isolated syndromes, no differences were noted between serum levels of nitrate and nitrite in patients with a progressive or relapsing course (63.7 µM (SD 34.1) vs. 74.3 µM (SD 33.7), p = not significant). No correlation was found between serum nitrate and nitrite levels and disability as measured by the EDSS. All 8 patients with a benign course had elevated serum nitrate and nitrite levels, i.e. greater than 2 SDs above the mean of the normal control subjects (median = 93.5 µM (interquartile range = 74.4–102.1).

In neither the normal control subjects or patient groups were differences noted in the serum levels of nitrate and nitrite between male and female subjects and there was no correlation with age (data not shown).

4. Discussion

To our knowledge this is the first study demonstrating raised levels of serum NO metabolites in patients with MS, and other inflammatory neurological diseases, supporting previous findings of raised CSF levels in patients with MS (Johnson et al., 1995) and other neurological conditions (Milstein et al., 1994). NO metabolites were raised in all clinical subtypes of MS, as well as in patients with benign MS and those with a clinically isolated syndrome compatible with a diagnosis of demyelination. As approximately two thirds of this latter group will go on to develop MS within 5 years (Morrissey et al., 1993), finding raised levels of nitrate and nitrite in their serum may indicate the presence of ongoing inflammation due to sub-clinical MS. Although serum nitrate and nitrite levels were unrelated to clinical disability in this study, any meaningful association will need to be assessed with a longitudinal study. Unfortunately, as none of the patients were sampled during a clinical exacerbation and MRI data is currently unavailable, the correlation between serum NO metabolites and disease activity could not be assessed. Interestingly, the raised levels of NO metabolites in patients with demyelinating disease were similar to
those found in patients with AIDS and neurological complications as a result of HIV-1 infection, implying a similar degree of iNOS activation in these diseases. Possible confounding factors in this study relate to differences in the age and sex ratios of the patient groups, as a result of the neurological controls being older and the striking male predominance in the patients with AIDS. We do not believe that these differences affected the study outcome as we found no evidence of a confounding influence for either age or sex on serum nitrate and nitrite levels in both the normal control or patient groups.

NO and its derivative peroxynitrite have been strongly implicated as non-specific inflammatory mediators of tissue damage causing cell death by lipid peroxidation (Beckman et al., 1990), depletion of intracellular energy stores due to inhibition of the mitochondrial electron transport chain (Bolaños et al., 1995), consumption of intracellular antioxidants (Barker et al., 1996), and damage to DNA (Inoue and Kawanishi, 1995). They are able to induce both neuronal (Boje et al., 1992; Bolaños et al., 1995) and oligodendroglial (Merrill et al., 1993; Mackenzie-Graham et al., 1994; Mitrovic et al., 1994) cell death in vitro, and have been strongly implicated in the pathogenesis of central and peripheral nervous system demyelination in the animal models of experimental allergic encephalomyelitis and neuritis (Cross et al., 1994; Zielasek et al., 1995). Increased iNOS activity (Bö et al., 1994) and iNOS mRNA (Bagasra et al., 1995) in lesions from patients dying with MS provides further support for a role of NO in the pathogenesis of MS. This study provides yet further evidence that NO is involved in the immunopathogenesis of MS.

As iNOS is upregulated by the pro-inflammatory cytokines IFNγ, TNFα and IL1 (Moncada and Higgs, 1993), all of which have been implicated in the immunopathogenesis of MS (Hartung et al., 1995). The measurement of serum nitrate and nitrite may be a suitable candidate as an inflammatory marker, and possibly as a surrogate marker, of disease activity in MS.

In HIV-1 infection levels of serum nitrate and nitrite correlate with disease stage, the CD4+ T-lymphocyte count, serum neopterin, and levels of soluble tumour necrosis factor alpha (TNFα) receptors (Zangerle et al., 1995). This suggests that levels of serum NO metabolites are useful as an inflammatory, as well as a surrogate, marker of disease activity in HIV-1 infection.

References


Cerebrospinal fluid and serum nitric oxide metabolites in patients with multiple sclerosis

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Departments of Clinical Neurology and Neurochemistry, Institute of Neurology, Queen Square, London WC1N 3BG, UK

Nitric oxide is hypothesised to play a role in the immunopathogenesis of multiple sclerosis. Raised cerebrospinal fluid and serum levels of the nitric oxide metabolites nitrate and nitrite have been described in patients with multiple sclerosis. Cerebrospinal fluid and serum nitrate and nitrite were measured in patients with multiple sclerosis, inflammatory and non-inflammatory neurological diseases, and correlated with the albumin quotient, an index of blood–brain–barrier dysfunction. Patients undergoing diagnostic lumbar and venepuncture were prospectively recruited, clinical data were obtained from the hospital records, and the CSF and serum nitrate and nitrite levels were measured by the nitrate reductase and Griess reaction methods. Nitrate and nitrite, were raised in the CSF and serum of patients with multiple sclerosis and other inflammatory neurological diseases compared to patients with non-inflammatory neurological disorders (median nitrate and nitrite: cerebrospinal fluid = 10.3 μM vs 15.4 μM vs 6.6 μM, P < 0.001, and serum = 49.0 μM vs 46.4 μM vs 38.8 μM, P = 0.02, respectively). CSF nitrate and nitrite levels correlated with the albumin quotient (n=59, r=0.42, P < 0.001). This study provides further evidence for a role of nitric oxide in the immunopathogenesis of diseases compared to patients with non-inflammatory neurological disorders.

Keywords: nitric oxide; multiple sclerosis; blood–brain–barrier dysfunction

Introduction

In inflammatory disorders of the central nervous system non-specific mediators are believed to be responsible for blood–brain–barrier damage and the demyelination and axonal loss that produces neurological disability. One of these mediators nitric oxide (•NO), is produced from L-arginine by the inducible form of nitric oxide synthase (iNOS).1 Animal models of intrathecal inflammation have demonstrated that blood–brain–barrier damage correlates with intrathecal •NO production2 and that iNOS inhibitors are able to prevent blood–brain–barrier damage.3 In cell culture, •NO and its reactive derivative peroxynitrite4 are toxic to oligodendrocytes4–7 and neurons.8 •NO induces cytotoxicity by several mechanisms, which include lipid peroxidation,9 inhibition of the mitochondrial electron transport chain,9 consumption of intracellular anti-oxidants,9 DNA damage,10 and inhibition of several key cellular enzymes like aconitase,11 glyceroldehyde-3-phosphate dehydrogenase12 and ribonucleotide reductase.13 Recent studies have demonstrated that iNOS induction in human foetal astrocytes and microglia, unlike rodent cells, is IL1-dependent14 and is augmented by the other pro-inflammatory cytokines tumour necrosis factor alpha (TNFs) and interferon-gamma (IFN-γ). Pathological studies in patients with MS, although conflicting, have demonstrated restricted iNOS expression and activity within the CNS that seems limited to macrophages/microglia15 and/or astrocytes.16 CSF and serum levels of nitrate and nitrite, the stable breakdown products of nitric oxide, are raised in patients with MS. We present further data on raised CSF and serum nitrate and nitrite levels in patients with MS and describe a correlation between CSF nitrate and nitrite levels and the CSF albumin quotient.

Methods

Nineteen patients with clinically definite MS,17 20 patients with inflammatory and 20 patients with non-inflammatory neurological disease were included in the study. All patients were recruited from inpatients undergoing diagnostic lumbar and venepuncture as part of their clinical investigations. CSF and serum samples were spun down, separated, coded, and stored at −20 C. After ultrafiltration CSF and serum nitrate and nitrite levels were measured blind, using the nitrate reductase and Griess reaction method.18 The assay's lower limit of detection is 2.5 μM, and has an average recovery of 95% (range=86–113%), and a mean intra-assay coefficient of variation of 14%. CSF and serum albumin were measured using a standard immunoelectrodiffusion technique.19 Continuous variables were first normalised using a logarithmic transformation, and then compared using a one-way analysis of variance. Non-parametric data was compared using the Yates corrected Chi-squared test. Simple linear regression model was used for the blood–brain–barrier correlations. Any P value less than 0.05 was considered statistically significant.

Results

Eighteen patients with MS had relapsing remitting disease and one patient a primary progressive course.20 The diagnoses of the patients with inflammatory and non-inflammatory neurological disease are presented in Table 1. Demographic data, and the CSF and serum

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results are presented in Table 2. The patients were of similar age but a difference was noted in the sex ratios, with significantly more patients in the inflammatory controls being male compared to the MS and non-inflammatory groups (P<0.001, Table 2). CSF and serum nitrate and nitrite, were significantly elevated in patients with MS and inflammatory neurological diseases compared to patients with non-inflammatory neurological diseases (P<0.001 and P=0.02 respectively, Table 2, Figure 1a and b). The CSF:serum nitrate and nitrite quotient was significantly elevated in the inflammatory control patients compared to the patients with MS and non-inflammatory neurological diseases (P<0.001, Table 2 and Figure 1c). This ratio was not elevated in patients with MS compared to patients with non-inflammatory neurological disease (P=n.s., Table 2 and Figure 1c). A linear correlation was found between the CSF nitrate and nitrite and the albumin quotient (n=59, r=0.42, and P<0.001; Figure 2a). A similar but weaker correlation was found between the CSF:serum nitrate and nitrite quotient and the albumin quotient (n=59, r=0.36 and P=0.006; Figure 2b).

Discussion

These data confirm previous studies which have demonstrated elevated levels of CSF" and serum •NO" metabolites in patients with MS, and provides further evidence for •NO in the immunopathogenesis of MS. An interesting finding was that patients with inflammatory neurological disorders had significantly higher CSF:serum nitrate and nitrite quotients than patients with MS and non-inflammatory neurological controls. Nitrate (NO$_3^-$) and nitrite (NO$_2^-$) are small molecules that exist as charged anions, which impedes their movement across the blood–brain–barrier creating a relative gradient and a quotient by which to assess intrathecal •NO production. The raised quotient in patients with inflammatory neurological disorders suggests that a greater quantity of intrathecal •NO is produced in the patients with inflammatory neurological diseases, which were mainly due to infective causes, than in MS. This is consistent with greater induction of iNOS in patients with infection and is supported by the observation of higher CSF nitrate and nitrite levels in patients with bacterial meningitis." A correlation between the absolute CSF nitrate and nitrite level, and intrathecal nitrate and nitrite synthesis, and the albumin quotient as an index of blood–brain–barrier dysfunction was found (Figure 2a and b). Whether this association between intrathecal •NO production and blood–brain–barrier breakdown is cause or effect, cannot be determined from these data. •NO may mediate some breakdown of the barrier, or the blood–brain–barrier breakdown itself causes the increase in the CSF levels of nitrate and nitrite. In support of the former, an in vitro study has shown that •NO results in perturbation of the blood–brain–barrier with opening of the endothelial tight junctions" and animal studies using intrathecal inoculation of live or inactivated bacteria, and lipopolysaccharide show a correlation between CSF nitrite levels and blood–brain–barrier breakdown.126

### Table 1 Control patients

| Inflammatory controls (n=20) | Bacterial Meningitis$^5$ | Cryptococcal Meningitis$^*$$^*$ | HSV Encephalitis$^6$ | Cerebral Toxoplasmosis$^7$ | Chronic inflammatory demyelinating polyneuropathy$^8$ | Guillain-Barré syndrome$^9$ | Neurosarcoidosis$^*$ | Mononeuritis multiplex$^*$ | Non-inflammatory controls (n=20) | Dementia of the Alzheimer's type$^*$ | Epilepsy$^*$ | Idiopathic dystonia$^*$ | Mitochondrial cytopathy$^*$ | Cerebellar degeneration$^*$ | Myopathy$^*$ | Benign intracranial hypertension$^*$ | Tension headache$^*$ | Conversion disorder$^*$ | Idiopathic Parkinson's disease$^*$ | Palilidoparalympidal degeneration$^*$ | Post-herpetic neuralgia$^*$ | Pallidopyramidal degeneration$^*$ | Tension headache$^*$ | Myopathy$^*$ | Benign intracranial hypertension$^*$ | Tension headache$^*$ | Conversion disorder$^*$ | Idiopathic Parkinson's disease$^*$ | Palilidoparalympidal degeneration$^*$ | Post-herpetic neuralgia$^*$ | Pallidopyramidal degeneration$^*$ | Tension headache$^*$ | Myopathy$^*$ | Benign intracranial hypertension$^*$ | Tension headache$^*$ | Conversion disorder$^*$ | Idiopathic Parkinson's disease$^*$ | Palilidoparalympidal degeneration$^*$ | Post-herpetic neuralgia$^*$ | Pallidopyramidal degeneration$^*$ |
|-----------------------------|--------------------------|-----------------------------|---------------------|---------------------------|---------------------------------|--------------------------|--------------------------|--------------------------|-----------------------------|---------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|

* AID$^*$ related

### Table 2 Patient's demographic details and CSF and serum nitrate and nitrite data

<table>
<thead>
<tr>
<th>Multiple sclerosis (n=19)</th>
<th>Inflammatory controls (n=20)</th>
<th>Non-inflammatory controls (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years) (s.d.)</td>
<td>44.7 (10.4)</td>
<td>41.1 (12.1)</td>
<td>38.4 (13.2)</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>9:10</td>
<td>18:2</td>
<td>10:10</td>
</tr>
<tr>
<td>Median CSF nitrate and nitrite (µM) (interquartile range)</td>
<td>10.3 (8.0–12.4)</td>
<td>15.4 (12.2–18.5)</td>
<td>6.6 (5.8–8.1)</td>
</tr>
<tr>
<td>Median CSF: Serum nitrate and nitrite quotient (interquartile range)</td>
<td>49.0 (44.0–74.7)</td>
<td>46.4 (27.5–87.3)</td>
<td>38.8 (28.2–42.7)</td>
</tr>
<tr>
<td>Median serum nitrate and nitrite (µM) (interquartile range)</td>
<td>0.180</td>
<td>0.302</td>
<td>0.185</td>
</tr>
<tr>
<td>Median CSF: Serum nitrate and nitrite quotient (interquartile range)</td>
<td>0.180</td>
<td>0.302</td>
<td>0.185</td>
</tr>
<tr>
<td>Median CSF: Serum albumin quotient (interquartile range)</td>
<td>0.0063</td>
<td>0.0057</td>
<td>0.0032</td>
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</table>

* $^*$AIDS related
Infusion of the iNOS inhibitor aminoguanidine attenuates blood–brain–barrier disruption in LPS induced experimental meningitis. In addition increased levels of TNFa, which augments and possibly induces eNO production, are associated with blood-brain-barrier dysfunction in patients with CNS inflammation.

Figure 1  Combined scatter and box and whisker plots of nitrate and nitrite levels in (a) CSF and (b) serum, as well as (c) CSF-serum nitrate and nitrite quotients in patients with multiple sclerosis (●, n=19), inflammatory neurological diseases (■, n=20) and non-inflammatory neurological diseases (▲, n=20). The box represents the 25th–75th quartile divided horizontally by the median, the whiskers, the range, and the adjacent scatter plots the individual values from which the box and whiskers are derived. *** P is significant.

Figure 2  Linear regression of the natural logarithm of the albumin quotient versus (a) the natural logarithms of the CSF nitrate and nitrite and (b) the CSF-serum nitrate and nitrite quotients in patients with MS (●, n=19). inflammatory neurological disease (■, n=20) and non-inflammatory neurological disease (▲, n=20).
a central nervous system, and supports a role for •NO as a possible mediator blood–brain–barrier dysfunction in CNS inflammation.

References

4 Beckman JS et al. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 97: 1620 – 1624.
Adaptation of the nitrate reductase and Griess reaction methods for the measurement of serum nitrate plus nitrite levels

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SUMMARY. Nitrite and nitrate determinations in biological fluids are increasingly being used as markers of nitric oxide production. We have modified a nitrate reductase and Griess reaction method for the measurement of serum nitrate and nitrite in ultrafiltrated samples using a microtitre plate. The recoveries of nitrate and nitrite were 95% (range = 86–113%) and 100% (range = 92–109%), respectively. The intra and inter assay coefficients of variation for nitrate plus nitrite in the concentration range 40–50 μM were 9.1% and 7.8%, and in the concentration range of 2.5–10 μM 23.4% and 25.5%, respectively. At its lower limit the assay is able to detect 125 pmol of nitrate plus nitrite in 50 μL of sample (2.5 μmol/L). A mean serum nitrate plus nitrite level of 32.8 μmol/L (SD 12.3) was measured in 24 healthy adult volunteers (12 men and 12 women), no age or sex differences were noted.

Additional key phrases: serum nitrogen oxides; nitric oxide

The majority of serum nitrogen oxides (nitrite and nitrate) are end-products of the endogenous synthesis of nitric oxide from the guanidino nitrogen of L-arginine. Increased production of nitric oxide and its metabolites nitrate and nitrite is associated with infections in human beings due to the induction of inducible nitric oxide synthase (iNOS). Nitric oxide plays an important role in non-specific immunity, has cytotoxic effects and hypothetically can cause significant tissue damage in several inflammatory and autoimmune diseases. Measuring levels of nitric oxide metabolites may be useful as an inflammatory marker, as well as providing information on the pathogenesis of inflammatory diseases, such as multiple sclerosis1 and HIV infection.2 We attempted to measure serum nitrogen oxides using the nitrate reductase and Griess reaction methods of Hevel and Marietta,3 but found their standard protocol for the assay, including a commercially available kit based on their methods, unsuitable for use in serum samples. We report on modifications to this assay which allow the reliable measurement of nitrate plus nitrite in serum and other biological fluids with a high protein concentration.

MATERIALS AND METHODS

Principles
The Griess reaction relies on a simple colorimetric reaction between nitrite, sulphanilamide and N-(l-naphthyl) ethylenediamine to produce a pink/magenta azo product with a maximum absorbance at 543 nm. Prior to the Griess reaction all nitrate is converted to nitrite using the bacterial enzyme nitrate reductase. The principles and steps of this assay are highlighted in Fig. 1.

Reagents
Nitrate and nitrite calibration standards (concentration range = 0–50 μmol/L) were prepared by diluting sodium nitrate (Sigma) and sodium nitrite (Sigma) in reagent grade water and stored at −20°C. Twenty units of nitrate reductase (Boehringer Mannheim, Lewes, UK) and 100 units of L-glutamic dehydrogenase (Sigma, Poole, UK) were reconstituted in 100 μL sterile water aliquoted and stored at −70°C. In our hands these enzymes have remained stable for 6

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Step 1 = Simple dilution
Serum diluted in phosphate buffered saline

Step 2 = Ultrafiltration
Ultrafiltration of diluted serum

Step 3 = Nitrate reductase step
\[
\text{NO}_3^- \rightarrow \text{NO}_2^- + \text{NADPH}
\]

Step 4 = NADPH consumption
\[
\text{a-Ketoglutarate} + \text{L-glutamic dehydrogenase} \rightarrow \text{L-Glutamate}
\]

Step 5 = Griess 5
\[
\text{NO}_2^- + \text{NH}_4^+ \rightarrow \text{Azo product} (\lambda_{\text{max}}=543\text{nm})
\]

Step 6 = Griess 2

Months. \(\beta\)-NADPH (Sigma) and \(\alpha\)-ketoglutaric acid (Sigma) 100 \(\mu\)mol/L and 12 mmol/L, respectively, were prepared in PBS (see below) for each assay. Prepared aliquots of 1·5 mol/L \(\text{NH}_4\text{Cl}\) (Sigma) in water were stored at \(-20^\circ\text{C}\). Griess reagent 1, 1% sulphanilamide (Sigma) in 5% concentrated phosphoric acid, and Griess reagent 2, 0·1% N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma) in water are stable for several months at 4°C in light proof containers. All sample dilutions and reagents were mixed in freshly prepared phosphate buffered saline (PBS) containing \(\text{NaCl} 8·0\text{g}, \text{KCl} 0·2\text{g}, \text{Na}_2\text{HPO}_4 1·15\text{g}\) and \(\text{KH}_2\text{PO}_4 0·20\text{g}/\text{L}\). Reagent grade water was used to avoid contamination from nitrogen oxides and was made using Milli-RO and Milli Q systems (Millipore, Watford, UK).

Sample preparation
Serum was diluted one in four with PBS, and 200-400 \(\mu\)L was ultra-filtered by centrifugation at 11 500 \(\times\) g for 30 min, using 10 000 kD molecular weight filters (Ultrafree-MC\(^\text{TM}\), Millipore).

If the filtrate was not clear and colourless, due to defects in the filters, the sample was re-filtered. Attempts at serum filtration without prior dilution were unsuccessful, due to the high protein concentration blocking the filters.

Assay
The assay was performed in a standard flat-bottomed 96-well polystyrene microtitre plate, containing 50 \(\mu\)L well of standard or sample. The assay was blanked against PBS. Fifty microlitres of nitrate reductase and \(\beta\)-NADPH were added to each well giving final concentrations of 300 U/L and 25 \(\mu\)mol/L, respectively. The plate was incubated at room temperature for 3 h. Excess \(\beta\)-NADPH was consumed by addition of 50 \(\mu\)L of PBS containing L-glutamic dehydrogenase, \(\alpha\)-ketoglutaric acid, and \(\text{NH}_4\text{Cl}\), (final concentrations 500 U/L, 4 mmol/L and 100 mmol/L, respectively) followed by a 10 min incubation at 37°C. The nitrite concentration

![Figure 2. Parallelism between calibrant (▲), ultrafiltrated (○) and untreated serum (■).](image)

![Figure 3. The effect on recovery of adding different concentrations of bovine serum albumin (BSA) to samples of phosphate buffered saline (PBS) containing either a fixed quantity of nitrite (≈25 \(\mu\)M) or no nitrite (0 \(\mu\)M). The points represent the mean and SEM.](image)
Serum nitrate plus nitrite

Figure 4. The effect of different diluents on the recovery of nitrite using the Griess reaction. □ = water, ▽ = 0.22 M ZnSO₄ in water, △ = phosphate buffered saline, ● = 0.22 M ZnSO₄ in phosphate buffered saline, ○ = 12% perchloric acid.

was then measured by the addition of 50 μL each of Griess reagents 1 and 2, and the absorbance read at 540 nm using a plate reader after a 10 min incubation at room temperature (Fig. 2).

Statistics
Where appropriate data were compared using a paired or unpaired Student's t-test, a P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Protein/serum effects
Doubling dilution of serum samples spiked with sodium nitrite were not parallel to the calibration curve (Fig. 2). The median nitrate plus nitrite concentration in 10 unfiltered serum samples diluted one in four with PBS with 65.5 μM (interquartile range = 60.7–80.1) compared with 26.8 μM (interquartile range = 23.0–34.9) in paired ultrafiltered samples (P = 0.03, paired t-test). This increase was highly variable and could not be corrected for by blanking against untreated samples (data not shown). These effects were variable and due presumably to serum components, which absorb light at 540 nm and/or interfere with the Griess reaction, and were removed by ultrafiltration.

In a separate experiment (Fig. 3), the addition of variable concentrations of bovine serum albumin (BSA) to samples of PBS containing either no nitrite or a fixed quantity of nitrite (25 μmol/L) significantly increases the recovery of nitrite at all concentrations of BSA (P < 0.006, two-tailed paired t-tests, n = 3). A significant effect was seen at all concentrations of BSA greater than 100 mg/L, but could be removed by ultrafiltration (data not shown).

Figure 5. The effect of the four salt constituents of phosphate buffered saline in the presence of ZnSO₄ on the recovery of nitrite using the Griess reaction. (a) ■ = NaCl, (b) ● = KCl, (c) △ = Na₂HPO₄, and (d) ♦ = KH₂PO₄. The points represent the mean and the SEM.

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Serum nitrate plus nitrite

The nitrate plus nitrite assay performed less well than that for the nitrite assay or Griess reaction only. This probably relates to the variability of the nitrate reductase step, and is much less of a problem in samples with elevated levels of nitrate plus nitrite.

Parallelism

Parallelism was assessed by assaying doubling dilutions of nitrate and nitrite calibration standards, and ultrafiltered and non-ultrafiltrated serum samples. The resulting optical densities obtained were normalized by expressing the absorbance obtained for each dilution as a percentage of that given by the highest value for that series. The results are shown in Fig. 2.

Sensitivity

As set out above the range of this assay is 2.5–50 µmol/L, representing 125 pmoles of nitrate plus nitrite in 50 µL of sample.

Normal levels

The mean serum nitrate plus nitrite levels measured in 24 healthy adult volunteers was 32.8 µmol/L (SD 12.3). Mean values in men [39.0 µM (SD 11.6)] and women [33.1 µM (SD 11.6)] were not significantly different, and no obvious age related changes were noted between 25 and 54 years. However, this will require confirmation with a larger number of subjects. Prior to the nitrate reductase step a significant number of normal subjects had undetectable levels of serum nitrite. This was probably a dilutional effect due to the ultrafiltration step.

Plasma and other biological fluids

Plasma was treated in an identical fashion to serum. In the 24 normal control subjects the serum levels of nitrate and nitrite were two to three times lower than paired plasma samples [32.8 µmol/L (SD 12.3) versus 81.0 µmol/L (SD 34.0) P < 0.001, two-tailed paired t-test]. The lower serum levels may result from protein nitrosylation during the process of coagulation. This assay is also suitable for measuring nitrate and nitrite concentrations in cerebrospinal fluid, urine and cell culture supernatants. Cerebrospinal fluid, because of relatively low levels of nitrates and nitrates, should not be diluted.4 Urine on the other hand, with high nitrate concentrations, should typically be diluted between 1/50 and 1/100. Cell culture supernatants must not contain high concentrations of phenol red, commonly used as a pH indicator in cell culture media, since this absorbs light at 540 nm.5 Supernatants with high concentrations of protein (>100 mg/L), should undergo ultrafiltration as above.

Conclusions

Nitrite and nitrate determinations in biological fluids are increasingly being used as markers for the activity of the nitric oxide synthases. We have adapted the methods of Hevel and Marletta6 for the determination of nitrates and nitrites using a microtitre plate. We identified several problems using the assay on serum samples. First, high concentrations of protein have an unpredictable and erroneous effect on the Griess reaction, which can be overcome by deproteinizing samples. Standard laboratory techniques for deproteinization using perchloric acid or zinc sulphate were found to be unsuitable, and therefore ultrafiltration was used. This effect of protein on the Griess reaction has also been noted by others.6 Interestingly, these investigators used ZnSO4 to deproteinize their plasma samples, but would have been unlikely to observe the interaction between Cl−, ZnSO4 and the Griess reaction as their standards were diluted in distilled water. Secondly, in contrast to the 30 min in the original methods,7 a longer incubation period of 3 h is required for the complete conversion of nitrate to nitrite. Using urine samples for nitrite determination alone, the Griess reaction performs well with low intra and inter coefficients of variation. However, with serum samples, with the addition of ultrafiltration and the nitrate reductase step, the assay performs less well. Reasons for this may relate to interactions of nitrate with protein, and the addition of the biological variability of the nitrate reductase enzymatic reaction. Previously published methods for this assay,8,9 including a commercial kit (Cayman Chemical, catalogue No. 780001), have omitted detailed performance data making comparisons difficult. Moshage et al.5 report an interassay coefficient of variation of less than 8%, but omit the intra-assay coefficient of variation. Gutman and Hollywood, using cadmium to convert nitrate to nitrite, report results not too dissimilar to ours with intra- and inter-assay coefficients of variation of 14% and 19%, respectively.8 In our hands the assay performs

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Daily urinary neopterin excretion as an immunological marker of disease activity in multiple sclerosis


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Summary
The aim of this study was to assess neopterin, a marker of interferon gamma (IFN-γ) induced macrophage activity, as a possible surrogate marker of inflammation in patients with multiple sclerosis. Urinary neopterin to creatinine ratios (UNCRs) were measured daily in 10 primary progressive (PP), 10 relapsing remitting (RR) and 11 secondary progressive (SP) patients with multiple sclerosis, and 14 normal control (NC) subjects, for periods of up to 12 weeks. After excluding measurements related to infection, the median of the individuals' average UNCRs was significantly higher in patients than in controls (P < 0.001 for all patients and P < 0.01 for each of the three groups of patients); the median UNCRs (and interquartile ranges) were 187 (135–231), 187 (165–277), 218 (164–517) and 134 (97–152) μmol/mol for PP, RR, SP patients and controls, respectively. Similarly, patients had a greater median proportion of days with a UNCR above normal (P < 0.001 for all patients and P < 0.01 for each group); the median percentages (and interquartile ranges) were 16 (6–62), 28 (21–36), 49 (14–86) and 0 (0–6)% for PP, RR, SP patients and controls, respectively. Nine relapses occurred in nine patients during the study, and all were associated with increased neopterin excretion, which tended to be greater than that on days not associated with a relapse. Three of the nine relapses were preceded by an upper respiratory tract infection. In eight out of 13 patients who had infections during the study, increased neopterin excretion was noted for periods of up to 6 weeks post-infection, significantly longer than that which occurred after infections in controls. This confirms infection as a potent inducer of symptomatic and asymptomatic disease activity in multiple sclerosis, and provides further support of a pivotal role for IFN-γ in the pathogenesis of multiple sclerosis. Urinary neopterin excretion is increased in patients with both progressive and relapsing multiple sclerosis, and therefore has potential as a surrogate marker of the inflammatory component of multiple sclerosis disease activity.

Keywords: neopterin; multiple sclerosis; interferon gamma

Abbreviations: EDSS = expanded disability status scale; IFN-γ = interferon gamma; PP = primary progressive; RR = relapsing remitting; SP = secondary progressive; TNF-α = tumour necrosis factor alpha; UNCR = urinary neopterin to creatinine ratio

Introduction
Multiple sclerosis is an inflammatory demyelinating disease of the CNS, characterized pathologically by an inflammatory infiltrate predominantly of macrophages, T and B lymphocytes, plasma cells and variable degrees of axonal loss and gliosis (Allen, 1991). The macrophages are not only phagocytic, clearing up myelin debris, but are also thought to act as antigen-presenting cells, sources of proinflammatory cytokines and producers of several potential myelinotoxic factors. Their activity is potently enhanced by the presence of gamma interferon (IFN-γ), a major T cell pro-inflammatory cytokine known to induce relapses in patients with multiple sclerosis (Panitch et al., 1987). Neopterin, a product of
IFN-γ activated macrophages, is an indirect measure of both the levels of IFN-γ and IFN-γ induced macrophage activity (Huber et al., 1984). Neopterin's production is also augmented by tumour necrosis factor alpha (TNF-α) (Werner-Felmayer et al., 1990). Neopterin is produced predominantly by cells of the monocyte lineage due to a functional block in the pathway synthesizing tetrahydrobiopterin, an essential co-factor for the inducible form of nitric oxide synthase (Kwon et al., 1989; Tayeh and Marletta, 1989). Neopterin’s role as a surrogate marker of inflammation has been well studied in infections, particularly HIV, malignancies, autoimmunity, especially rheumatoid arthritis and transplantation (Fuchs et al., 1992). Elevated levels of neopterin have been found in the CSF (Fierz et al., 1987; Fredrickson et al., 1987; Ott et al., 1993; Shaw et al., 1995) and serum (Ott et al., 1993) of patients with multiple sclerosis. To date, there are no reports of studies on its longitudinal role as a potential surrogate marker of disease activity in multiple sclerosis. The need for a reliable and practical surrogate marker of disease activity in multiple sclerosis cannot be over-emphasized, as the majority of disease activity in multiple sclerosis is asymptomatic (Miller et al., 1988; Koopmans et al., 1989; Willoughby et al., 1989; Capra et al., 1992). It could be argued that MRI is an excellent surrogate marker of inflammation in relapsing patients, but not in patients with progressive multiple sclerosis who do not have clinical relapses, and have little, if any, evidence of active inflammation on MRI (Thompson et al., 1991). Neopterin also provides an indication of the role of IFN-γ, a notoriously difficult cytokine to measure in vivo, in the immunopathogenesis of multiple sclerosis.

Neopterin, which is relatively stable, is excreted in the urine; dividing its concentration by that of creatinine to correct for any dilutional effects allows one to assess its production and excretion accurately (Fuchs et al., 1992). We therefore performed a study to assess the potential use of serial urinary neopterin to creatinine ratios (UNCRs) as an immunological marker of disease activity in multiple sclerosis, and to delineate the role of IFN-γ in patients with multiple sclerosis further. Patients with relapsing as well as progressive multiple sclerosis were studied and compared with a group of healthy normal control subjects.

**Methods**

**Subjects**

Fourteen normal control subjects and 31 patients with clinically definite multiple sclerosis (Poser et al., 1983) were asked to collect consecutive daily urine specimens for periods from 2 to 12 weeks in duration. An earlier pilot study, comparing results from 24 h urine collections with those of spot urine specimens, demonstrated that 24 h collections were too inconvenient and unreliable for such intensive monitoring. Normal control subjects were recruited as volunteers from laboratory staff and the general population, with the aim of obtaining equal numbers of male and female subjects of similar age to the patients with multiple sclerosis. They were only included if they had no underlying medical problems. All patients were recruited from the National Hospital for Neurology and Neurosurgery, London. The study was approved by the hospital’s Ethics Committee and written informed consent was obtained from all participants. Subjects were instructed to collect daily specimen urine of which 1–2 ml were placed in small polypropylene tubes, labelled and immediately home frozen in a light-proof container. A criterion for inclusion in this study was the availability of a home freezer. As the urinary excretion of neopterin shows a diurnal variation, with an ~50% fluctuation in baseline levels (Auzéby et al., 1988), we instructed subjects to collect the first specimen passed each day. This period includes the time of peak excretion from 02.00 to 06.00 (Auzéby et al., 1988), and eliminates any confounding effects of diurnal fluctuations. All specimens from individual subjects were brought to the laboratory at the end of the study period. In addition to collecting urine, all subjects had to keep a detailed diary throughout the study period. The diary used a fixed format documenting any infections, intercurrent medical illnesses, use of corticosteroids or immunosuppressive agents, and comments on changes in neurological function. At the end of the study each patient underwent a structured interview to discuss the significance of any entries in the diary; they also had a neurological examination so that physical disability could be rated according to Kurtzke’s functional systems and expanded disability status scale (EDSS) (Kurtzke, 1983). Finally, on the basis of disease course, patients were classified into one of three groups, as either primary progressive (PP), relapsing remitting (RR) or secondary progressive (SP). The diagnostic criteria of these clinical subtypes have been described previously (Thompson et al., 1991). For the purpose of this study a clinical relapse was defined as the occurrence of a symptom or symptoms of neurological dysfunction, with or without objective confirmation, lasting >24 h (Poser et al., 1983). Remission was defined as a definite improvement of signs, symptoms, or both, present for periods of ≥ 24 h which occurred regularly for ≥ 1 month (Poser et al., 1983). The presence of excess urinary nitrates was used as a screen to test for bacterial colonization of the urinary tract.

**Neopterin and creatinine assays**

Urine neopterin and creatinine were measured by HPLC (high pressure liquid chromatography) (Niederweiser et al., 1982), using a reverse-phase Anasil 80, 5 μm ODS column (Anachem). Elution was performed using degassed 15 μmol/l potassium phosphate buffer, pH 6.4, at a flow rate of 1 ml/min. Neopterin detection was by native fluorescence using an excitation wavelength of 353 nm and an emission wavelength of 438 nm. Creatinine was measured using UV absorption at 235 nm. The fluorescence and UV detector were linked serially to allow the determination of neopterin and creatinine on the same chromatographic run. Urine samples...
were prepared by diluting them 1:10 with the 15 mmol/l potassium phosphate buffer, pH 6.4, containing 5.4 mmol/l EDTA (ethylenediaminetetra-acetic acid) to dissolve any urinary sediments. A fixed volume sample loop of 20 μl was used. The within-batch, and batch-to-batch, coefficients of variation for the UNCR were 3.9% and 7.6%, respectively, using this method. Experiments done in triplicate, on three urine specimens protected from exposure to light showed that the UNCR was stable for 48 h at room temperature, for >72 h at 4°C, and for >4 months at –20°C. Exposure of these urine specimens to ambient light resulted in a progressive fall in the UNCR which became significant after 2 h. Experiments to study the effect of multiple freeze-thaw cycles on urinary neopterin levels, demonstrated no significant effect on the UNCR with up to four freeze-thaw cycles.

Data manipulation and statistics
Due to a smaller muscle mass and lower creatinine excretion, females have on average a 20% higher UNCR than males, therefore all UNCRs from female subjects were divided by a factor of 1.2 to correct for this, thereby allowing direct comparisons between male and female subjects. This correction factor was obtained empirically, from data collected on 40 control subjects (data not shown) and corresponds exactly to that published in the literature (Fuchs et al., 1992).

In normal subjects, we found that infections can cause an increase in neopterin production that may precede the onset of symptoms by 24-48 h, therefore all measurements on days of documented infection including the 2 days prior to the symptomatic phase of the infection were excluded from the analysis. Due to the nature of this study, infective episodes were not documented objectively, and subjects were given the benefit of the doubt with regard to their own assessment and interpretation of infection. The UNCRs were then processed to generate several variables for each subject. First, the mean of all their UNCRs was used as an overall indicator of neopterin production. Secondly, the variance of the individual’s UNCRs was used as an indicator of intra-

individual variability in neopterin excretion, as this would uncover fluctuations in day-to-day neopterin production that would not necessarily be associated with well defined peaks in the serial levels or with an elevation in the mean above normal. Thirdly, all peaks in the UNCR versus time curve were identified and counted. Their identification was achieved by taking the second derivative of the UNCR time-course curve; this derivation was checked visually and if any peaks were missed by the algorithm, they were manually flagged. The duration of a peak had to be >2 days, and its maximum height >50% greater than that of the preceding baseline, for it to be considered significant. A baseline was defined as a period of >3 days that was not associated with a large change in the slope, during which all UNCR values were within 50% of each other. All peaks identified were checked with a mathematical algorithm to ensure that they complied with the above definition. Other variables analysed were related to the specific pattern of elevated neopterin excretion, and included the proportion of days on which the UNCRs were above normal or whether these elevated levels were involved in definite peaks or merely as part of an elevated baseline.

Data involving serial measurements were computed as subject means using all readings available for that particular subject, after the exclusion of those measurements related to infection. The data tended not to be normally distributed in its native form and in many cases required a log transformation to achieve this aim. If normally distributed before or after transformation it was compared using a one-way analysis of variance and, if not, the Kruskal–Wallis one-way analysis of variance was used. Between-group comparisons were carried out using the Student’s t-test, and non-parametric data was compared using the Yates corrected χ² test, and if an expected value was <5 the Fisher exact test was used. A P-value of <0.05 was considered statistically significant.

Results
Clinical features
The clinical data are presented in Table 1. Ten patients had PP multiple sclerosis, 10 had RR disease and 11 SP disease.

Table 1 Clinical data

<table>
<thead>
<tr>
<th></th>
<th>Normal controls</th>
<th>Patients</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>PP group</td>
<td>RR group</td>
<td>SP group</td>
</tr>
<tr>
<td>Subjects (n)</td>
<td>14</td>
<td>10</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Sex ratio (M:F)</td>
<td>7:7</td>
<td>8.2</td>
<td>2.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.1 ± 9.4</td>
<td>47.5 ± 9.5</td>
<td>31.8 ± 8.0</td>
<td>37.7 ± 6.4</td>
</tr>
<tr>
<td>Age at disease onset (years)</td>
<td>37.5 ± 10.2</td>
<td>27.0 ± 6.9</td>
<td>26.4 ± 6.7</td>
<td>0.006</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>9.9 ± 4.0</td>
<td>4.7 ± 2.9</td>
<td>11.3 ± 5.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Disability: median EDSS (range)</td>
<td>6.5 (4.5–7.5)</td>
<td>2.0 (0–3.5)</td>
<td>6.0 (6.0–8.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relapses</td>
<td>0.6 ± 0.8</td>
<td>0.2 ± 0.3</td>
<td>0.3 ± 0.4</td>
<td>0.2 ± 0.3</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD where appropriate. *Differences between the four groups of subjects; †comparing the PP group with all other subjects; ‡comparing the PP group with the RR and SP groups combined; §comparing the RR group with the PP and SP groups combined.
Colonization of the lower urinary tract was detected in four subjects, all of whom were using intermittent self-catheterization. Asymptomatic bacterial infections occurred in 21 of the 45 subjects during the study period. No other patients received any other immunosuppressive or immunomodulatory therapy. Twenty-nine infections occurred in 21 of the 45 subjects during the study. These all involved the upper respiratory tract except for two urinary tract infections and one episode each of gingivitis, herpes labialis and a superficial skin infection. When the numbers of infections occurring in each group were normalized for the duration of the study no significant differences were noted in the incidence of infection between the study groups, although the multiple sclerosis patients tended to have fewer infections (Table 1). Asymptomatic bacterial colonization of the lower urinary tract was detected in four subjects, all of whom were using intermittent self-catheterization.

### Table 2: Urinary Neopterin:Creatinine Ratio (UNCR) Data

<table>
<thead>
<tr>
<th></th>
<th>Normal controls</th>
<th>Patients</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PP</td>
<td>RR</td>
</tr>
<tr>
<td>Collection days per subject</td>
<td>30±16</td>
<td>64±29</td>
<td>49±33</td>
</tr>
<tr>
<td>Days excluded per subject due to recorded infection</td>
<td>2.9±3.9</td>
<td>1.6±2.1</td>
<td>3.7±5.2</td>
</tr>
<tr>
<td>Mean UNCR (μmol/mol)</td>
<td>134 (97-152)</td>
<td>187 (135-231)</td>
<td>187 (165-277)</td>
</tr>
<tr>
<td>Log₁₀ (UNCR variance)</td>
<td>6.5±1.0</td>
<td>8.2±1.8</td>
<td>9.2±1.6</td>
</tr>
<tr>
<td>Days with UNCR &gt; normal (%)</td>
<td>0 (0-6)</td>
<td>16 (6-62)</td>
<td>28 (21-36)</td>
</tr>
<tr>
<td>Subjects with an elevated baseline</td>
<td>0/14</td>
<td>3/10</td>
<td>2/10</td>
</tr>
<tr>
<td>Subjects with peaks in serial UNCR unrelated to infection</td>
<td>3/14</td>
<td>8/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Peaks in serial UNCR/subject/month</td>
<td>0.2±0.6</td>
<td>2.1±1.8</td>
<td>3.0±1.7</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD, median (interquartile range), percentages or ratios as appropriate. *p values in the table all refer to comparisons between normal controls and the three patient groups using ANOVA. †All comparisons of normal controls with the individual patient groups are significant (P < 0.01); ‡comparison of secondary progressive patients with controls is significant (P = 0.009).

There were significant differences in the sex ratios of the four groups (P = 0.03, Table 1). The mean age, calculated on the final day of urine collection for each subject, differed between groups, with the PP patients being significantly older than the other subjects (P = 0.001, Table 1). Similarly, the mean age of disease onset of the PP patients was significantly older than the RR and SP patients (P = 0.006, Table 1), which is in accordance with previously published observations (Confavreux et al., 1980). As expected, the disease duration and level of disability were significantly greater in the progressive multiple sclerosis groups (PP and SP versus RR, P = 0.004 and P < 0.001 for disease duration and disability, respectively, see Table 1). Nine clinical relapses occurred in nine patients during the study period: five in the RR group and four in the SP group. Two of these relapses were treated with high dose intravenous corticosteroids (1 g methylprednisolone intravenously for 3 days). In addition to these relapses, two patients in the SP group received intravenous methylprednisolone, one patient 3 weeks prior to commencing the study and another patient during the study period. A single patient in the PP group was on low dose oral prednisone (10-25 mg/day) throughout the study period. No other patients received any other immunosuppressive or immunomodulatory therapy. Twenty-nine infections occurred in 21 of the 45 subjects during the study. These all involved the upper respiratory tract except for two urinary tract infections and one episode each of gingivitis, herpes labialis and a superficial skin infection. When the numbers of infections occurring in each group were normalized for the duration of the study no significant differences were noted in the incidence of infection between the study groups, although the multiple sclerosis patients tended to have fewer infections (Table 1). Asymptomatic bacterial colonization of the lower urinary tract was detected in four subjects, all of whom were using intermittent self-catheterization.

### Urinary Neopterin to Creatinine Ratios

Daily urine specimens were collected for 4 weeks in control subjects and for 7-9 weeks in patients with multiple sclerosis (Table 2), with a minimum collection period of 14 days. Similar numbers of days were excluded due to infections in all groups (Table 2). After measurements related to symptomatic infections were excluded, the subject's mean UNCR over the study period was significantly higher in patients with multiple sclerosis than in normal controls (P < 0.001, Table 2 and Fig. 1A). To analyse the pattern of this observed cross-sectional difference we looked at the other variables relating to the individual UNCR time-course plots described above. First, the natural logarithm of the variance of the mean UNCR for each subject was greater in patients than in controls (P < 0.001, Table 2 and Fig. 1A), which indicates greater day-to-day fluctuations in neopterin excretion or increased variability of the baseline of the UNCR time-course plot. Secondly, the proportion of days (as a percentage) on which the UNCR was greater than normal, i.e. more than the 'mean + 2.5X SD' of the UNCRs for the normal controls, namely >198.1 μmol/mol. This proportion was significantly elevated in all patient groups (P < 0.001, Table 2 and Fig. 1C). By using a cut-off, with ≥50% of the serial UNCR measurements above the upper limit of normal, three out of 10 PP, two out of 10 RR and five out of 11 SP had elevated baselines, with only the SP group significantly different from controls (P = 0.009, Table 2).

In three out of 14 of the control subjects, three small peaks in the serial UNCRs were found in 14 months (424 days) of urine testing, that could not be attributed to a symptomatic infection. This contrasted with 143 peaks in 59 months (1759 days) of serial urine testing that were found in all except two of the 10 PP patients (P < 0.001). When expressed as a rate, the controls had significantly fewer peaks unrelated
Fig. 1 Combined box and whisker scatter plots for (A) the mean UNCRs, (B) the natural logarithm (log) of the variance of the serial UNCRs, (C) the number of peaks per month in the serial UNCR time-course plots unrelated to infection, and (D) the percentage of days with a UNCR greater than normal, for normal control subjects and patients with PP, RR and SP multiple sclerosis. These measurements excluded all UNCRs that were associated with symptomatic infections. The box represents the interquartile range, divided horizontally by the median, the whiskers, the range; and the adjacent scatter plot, the individual values from which the box and whiskers are derived.
to infection than the multiple sclerosis patient groups ($P < 0.001$, Table 2 and Fig. 1D).

Although the SP patients tended to have higher mean UNCRs, greater variability and elevations in their baselines, with more peaks than the PP and RR patients, these comparisons did not reach statistical significance (Table 2 and Fig. 1A–D).

**Relapses**

Five of the nine relapses involved the sensory pathways, two the brainstem and one each the cerebellum and optic nerve. All patients who had relapses had elevated mean UNCRs. Seven of the relapses were confirmed by clinical examination and two were diagnosed retrospectively on history alone. The median of maximum UNCRs measured during a relapse was 559 µmol/mol (range 218–13329 µmol/mol). The median UNCR on the symptomatic days of the relapses (215 µmol/mol; interquartile range, 179–358) tended to be higher than the median levels of days not associated with the relapse in the same patients (160 µmol/mol; interquartile range, 152–189), but was not significant. All patients had increased variability in their baseline UNCRs during the relapse, the median natural logarithm of the variance of the UNCR on the symptomatic days of the relapse was 9.7 (range 7.6–16.4); although greater than the variability during other periods of the study and greater than variability of the patients without clinical relapse, it did not reach statistical significance either. Five relapses were time-related with peaks in the baseline, one of which was the highest detected in the study (Fig. 3, patient SP-4). These peaks preceded the onset of symptoms in four out of five cases by a variable period of time (range 1–14 days). In one case, however, the onset of symptoms preceded the neopterin peak by 5 days. Of the four relapses not associated with a clear peak in the UNCR, three had elevated baselines and one had a single elevated UNCR measurement during the relapse. This latter relapse involved a recurrence of symptoms related to a previous brainstem episode with vertigo, dysequilibrium and facial myokymia. No correlation could be found between the clinical severity of the relapses and the levels of elevated neopterin excretion (data not shown).

**Corticosteroids**

A brainstem relapse associated with a modest increase in the UNCR (maximum UNCR = 291 µmol/mol) was treated with high dose intravenous corticosteroids (1 g methylprednisolone intravenously for 3 days). The UNCR normalized to levels below 100 µmol/mol for a 6-day period, from day 3 of the infusion, but then increased to pre-treatment levels for the remainder of the study. The second patient who received intravenous corticosteroids (for a sensory spinal cord relapse associated with a positive Lhermitte’s sign) had a dramatic fall in her UNCR from 1467 to 168 µmol/mol on day 2 of the infusion. This response was maintained for 7 days before the UNCR increased to levels of >300 µmol/mol that coincided with an upper respiratory tract infection. The patient with SP multiple sclerosis who received intravenous corticosteroids 3 weeks prior to entry into the study, had a persistently elevated UNCR from day 1 of the study and therefore no effects of the corticosteroid treatment on this UNCR could be inferred. The other SP patient, who had a severe cerebellar tremor but no evidence of a clinical relapse, received intravenous methylprednisolone when her baseline UNCRs were normal; no effects were noted. Finally, the patient with PP multiple sclerosis who was on low dose oral prednisone had significantly elevated UNCRs throughout the study.

**Infections**

During the study, 10 infections were recorded by eight control subjects, and 19 by 13 patients with multiple sclerosis. To see if infection precipitated relapse or an increase in urinary neopterin excretion, we analysed the ‘at risk period’ related to these infectious episodes. This is defined as the 6-week period starting 1 week prior to, and ending 5 weeks after, the onset of the infection (Sibley et al., 1985). As this period includes the episode of infection, a known stimulus for neopterin production, we were unable to use the mean and variance of the UNCR. We therefore analysed the proportion of days associated with an elevated UNCR in relation to the onset of the infection. Not all subjects had a complete set of data points for each infectious episode and in two cases this period had to be modified as a result of a second infection occurring prior to the end of the first ‘at risk period’ period. In these cases we terminated the first ‘at risk period’ the day prior to the onset of the second infection and started the next ‘at risk period’ the following day. We therefore avoided using any data point more than once in the analysis. Eight out of 13 patients had clearly defined UNCR peaks following on documented infections. This inflammatory activity tended to persist following on the infection for variable periods of time of up to 6 weeks. In comparison, control subjects had a brisk return to baseline, usually by day 10, from the elevated levels associated with the infectious episode (Fig. 2). Of note, three of the nine relapses were temporally associated with infections, becoming symptomatic in the ‘at risk period’ on days 23, 31 and 33 post-infection. Two such examples are given in Fig. 3, as patients SP-3 and SP-4. Of the four subjects with asymptomatic bacterial colonization of the lower urinary tract, one had a normal baseline with two small peaks, two had elevated baselines with increased variability and one had a normal baseline with intermittent activity that was associated with a clinical relapse.

**Individual profiles and frequency of testing**

As daily monitoring would be impractical for long term follow-up we compared it with alternate daily and weekly urine sampling frequencies (Table 3). No differences are
Neopterin excretion in multiple sclerosis

Infections in patients with MS, n = 19
Infections in normal controls, n = 10

**Fig. 2** Longitudinal serial scatter plot of the proportion of days with a UNCR greater than normal, in relation to the onset of a symptomatic infection, for 10 and 19 infections in eight normal control subjects (triangles) and 13 patients with multiple sclerosis (MS), respectively. The shaded area represents the approximate period related to the infectious episode. Day 0 represents the day of onset of symptoms related to the infection.

### Table 3 Comparison of the urinary neopterin:creatinine ratio (UNCR) data for daily, alternate day and weekly sampling

<table>
<thead>
<tr>
<th></th>
<th>Normal controls</th>
<th>Patients</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>median UNCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (μmol/mol)</td>
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<tr>
<td>(Interquartile range)</td>
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<tr>
<td>Daily</td>
<td>134 (97–152)</td>
<td>187 (135–231)</td>
<td>218 (164–517)</td>
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<tr>
<td>Alternate days</td>
<td>127 (98–151)</td>
<td>188 (135–231)</td>
<td>247 (162–468)</td>
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<tr>
<td>Weekly</td>
<td>126 (95–140)</td>
<td>184 (130–240)</td>
<td>237 (145–444)</td>
</tr>
<tr>
<td>Log_{e} UNCR variance: mean ± SD</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Daily</td>
<td>6.5 ± 1.0</td>
<td>8.2 ± 1.8</td>
<td>10.3 ± 3.2</td>
</tr>
<tr>
<td>Alternate days</td>
<td>6.4 ± 1.1</td>
<td>8.3 ± 2.0</td>
<td>10.2 ± 3.3</td>
</tr>
<tr>
<td>Weekly</td>
<td>4.9 ± 2.7</td>
<td>7.6 ± 1.6</td>
<td>9.5 ± 3.1</td>
</tr>
</tbody>
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observed between these different sampling frequencies with regard to the cross-sectional data analysis, except the degree of variability in the baseline is reduced with fewer samples. However, the detailed time course and peak analysis becomes more difficult with smaller numbers of samples as some peaks are short lived and may be missed by infrequent sampling. Daily serial individual UNCR profiles of three control subjects and 10 patients with brief case histories are shown in Fig. 3. They illustrate the different patterns of elevated UNCR found in patients with multiple sclerosis, and demonstrate the effects of clinical relapses and infections on the UNCR time-course plot.

**Discussion**

Neopterin is a well established marker of cell mediated inflammation and preliminary studies have already shown elevated levels in both the CSF and serum of patients with multiple sclerosis (Fierz et al., 1987; Fredrickson et al., 1987; Ott et al., 1993; Shaw et al., 1995). The primary aim of our study was to investigate whether urinary neopterin excretion would be sensitive enough to detect inflammation in multiple sclerosis and to see if it had any potential as a surrogate inflammatory marker. We chose to study urinary neopterin, because urine is more suitable for frequent self-collection, home freezing and storage, and the intensive daily monitoring used in this study would not have been possible with plasma or CSF. Neopterin is excreted in the urine in a concentration several hundred fold higher than that which is found in plasma or CSF, making it easier to detect significant changes in production (Fuchs et al., 1992). The kidneys therefore act as an integrator of neopterin production, and provide more reliable information than the brief snapshot obtained from a plasma or CSF level. In addition, the extended collection periods used in this study allow an accurate intra-subject assessment of the day-to-day variability in levels of neopterin excretion.

As a result of confounding factors, the raw UNCR data had to be manipulated prior to analysis. First, women who have a lower muscle mass and therefore a lower creatinine
excretion and higher UNCRs than men, had their UNCRs reduced by a correction factor to allow a direct comparison with male subjects. This correction factor only affected the mean UNCR levels and did not have any significant effect on the variability of the UNCR or the detection of peaks in the time-course plot. Analysis of the mean UNCR prior to this correction produced similar results. This correction was performed to minimize any bias due to differences in the sex ratio between the patient groups which would have raised the mean levels in the RR and SP groups compared with patients with PP disease. Secondly, the sensitivity gained from monitoring urinary neopterin excretion is offset by its lack of specificity, especially as common viral infections give rise to increased urinary neopterin excretion. We therefore excluded UNCR measurements associated with symptomatic infections. These infections were reported by the subjects in the study and were not confirmed clinically or by laboratory investigations. The reliability of the self-reporting of common viral infections has not been tested, and is therefore unknown. If any under-reporting of infections occurred in the patient groups compared with control subjects, it would bias the results. Thirdly, we manipulated the UNCR time-course plot by taking the second derivative to identify any peaks in the excretion of urinary neopterin. This is a mathematical technique which recognizes large changes in the slope of the UNCR time-course plot and hence identifies peaks. Although this manipulation was effective and reproducible, it occasionally failed to detect peaks that were obvious to the naked eye when many fluctuations were present in the baseline, i.e. when a peak occurred on a noisy baseline. All peaks were checked mathematically to ensure compliance with the definition. The mathematical manipulations were entirely reproducible. The reproducibility of the manual flagging technique was not assessed. However, this is unlikely to affect the results of the study as the automated detection of peaks only failed in subjects with a noisy baseline which tended to occur in the patients and not the normal controls. Therefore the manual inspection would have been more likely to miss peaks in the patients, resulting in an underestimation of the differences noted between normal controls and patients with multiple sclerosis.

Patient groups could not be age- or sex-matched as PP multiple sclerosis is more common in males than in females, and has greater age of disease onset (Confavreux et al., 1980; Compston, 1991). The differences between the ages and sex ratios of the groups could, however, have possible confounding effects. First, the UNCR is ~20% higher in subjects over the age of 65 years (Fuchs et al., 1992), but as only one subject in our study was older than 65 years, this would be unlikely to affect the results. Secondly, a correction was made for the higher UNCR that occurs in females and following this, no differences were found between male and female control subjects (data not shown). As patients and controls were not randomly selected or age- and sex-matched, other unidentified confounding variables may be present.

Neopterin excretion was clearly raised in the majority of our patients, particularly in those with SP multiple sclerosis, who tended to have the highest levels, followed by the patients with RR and PP disease. This inflammatory hierarchy, between the different patient subgroups, corresponds approximately to the one found using gadolinium-enhanced T1-weighted MRI as the surrogate marker of CNS inflammation (Thompson et al., 1991, 1992). In addition, clinical activity in the form of a relapse was, without exception, associated with some elevation in neopterin excretion, albeit modest in some cases. Neopterin excretion tended to be higher during clinical relapse, implying that detection of inflammation with UNCRs is more likely during a relapse than during clinical remission. This has been demonstrated by MRI studies which show that the majority of patients in relapse (80%), compared with ~25% of patients in remission have detectable gadolinium-enhancement (Thompson et al., 1991, 1992). Due to the small number of relapses which occurred during this study, it was not possible to assess the association between the level of neopterin excretion and the severity of clinical relapse, or whether increases in neopterin excretion can predict the onset of a clinical relapse. An important thing to consider is that the clinical severity of a relapse may not relate to the size of the offending lesion, or to its inflammatory mass, but rather to its location in sites which may or may not be clinically eloquent. Therefore, the level of neopterin excretion, and indeed other inflammatory markers, may bear no relation to the severity of a clinical relapse. Although two relapses were not confirmed clinically, we felt confident with a retrospective diagnosis, as self-reported neurological dysfunction correlates well with that of a formal neurological assessment (Verdier-Taillefer et al., 1994).

Asymptomatic elevations in neopterin excretion in the form of a persistently elevated baseline with or without superimposed peaks, or a normal baseline with intermittent peaks was common in patients with multiple sclerosis, supporting the MRI data that much of the disease activity in multiple sclerosis is asymptomatic (Miller et al., 1988; Koopmans et al., 1989; Willoughby et al., 1989; Capra et al., 1992). Two PP patients who had been clinically stable for several years had normal neopterin excretion, suggesting that there was no inflammatory component to their disease process during the study period. Finding elevated levels of neopterin

Fig. 3 Longitudinal profiles of serial UNCRs for three normal subjects and 10 patients with multiple sclerosis, for periods of 6 and 12 weeks, respectively. The dotted line represents the upper limit of the normal UNCR (198 µmol/mol); the thick bars represent episodes of symptomatic infection; and the open squares, clinical relapses. The episodes of infection and clinical relapse have been highlighted with areas of shading.
excretion in some patients with PP multiple sclerosis, indicates that ongoing inflammation is occurring in these patients and supports a recent pathological study demonstrating low grade inflammation in this subgroup of patients (Revesz et al., 1994). The fact that inflammatory activity can be detected by measuring urinary neopterin is very important, as neopterin may provide a means of monitoring inflammation in these progressive patients who characteristically lack MRI activity (Thompson et al., 1991; Kidd et al., 1996). However, a caveat to be kept in mind is that the pathogenesis of progressive disability in multiple sclerosis is uncertain; several lines of evidence suggest that it may be due to progressive axonal loss (Arnold et al., 1994; Davie et al., 1994; Filippi et al., 1994; Lassmann et al., 1994), but whether this is a consequence of persistent, low grade inflammation is currently unknown. Although our study was not powered to investigate the association between inflammatory activity and disability, the patients with rapidly progressing disability appeared to have higher and more persistently elevated levels of neopterin excretion compared with clinically stable patients. Further studies are needed to ascertain the relationship between neopterin levels, clinical course and putative MRI markers for axonal loss. Importantly, urinary neopterin excretion appears to be sensitive enough to detect relatively small volumes of inflammation of the order of magnitude of that occurring in multiple sclerosis.

Although only a few patients received high dose intravenous corticosteroids, it appears that this form of treatment suppresses the production of neopterin for a short period of time. This is similar to the transient suppression of gadolinium-enhancement (< 1 week) seen after high dose intravenous methylprednisolone (Miller et al., 1992), and to that which occurs in renal and bone marrow transplant rejection, where the administration of high dose methylprednisolone exerts a dramatic suppression of neopterin production (Wachter et al., 1992). However, the transplant scenario, in which neopterin excretion is used to monitor immunosuppressive therapy, is confounded by the persistent use of other immunomodulatory drugs that, if successful, cause a sustained suppression of neopterin excretion. The effects of high dose corticosteroids and other immunomodulatory therapy on the time course of neopterin excretion in patients with multiple sclerosis requires assessment. Unfortunately, neopterin may be unsuitable for monitoring the effects of the type I IFNs (α and β) which are currently in clinical use. They have overlapping actions with those of IFN-γ, and cause a non-specific increase in neopterin production by monocytes and macrophages (Chiang et al., 1993; Liberati et al., 1994).

Neopterin is a sensitive marker of IFN-γ induced monocyte/macrophage activity, and T cells that produce the IFN-γ are strongly implicated in the immunopathogenesis of multiple sclerosis. Prior to clinical relapses, mitogen-stimulated peripheral blood cells of patients with multiple sclerosis produce greater quantities of IFN-γ (Beck et al., 1988). IFN-γ production has also been demonstrated in actively inflamed multiple sclerosis plaques (Traugott and Lebon, 1988; Cannella and Raine, 1995), and the administration of IFN-γ to patients with multiple sclerosis significantly increased the number of clinical exacerbations (Panitch et al., 1987). Since infections, particularly viral, result in increased IFN-γ and neopterin production (Wachter et al., 1992), could the elevated levels that we detected have been due to subclinical or low grade chronic infections? We feel that this is unlikely for several reasons (unless multiple sclerosis is caused by an unidentified viral infection). First, in normal controls only three peaks in 14 months of daily urine testing could not be accounted for by a symptomatic infection. Secondly, the number of subclinical infections would have had to have been very frequent (2–3 per month) and thirdly, patients were instructed to document all ailments, however minor, and were always given the benefit of the doubt with regard to their interpretation of an infection. Fourthly, after excluding urinary tract infections, there is no evidence that patients with multiple sclerosis are more predisposed to infection than normal controls. On the contrary, patients with multiple sclerosis have been documented to have significantly fewer viral infections than healthy subjects (Sibley et al., 1985), a trend noted in our study (Table 1). Finally, neopterin levels are higher in the CSF than in the serum of patients with multiple sclerosis, a finding opposite to that in non-inflamatory control patients (Shaw et al., 1995). This indicates that neopterin is synthesized intrathecally, which would be incompatible with a non-CNS infection causing the increase in neopterin production. Taking these points together, we feel that the increase in urinary neopterin excretion is due to inflammatory activity within the CNS. We are currently setting up clinical experiments to assess the relative contributions of the CNS and systemic compartment to the increased neopterin production observed in patients with multiple sclerosis.

The observation that infection is associated with clinical relapse in patients with multiple sclerosis has been known for several years (Sibley et al., 1985; Andersen et al., 1991; Panitch, 1994). We have confirmed this and have demonstrated that this effect is not limited to symptomatic relapses. A third of the relapses in this study occurred in the 'at risk' period and 62% of subjects had a persistent increase in neopterin excretion post-infection for a period much longer than expected (Fig. 2). From this data, it appears infections are a potent stimulus for causing prolonged elevations in neopterin excretion beyond the time period expected for an uncomplicated infection. This elevation in neopterin production, which occurs for up to and possibly beyond 6 weeks post-infection, is presumably due to an increase or precipitation of both symptomatic and asymptomatic disease activity. As this was not seen in the control subjects it suggests a phenomenon related to disease activity. How infections precipitate disease activity is unknown, but it may result from the systemic induction of IFN-γ. Our data also suggests that the at risk period may be longer than that defined in the literature (Sibley et al., 1985).

Our study demonstrates the informative nature of daily
longitudinal data on individual patients that is unobtainable from simple cross-sectional analysis. If urinary neopterin excretion proves to be a useful surrogate inflammatory marker in multiple sclerosis, how frequently should it be measured? We have presented a relatively extreme scenario of daily UNCR measurements, which we feel would be impractical and unnecessary for a long-term study. A comparative analysis demonstrates that alternate day and weekly sampling is sufficient to detect inter-group differences, but a sampling frequency of less than this would miss some of the short lived, intermittent inflammatory activity. We therefore recommend for the further long-term assessment of neopterin, as a surrogate inflammatory marker in patients with multiple sclerosis, that a weekly sampling frequency be used.

In conclusion, neopterin is a novel, sensitive marker for the in vivo production of IFN-γ and other pro-inflammatory cytokines in multiple sclerosis. We believe neopterin is a potential marker for the frequent long-term monitoring of active inflammation in patients with multiple sclerosis. It is apparent that there is heterogeneity within multiple sclerosis patient population, with not all patients demonstrating elevated levels of neopterin production. Neopterin may therefore provide a way to detect which patients have active inflammation and are therefore more likely to benefit from immune-modulating therapy. However, to be a useful surrogate marker of disease activity, it needs to be shown that high neopterin levels are predictive of future increases in disability.

References


Appendix: Case histories

Control-1. A healthy 30-year-old male, with a flat baseline, limited variability and no peaks.


Control-3. A normal 33-year-old female with a UNCR peak related to a mild upper respiratory tract infection, which was followed by a second peak that could not be attributed to a symptomatic infection. Only three such peaks occurred in 14 months of serial testing in the normal control subjects and they probably represent episodes of subclinical viral infection.

PP-1. A 50-year-old male with PP multiple sclerosis of 12 years duration and a Kurtzke’s EDSS of 7.0. He demonstrates a slight increase in variability in the baseline with some peaks unrelated to infection.

PP-2. A 41-year-old female with PP multiple sclerosis of 8 years duration, an EDSS of 6.5 and a persistently elevated UNCR throughout the study period. She had progressed from 4.5 to 6.5 on the EDSS in the 15 months preceding this study.

PP-3. A 43-year-old male with PP multiple sclerosis of 7 years duration, an EDSS of 4.5 and a normal UNCR time-course plot. His disability has not progressed in the last 3 years.

PP-4. A 46-year-old male with PP multiple sclerosis of 20 years duration and an EDSS of 7.5. His disability has also not progressed in the preceding 3 years.

RR-1. A 27-year-old female with RR multiple sclerosis of 2.5 years duration and an EDSS of 1.5 demonstrates intermittent increases in neopterin excretion with a large peak occurring in the ‘at risk period’ of a symptomatic infection.

RR-2. A 29 year female with RR multiple sclerosis of 6 years duration, an EDSS score of 3.5 and an intermittent elevation in neopterin excretion with a variable baseline and numerous peaks.

SP-1. A 39-year-old female with SP multiple sclerosis of 8 years duration and an EDSS of 6.0. Her plot demonstrates a persistently elevated baseline, increased variability and clear peaks occurring in the post-infection ‘at risk’ periods. She did not develop any new neurological symptoms during these periods but did complain of increased fatigue. She was one of the more rapidly progressive patients moving from an EDSS of 4.5 to 6.0 in the 12 months preceding this study.
SP-2. A 40-year-old female with SP multiple sclerosis of 10 years duration and an EDSS of 6.0. She had the highest elevation in neopterin excretion throughout the study period. Although she did not have any neurological symptoms that could be classified as a relapse, she complained of numerous intermittent symptoms, marked day-to-day fluctuations in activities of daily living and severe fatigue. Her disability, however, had not changed in the preceding 12 months.

SP-3. A 35-year-old female with SP multiple sclerosis of 16 years duration and an EDSS of 6.0. She had a persistently elevated baseline with numerous peaks and a symptomatic spinal cord relapse, with sensory symptoms and an associated Lhermitte's sign. Her relapse and increased neopterin excretion was probably precipitated by the preceding viral infection of the upper respiratory tract.

SP-4. A 46-year-old female with SP multiple sclerosis of 21 years duration, an EDSS of 6.5 and a peak in her UNCR associated with a mild episode of optic neuritis.
Increased Urinary Neopterin Excretion in Vaccinated Adults

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Summary

Serial urinary neopterin:creatinine ratios of 58 healthy adult subjects were measured from 2 days prior to and up to 12 days post vaccination. An increase in the urinary neopterin of at least twice the baseline was detected in 24/46 subjects who received either a Mantoux skin test or a live viral vaccine (cell mediated immune group, median average increase above baseline = 206%; interquartile range 142-315%), and in 5/12 who received non-live vaccines known to stimulate mainly humoral immunity (humoral immune group, median average increase above baseline = 165%; interquartile range 142-417%). The peaks in the urinary neopterin in the cell mediated group occurred between days 5 and 10 post-vaccination, with the mode on day 5. In contrast the 5 subjects in the humoral immune group all developed their peaks on day 7 or 8. Three of these subjects received an influenza vaccine, one a typhoid vaccine and one a tetanus toxoid. No association was found between local or systemic side-effects of vaccination and raised urinary neopterin. In conclusion, the monitoring of urinary neopterin excretion in adults is sensitive enough to detect an immunological response to vaccination with immunogens that induce either cell mediated or humoral immune responses.

Key words: Neopterin, Vaccination, Purified protein derivative, Skin tests.

Introduction

Neopterin is a relatively stable product of interferon gamma (INF-γ) induced macrophage activity and is an indirect measure of both INF-γ levels and macrophage activity(1). It is produced almost exclusively by macrophages, due to a functional block in the pathway synthesising tetrahydrobiopterin(2), an essential co-factor for the inducible form of nitric oxide synthase(3, 4). Neopterin's role as a surrogate marker of inflammation has been well studied in infections (particularly HIV), malignancies, autoimmunity and transplantation(1). To determine how sensitive neopterin is as a marker of inflammation in adults, we measured serial neopterin excretion in a group of adult subjects undergoing various vaccinations or Mantoux testing with tuberculin purified protein derivative (PPD).

Materials and Methods

Fifty eight healthy adults who were either receiving a vaccination or undergoing a Mantoux skin test with tuberculin PPD were included in the study. They were divided into two groups depending on whether the predominant immunological stimulus was cell mediated or humoral.
Forty five subjects undergoing Mantoux skin test with tuberculin PPD (Evans Medical) and one subject who had a live rubella vaccine (Evans Medical) constituted the cell-mediated immune (CMI) group. Twelve subjects undergoing vaccination with non-live vaccines were included in the humoral immune group. These latter vaccinations included 5 influenza (Fluvirin®, Evans Medical), 2 hepatitis B (Engerix B®, SmithKline Beecham), 2 typhoid (Typhim Vi®, Pasteur Mérieux), 1 hepatitis A (Havrix®, SmithKline Beecham) and 2 tetanus toxoid (Wellcome). A disproportionately large number of patients undergoing Mantoux skin tests were recruited, to allow us to compare differences in subjects with positive or negative skin reactions. Subjects were instructed to collect daily urine specimens starting 2 days prior to and finishing 10 to 12 days after the vaccination or skin test. As urinary neopterin excretion has a normal diurnal variation (5), we elected to collect the first specimen of the day. Urine was collected in small polypropylene tubes, labelled and immediately home frozen in a light-proof container to prevent spontaneous degradation of neopterin from exposure to light. Patients were also instructed to keep a detailed daily diary documenting and describing any local or systemic side-effects to the vaccination as well as any infections during the study period.

To correct for the variable concentration of urine, the neopterin concentration is expressed as a ratio to creatinine. Both neopterin and creatinine were measured by high pressure liquid chromatography (HPLC) (6). Due to a smaller muscle mass and lower creatinine excretion, females have on average a 20% higher urinary neopterin:creatinine ratio than males, therefore all urinary neopterin levels on females subjects were divided by a factor of 1.2 to correct for this. This correction factor was obtained from data collected on 40 control subjects (data not shown) which corresponds to that published in the literature (1).

A baseline threshold was established for each subject by taking the mean of the urinary neopterin from days 2 pre to day 3 post-vaccination inclusive. Subjects were only considered to have a significant increase in their neopterin excretion if at least one urinary neopterin after day 3 was more than double their calculated baseline threshold. A time course plot was then generated for each group by plotting the mean daily corrected urinary neopterin against time. Peaks were identified and only considered significant if their peak height was at least double that of the baseline. Data was compared using the Student T, Kruskal-Wallis, Chi-squared and Fisher Exact tests as appropriate. A p-value of less than 0.05 was considered significant.

Results

The mean ages of the cell mediated and humoral immune groups were 36.6+11.6 years and 42.0+13.3 years respectively. Twenty six out of 46 subjects in the CMI and 10/12 in the humoral immune group were females. These differences are not significant.

Post-vaccination, most subjects had an increase in their urinary neopterin (fig. 1). The cell mediated group had a median average increase of 206% (interquartile range 150-238%) in their peak urinary neopterin from a median baseline of 138 µmol/mol (interquartile range 94-209) to a median peak of 232 µmol/mol (interquartile range 190-348) and the humoral group a median average increase of 165% (interquartile range 147-212) from a median baseline of 124 µmol/mol (interquartile range 92-182) to a median peak of 227 µmol/mol (interquartile range 140-404). Only 24/46 (52%) subjects in the CMI group and 5/12 (42%) subjects in the humoral immune group had clear peaks in their urinary neopterin (fig. 1) as defined above. In the CMI group, the maximum levels were all detected from day 5 to 10 post skin test, with the mode on day 5. In contrast the 5 subjects in the humoral immune group all developed their peaks on day 7 or 8. Three of the latter subjects received influenza vaccines, one a typhoid vaccine, and one a tetanus toxoid.

Eighteen out of 45 subjects receiving Mantoux skin tests had had a previous BCG vaccination, and no patient had a previous history of tuberculosis. Eight out of 18 subjects (44%) who had previously had a BCG vaccination developed clearly defined peaks in response to PPD, compared to 15/27 subjects (56%) who had not had a previous BCG vaccination (p = n.s.). The former patients tended to have a lower median urinary neopterin peak than the latter patients (179 µmol/mol (interquartile range 160-283) vs. 248 µmol/mol (interquartile range 144-346), p = 0.07). All Mantoux skin tests were read at 48 hours and considered positive if the diameters in two planes were greater than 5 mm. Nineteen skin tests were negative and 26 positive. Eleven out of 19 (58%) subjects with a negative Mantoux skin test, compared to only 10/26 (38%) subjects with a positive Mantoux skin test, had a peak in their urinary neopterin as defined above (p = n.s.). The subjects with negative Mantoux

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Figure 1. Scatter and box and whisker plot of mean baseline urinary neopterin: creatinine ratio (UNCR) (day 2 pre to day 3 post-vaccination) and maximum peak UNCR (days 4 to 10 post-vaccination) in 46 subjects receiving vaccinations known to stimulate cell mediated immunity (cell mediated group) and 12 subjects receiving vaccines known to stimulate humoral immunity (humoral group). The boxes represent the 25th to 75th percentiles divided by the median and the whiskers the maximum and minimum values.

Skin tests tended to have higher median peak urinary neopterin levels (257 μmol/mol, range 170 to 360), than subjects with positive Mantoux skin tests (198 μmol/mol, range 145 to 288, p = n.s.).

No association was found between the local or systemic side-effects of vaccination and raised neopterin levels. Thirty one patients documented local reactions of pain, swelling or erythema due to vaccination. Their average median peak urinary neopterin from day 5 to 10 was slightly lower than patients without local reactions, but did not reach statistical significance (205 μmol/mol, range 163 to 313, vs. 256 μmol/mol, range 173 to 360, p = n.s.). Similarly 31 subjects who complained of mild systemic symptoms like fever, myalgias, headache and tiredness due to the vaccination, tended to have an average median peak urinary neopterin level that was lower than the 27 subjects without systemic reactions (201 μmol/mol, range 145 to 313, vs. 258 μmol/mol, range 109 to 360, p = n.s.).

The average time course plots for the CMI and humoral immune groups are presented in figs 2a and 2b respectively. In the CMI group a clear peak occurred between days 4 and 10, with the maximum height on day 5. The mean area under the graph from days 4 to 9 post-vaccination was significantly greater than the area under the graph from days 2 pre-to day 3 post-vaccination (p = 0.009). In the humoral immune group the post-vaccination peak is noted on day 8, with the area under the curve from days 4 to 9 being statistically greater than the area under the curve graph from days 2 pre-to day 3 post vaccination (p = 0.04).

Discussion

In contrast to Wildgrube, et al (7) who were unable to demonstrate increased neopterin production in response to various skin tests in 25 subjects, we have shown that neopterin is sensitive enough in adult subjects to detect the CMI response to low dose PPD as well as vaccines known to stimulate a humoral response. Fuchs et al(8), have demonstrated previously that the vaccination of 4 children with live measles/mumps vaccine induced a marked and brisk increase in neopterin excretion that peaked between day 8 and day 11. This time course was similar to the 5
subjects in the humoral group but differed from subjects in the cell mediated group, who tended to have an earlier peak between day 4 and day 8. This difference in time courses is probably related to the fact that the immune response in the 4 paediatric cases and subjects in our humoral group was a primary response to a new set of antigens. This would therefore be delayed compared to a secondary response to a previously recognised set of antigens, like PPD in the CMI group. It is worth noting that children have higher baseline urinary neopterin than adults(1). The peak urinary neopterin (1000-2000 μmol neopterin/mol creatinine) found by Fuchs et al(8) in their 4 paediatric subjects are higher than those in our CMI group (±350 μmol neopterin/mol creatinine). This may reflect the baseline differences between children and adults.

Surprisingly, 3 subjects receiving influenza vaccine, 1 a typhoid and 1 a tetanus toxoid booster had an increase in their urinary neopterin. Although the primary aim of these vaccines is to induce a good humoral IgG response which depends on the Th2 cytokine IL-4, some IFN-γ is also produced. It therefore seems highly unlikely that the Th1 and Th2 pathways are mutually exclusive in vaccination, and the various contributions of the Th1 and Th2 pathways to a specific set of antigens depends on other factors that are probably host-related.

A striking and very interesting observation is the elevated level of neopterin production in subjects who have negative skin reactions to PPD. These subjects are able to generate a systemic response that is unaccompanied by a positive skin reaction. These patients may have low or absent circulating memory T-cells to PPD, and when exposed to the antigen they mount a more vigorous primary immune response. In contrast the patients with positive skin reactions have a good pool of circulating memory T-cells and are able to localise the immune reaction to the site of inoculation as a secondary immunological response. Against this suggestion is the similar time course of neopterin production in patients without and with positive skin reactions, as one would expect the neopterin production of a primary immunological response to be delayed relative to a secondary one. Another possibility is that PPD contains sufficient quantities of mycobacterial heat shock proteins, which can activate large numbers of T-cells non-specifically as superantigens(9), resulting in systemic IFN-γ production.

The purpose of undertaking this study was not to determine the different immunological responses to various antigens, but to see how sensitive neopterin is as an immunological marker. Our results show that urinary neopterin excretion, although non-specific, is a very sensitive marker. Monitoring urinary neopterin excretion is therefore capable of detecting minor degrees of inflammation, of the order of magnitude associated with vaccinations or immunological skin tests.

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Urinary markers of disease activity in multiple sclerosis

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Numerous markers of disease activity, representing different aspects of the inflammatory cascade and pathogenic process in multiple sclerosis, can be detected in the urine. Urinary monitoring provides distinct advantages over blood and cerebrospinal fluid: it is easier to collect, allows frequent sampling and acts as a natural integrator by capturing the excretion of a substance over a prolonged period of time. We will discuss the principles, advantages, and pitfalls of urinary monitoring in relationship to multiple sclerosis.

Keywords: multiple sclerosis; urine; neopterin

Introduction
Multiple sclerosis is a putative T-cell mediated organ-specific autoimmune disease. Pathologically the disease is characterised by plaques of demyelination, and depending on the age of the lesion, a perivascular inflammatory infiltrate consisting of lymphocytes, macrophages and occasional plasma cells, and variable degrees of axonal loss and gliosis. The clinical course is heterogeneous with inflammatory disease activity varying over time. Currently clinical relapses and MRI activity are used as outcome measures in clinical trials, but cannot be used in patients with non-relapsing progressive disease who by definition have no clinical relapses and little if any MR activity. Sensitive and reliable surrogate markers of disease activity are needed in MS to study further the natural history of the disease and hopefully to evaluate the efficacy of various therapeutic agents. Of the body fluids urine is the least well-studied in MS. This paper will discuss the advantages, disadvantages and principles behind the use of urine for immunological monitoring in patients with MS.

Why urine?
Urine has several advantages over other body fluids.

Route of excretion
If a substance is excreted in the urine, its high fractional excretion (see Figure 1 for formula) results in a relatively greater concentration than that found in CSF or blood. Therefore it is easier and more reliable to monitor levels of these substances in the urine. Numerous products of inflammation are excreted in the urine, however few have been studied in detail (Table 1).

Sample collection
Urine is easy to collect and allows for more frequent sampling with little discomfort to the patient. This has major advantages when attempting to study disease activity that is more often than not asymptomatic and variable. Serial (monthly) MR studies have shown that for every clinical relapse there are between five and 10 asymptomatic lesions and disease activity occurs in cycles with new lesions typically occurring in clusters. In addition disease activity in the spinal cord rarely occurs in isolation and is usually associated with simultaneous activity in the brain. This makes single or infrequent measurements very difficult to interpret.

Frequency of urine sampling
There is so far little evidence supporting the clinical utility of frequent body fluid sampling in patients with MS. Ethical considerations and patient compliance limit the frequency of invasive procedures such as lumbar puncture and venesection for research rather than diagnostic purposes. Urine sampling, however, has distinct advantages in that it is non-invasive, can be performed by patients at home and does not require trained personnel to perform. Taking advantage of these points we performed a longitudinal study on 29 patients with multiple sclerosis in which we measured daily urinary neopterin (a well-defined inflammatory marker of interferon-gamma activated macrophage activity) excretion. The study revealed that neopterin excretion, and by inference the inflammatory reaction, is highly variable and dynamic in MS. Changes in neopterin excretion occur over a time course of days rather than weeks. An example of a time course profile of a patient with secondary progressive MS is presented in Figure 2. The inflammatory profiles of biweekly or monthly sampling detect very little of the inflammatory activity compared to daily or weekly sampling. In addition the proportion of MS patients, selected at random, with elevated urinary neopterin excretion is significantly lower than the proportion of patients with elevated mean levels from a series of specimens collected over a 2–3 month period (Figure 3).

Natural integrator
The bladder functions as a urinary reservoir, collecting and storing urine over a period of hours. Any
substance measured in a spot urine specimen represents an average of its excretion over a particular period of time. A urinary level is therefore an integral of the production of a substance i.e. the area under the time curve (Figure 4). This has particular advantages in relation to diurnal variation (see later) and selecting the most suitable time for sampling.

Diurnal variation
Most biological processes are affected by diurnal rhythms, the immune system being no exception. For example IL6 levels may vary by as much as 350% during 24 h. Similarly levels of urinary neopterin excretion can vary by as much as 50% during 24 h. Figure 5 demonstrates the diurnal variation in urinary neopterin excretion in six normal control subjects over

Table 1 Some inflammatory markers detectable in urine

<table>
<thead>
<tr>
<th>Marker</th>
<th>Pathological process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neopterin*23</td>
<td>Macrophage activity</td>
</tr>
<tr>
<td>Nitrate and nitrite*22,24</td>
<td>Inducible nitric oxide activity</td>
</tr>
<tr>
<td>Prostaglandin metabolites*25</td>
<td>Inducible cyclo-oxygenase activity</td>
</tr>
<tr>
<td>B2-microglobulin*26</td>
<td>MHC Class I expression</td>
</tr>
<tr>
<td>Immunoglobulin light chains*27</td>
<td>β and plasma cell activity</td>
</tr>
<tr>
<td>IL-3*28</td>
<td>Macrophage activity</td>
</tr>
<tr>
<td>IL-β*28</td>
<td>T cell proliferation</td>
</tr>
<tr>
<td>Soluble IL-2 receptor*31</td>
<td>T cell proliferation</td>
</tr>
<tr>
<td>IL-6*35</td>
<td>Th1 cytokine inflammatory marker</td>
</tr>
<tr>
<td>II, R*31</td>
<td>Th2 cytokine inflammatory marker</td>
</tr>
<tr>
<td>Mvelin basis protein like material*34</td>
<td>Demyelination and/or failure of remyelination</td>
</tr>
</tbody>
</table>

Figure 1 The formula to calculate the fractional excretion of a substance in urine

\[
\text{Fractional excretion of substance } y = \left( \frac{\text{area of } (\text{crop} \times \text{time})}{\text{concentration of } y \times \text{time}} \right) \times 100\%
\]

As GFR can be estimated by the endogenous clearance of creatinine the fractional excretion can thus be simplified.

\[
\text{Fractional excretion of substance } y = \left( \frac{\text{area of } (\text{crop} \times \text{time})}{\text{concentration of creatinine} \times \text{time}} \right) \times 100\%
\]

Figure 2 Longitudinal profiles. A daily, weekly, biweekly and monthly longitudinal profile of the serial urinary neopterin:creatinine ratios for a 35 year-old female with secondary progressive multiple sclerosis. The profiles are derived from the same data. The dotted line represents the upper limit of normal for the urinary neopterin:creatinine ratios (198 μmol/mol)
a period of 48 h. CSF dynamics are also affected by a diurnal variation. CSF production is not homogenous as a function of time of day. CSF aqueductal flow rates vary by as much as a factor of 3.5, with minimum CSF production occurring around 18 h 00 (12 ± 7 mL/h) and a maximum at approximately 02 h 00 (42 ± 2 mL/h). As CSF is a major outflow conduit for inflammatory markers produced in the central nervous system sampling during maximal CSF flow would seem logical. The first urine specimen of the day has the advantage of integrating events over this period of maximal CSF flow.

Figure 3: Cross-sectional versus a longitudinal study. Due to the dynamic nature of disease activity in MS, the proportion of randomly selected patients with a raised urinary neopterin:creatinine ratio (greater than 198 nmol/mmol) is significantly greater in a longitudinal study (daily sampling for 8 weeks) compared to a cross-sectional study (one sample).

Figure 4: Natural integrator. A 24 h time course plot of the average volume of urine produced per hour in a normal adult. The diurnal variation is due to a nocturnal increase in the production of antidiuretic hormone (ADH). An early morning spot specimen (07 h 00) captures the nocturnal excretion of substance over a prolonged period of time (shaded light grey area). This allows urine to act as a natural integrator providing information on the average excretion of a substance over a defined period (area under the curve).
Anatomical constraints of CSF compared to urine

The lumbar subarachnoid space is a cul-de-sac. CSF obtained from lumbar punctures cannot realistically provide accurate information on inflammatory events in the brain which occur distal to the CSF outflow foramina of the fourth ventricle, i.e. inflammation in relation to the surface of the brainstem, cranial nerves, and cerebral hemispheres. In addition, the extracellular space of intraparenchymal lesions may not necessarily communicate with the free CSF space, preventing the detection of inflammatory mediators in the CSF. Substances measured in the blood and urine are not affected by such anatomical constraints and represent a summation of brain-derived products. Whether plasma or urinary levels of a particular substance accurately reflect disease activity in the central nervous system is a moot point. Combined CSF, blood and urine studies may help to answer this question, although more invasive human and/or animal studies are still required.

Twenty-four hour versus a spot urine sample

A 24 h urine collection should, at least theoretically, provide a better index of the daily production of an inflammatory marker than a spot urine specimen. Unfortunately 24 h urine collections are laborious, impractical and notoriously unreliable. It is very difficult to ensure patient compliance especially if frequent collections are planned. We have found that the total excretion of neopterin over a 24 h period correlates closely with the neopterin : creatinine ratio of an early morning spot urine specimen collected on the same day (Figure 6). Therefore, in the case of neopterin a spot urine specimen is sufficient and 24 h collections are unnecessary. The first specimen of the day also has the advantage of capturing the overnight period during which the production of neopterin is maximal. Bacterial contamination and growth in 24 h samples stored at room temperature can significantly affect results. For example, we have found a progressive increase in urinary nitrite levels (presumably due to bacterial production of nitrite) in some urine specimens maintained at room temperature over a 24 h period (unpublished observations).

Variable concentration of urine

As total urinary volume is highly variable (1.0-3.5 litre/24 h) the concentration of a substance will depend on urine output. Therefore it is common practice to use an internal reference, such as creatinine, to correct for urinary concentration. Creatinine is excreted by glomerular filtration, with an additional 10-20% by tubular secretion. No significant tubular reabsorption occurs therefore creatinine is a gross indicator of urinary concentration, and an ideal internal reference for other substances excreted in the urine which do not undergo tubular reabsorption. Using creatinine as an internal reference for proteins or peptides, however, may give unreliable results because peptides undergo significant tubular reabsorption. If tubular flow is decreased for example by dehydration, increased peptide reabsorption and increased tubular excretion of creatinine will lower the peptide : creatinine ratio. The opposite would occur with high tubular flow rates. In addition, urinary protein excretion is affected by factors which do not necessarily alter renal creatinine excretion such as mild glomerular and tubular dysfunction, erect posture, and physical exertion which all increase urinary protein excretion.
Using a serum protein, e.g. albumin, as an internal reference for urinary proteins and peptides will eliminate these problems. Despite these reservations there is a reasonable correlation between urinary creatinine and albumin levels (Figure 7). This correlation however only holds for patients with normal renal function: it is lost in patients with significant proteinuria due to tubular or glomerular dysfunction (unpublished observations).

Whitaker et al. have reported a positive correlation between urinary creatinine concentration and disability in patients with MS. We have confirmed their findings using early morning spot specimens (unpublished data). The reasons for the correlation between disability and urinary creatinine levels are currently speculative. It is unlikely to be due to a change in muscle mass. MS patients with urinary symptoms are known to restrict their fluid intake to control
troublesome urinary frequency. As these patients tend to be more disabled the higher urinary creatinine concentrations may simply reflect voluntary dehydration. Finally, creatinine levels are related to muscle mass, with males having a urinary creatinine concentration approximately 1.2–1.4 times higher than females. It is therefore important to correct for this when making comparisons between male and female subjects. We use an empirical correction factor of 1.2 which has been derived from 40 normal control subjects (unpublished data).

Types of mediators
Although urinary measurement is best suited to substances that are excreted in the urine with minimal tubular reabsorption, e.g. neopterin and nitrate plus nitrite, proteins and peptides can also be measured (Table 1).

Urinary tract infections
Asymptomatic bacterial colonisation of the bladder and urinary tract infection is common in patients with MS. This is a particular problem in those with bladder dysfunction who are unable to void completely. In addition some patients require intermittent self-catheterisation or rarely an indwelling catheter. These factors encourage bacterial growth and metabolism, which could affect metabolites that are present in urine. This has to be considered and controlled for when measuring urinary metabolites, particularly proteins or peptides. We have found that nitrite, measured with the Griess reaction, is a useful screening test for bacterial colonisation of urine and one which can be performed on stored samples. It does, however, have a false negative rate of approximately 30%.

Conclusions
Monitoring the urinary excretion of various markers of inflammation and demyelination is a relatively unexplored area in MS research. It has distinct advantages over other body fluids in that it is more accessible, can be sampled more frequently, is a natural integrator and is ideally suited for substances that are excreted in the urine with minimal tubular reabsorption, e.g. neopterin and nitrate plus nitrate, proteins and peptides can also be measured (Table 1).

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