MECHANISMS OF MYELIN DEGRADATION
IN MULTIPLE SCLEROSIS

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ABSTRACT

The role of T cells and their products in the pathology of multiple sclerosis (MS) has been studied. A quantitative method has been used to investigate the breakdown of cell free human myelin in vitro. The assay measures the release of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), an intrinsic myelin component.

Sera from MS and other neurological diseases (OND) patients was generally devoid of any myelin degradative activity. However, peripheral blood mononuclear cells (PBMC) from 42% (41/97) of MS patients caused significant losses of CNPase activity from the myelin as did PBMC from 58% (15/26) of control patients with another organ-specific autoimmune disease, rheumatoid arthritis (RA). No correlation was seen in the MS group between in vitro cellular myelin degradative activity and disease status. Significant myelin degradation by cells occurred with only 10% (2/20) of OND PBMC samples and with none of the healthy control samples (n=30).

To further investigate the factor(s)/mechanism responsible for in vitro myelin degradation by MS PBMC the possibility that loss of CNPase activity was caused by activated T cells was investigated. Expression of the activation markers, interleukin-2 (IL-2) receptor and MHC class II antigen (HLA-DR) was measured. No correlation was apparent between activation of cells and loss of CNPase activity caused by these cells. Cell supernatants from unstimulated, mitogen stimulated (PHA and Con-A) and antigen stimulated (human myelin basic protein and tuberculin purified protein derivative) MS PBMC were generally unable to cause myelin degradation. The possibility of direct cytokine involvement in myelin damage was investigated by addition of recombinant cytokines to myelin but, no significant loss in CNPase activity was seen.

A possible relationship between myelin basic protein (MBP) T cell reactivity (as measured by thymidine incorporation) and cellular myelin degrading activity was also investigated however, no correlation was seen. Therefore two human MBP specific T cell lines derived from a healthy donor and an MS patient were added to myelin in the presence of autologous antigen presenting cells to see whether myelin antigen specific cells were capable of myelin damage in vitro. Neither of the cell lines caused a significant loss of CNPase activity from myelin.
Finally, in order to identify the cell type(s) required for in vitro myelin degradation, the T cell population was enriched and added to myelin with or without autologous macrophages. Macrophages alone at a concentration similar to that observed in a PBMC sample were unable to cause a significant loss in CNPase activity of the myelin. However, when an enriched population of T cells was added, significant myelin degradation was observed. Preliminary experiments investigating serine esterase (SE) expression by MS and healthy individuals' PBMC showed that there was an increase in the number of MS CD8+ lymphocytes expressing SE after 24 h incubation with myelin. The expression of SE by healthy individuals' CD8+ lymphocytes did not change after incubation with myelin.

In this thesis, I aim to test the hypothesis that the myelin degrading activity seen with both MS and RA PBMC is a non-specific cellular response shown by chronically activated immune cells or their products occurring in chronic inflammatory autoimmune diseases, and is not confined to MS.
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4. Tubercul in purified protein derivative
5. Concanavalin - A
6. Lipopolysaccharide
7. Phytohaemagglutinin - P

2.2. CYTOKINES

1. Interleukin-1, human recombinant
2. Interleukin-2, human recombinant
3. Interleukin-2, natural human
4. Interferon y, rat recombinant
5. Tumour necrosis factor α, recombinant

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<td>adherent cells</td>
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<td>AET</td>
<td>aminoethylisothio-uronium bromide</td>
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<td>AMLR</td>
<td>autologous mixed lymphocyte reaction</td>
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<td>APAAP</td>
<td>alkaline phosphatase-anti alkaline phosphatase</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<td>BBB</td>
<td>blood brain barrier</td>
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<td>BBG</td>
<td>bovine brain gangliosides</td>
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<td>BLT</td>
<td>benzylxocarbonyl-L-lysine thiobenzyl ester</td>
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<td>BP</td>
<td>basic protein</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CD</td>
<td>cluster designation</td>
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<td>CFA</td>
<td>complete Freund's adjuvant</td>
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<td>CNPase</td>
<td>2',3'-cyclic nucleotide 3'-phosphodiesterase</td>
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<td>CNS</td>
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<td>Con-A</td>
<td>concanavalin-A</td>
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<td>CPM</td>
<td>counts per minute</td>
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<td>chronic relapsing experimental allergic encephalomyelitis</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>CVA</td>
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<td>EDDS</td>
<td>expanded disability status score</td>
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<td>ELISA</td>
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<td>GFAP</td>
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<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<td>human T-lymphotropic virus</td>
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<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IFN</td>
<td>interferon</td>
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<td>interleukin</td>
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<td>KLH</td>
<td>keyhole limpet-haemocyanin</td>
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<td>LCA</td>
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<td>lipopolysaccharide</td>
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<td>MAb</td>
<td>monoclonal antibody</td>
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<td>MAC</td>
<td>membrane attack complex</td>
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<td>Acronym</td>
<td>Description</td>
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<td>MAG</td>
<td>myelin associated glycoprotein</td>
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<td>myelin basic protein</td>
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<td>major dense line (myelin)</td>
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<td>major histocompatibility complex</td>
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<td>MND</td>
<td>motor neurone disease</td>
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<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>NA</td>
<td>non-adherent</td>
</tr>
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<td>NAWM</td>
<td>normal appearing white matter</td>
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<td>NBBS</td>
<td>new born bovine serum</td>
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<td>N-CAM</td>
<td>neural cellular adhesion molecule</td>
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<td>NEAA</td>
<td>non-essential amino acids</td>
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<td>NF</td>
<td>neurofilament</td>
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<td>NHS</td>
<td>normal human serum</td>
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<td>NK</td>
<td>natural killer</td>
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<tr>
<td>NOD</td>
<td>non obese diabetic</td>
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<td>OND</td>
<td>other neurological disease</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PBL</td>
<td>peripheral blood lymphocyte</td>
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<td>peripheral blood mononuclear cells</td>
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<td>phosphate buffered solution</td>
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<td>prostaglandin E</td>
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<td>polymerase chain reaction</td>
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<td>PHA-P</td>
<td>phytohaemagglutinin</td>
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<td>PLP</td>
<td>proteolipid protein</td>
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<td>PPD</td>
<td>tuberculin purified protein derivative</td>
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<tr>
<td>PWM</td>
<td>pokeweed mitogen</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<td>REAE</td>
<td>relapsing experimental allergic encephalomyelitis</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SE</td>
<td>serine esterase</td>
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<td>SI</td>
<td>stimulation index</td>
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<td>sIL-2R</td>
<td>soluble interleukin-2 receptor</td>
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<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<tr>
<td>SN</td>
<td>supernatant</td>
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<td>SRBC</td>
<td>sheep red blood cells</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler's murine encephalomyelitis virus</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TSP</td>
<td>tropical spastic paraparesis</td>
</tr>
<tr>
<td>VLA</td>
<td>very late antigen</td>
</tr>
<tr>
<td>VP</td>
<td>ventriculopuncture</td>
</tr>
<tr>
<td>WP</td>
<td>Wolfgram protein</td>
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ACKNOWLEDGEMENTS

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CHAPTER 1

CELLULAR IMMUNITY IN MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). It affects approximately one in 10,000 people in this country and is a particularly devastating disease as it primarily affects young adults, is chronic and has an unpredictable course. The first comprehensive account of MS was by Jean Charcot in 1877 who identified the clinical features of the disease 'la sclerose en plaques' as visual defects, dysarthric speech, ataxia, tremor and paresis. These symptoms reflect the high incidence of lesions occurring in areas such as the optic nerve, spinal cord, brain stem and cerebellum. The clinical symptoms can, however, be highly variable which makes diagnosis difficult, and despite recent advances in the refinement of cerebrospinal fluid (CSF) analysis, evoked potentials and magnetic resonance imaging (MRI), diagnosis is largely based on clinical criteria.

Although first described over a century ago the aetiology and pathogenesis are still unknown, however, an immunological process is thought to be one of the major elements involved in the disease with contributing environmental and genetic factors. The purpose of this chapter is to review the current hypotheses on the pathogenesis of MS based on both past and present research.

1.1 EPIDEMIOLOGY

Numerous epidemiological studies have provided evidence to suggest that an environmental factor (possibly an infectious agent) may contribute to the aetiology of MS. Geographical variations in the prevalence of MS indicate that western and northern Europe, southern Canada and northern USA have increased frequencies of between 30 and 80 MS cases per 100,000 of the population, the prevalence in southern Europe, southern USA and Australia in comparison is 5-15 cases per 100,000. The lowest rate of 0-4 MS cases per 100,000 individuals has been reported in Asia and Africa (reviewed by Kurtzke, 1983). This variation of incidence with latitude may therefore reflect the involvement of an environmental agent. Migration studies also support the hypothesis that environmental factors may be involved in
MS. Reports from South Africa showed a relatively high risk of developing the disease amongst northern European migrants who had settled in South Africa in adult life, whereas the prevalence was low in those who had been born there. It was thus concluded that the critical age for migration was 15 years and that people migrating below this age carried the risk level from their native land for developing MS. Similar observations have been reported from Israel and the USA (reviewed by Compston, 1986).

The recognition of clusters of cases in restricted geographical regions within a relatively circumscribed period of time also favours the hypothesis of an environmental factor. In the Faroe Islands, MS apparently appeared for the first time during the occupation by British troops in the second world war with a peak of 64 cases per 100,000 individuals occurring by 1960, and subsequently declining to 30 cases per 100,000 in 1980 (Kurtzke, 1986). A similar though less striking epidemic may also have occurred in Iceland (Kurtzke et al., 1982). These epidemiological studies all support the theory that an environmental factor may be involved in the aetiology of MS, however there is no indication as yet as to what the infective agent might be.

1.2 GENETIC FACTORS

Irrespective of the latitudinal prevalence rates, certain racial groups have a resistance to MS whilst others are more susceptible. For example, the prevalence of MS is high in people of Scandinavian origin, whereas the disease is rare in the Chinese and Japanese and other ethnic groups where the frequency of cases would be expected to be higher due to the reported relationship between latitude and prevalence of the disease (Compston, 1986).

In a Canadian study of twins, Ebers and colleagues (1986) showed that there was a concordance rate of 25.9% in monozygotic twins but only 2.3% in dizygotic twins which was similar to that for siblings (1.9%). In addition, MS has been found to occur at higher rates in first degree relatives (reviewed by Kurtzke, 1983). It has also been observed that clinically unaffected monozygotic twins may have CSF oligoclonal bands (a hallmark of MS) similar to those found in the concordant pair (Xu and McFarlin, 1984), and MRI of the CNS of unaffected twins showed abnormalities consistent with MS (Ebers et al., 1986).
The putative genes encoding susceptibility to MS, have however, yet to be identified although much emphasis has been placed on the genes coding for the human leukocyte antigen (HLA) molecules which are involved in the immune response.

The major histocompatibility complex (MHC) located on chromosome 6 consists of a series of loci encoding the highly polymorphic polypeptide chain of the HLA class I (A, B and C) molecules, and the alpha and beta polypeptide chains of the Class II molecules (DP, DQ, DR and DZ) (Moeller, 1983). Class I antigens generally act as restricting elements for cytotoxic/suppressor CD8+ T lymphocytes and Class II molecules for helper/inducer CD4+ T cells, however this classification is not absolute as some CD4+ Class II restricted T cells are cytotoxic (Jacobson et al., 1984). The first association in MS between susceptibility and specific HLA haplotypes was with the Class I molecules A3 and B7 (Jersild et al., 1973). In northern Europeans a strong association between the Class II molecules DW2 and DR2 (which are both in linkage disequilibrium with A3 and B7) was later seen (Compston et al., 1986a). More recently a weak association with DQw1 has been shown (Francis et al., 1987). The significance of these associations remains unclear as there are many exceptions to the genes for DR2 or DQw1 conveying susceptibility. There does however, seem to be mounting evidence to suggest that gene products encoded within the HLA complex such as tumour necrosis factor (TNF) and complement may influence susceptibility to MS through an effect on regulation of the immune response.

1.3. EVIDENCE FOR A VIRAL AETIOLOGY IN MULTIPLE SCLEROSIS

Epidemiological investigations have suggested that an environmental agent may be a causative factor in MS, and much research has focused on a viral involvement. Many viruses have been suggested as being involved in the pathogenesis of MS, although no single one has yet gained universal acceptance as a causative factor.

Much research has concentrated on the measles virus ever since the initial report of elevated levels of measles antibody in the sera of MS patients (Adams and Imagawa, 1962). Elevated intrathecal production of measles virus antibody has been detected as well as viral ribonucleic acid (RNA) in some post-mortem MS brains (Norrby et al., 1974; Haase et al., 1981). There is however, increased synthesis of antibodies in the CSF directed against other viruses such
as vaccinia, rubella, mumps, herpes simplex type I, Rous sarcoma, Epstein-Barr and the corona viruses (Norrby, 1978). The relevance of these raised antibody titres is questionable since elevated antibody levels can also be found in control patients’ CSF suggesting the response is not specific for MS (Forghani et al., 1978). Measles and herpes simplex genomic material, which has been detected in MS brain tissue, has also been found in controls (Fraser et al., 1981; Haase et al., 1981).

More recently, human T-lymphotropic viruses (HTLV) have been implicated in the aetiology of MS. Tropical spastic paraparesis (TSP) is a chronic inflammatory demyelinating disease of the spinal cord that causes paralysis of the legs and has been associated both serologically and with a cell mediated immune response to HTLV-I (Jacobson et al., 1990). The possibility that MS, which resembles TSP in some cases, is associated with HTLV-I infection has generated considerable interest. Koprowski and colleagues (1985) reported elevated anti HTLV antibodies in CSF and sera of some MS patients and also detected HTLV-I RNA in the CSF cells of a few patients. Hauser and co-workers (1986a) however, failed to detect any HTLV-like sequences in MS CNS tissue, or in cells isolated from the CSF or peripheral blood, and found no difference between serum reactivity to HTLV-1 in MS and control groups. Also, no difference was found between MS and control levels of CSF HTLV-I antibody which was confirmed by Karpas and colleagues (1986) who failed to detect antibodies to HTLV-I in sera and CSF of MS patients. As elevated antibody titres to other viruses are frequently found in MS CSF, as well as viral genomic material in the CNS, the significance of Koprowski’s findings was doubtful especially since only two healthy controls were analysed for HTLV-I genomic material.

It was not until 1989 that the relationship between MS and a retrovirus reappeared. Two reports extended the original study of Koprowski and identified HTLV-I deoxyribonucleic acid (DNA) in peripheral blood mononuclear cells (PBMC) of all 6 MS patients tested, but in only one of the healthy individuals tested (n=35) (Reddy et al., 1989). Greenberg and colleagues (1989) also detected sequences homologous to retroviral DNA in the PBMC of 6 of the 21 MS patients they tested (none had serological evidence of a retroviral infection) but not in the PBMC of 21 healthy individuals tested. Subsequent studies have amplified HTLV-I DNA sequences from brain and spinal cord tissue of 3 of 10 MS patients analysed (Antel et al., 1990). Both groups used the polymerase chain reaction (PCR) which detects and amplifies low concentrations of viral material, allowing molecular cloning and sequence
analysis of the amplified DNA. One problem with the PCR technique is the possibility that the test system is contaminated with retrovirus already present in the analysing laboratory. Since Reddy and co-workers reported that the amplified DNA material detected was identical in each of the 6 patients, the results should be treated with caution until the association is confirmed by other laboratories. Subsequent studies have failed to confirm the findings of both Reddy and Greenberg (Bangham et al., 1989; Richardson et al., 1989; Nishimura et al., 1990; Oksenberg et al., 1990) and at present there is insufficient evidence to indicate that HTLV-I is involved in the pathogenicity of MS. The possibility that the published positive findings may involve patients who have TSP rather than MS should also be considered. Recently, Perron and colleagues (1991) reported on the isolation of a new retrovirus from an MS patient’s CSF which was antigenically different from any known human retrovirus. This however has yet to be confirmed.

Other viruses such as the paramyxovirus SV5 have also been implicated in the aetiology of MS, and this will be discussed later.

1.4 HISTOPATHOLOGY

The main pathological finding in MS is the presence throughout the white matter of scattered lesions characterised by the breakdown of the myelin sheath with relative sparing of axons. The extent and localisation of the plaques of demyelination are relatively unpredictable but there are distinct regions of predilection, such as the periventricular areas and optic nerve. Interestingly, typical lesions apparently without clinical expression in life, are commonly found in MS brains by MRI and at post mortem (McDonald & Barnes, 1989).

In active lesions, where perivascular inflammation is associated with myelin loss, the cuff consists predominantly of T lymphocytes and macrophages, with varying numbers of plasma cells being present (Nyland et al., 1982). A higher proportion of CD8^+ T cells as compared to CD4^+ are generally seen at the plaque edge (Traugott et al., 1983; Hauser et al., 1986b; McCallum et al., 1987). However, there have been reports of both CD4^+ cells penetrating the normal appearing white matter (NAWM) adjacent to the lesion (Traugott et al., 1983) and CD8^+ cells being the predominant cell type (Woodroffe et al., 1986; Hauser et al., 1986b; Hayashi et al., 1988). This conflicting immunohistological data could well reflect the
heterogeneity of the MS lesions. Apart from these differences, a decreased number of CD4+ cells reacting with the anti-2H4 monoclonal antibody (MAb) has been reported (Sobel et al., 1988; Hayashi et al., 1988). This MAb selectively stains a subpopulation of T cells thought to be suppressor-inducers (Morimoto et al., 1985a), suggesting that there may be a defect in the down-regulation of the local immune response.

Activated T cells expressing the interleukin-2 (IL-2R) receptor (CD25) as well as lymphocytes containing IL-2 have been identified in the perivascular infiltrates of active lesions as well as other immunoregulatory mediators, interleukin-1 (IL-1) and interferon-gamma (IFN-γ) suggesting an active immune mechanism in the pathogenesis of MS (Bellamy et al. 1985; Woodroffe et al., 1986; Hofman et al., 1986; Traugott and Lebon, 1988a). More recently Hofman and colleagues (1989) have identified TNF positive cells at the edge of chronic active plaque lesions in six MS brains. Double staining indicated the TNF was predominantly associated with astrocytes, although macrophages also stained positive, and until in situ hybridisation with molecular probes for the TNF is carried out, the cellular source of the TNF cannot be confirmed.

MHC class II positive cells, which are required for local antigen presentation, are normally not expressed in the CNS, although there have been some reports of scattered class II in normal human white matter (Lampson & Hickey, 1986). Hayes and colleagues (1987) also reported the presence of class II positive microglial cells in the NAWM of control brains. Whether this expression is constitutive or a modulation in response to an unknown insult is uncertain. However, in MS, class II positive cells have been identified in abundance at the hypercellular edge of plaques where demyelination is ongoing, with a decrease in number towards the plaque centre and in adjacent white matter. A range of cell morphologies has been identified in MS CNS expressing class II antigens which includes, lymphocytes, macrophages, astrocytes and more recently endothelial cells (Traugott et al., 1985; Hofman et al., 1986; Hayashi et al., 1988).

The presence of immunoregulatory mediators, as well as activated T cells and class II positive cells, all suggest that a local immune response directed against an unidentified antigen is involved in the pathogenesis of MS.
1.5. IMMUNE ABNORMALITIES IN MULTIPLE SCLEROSIS

Various proposals have been made implicating cellular and humoral factors in the pathogenesis of MS which suggest a possible autoimmune basis for the disease. It is an inflammatory condition exclusively affecting the white matter of the CNS, with a characteristic hallmark of increased intrathecal synthesis of oligoclonal immunoglobulin G (IgG) and histologically perivascular inflammatory cuffs close to the demyelinating lesions containing activated T cells, immunoregulatory mediators and antigen presenting cells. The CSF also contains increased numbers of activated T cells and irregularities in CD4^+/CD8^+ ratios. Although all this evidence points to a localised immune reaction within the CNS there are also immune irregularities associated with the peripheral immune compartment.

1.5.1. Intrathecal production of IgG and IgM

The increased intrathecal synthesis of IgG and IgM and presence of oligoclonal IgG in almost all MS patients is the most consistent immunological change found in MS (reviewed by Walsh and Tourtellotte, 1983). Due to the oligoclonal nature of the immunoglobulin it has been suggested that the specificity might be directly related to the disease process, implicating an infectious agent in the aetiology of MS. However, high levels of numerous different virus specific antibodies exist in the CSF and in most cases represent only a small fraction of the total Ig present (Vartdal et al., 1980). Nevertheless, antibody directed against the measles virus appears to be the most consistent antiviral antibody found in MS CSF (reviewed by Norrby 1978).

Goswami and colleagues (1987) suggested that the paramyxovirus SV5, a Simian virus closely related to human parainfluenza type 2 virus, may be a major intrathecal immunogen in MS, as in some MS patients' CSF antibodies against SV5 represent a significant proportion of the oligoclonal IgG. This finding has not been confirmed, and even though Vandvik and Norrby (1989) found that the CSF of 7/30 MS patients contained antibodies reacting with SV5, they were not associated with the oligoclonal IgG and could be explained as potentially cross-reacting antibodies to other paramyxoviruses known to be human pathogens.

Antibodies and plasma cells secreting antibodies to the myelin components, myelin basic protein (MBP), myelin associated glycoprotein (MAG), proteolipid protein (PLP) and myelin
oligodendrocyte glycoprotein (MOG) have also been identified in MS CSF (Emerudh et al 1987; Moller et al., 1989; Sun et al., 1991a) and their possible role in the demyelinating process will be discussed later.

The significance of the oligoclonal antibody production in MS has therefore yet to be elucidated. It is not known whether it represents a specific response to an autologous or exogenous antigen, or is due to non-specific activation of B cells since it has been demonstrated that there are intrathecal antibody responses to unrelated antigens in patients with other CNS inflammatory diseases.

1.5.2. Activated T lymphocytes in cerebrospinal fluid and peripheral blood

The CSF also contains significantly increased numbers of activated T lymphocytes in comparison to the peripheral blood. Noronha and colleagues (1980) using flow cytometric techniques were the first to show increased numbers of lymphoblastoid cells in MS patients' CSF as measured by RNA and DNA content. They later demonstrated that over half of activated CSF cells were CD4^ lymphocytes (Noronha et al., 1985). Using the MAb anti-Tac, specific for the IL-2 receptor (Uchiyama et al., 1981) it was shown that the expression of IL-2 receptors is significantly greater in lymphocytes from MS patients' CSF compared with those from peripheral blood suggesting that a localised immune response may be occurring (Bellamy et al., 1985). Bellamy and colleagues also reported that there was a small but significant increase in the proportion of IL-2R^ peripheral blood lymphocytes (PBL) in MS patients compared to healthy controls or those with non-infectious disease. However, no correlation was found between MS disease activity and frequency of IL-2R bearing lymphocytes in blood. These results were confirmed by Tournier-Lasserve and co-workers (1987) who reported increased numbers of CSF cells expressing IL-2 receptors and HLA class II molecules. Hafler and colleagues (1985c) could not demonstrate increased IL-2R expression, but did observe a high proportion of Tal^ cells in the CSF as compared to the blood. Tal is a marker of post differentiated T cells and indicates the presence of memory cells within the CSF (Hafler and Weiner, 1987). In another study the concentration of soluble IL-2 receptors (sIL-2R) was quantified in MS serum and CSF and was shown to be higher in patients in relapse as compared to those in remission or to controls (Adachi et al., 1990). Soluble IL-2 receptors are released from activated T cells with IL-2 (Rubin, 1985) and the levels are increased in autoimmune disorders such as systemic lupus erythematosus.
(SLE) and rheumatoid arthritis (RA) (Wolf and Brelsford, 1988; Manoussakis et al., 1989). Increased serum and CSF IL-2 has been correlated with increased disease activity in MS (Gallo et al., 1988) and it has therefore been suggested that sIL-2R may be a useful marker of disease activity in MS.

1.5.3. Phenotypic and functional abnormalities of cerebrospinal fluid and peripheral blood lymphocytes

Studies of MS peripheral blood and CSF mononuclear cells, in comparison to those of other neurological disease (OND) and healthy individuals, have shown irregularities in the ratio of CD4⁺ to CD8⁺ lymphocytes in MS patients. It has been demonstrated that there is a selective decrease in CD8⁺ cells and an increase in the CD4⁺/CD8⁺ ratio in patients with active disease as compared to other neurological diseases (OND) and controls (Reinherz et al., 1980; Bach et al., 1980). Subsequent studies have since confirmed the correlation between reduced CD8⁺ levels and clinical activity (Huddlestone and Oldstone, 1982; Compston et al., 1983; Paty et al., 1983).

In addition, in vitro T cell function as assessed by response to conconavalin-A (Con-A) and pokeweed mitogen (PWM) have been shown to be abnormal in MS patients leading to suggestions that there is an abnormality in immunoregulation in MS. These studies were extended to assess Con-A induced PBMC suppression of the mitogenic response of autologous PBMC or PWM induced IgG secretion by B cells in MS. It was concluded that there were abnormalities of T cell function in MS which could not however, be correlated with the CD8⁺ phenotype (Antel et al., 1984; Tjernlund et al., 1984; Oger et al., 1986; Hughes and Compston, 1988).

Another reported T cell irregularity in MS patients is a decreased response in the autologous mixed lymphocyte reaction (AMLR) of progressive patients (Hafler et al., 1985b; Hirsch et al., 1986; Baxevenis, 1987). The AMLR measures the response of T cells to self MHC molecules and as the CD4⁺ lymphocyte is the major population that proliferates it was suggested there may be a functional deficit in the CD4⁺ subset (Hafler et al., 1989).

Whether T cell irregularities in immunoregulation can be associated with a particular lymphocyte phenotype and correlated with the clinical course of MS still remains to be
determined.

In attempts to understand the role of lymphocyte subsets in the regulation of the immune response the T cell surface markers CD45 and CDw29 have recently been studied and characterised (Morimoto et al., 1985a&b; Smith et al., 1986; Takeuchi et al., 1987). CD45R is part of the leucocyte common antigen (LCA) family and CDw29 is a member of the adhesion molecule family (Sanders et al., 1988). CD45 is expressed in two different isoforms, CD45RA and CD45RO and T cell activation results in a conversion of CD45RA to the CD45RO isoform of the LCA (Akbar et al., 1988; Sanders et al., 1988).

CD45RA/RO and CDw29 can be present at low or high levels on the T cell membrane and their expression is thought to characterise different T cell subpopulations. CD45RA is thought to be a marker of suppressor-inducer cells and CD45RO/CDw29 a marker of helper-inducer T cells (Morimoto et al., 1985a&b). More recently, an alternative interpretation is that the expression of CD45RA and CDw29 indicate different stages of T cell maturation rather than distinguishing between separate functional lineages. The T cells expressing high levels of CD45RA are thought to be 'naive' CD4+ cells that do not respond to recall antigens whereas CD45RO / CDw29 high co-expression on T cells is suggestive of 'memory' lymphocytes that have been activated by prior exposure to antigen (Sanders et al., 1988). Support for the latter concept is that CD45RA cells can be converted to CD45RO / CDw29 upon activation with mitogen.

Rose and colleagues (1985) were the first to show that the frequency and absolute levels of CD4+ CD45RA peripheral blood lymphocytes (PBL) were significantly reduced in MS patients with active disease. This selective decrease also occurred in progressive MS patients but not in patients with stable disease activity, ONDs and healthy individuals (Morimoto et al., 1987).

Rose and co-workers (1988) then studied the expression of CD45RA serially in a group of patients and controls over one year. They found significant depletions in the CD4+ CD45RA subpopulation resulting in an increased CD4+ CD45RO / CD4+ CD45RA ratio in 7/9 relapsing-remitting patients within one month of clinical symptoms being reported, whereas no significant changes were seen in the control group. They concluded that the loss in CD4+ CD45RA subset may be linked to a functional loss of suppression which correlated with
increased disease activity. Chofflon and colleagues (1988) also found a decrease in CD4+ CD45RA cells in progressive patients which they correlated with a loss in suppression as measured by AMLR and AMLR-induced suppression of IgG synthesis. More recently it has been demonstrated that CD4+ CDw29 cells predominate in the peripheral blood of patients during the attack phase of relapsing-remitting MS (Porrini et al., 1992).

Irregularities in CSF T cell ratios have also been noted in MS, the predominant cell type being the CD4+ lymphocyte (Cashman et al., 1982; Hauser et al., 1983). It has been shown that there is a marked reduction in CD4+ CD45RA cells in the CSF as compared to the blood in MS patients. In addition, there is an increase in CD4+ CD45RO / CDw29 cells (Chofflon et al., 1989; Hedlund et al., 1989; Matsui et al., 1990). These phenotypic irregularities are not confined to MS as decreases in CSF CD4+ CD45RA and increases in CD4+ CDw29 cells have been seen in the CSF of both inflammatory and non-inflammatory OND patients. In addition, an increase in CSF CD4+ CD45RO / CDw29 has been reported in healthy individuals (Salonen et al., 1989; Hedlund et al., 1989; Matsui et al., 1990). The high proportions of CD4+ CD45RO / CDw29 T cells and low proportion of CD4+ CD45RA cells have also been observed in the CSF of patients with other inflammatory diseases including Guillain-Barré syndrome (GBS) and SLE and in the synovial fluid of RA patients (Chofflon et al., 1989). It has also been shown that there is a decrease in CD4+ CD45RA cells in the CNS of patients who had died of MS compared to the CNS of patients who had died from viral encephalomyelitis (Sobel et al., 1988). Whether this decrease in a particular CD4+ phenotype is more common in autoimmune diseases requires further studying.

Decreased numbers of CD4+ CD45RA cells in the blood and CSF of MS patients (and in diseases of autoimmune origin) and the increase in CD4+ CD45RO / CDw29 cells in the CSF may thus suggest that the T cells have previously been exposed to antigen. Hafler and colleagues, (1985c) reported an increase in MS CSF cells expressing Ta1, a marker for previously activated T cells including memory cells, and the reports of increased numbers of IL-2R+ T cells in the CSF compared to the blood are both in agreement with this hypothesis.

1.5.4. T Cell reactivity

Further evidence to support an autoimmune process in the pathogenesis of MS would be the identification of CNS autoantigen-specific T cells in either blood, CSF or CNS tissue. The
similarity of MS to chronic relapsing experimental allergic encephalomyelitis (CREAE) which can be produced in mice by injection of MBP-specific T cell lines or clones (Raine et al., 1984; Zamvil et al., 1985), has led to the search for MBP specific T cells in MS, as this myelin specific protein might be one of the target antigens in MS.

Using a variety of in vitro techniques many investigators have demonstrated T cell responses to MBP among the PBMC of MS patients (Hughes et al., 1979, Traugott et al., 1979; Brinkman et al., 1982; Ilyas and Davison, 1983; Lisak and Zweiman, 1985; Tournier-Lasserve et al., 1986; Selmay et al., 1988b). CSF T cell reactivity to MBP has also been observed (Lisak and Zweiman, 1977; Colby et al., 1977; Vandenbark et al., 1979; Offner et al., 1979; Richert et al., 1983; and Selmay et al., 1988b). Nevertheless, others have been unable to detect any myelin reactive T cells in the CSF when using single cell cloning techniques (Fleischer et al., 1984; Hafler et al., 1985a) which led to the suggestion that the myelin-specific T cells are sequestered at the lesion site. Hafler and co-workers (1987) investigated the specific myelin antigen reactivity of T cells cloned from the blood, CSF and brain of an MS patient. The clones showed no reactivity to MBP, PLP, or whole myelin, whereas there was significant reactivity to MBP in the CSF of a patient with post viral encephalomyelitis where MBP is thought to be the target antigen (Lisak and Zweiman, 1977; Johnson et al., 1984). The significance of not finding MBP specific T cells at the site of demyelination is questionable since it has been shown that in experimental allergic (EAE) the actual frequency of MBP reactive T cells in the CNS is low compared to that measured in the lymph nodes draining the MBP inoculation site, indicating that only a very small number of MBP T cells are required in the CNS for disease initiation. (Fallis et al., 1987). They suggest that the lymph nodes and spleen act as reservoirs for MBP reactive cells during the chronic phase of EAE. Also it could be argued that it is only the lymphocytes obtained from the CNS at the very earliest stages of disease which might have myelin specificities and that with time, the frequency of MBP reactivity may be lost and replaced by a chronic nonspecific inflammatory reaction. How these MBP reactive cells are involved in the pathogenesis of the EAE is not known but will be discussed later.

The relevance of myelin specific T cells in the pathogenesis of MS is also unclear as OND patients and healthy individuals also display peripheral blood reactivity to MBP (Hughes et al., 1979; Burns et al., 1983; Lisak and Zweiman, 1985; Johnson et al., 1986; Tournier-Lasserve et al., 1988). Schluesener and Werkerle (1985) showed that MBP specific T cells
could be obtained from non-immunized rats and that they were encephalitogenic when transferred into a syngeneic recipient. Thus the existence of MBP-specific T cells in individuals other than patients suffering from MS does not rule out the possibility of their pathogenic role in MS, particularly if there is a defect in T cell suppression in MS patients.

Recent studies suggest that MS patients differ in their reactivity to specific T cell epitopes of the MBP molecule when compared to healthy individuals and OND patients (Baxevenis et al., 1989; Richert et al., 1988) and that T cell recognition of these potentially encephalitogenic epitopes is genetically restricted as in EAE (see chapter 1.7.2). Chou and co-workers (1989) suggested that there may be multiple determinants on the MBP molecule made immunodominant and encephalitogenic by their ability to associate with certain MHC Class II molecules such as DR2 which has been associated with disease susceptibility. Ota and colleagues (1990) reported a higher frequency of T cell lines reactive with the MBP residues 84-102 from MS patients with a DR2^ haplotype than in controls with the same haplotype, suggesting that this immunodominant region may be encephalitogenic in some DR2^ individuals. In fact HLA DR2^ has been described as a restriction element for up to eight different MBP epitopes to T cells isolated from MS patients (Chou et al., 1989). Similar studies have identified class II restricted CD4^ cytotoxic T cell lines reactive to the MBP immunodominant region 87-106 in HLA-DR2^ and DR4^ MS patients and controls. In contrast other T cell lines recognised peptide 154-172 and were from DR2^ controls, DR1^ and DR4^ MS patients, and DR6^ patients and controls (Martin et al., 1991). Whether an immunogenic epitope is pathogenic is difficult to confirm but studies of EAE have shown that encephalitogenic MBP epitopes are not always immunodominant and can sometimes be protective (Bell and Steinman, 1991).

Recently the T cell receptor (TCR) repertoire of MBP specific T cells has been investigated to determine whether there are any common rearrangements used to preferentially recognise immunodominant regions of MBP that may predict susceptibility to MS and therefore offer possible opportunities for immunotherapy.

Using the PCR technique, amplification of the TCR Vβ gene sequences derived from T cell lines and clones specific for immunodominant regions of MBP (84-102 and 143-168) was performed (Wucherpfennig et al., 1990). It was found that the frequency of usage of the Vβ17 gene was increased in 77% of the independent T cell lines generated from a
DR2+/DR7+ MS patient and also by some of the T cell lines generated from four other MS patients. Other TCR V gene segments, VB12 and VB7, were also more frequently used in recognition of the MBP region 84-102 but were less frequently used in the recognition of the other immunodominant region tested (143-168) where VB14 usage was more frequent. T cell lines specific for region 84-102 of MBP from four of the five healthy individuals also frequently used VB12 and VB17 TCR genes indicating that recognition of this immunodominant region involves common TCR gene usage. Counter to these observations, using the CD4+ cytotoxic T cell response to another epitope of MBP (87-106) that is frequently recognised by MS patients and controls, Martin and colleagues (1991) demonstrated that the MBP reactivity was restricted by four different HLA-DR molecules and that the VB and Va gene usage was dissimilar in the MS T cell lines tested. Later experiments showed that when the TCR VB gene usage of 15 CD4+ cytotoxic T cell lines from four MS patients and two healthy relatives was investigated, there was heterogeneous TCR usage for lines that differed in peptide specificity and also for some that showed identical responses and were restricted by the same HLA-DR antigen (Martin et al., 1992). In addition, Ben-Nun (1991) recently demonstrated a diverse Va and VB usage by MBP clones from different MS patients. However, there was a restricted gene usage by MBP clones of the same patient but no clear association of TCR genes and MBP epitope specificity.

Investigation into the expression of TCR genes of T cells infiltrating the brains of MS patients has also been performed. RNA was isolated from demyelinating plaques of three MS brains and three control brains and complimentary DNA synthesised. Gene amplification using the PCR method was done using TCR Va primers. Interestingly in all the MS brains only 2-4 rearranged Va transcripts were detected and no Va transcripts were detected in the control brains (Oksenberg et al., 1990b). They then went onto show that certain VB rearrangements detected in MS brain autopsy samples may be preferentially expressed in certain HLA haplotypes (Oksenberg et al., 1991). This was also confirmed by Hvas and colleagues (1991) who showed a restricted gene usage in MS brains expressing certain haplotypes previously associated with susceptibility. They also showed that there was restricted usage of one to four rearranged TCR Vy and Vδ transcripts. Whether there is a selective traffic of MBP T cells with specific TCR genes into the brain of MS patients is not known and requires further investigation.
Nevertheless, the hope for using immunotherapies targeting TCR V region genes may be more complicated than originally anticipated in the treatment and prevention of MS due to the heterogeneity of TCR V α and β gene usage between different patients. It will also have to be clearly demonstrated that the MBP reactive T cells bearing specific TCR’s are involved in the pathogenesis of MS.

There are other autoantigens besides MBP that might play a role in the pathogenesis of MS. Peripheral blood T cell responses to MAG and PLP and its selected peptides, have been detected (Johnson et al., 1986; Trotter et al., 1991) and ganglioside specific T cell clones have been isolated from the CSF of an MS patient (Bellamy et al., 1986). PLP and MOG reactive T and B cells have also been detected in the CSF of MS patients at a higher frequency than that found in blood (Sun et al., 1991a) Peripheral blood reactivity but rarely CSF T cell activity was also seen with control patients’ (OND) lymphocytes, but at a much lower frequency (Sun et al., 1991b). Burns and Littlefield (1989) identified another myelin protein other than MBP that was immunogenic, suggesting that there are other myelin antigens that have not yet been identified that could be involved in the pathogenesis of disease.

However, even if MBP is shown to be an autoantigen in MS and T cells are involved, the exact role of these auto-reactive T cells in the demyelinating process has still to be determined.

1.6 EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS AS A MODEL FOR MULTIPLE SCLEROSIS

EAE is an organ specific autoimmune disease of the CNS causing transient paralysis and is inducible in both rodents and primates and characterised by CNS inflammation, and in some species, demyelination. Both an acute form of EAE (acute onset of paralysis) and a relapsing form (REAE) can be induced in genetically susceptible strains of animals by immunisation with CNS antigens emulsified in appropriate adjuvant, or more recently by immunisation with both MBP and PLP-specific T cells. EAE is an acute monophasic disease characterised pathologically by inflammation of the CNS with absent or minimal demyelination thereby limiting its usefulness as a model for MS. Chronic-relapsing EAE (CREAE) as the name implies, resembles the relapsing-remitting course of MS and is accompanied by CNS demyelination and therefore, is a better model for MS than the
monophasic form of the disease. Although there are dissimilarities between MS and CREAE, the model allows the study of auto-reactive T cells and their ability to induce inflammation and subsequent demyelination in the CNS and also allows the testing of novel forms of immunotherapy.

1.6.1. Induction

EAE can be induced in genetically susceptible animals by inoculation with CNS tissue (spinal cord homogenate, whole white matter or purified myelin) emulsified in adjuvant containing mycobacterium. MBP was identified as being an encephalitogenic antigen in CNS tissue by Einstein and colleagues (1962) and has since been used to induce acute EAE in a wide variety of animals when injected subcutaneously in adjuvant. Now that the amino acid sequence of MBP from various species has been determined the encephalitogenic activity of various MBP fragments has been studied using different animal models of EAE. The nonapeptide, 114-122, induces EAE in guinea pigs, whereas fragment 68-88 is encephalitogenic in rats, and peptides 1-37 and 89-169 are encephalitogenic in different inbred mouse strains. Even within the 89-169 region of MBP there are four overlapping encephalitogenic peptide epitopes for the SJL/J mouse strain (reviewed by Zamvil and Steinman, 1990).

Alternatively EAE can be induced in naive recipient animals by adoptive transfer of lymphocytes from syngeneic animals immunised to whole CNS tissue or MBP. Petinelli and McFarlin (1981) demonstrated that it was the Lyt 1^ 2' subclass of T cells (CD4^) from the lymph nodes of MBP sensitised mice that were responsible for inducing disease. EAE and REAE have since been adoptively transferred using activated MBP specific T cell lines and peptide specific CD4^ T cell clones (Ben-nun and Lando 1983; Mokhtarian et al, 1984; Zamvil et al, 1985).

Until recently it was assumed that MBP was the predominant encephalitogen responsible for EAE however, REAE has also been induced in SJL/J mice by PLP, the major integral membrane protein of the myelin sheath, and acute EAE induced with a PLP synthetic peptide (139-151) (Satoh et al., 1988; Tuohy et al., 1989). PLP specific T cells and peptide specific T cell lines can also transfer acute and relapsing disease to naive recipients (Van der Veen et al., 1989; Whitham et al., 1991). Further evidence confirming the critical role for
CD4$^+$ T cells in EAE is the observation that anti-CD4 MAb can inhibit or reverse ongoing EAE (Brostoff and Mason, 1984; Waldor et al., 1985) and that CD4$^+$ cells are also found in abundance in inflammatory EAE lesions in the CNS (Traugott et al., 1986).

1.6.2 Susceptibility and MHC expression

The exact immune mechanisms which regulate susceptibility and resistance to EAE are still undetermined. MHC class II expression on accessory cells, has been shown to be involved in the T cell mediated response to encephalitogenic antigen and it is class II restricted $T_{MBP}$ and $T_{HF}$ cells that induce EAE. It may be due to the way different MHC molecules present antigen as there are multiple discrete encephalitogenic epitopes as well as non-encephalitogenic epitopes of MBP and T cell recognition of each occurs with different alleles of the Class II gene. However, the PVG rat (haplotype RT-1®), which is relatively resistant to EAE induction, responds to the same MBP peptide as the Lewis rat (RT-1$^+$) in which the peptide is encephalitogenic (Vandenbark et al., 1985 & 1989). Therefore, other factors are likely to be involved in disease susceptibility other than the MHC alone.

Experiments performed by Matsumoto and colleagues (1990) using thymectomised rat chimeras that possessed thymuses from EAE susceptible (LEW) or resistant (BN) strains revealed that chimeras with thymuses from the resistant strain developed EAE after active immunisation. This suggested that it was not the strain specific T cell repertoire alone that was involved in regulating EAE susceptibility. When further experiments were done with the chimeras reconstituted with $F_1$ (LEW x BN) T cells and bone marrow cells from either strain they showed that it was the MHC molecules expressed on the accessory cells which primarily determined susceptibility or resistance to EAE. They argued that the strain specific T cell repertoire for MBP, which is influenced by the MHC molecule expressed on the thymic epithelial cells does not determine EAE susceptibility.

Increased class II expression is also thought to play a role in the immunopathogenesis of autoimmune disorders as aberrant expression in areas where it is not constitutively present may initiate disease (Bottazo et al., 1985). Male and Pryce (1989) showed a relationship between disease susceptibility and inducibility of MHC class II, as rat strains that were susceptible to EAE (LEW and DA) could be induced to express higher levels of class II molecules than EAE resistant strains (PVG, BN). Cerebral endothelial cells of the SJL
susceptible mouse strain have also been shown to express more class II than the resistant B10 strain (Jemison et al., 1991).

1.6.3 T cell receptor \( V_\beta \) genes and susceptibility to EAE

The MHC class II gene is not the only factor involved in susceptibility as the TCR repertoire has also been implicated. After the discovery of multiple encephalitogenic epitopes of MBP for induction of EAE, it was noted that the T cell response to each epitope was limited to a discrete population of T cells. Nevertheless, these cells could not be distinguished from one another on the basis of their peptide specificity or class II restriction suggesting that the heterogeneity of the TCR repertoire of encephalitogenic T cells was limited. This supported the concept that the immune response genes for expression of the MHC and TCR act together to initiate disease. Investigations into the expression of limited T cell receptor repertoires has revealed that T cells of both rat and mouse preferentially utilise the \( V_\beta 8 \) gene (rat \( V_\beta 510 \) has 79% homology with the mouse \( V_\beta 8 \) gene) and to a lesser extent the \( V_\alpha 2 \) gene (rat \( V_\alpha 5 \) has 77% homology with the mouse \( V_\alpha 2 \) gene) for recognition of encephalitogenic epitopes of MBP (Acha-Orbea et al., 1988; Burns et al., 1989). Other families have also been identified such as the \( V_\beta 4 \) and \( V_\beta 17 \) in the SJL/J mouse (the \( V_\beta 8 \) gene is lacking in this strain) but such usage is not essential for induction of EAE (Sakai et al., 1988; Padula et al., 1991). Nevertheless \( V_\beta 8 \) usage is the most consistently reported gene usage for encephalitogenic T cells from both the rat and mouse model of EAE. Recently more detailed studies have investigated the \( V_\beta \) gene usage of MBP synthetic peptide T cell clones isolated from the spinal cord of EAE affected Lewis rats. \( V_\beta 6 \) usage predominated but lymph node T cell clones reactive to the same peptide expressed multiple \( V_\beta \) genes including \( V_\beta 6 \) suggesting that maybe certain TCR \( V_\beta \) gene rearrangements are preferentially sequestered in the CNS and thus involved in disease pathogenesis (Gold et al., 1992).

An alternative role for restricted TCR \( V_\beta \) gene usage in demyelinating diseases has recently been proposed and is illustrated by the following experiments. Rodriguez and colleagues (1992) investigated the role of TCR \( V_\beta \) genes in the pathogenesis of an immune-mediated demyelinating disease, induced by intracerebral injection of Theiler's murine encephalomyelitis virus (TMEV). As with EAE, susceptibility / resistance to chronic demyelination after viral infection is partly influenced by MHC genes and more recently the genes encoding the constant region of the \( V_\beta \) chain of the TCR have been implicated.
Using an inbred mouse strain which has deletions of approximately 60% of the known Vβ genes of the TCR, Rodriguez demonstrated that these mice had significantly more and extensive demyelinating lesions in the spinal cord than mice which had no deletions in the VB loci. Paradoxically, demyelination has been considered in part to be mediated by T cells and the authors therefore suggest that T cells expressing Vβ genes not encompassed by the deletion are involved in demyelination. To explain the increased demyelination in this model, they conclude that in all diseases where a role for limited TCR Vβ gene usage has been implied in disease pathogenesis, such as MS, EAE and TMEV induced demyelinating disease, T cells of limited Vβ usage may function as both effectors for induction of disease while another subpopulation of T cells with a different Vβ gene usage may protect against disease induction. A balance between these suggested subpopulations may determine susceptibility versus resistance and even the extent of pathological damage.

1.6.4 Immunopathogenesis

How do CD4+ encephalitogenic cells cross the blood brain barrier (BBB) and enter the CNS from the periphery and then once within the CNS initiate disease? Recent studies using the Lewis rat model of EAE indicate that T cell entry into the CNS is primarily dependent upon the activation state of the lymphocytes as T lymphoblasts enter the CNS whilst unactivated T cells are excluded (Hickey et al., 1991). Antigen specificity, MHC compatibility, T cell phenotype and TCR gene usage all appear unrelated to the T cells ability to cross the BBB and enter the brain parenchyma. It has been proposed that entry of activated cells is a result of membrane changes on the activated T cell rather than an alteration in the expression of adhesion receptors, as in EAE, activated syngeneic cells gain entry into the CNS tissue within 3 h of being introduced intravenously. In addition, there have been reports that activated lymphocytes produce increased levels of heparin sulfate endoglycosidase which may help in the migration of cells across the BBB (Naparstek et al., 1984). In vitro, models of lymphocyte adhesion and migration have shown that lymphocyte adhesion depends primarily on the activation state of the lymphocyte and secondarily on cytokine stimulation (TNFα and IFNγ) of adhesion molecule expression by the endothelium (Male et al., 1990; Male et al., 1992). Also, no brain specific lymphocyte-endothelial receptor systems have been identified which would specifically influence T cell homing to the CNS. Nevertheless, in vivo it has been shown that upregulation of adhesion molecules on leukocytes and endothelial cells in the CNS occurred during the acute stage of EAE. Whereas during remission, expression was
down regulated and each subsequent relapse was accompanied by a corresponding upregulation of adhesion molecules (Cannella et al., 1990). This suggests that the facilitation of adherence of lymphocytes to brain endothelium may be fundamental to the inflammation and subsequent demyelination that occurs in EAE. Whether the upregulation of Ia on endothelium is a consequence of cytokine secretion from activated circulating T cells that gain entry into the CNS. Or whether Ia expression by endothelial cells aids the T cell entry is unclear. However, in this model system activated non-CNS specific T cells could not be detected in the CNS which is in conflict with the reports that any activated cell can traffic through the CNS.

In support of the importance of expression of adhesion receptors it has recently been demonstrated that antibodies against α4β1 integrin (also known as very late antigen-4 (VLA-4) which is expressed on activated T cells) when injected intra-peritoneally two days after the transfer of syngeneic T<sub>M</sub> clones can prevent the accumulation leukocytes within the CNS and the development of EAE (Yednock et al., 1992).

Once within the CNS what is the role of the encephalitogenic CD4<sup>+</sup> T cells in the immunopathogenesis of EAE? Are they only important in the induction phase or are they also involved in the continuation of disease? The CNS has always been assumed to be an immunologically privileged site as it lacks a lymphatic drainage and there is a paucity of immunological cells present in the CNS due to the shielding effect of the BBB. There is also a lack of cells expressing MHC molecules which are required for antigen presentation to T cells. It has since been reported that the CNS undergoes routine immune surveillance as activated T cells irrespective of their antigen specificity can cross the BBB (Wekerle et al., 1986; Hickey 1991). Once in the CNS if the activated T cells encounter specific antigen in association with antigen presenting cells of which the following are candidates, astrocytes, endothelial cells and microglia then they remain and undergo clonal expansion and initiate an immune response.

Astrocytes were the first cells that were shown in vitro to have inducible MHC class II molecules and present MBP to T cells, they also produce IL-1 and TNFα that have important roles in immunological responses (Fontana et al., 1984; Fontana et al., 1982; Sawada et al., 1989). Nevertheless, reports on the identification of class II positive astrocytes histologically in the CNS of EAE animals have been conflicting (Vass et al., 1986; Traugott et al., 1986;
The endothelial cell is also a likely candidate for initiating inflammation in the CNS by its potential role as an antigen presenting cell (APC) in EAE. *In vitro* studies have confirmed the ability of endothelial cells to present MBP to T cells (McCarron *et al.*, 1985) although this could not always be confirmed by others (Risau *et al.*, 1990). Induction of class II molecules on brain endothelium has also been related to susceptibility to EAE (Male and Pryce, 1989). Nevertheless, identification of MHC class II bearing brain endothelial cells *in vivo* is contradictory (Sobel *et al.*, 1985; Matsumoto *et al.*, 1986). The possibility exists that encephalitogenic T cells could release IFNγ thereby stimulating the expression of class II antigens and adhesion molecules on the brain endothelial cells thus leading to T cell adhesion and possible antigen specific activation of T cells at the BBB. As a result, alterations in the permeability of the BBB could occur leading to the migration of cells into the CNS. Antigen specific CD4⁺ lymphocytes have been shown to be cytolytic towards brain vascular endothelial cells in an antigen specific manner (Sedgewick *et al.*, 1990) and it was recently demonstrated that with repeated passages of encephalitogenic T<sub>MBP</sub> cell lines the ability to cause *in vitro* endothelial cell lysis diminished as did the ability for the cells to transfer disease thus indicating the importance of the T cell-endothelial cell interaction in the pathogenesis of EAE (McCarron *et al.*, 1991). In contrast experiments by Hinrichs and colleagues (1987) using rat bone marrow chimeras in which the endothelial cells of the host animal were non-syngeneic to the antigen presentation requirements of the inducing T cells demonstrated that the animals were capable of developing disease. This therefore argued that the endothelial cell was not a critical cell for antigen presentation and disease induction. Nevertheless, other experiments with thymus grafted chimeras provided evidence that class II expression by accessory cells was important in regulating susceptibility to EAE (Matsumoto *et al.*, 1990).

The final candidate for a CNS APC is the microglial cell. As with the astrocyte and endothelial cell, microglial cells can present antigen to T cells *in vitro* (Frei *et al.*, 1987) and they have been identified *in vivo* as expressing class II molecules (Matsumoto *et al.*, 1986). Therefore, three different cellular possibilities exist for local antigen presentation within the CNS the astrocyte, endothelial cell and microglial cell.

Once the encephalitogenic CD4⁺ lymphocytes have entered the CNS how do they initiate inflammation and demyelination? Re-stimulation within the CNS of the encephalitogenic
CD4+ cells with antigen and the appropriate MHC molecule on the surface of the APC is thought to occur resulting in the persistence of the encephalitogenic cells in the CNS and subsequent clonal expansion with the initiation of an immune response. Cytokines are produced which can influence BBB permeability, adhesion molecule expression, class II expression and also act as chemoattractants for further inflammatory cells. Once the BBB is opened, inflammatory cells could accumulate within the CNS along with immunoglobulins and serum complement factors which have all been shown to be potential mediators of demyelination \textit{in vitro} (see next section 1.7 mechanisms of demyelination). Inflammation and demyelination is therefore initiated.

Interestingly, not all activated MBP specific T cells are encephalitogenic and since one cannot distinguish between encephalitogenic and non-encephalitogenic T cell clones on the basis of their fine MBP specificity there must be other properties of these cells that contributes to their encephalitogenicity. If all activated T cells are able to gain entry to the CNS there may possibly be a defect in non-encephalitogenic T cells ability to cross the BBB. A recent study however, showed that both encephalitogenic and non-encephalitogenic labelled CD4+ T\textsubscript{MNP} cell lines could be localised to the spinal cord 3 days after injection. However, by 4 days post injection the number of non-encephalitogenic T\textsubscript{MNP} cells identified had returned to almost basal levels whereas greater numbers of EAE inducing T cells were identified in the spinal cord (Barbarese \textit{et al.}, 1992). The authors suggested that the differentiation between encephalitogenic and non-encephalitogenic cells occurred within the first 3 days and that there was possibly a difference in the secretion of cytokines by certain T cells. Thus only the encephalitogenic T cells are able to initiate inflammation, subsequent demyelination and relapsing paralysis. Recent experiments have demonstrated a positive correlation between T cell encephalitogenic potential and expression of the TNF\beta gene and production of protein although the exact role of certain cytokines in autoimmune demyelination is as yet unknown (Powell \textit{et al.}, 1990). Nevertheless antibodies that neutralise TNF\alpha and \beta can prevent the transfer of clone-mediated EAE (Ruddle \textit{et al.}, 1990).

1.6.5 Immunotherapy

There are many possibilities of immune intervention in the treatment and prevention of EAE as the trimolecular complex of MHC, TCR and antigen provides multiple sites at which to intervene in the immune response to autoantigen. Monoclonal antibodies to MHC class II
gene products associated with disease susceptibility have been used to successfully prevent acquisition of EAE (Steinman et al., 1981) and also reduce the number of relapses and mortality in CREAE (Sriram & Steinman, 1983). This type of therapy is however, only partially specific as it blocks responses to a restricted class II isotype. Blocking of the accessory molecule CD4 has also been shown to reverse EAE in rats and mice (Brostoff & Mason, 1984; Waldor et al., 1985), but again this type of therapy is non-specific. Non-immunogenic peptides which are thought to inhibit the presentation of encephalitogenic antigen to the auto-reactive T cells by competitive blockade of the MHC peptide binding site have also been used to effectively prevent the induction of EAE (Sakai et al., 1989).

Nevertheless, both these types of immunotherapy are non-specific and result in a general immunosuppression which might mask the specific mechanisms involved in preventing EAE induction.

An alternative approach to immunotherapy is by targeting the TCR. Vaccination with attenuated cloned encephalitogenic T cell lines was shown to immunise rats against EAE and the TCR was thought to be the target for the vaccination (Ben-Nun et al., 1981). It was later shown that T cells from the popliteal lymph nodes of rats vaccinated with sub-encephalitogenic doses of a basic protein (BP) - specific T cell clone (Z1a) were the mediators of the subsequent resistance to EAE induction (Lider et al., 1988). Analysis of the specificity of these T cells (both CD4+ and CD8+ T cells) showed that they recognised clone Z1a and were able to cause stimulation of the BP specific clone Z1a in the absence of antigen (CD4+ T cells) and also suppression of the response to BP (CD8+ T cells). This anti-idiotypic response was thought to be directed against the TCR (idiotype) for MBP.

Since it has been recognised that there is a restricted TCR Vβ gene repertoire of encephalitogenic T cells, a Vβ-specific antibody (F23.1) has been used to prevent and treat EAE where disease was induced by a T cell clone expressing Vβ8.2 (Acha-Orbea et al., 1988). The antibody successfully reversed the symptoms of EAE by depleting 98% of the Vβ8 bearing T cells. However, when multiple encephalitogenic epitopes are present in the inoculating antigen the reversal of disease by the antibody was not as successful due to more than one TCR Vβ usage. This led to the use of mixtures of Vβ specific antibodies to prevent and treat EAE induced by inoculation of MBP in adjuvant (Zaller et al., 1990). Nevertheless, immunotherapy by this method is more complicated than first anticipated due to the use of more than one TCR V gene and thus a cocktail of antibodies is probably required.
More recently, EAE in rats has been prevented and reversed by active immunisation with peptide sequences of the TCR variable chain of pathogenic T cell clones (Vandenbark et al., 1989; Offner et al., 1991). Anti-Vβ T cells and antibodies are thought to be responsible for this effect, similar to the anti-idiotypic T cells thought to be involved when attenuated pathogenic T cell clones were used previously (Lider et al., 1988).

Alternative approaches to immunotherapy have been tolerisation with immunodominant peptides of MBP of neonates which confers protection to the adult animal (Clayton et al., 1989). Oral administration of MBP has also been shown to be successful in the prevention of EAE (Lider et al., 1989) and is thought to involve an immunoregulatory pathway that is dependent on transforming growth factor β (TGFβ) (Miller et al., 1992). In vitro, TGFβ inhibits the activation and proliferation of MBP sensitised lymph node cells (LNC) thereby reducing their ability to transfer disease. Also, TGFβ when given in vivo after disease induction improves the clinical course of the disease in SJL/J mice. In fact there was a marked decrease in inflammation and the expression of accessory molecules (MHC class II and lymphocyte function associated-antigen-1 (LFA-1)) in the CNS (Racke et al., 1991).

Miller and colleagues (1992) have demonstrated that in the suppression of EAE by oral administration of MBP, the suppression is mediated by CD8+ T cells which can adoptively transfer protection and also suppress immune responses in vitro. These modulator cells produce a suppressor factor upon stimulation with MBP in vitro, that is specifically inhibited by anti-TGFβ neutralising antibodies. However, Weinberg and colleagues (1992) in contrast have shown that TGFβ enhances the in vivo effector function of encephalitogenic T cells in the adoptive transfer of EAE.

1.7 MECHANISMS OF DEMYELINATION

The mechanisms underlying the myelin damage that occurs in MS are still mostly speculative, but immunological factors are thought to be the major mediators due to the abundance of infiltrating lymphocytes and macrophages within actively demyelinating plaques and the fact that EAE can be transferred by myelin specific CD4+ lymphocytes. Involvement of both humoral and cell mediated immune mechanisms of demyelination have been proposed and will be discussed below.
A role for anti-myelin antibodies in the pathogenesis of demyelination has been implied from extensive in vitro studies looking at the effect of sera from animals with EAE or sera from MS patients on mouse or rat myelinated cerebellum tissue cultures. Due to the complex nature of sera, the mechanisms for the myelinotoxic and cytotoxic effects have yet to be elucidated however, complement-dependent anti-myelin antibodies have been proposed as being responsible for the in vitro demyelination seen. Thus suggesting that circulating antibodies in EAE may play a pathogenic role in the production of demyelination with suggestions that a similar mechanism is occurring in MS (Bornstein and Appel, 1965; Seil, 1977). However, serum from healthy individuals and OND patients can also induce tissue culture demyelination and it has been suggested that complement-dependent antibody-independent factors in human sera compared to antibody-dependent factors in EAE sera are responsible for the in vitro demyelination reported (Wolfgram, 1978; Silberberg et al., 1984; Bradbury et al., 1985). Immunoglobulins have nevertheless been implicated in the immunopathogenesis of the MS plaque as direct activation of myelin neutral proteases by Ig isolated from MS brain have been shown to split the lamellae and disrupt the myelin sheath (Kerlero de Rosbo and Barnard, 1989).

The most convincing role for antibody-mediated demyelination so far however, is the observation that when antibody to the myelin constituent, MOG is given to rats in conjunction with syngeneic CD4+ T\textsubscript{MBP}, there is widespread demyelination within the CNS unlike the acute inflammatory encephalomyelitis with little loss of myelin that is normally seen when T\textsubscript{MBP} are given alone. The demyelination occuring is thought to be due to a combination of oligodendrocyte-specific complement activation and antibody mediated phagocytosis (Lingtonton et al., 1988).

Cultured oligodendrocytes are sensitive to normal serum, and when exposed become osmotically disrupted and eventually lyse (Scolding et al., 1989a). The complement within serum becomes activated antibody-independently by the classical pathway on the oligodendrocyte surface, with the formation of activation products and membrane attack complexes (MAC). The MAC's then insert themselves into the oligodendrocyte membrane and act as a calcium ionophore. An increase in intracellular calcium is thus caused which can eventually lead to cell lysis. The increase in calcium does not always lead to lysis as the oligodendrocyte can recover by shedding the MAC on the surface by vesicular extrusions (Scolding et al., 1989b).
Consistent with the hypothesis of complement-dependent injury of oligodendrocytes and their myelin membranes is the finding that in the CSF of MS but not normal controls or patients with structural CNS damage, there are vesicles reactive with antibody to the complement components C8, C9, MAC, and galactocerebroside (GalC). As mentioned earlier these MAC vesicles have been shown to be shed by oligodendrocytes undergoing recovery from complement mediated attack (Scolding et al., 1989b).

All of the above reports have concentrated on damage to oligodendrocytes as being the initiating event in demyelination. Direct myelin destruction by macrophages through receptor-mediated endocytosis of the myelin lamellae via clathrin-coated pits (on the macrophage surface) has also been demonstrated in vivo (Raine et al., 1981), as well as direct myelin damage by complement in vitro (C Yong et al., 1982; Vanguri et al., 1982; Silverman et al., 1984). Also as there are many circulating myelin/oligodendrocyte specific antibodies within MS CNS these could serve as an opsonin for both compact myelin or oligodendrocyte phagocytosis. Nevertheless, whether demyelinating antibodies are primarily involved in demyelination in co-operation with cell-mediated responses or are produced as a consequence of myelin breakdown is not known.

T cell mediated cytotoxicity is another possible mechanism of demyelination in vivo via both a direct oligodendrocyte cytotoxic effect and an indirect cytotoxic and myelinotoxic role of T cell secreted products (cytokines and lytic granules). In vitro, oligodendrocytes are susceptible to cytotoxic attack by T lymphocytes (Jewtoukoff et al., 1989; Kawai and Zweiman, 1988; Ruijs et al., 1990). Whether the damage is due to a direct effect of cytotoxic lymphocytes where there is a necessity for class I expression by the target cell is not known. Reports are conflicting as to whether oligodendrocytes can be induced to express MHC class I (Wong et al., 1984; Suzumura et al., 1986; Kawai and Zweiman, 1988; Grenier et al., 1989). Nevertheless, GalC and MBP antigen specific lymphocytes have been shown to selectively damage oligodendrocytes in vitro in a tissue specific manner as astrocytes and fibroblasts were unaffected (Niedeck and Lohmann, 1981) as were non-histocompatible oligodendrocytes (Kawai and Zweimen, 1988). Jewtoukoff and colleagues (1989) selected oligodendrocyte specific CD8+ clones from mouse splenocytes sensitised in vitro by rat oligodendrocytes and tested their cytotoxicity towards oligodendrocytes. The cytosis observed required both the CD3-TCR complex and the CD8+ molecule with a lack of involvement of MHC molecules. Antibodies directed against MOG fully blocked the cytotoxicity suggesting that this surface
antigen may be directly and specifically recognised by autoreactive T cells, resulting in cell death without MHC restricted killing. In contrast, Ruijs and colleagues (1990) demonstrated that in vitro, human oligodendrocytes express class I antigens and are susceptible to class I restricted lysis. Therefore the reported in vitro oligodendrocyte cytotoxicity by T cells may be a combination of MHC restricted and non-MHC restricted killing.

Histological studies of the MS plaque are conflicting since it is unknown whether myelin breakdown occurs secondary to oligodendrocyte loss. In acute lesions some reports suggest oligodendrocytes survive in large numbers in active plaques and that if there is loss it is secondary to myelin damage (Lassman et al., 1983). It was suggested that the absence of any marked reductions in oligodendrocytes in acute lesions is due to a proliferative response following a few weeks of acute destruction of both myelin and oligodendrocytes. There are many histological studies which describe oligodendrocytes as present or absent without any reference to changes in these cells as they degenerate. Others however, have reported nuclear pyknosis and cytoplasmic swelling, but since this is a common post-mortem effect, the significance of these changes to MS is questionable (reviewed by Prineas, 1985). Similarly, in older studies it is not known whether the changes seen were fixation artifacts rather than true cell damage, since more recent reports show the majority of oligodendrocytes are normal ultrastructurally (Prineas, 1984). Raine (1981) reported that the number of oligodendrocytes in areas of active demyelination was double that found in NAWM. He interpreted this as evidence of oligodendrocyte survival and proliferation and that there is no depletion of oligodendrocytes during or shortly after CNS demyelination. In contrast, Prineas (1984) reported a decrease in the numbers and degeneration of oligodendrocytes in active plaques which showed evidence of cellular disorganisation and plasma membrane rupture. These affected cells were seen in the presence of normal appearing oligodendrocytes, and were seen in contact with macrophages and lymphocytes which was interpreted as possible lytic attack by cytotoxic lymphocytes. Alternatively antibody-dependent cell-mediated cytotoxicity or killing by phagocytes could have been occurring (complement antibody-mediated phagocytosis).

In summary, multiple effector mechanisms operate to produce the tissue damage that is observed in the CNS of MS patients. It is not known whether the disease is initiated centrally or peripherally, nevertheless early in the course of the disease there is probably an influx into the CNS of serum proteins and migration of inflammatory cells from the
Peripheral immune compartments. Once focused into the lesion these pathogenic demyelinating factors and inflammatory cells further add to the immune mediated demyelination that has already been initiated. It still remains unknown how demyelination is initiated in the CNS and what cell or factors are responsible. In this thesis using an in vitro model the specific role of T cells in myelin degradation will be investigated as this could provide insight into the possible relevance of T cells in the pathogenesis of demyelination in MS.
2.1 ANTIGENS & MITOGENS

1. Bovine brain gangliosides
Bovine brain gangliosides Type III (BBG) (Sigma Chemical Co., Poole, U.K.) were dissolved in phosphate buffered saline pH 7.2 (PBS) filter sterilised and further diluted (ng-µg/ml) in culture medium for use.

2. Guinea pig myelin basic protein
Guinea pig myelin basic protein (GPMBP) was prepared from isolated myelin according to the method of Dunkley and Carnegie (1974) (see Chapt 2.5.22). Briefly basic protein was extracted from guinea pig myelin membranes by extraction in HCL followed by separation on a G50 chromatography column. The purity was confirmed on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) (Plate 2.2). Lyophilised protein was stored at -20°C.

3. Human myelin basic protein
Human myelin basic protein (HMBP) was a kind gift of Dr Paul Glynn and was prepared and stored as for GPMBP.

4. Tuberculin purified protein derivative
Tuberculin purified protein derivative (PPD) (100,000 units/ml) prepared from human strains of mycobacterium tuberculosis (Evans Medical Ltd. U.K.) was dialysed in PBS overnight and stored at -20°C.

5. Concanavalin - A
Con-A Type III (Sigma Chemical Co.) was dissolved in PBS to give a stock concentration of 5 mg/ml and stored at 4°C. The solution was sterilised by passage through a 0.2 µm filter and used at a final concentration of 10 µg/ml.

6. Lipopolysaccharide
Lipopolysaccharide (LPS) *E.Coli* 026.B6 (Sigma) was freshly prepared by addition of sterile
distilled water and used at a final concentration of 1 ng - 50 µg/ml.

7. Phytohaemagglutinin - P
Phytohaemagglutinin - P (PHA-P) (Difco Labs, West Molesey, UK) was rehydrated in distilled water to give a stock concentration of 1 mg/ml. After 0.2 µm filter sterilisation it was stored in aliquots at -20°C and used at 1 µg/ml final concentration.

2.2. CYTOKINES

1. Interleukin-1 α, human recombinant
Human recombinant IL-1α, specific activity 1 x 10⁶ units/mg (Hoffman La Roche, Basle, Switzerland) in 0.1% bovine serum albumin (BSA) PBS was stored at -70°C.

2. Interleukin-2, human recombinant
Human recombinant IL-2, 200 units/ml (Boehringer Mannheim, Lewes, U.K.) in PBS with 0.5 mg/ml BSA was stored in aliquots at -20°C and used at a final concentration of 20 units/ml.

3. Interleukin-2, natural human
IL-2 supernatant (lectin free), produced by PHA stimulation of human mononuclear cells from healthy donors, was stored frozen in aliquots at -20°C.

4. Interferon gamma, rat recombinant
Rat recombinant IFNγ, specific activity 1-2 x 10⁷ U/mg (Holland biotechnology bv, Holland) when measured in a bioassay using mouse L cells, and containing less than 0.016 U/mg endotoxin was reconstituted in 0.1% BSA PBS and kept at 4°C until required (1 month maximum).

5. Tumour necrosis factor α, recombinant
Human recombinant TNFα, specific activity 2 x 10⁶ U/mg as measured by its cytolytic effect on murine (L929) cells in the absence of actinomycin D (British Biotechnology Ltd., Oxford, U.K.) was reconstituted in 0.1% BSA PBS and kept at 4°C until required.
2.3. ANTISERA

A list of the MAb's used and their sources are shown in Table 2.1. Fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin (RAMIgFITC) was obtained from Nordic Immunological Laboratories (Maidenhead, U.K.). All antibodies were diluted in 10% foetal calf serum (FCS) containing medium and used at optimal concentrations determined by previous titration experiments.

2.4. TISSUE CULTURE MEDIA

1. Culture medium for long term culturing of human peripheral blood mononuclear cells.
For culturing human PBMC's and CSF lymphocytes, Roswell Park Memorial Institute (RPMI) 1640 was supplemented with 25 mM Hepes buffer, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% (v/v) non-essential amino acids (NEAA) and 50 units penicillin/50 μg streptomycin ml⁻¹, (all Flow Laboratories, Rickmansworth, U.K.). Heat inactivated (56°C, 45 min) human type AB⁺ serum (Sigma Chemical Co.) was added at 10% (v/v).

2. Culture medium for overnight culturing of human peripheral blood mononuclear cells
For short term culturing and washing of human PBMC, RPMI 1640 was supplemented with 10% heat inactivated FCS (Gibco Europe, Paisley, U.K.), 25 mM Hepes, 2 mM L-glutamine and 50 units penicillin/50 μg streptomycin ml⁻¹.

3. Immunofluorescent staining medium
When staining either human PBMC or mouse lymphocytes, ice cold RPMI 1640 was supplemented with 5% newborn bovine serum (NBBS)(Flow Labs.) and 0.2% sodium azide.

4. Culture medium for mouse lymph node lymphocytes
Mouse lymph node lymphocytes were cultured in RPMI 1640 supplemented with 10% heat inactivated FCS, 25 mM Hepes, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% (v/v) NEAA, 50 units penicillin/50 μg streptomycin ml⁻¹ and 5 mM 2-mercaptoethanol (Sigma Chemical Co.).
2.5 DONOR PERIPHERAL BLOOD

Heparinised and clotted blood samples were collected from MS and OND patients attending the National Hospital for Neurology and Neurosurgery, Queen Square, London. Clinical assessments were made by Professor Ian McDonald, Dr Peter Rudge and Dr Alan Thompson.

1. Multiple Sclerosis
PBMC were obtained from 97 patients identified as having clinically probable MS by the criteria of McDonald and Halliday (1977). Patients were classified as in relapse when a new clinical sign had developed within the last three weeks; those with active disease had a clear previous history of new signs or symptoms within the last three months. Patients with stable disease had no new sign or symptom for six months or more. Progressive cases had evidence of two or more separate CNS lesions and a history of progressive paraplegia.

2. Other neurological diseases
Sera and PBMC samples were obtained from 31 patients with OND (14 matched PBMC & sera samples, 6 PBMC samples alone and 11 sera samples alone). The patient population was as follows; 3 Guillain-Barré syndrome, 7 infected ventriculopuncture (VP) shunt, 1 Graves disease, 4 epilepsy, 2 non-demyelinating peripheral neuropathy, 4 CNS tumour, 6 motor neurone disease (MND), 1 cerebrovascular accident (CVA), 2 cerebellar ataxia, 1 Parkinsons disease.

3. Rheumatoid Arthritis
Heparinised and clotted blood samples were collected from rheumatoid arthritis (RA) patients attending clinics at Guy's Hospital (Dr M Corkhill), West London Hospital (Dr P Venables) and the Hammersmith Hospital (Dr Walport). All patients satisfied the American Rheumatism Association criteria for classical disease (Silman et al., 1988). All but 3 of the patients were on some form of immunosuppressive therapy.

4. Healthy individuals
Blood was collected from laboratory and medical staff at the Institute of Neurology and National Hospital for Neurology and Neurosurgery.
2.6. METHODS

1. Isolation of peripheral blood mononuclear cells
Peripheral blood collected in lithium heparinised containers was diluted 1:1 (v:v) with RPMI 1640 and layered onto Ficoll-paque, (Pharmacia, Milton Keynes, U.K.) in a ratio of 3 parts diluted blood to 1 part Ficoll according to the method of Boyum (1968). After centrifugation at 500 x g for 35 min, PBMC were isolated from the interface layer and washed three times in FCS containing washing medium at 100 x g for 10 min. The preparation contained not more than 3% polymorphonuclear granulocytes.

Mononuclear cell viability was assessed by diluting the cell suspension 1:1 (v:v) in 0.5% trypan blue (Flow Labs.) which is excluded from viable cells. Cell counts were made on an improved Neubauer haemocytometer using X 40 objective lens. At least 100 cells were counted in the large triple-ruled squares.

Number of viable cells/ml:
\[
\frac{\text{no. lymphocytes counted}}{\text{no. triple-ruled squares}} \times 25 \times 10^4 \times \text{original dilution}
\]

2. Isolation of serum
Fresh serum was collected from blood which had been allowed to clot at room temperature and then spun for 5 min at 100 x g. The serum was always assayed within a few hours of the blood being taken (frozen serum was never used).

3. Isolation of cerebrospinal fluid lymphocytes
Approximately 10 ml or more of fresh (not more than 30 min after collection) CSF was centrifuged at 100 x g for 5 min. The cell free supernatant was removed, divided into aliquots and stored at -20°C. The cell pellet was washed once and resuspended in 1 ml of 10 % AB+ culture medium and a cell count made on an improved Neubauer haemocytometer.

4. Isolation of granulocytes from whole blood
Diluted heparinised peripheral blood was separated on Ficoll-paque density gradient (see 2.5.1). The resulting precipitate which contained erythrocytes and mature granulocytes was suspended in an equal volume of autologous plasma and layered on top of an equal volume
of Ficoll-paque. The preparation was left at room temperature for 40 minutes and the interface layer washed 3 times to obtain mature granulocytes of approximately 98% purity (2% lymphocytes) assessed by haematoxylin staining.

5. Removal of monocytes from PBMC
PBMC suspensions in 10% FCS containing culture media were plated onto 90 mm tissue culture petri dishes (Nunc, Gibco Europe) at a concentration of approximately $5 \times 10^6$/ml and incubated for 1-2 h 37°C, 5% CO$_2$95% air atmosphere. Non adherent (NA) cells were removed from the dish by tilting the plate and gently pipetting. The adherent (ADH) cells were washed twice with medium and then gently resuspended using a rubber cell scraper.

6. Lysing of erythrocytes
Ammonium chloride 0.83% (9 vol) was added to 0.17 M Tris buffer pH 7.6 (1 vol) (all BDH, Poole, U.K.) and 0.2 μm filter sterilised. 1 ml was added to the cell pellet containing erythrocytes and gently shaken for 1-2 min. The cells were washed and the process repeated until all erythrocytes were lysed. The resultant leucocytes were washed three times.

7. Isolation of mouse peritoneal exudate macrophages
SJL/J mice peritoneal macrophages were collected by peritoneal lavage using iced cold RPMI, cells were washed twice in FCS containing medium and cultured at 37°C, 5% CO$_2$95% air atmosphere. After 3 h the medium containing the NA cells was aspirated, the ADH cells were washed twice with medium and then gently resuspended using a rubber cell scraper.

8. Isolation of T cells from a mixed cell population by nylon wool adherence
T cells were isolated from human PBMC or mouse lymph node or spleen cell suspensions (after removal of erythrocytes by hypotonic lysis, 2.5.6.) on the basis of their lack of adherence to nylon fibres. At 37°C in the presence of serum, B lymphocytes will bind avidly to nylon wool columns, giving an effluent population enriched for T lymphocytes.

Monocytes/macrophages were initially removed from the cell suspension by petri dish adherence (Chapt. 2.5.5). NA cells were washed and resuspended in 1 ml of 20% FCS-containing media before addition to the nylon wool column (maximum of $10^8$ cells per column). Nylon wool columns were prepared as follows. Nylon wool was boiled in three
changes of distilled water and air dried. Approximately 0.6 g of nylon wool was loosely packed into a 10 ml plastic syringe barrel, which was subsequently sealed and autoclaved to sterilize. Before use, each column was washed through with 20 ml warm RPMI 1640 to remove air bubbles followed by 10 ml warm 20% FCS containing media to thoroughly wet the column and incubated for 30 min at 37°C. The concentrated NA cell suspension (in 1 ml) was added drop wise to the column and further incubated at 37°C, 5% CO2, 95% air atmosphere for 30-45 min. Unbound cells were eluted from the column with <20 ml of prewarmed 20% FCS-containing media. The effluent population was centrifuged x 100 g for 10 min, resuspended in 1 ml and added drop wise to a second column. After a further incubation for 30 - 45 min the unbound cells were eluted as detailed above, washed and counted.

The resultant cell population contained approximately 86% CD3+ lymphocytes and < 2% CD19+ lymphocytes as measured by indirect immunofluorescence (Chapt. 2.5.15).

9. Isolation of T cells from PBMC by the rosetting technique

The ability of T cells to bind sheep red blood cells (SRBC) via CD2 was used to isolate T cells from a mixed population. At least one day prior to T cell separation, SRBC in Elsevers (Tissue Culture Services, Buckingham, U.K.) were washed four times with PBS pH 7.2. Four volumes of filter sterilised 143 mM 2-aminoethylisothio-uronium bromide (AET) (Sigma Chemical Co.) pH 9.0 was added to the SRBC pellet and incubated for 30 min at 37°C with occasional mixing. The AET coated SRBC were washed four times with PBS and resuspended in an equal volume of 10% FCS containing medium.

AET coated SRBC were added to PBMC (depleted of monocytes by petri dish adherence) in a ratio of 300 µl 50% AET-SRBC : 20 x 10⁶ PBMC in 10 ml 10% FCS containing medium. The cells were vortexed and spun for 5 min at 100 x g before incubating on ice. After 1 h the SRBC-PBMC pellet was slowly resuspended by inversion and layered onto Ficoll-paque and spun 30 min at 500 x g. The erythrocyte negative (E⁻) cells were removed from the interface layer and washed three times. The erythrocyte positive (E⁺) cell pellet was depleted of SRBC by hypotonic lysis. The remaining cells (E⁺) were washed three times in 10% FCS containing medium. The resultant cell population was analysed immunocytochemically with MAb and the alkaline phosphatase-anti alkaline phosphatase (APAAP) method of staining (Table 2.1 & method 2.6.7). E⁺ cells, 90-95% CD3⁺, 2%
10. Interleukin-2 producing cell line
A primate cell line MLA 144 (a gift of Dr. P.C.L. Beverley, University College Hospital, Gower Street, London.) known to release a soluble factor with properties of IL-2 (Rabin et al., 1981) was cultured at 4 x 10^5 cells/ml in 10% FCS containing medium and the supernatant (SN) collected after 48 h in culture. After 0.2 μm filter sterilisation, the SN was tested for its ability to induce proliferation of lymphocytes (Chapt.25.11) and stored at 4°C (see Fig. 2.1).

11. Interleukin-2 assay
In order to test the lymphoproliferative properties of the MLA 144 SN batches and human recombinant IL-2, an IL-2 dependent cell line was established. Briefly, PHA blasts were prepared from human PBMC and cultured with PHA-P 1 μg/ml to induce blast formation. These cells are dependent on exogenous IL-2 for further proliferation. After 4 days the cells were washed twice to remove mitogen and restimulated with IL-2 every 3 - 4 days. Resting cells were resuspended at 2 x 10^6/ml in 10% FCS containing media. The cells were plated out in triplicate at 2 x 10^5 cells per well in 96 well flat bottomed tissue culture plates in 100 μl volumes. Dilutions of the MLA 144 SN and rIL-2 (5%, 10%, 25%, 50%) (v/v) were made in culture medium and added to the cells in triplicate in 100 μl aliquots. Cells incubated with media alone were also included as a control to check for background proliferation. A proliferative response was measured after 48-72 h incubation at 37°C 5% CO₂/95% air atmosphere by pulsing each well with 1 μCi ³H-thymidine (Amersham International, Aylesbury, U.K.) for the final 8 h. The cells in each well were harvested using a cell harvester (Titertek, Flow Labs.) and counted on a beta scintillation counter (Packard Instruments). Mean counts per minute (CPM) were calculated for each triplicate and plotted against IL-2 concentration (%). The IL-2 dilution that induced the maximum proliferation was used for maintaining IL-2 dependent cell lines.

An example of a typical growth response curve for IL-2 is shown in Fig 2.1. For optimal growth of human T cell lines, either recombinant IL-2 or fresh MLA-144 SN was added every 3 days at a concentration of 10% or 25% (v/v) respectively.

12. Human lymphocyte proliferation assay

CD19+, <3% CD14+. 
PBMC in human culture medium were plated out in triplicate at $4 \times 10^5$ cells per well in 96 well flat bottomed tissue culture plates (Nunc) in the presence of antigen (concentration range ng - µg) to be tested. Cells plus PHA or Con-A were also plated out in triplicate as a positive control for cell proliferation at 1 µg/ml and 10 µg/ml respectively. Cells incubated with media alone were also included as a control to check for background proliferation. A proliferative response was measured after 5 days incubation at 37°C 5% CO₂/95% air atmosphere by pulsing each well with 1 µCi [%H-thymidine (Amersham International, Aylesbury, U.K.) for the final 8 h. The cells were harvested using a cell harvester and radioactivity counted on a beta scintillation counter as described above. Mean counts per minute were calculated for each triplicate and plotted against antigen concentration. The proliferative response to antigen was expressed as a stimulation index (SI) (proliferation to antigen / background proliferation) where a significant proliferative response was said to have occurred when the proliferation to antigen was at least two times greater than that of cells incubated without antigen (SI ≥ 2).

13. Culturing of human myelin basic protein reactive T cell lines

Human myelin basic protein reactive lines and autologous Epstein Barr Virus (EBV) transformed B cells, a kind gift of Dr M A Bach (Institute Pasteur, Paris, France.), were isolated from a the PBMC of a healthy volunteer and an MS patient as previously described (Tournier-Lasserve et al., 1988). The cell lines were cultured in 10% AB⁺ human serum supplemented with IL-2 were restimulated every 10 days with IL-2 and HMBP (50 µg/ml) plus irradiated (8000 Rads) autologous EBV transformed B cells acting as antigen presenting cells (10⁶ EBV B-cells : 4x10⁵ T cells).

14. Preparation and culturing of short term mouse T cell lines

SJL/J mice, 6-8 weeks old, were injected subcutaneously on each abdominal flank with a total of 0.1 ml of inoculum consisting of either 100 µg of keyhole limpet haemocyanin (KLH) in PBS emulsified 1:1 (v/v) with complete Freund's adjuvant (CFA) or 200 µg of GPMBP in PBS emulsified 1:3 (v/v) with CFA supplemented with 50 µg Mycobacterium tuberculosis H37 Ra.

At 7-10 days post inoculation, the inguinal and axillary lymph nodes were excised and the tissue disrupted through a 0.5 mm mesh sieve in RPMI. The resulting lymph node cell (LNC) suspension was washed three times with 10% FCS and the cells plated out in mouse
complete medium (see 2.4.4) at 2 x 10⁴ cells /ml in 24-well tissue culture plates with the appropriate antigen (10 µg/ml KLH or 5-25 µg/ml GPMBP). At day 7 the cells were restimulated with antigen plus syngeneic irradiated (3000 rads) spleen cells (feeder cells), at a ratio of 1:15 - 1:20 LNC : feeder cells plus IL-2. The cells were subsequently restimulated with IL-2 (MLA 144 SN) at 3 to 4 day intervals with restimulation by antigen/feeder cells every 7 days.

After 4-5 weeks in culture, and at least 7 days after the last addition of feeder cells, and antigen, the lymph node cells were purified by double passage over nylon wool columns at 37°C in the presence of serum (20% FCS)(see 2.6.8). The effluent cell population, enriched for T lymphocytes, was washed once with RPMI plus 10% FCS and the cell density was adjusted as required for incubation with myelin.

15. Indirect immunofluorescent staining of PBMC
To block Fc receptors expressed by some PBMC, the cells (2 x 10⁶) were incubated with RPMI 1640 medium containing 5% NBBS in flexible 96 well microtitre plates for 20 min at 4°C. The plate was centrifuged 100 x g for 3-4 min and supernatant removed. The cell pellets were resuspended in 40 µl of MAb used at optimal concentrations as determined by previous titration experiments (example, Table 2.2). After 30 min incubation at 4°C the cells were washed 3 times in staining medium to remove any unbound first layer antibody before adding 40 µl of fluorescein conjugated rabbit anti-mouse IgG. After a further 30 min at 4°C the cells were washed 3 times and resuspended in 10 µl of medium and added to a glass microscope slide. The stained cells were sealed with a glass coverslip and nail varnish and counts of at least 200 cells per slide were performed immediately using a Zeiss universal microscope (Oberkochen; FRG). Plate 2.1 shows human PBL PHA blasts stained with FTTC conjugated anti-Tac (CD25) MAb. Five of the nine cells are stained positively for the expression of the IL-2 receptor.

16. Indirect immunofluorescence of CSF lymphocytes
The CSF cell pellet was resuspended in RPMI 1640 and 200 µl aliquots were centrifuged onto microscope slides using a Shandon cytocentrifuge (Shandon Southern Products Ltd, Runcorn, U.K.) 100 x g for 5 min. The slides were allowed to air dry and then fixed in acetone for 6 min and washed in PBS before Fc receptor blocking with 5% NBBS containing medium for 20 min at 4°C. First layer MAb was applied for 30 min in a 50 µl volume at 4°C.
The slides were then washed 3 times with staining medium to remove all unbound antibody before applying the second layer antibody, fluorescein conjugated rabbit anti-mouse Ig for a further 30 min at 4°C. After washing 3 times the cells were sealed with a glass coverslip and nail varnish and counts of 100 cells or more per slide were made as above.

17. Single alkaline phosphatase anti-alkaline phosphatase (APAAP) staining of leucocytes. Cytocentrifuge slides of PBMC or isolated suspensions were prepared by centrifugation of 1 - 2 x 10^6 cells onto microscope slides using a Shandon cytocentrifuge. The slides were allowed to air dry overnight and either stained immediately or stored frozen at -20°C until required. The air dried cytospins were fixed with 50 µl 1 % paraformaldehyde (w/v) (BDH) for 1 min and washed with PBS pH 7.2. Slides were sequentially incubated in the dark with primary MAb followed by rabbit anti-mouse Ig followed by APAAP complex (both DAKO Ltd, High Wycombe, U.K.), a 5 minute wash was carried out between each 30 minute incubation. All MAb were diluted in 10% FCS containing medium to optimal concentrations determined by previous titration experiments. Slides were rinsed, dried and incubated with 0.1 mg/ml Fast Red TR salt in Tris HCL buffer, pH 8.1 including 0.025 % Naphthol AS-MX phosphate (all Sigma Chemical Co.) until a delicate pink colour developed. Slides were immersed in tap water to rinse excess reaction mixture and left to air dry before counter staining with Mayers haematoxylin (BDH) for 1 min. Slides were rinsed, dried and mounted under a coverslip with a 50 % glycerol/isotonic saline (v/v) mixture and at least 200 cells counted per slide using a bright field microscope.

18. Localisation of serine esterase in leucocytes cytochemically
Cytocentrifuge slides prepared and fixed as above were stained for the trypsin-like serine esterase (SE) enzyme according to the method of Wagner and colleagues (1991). Slides were incubated at 37°C with 2 x 10^{-4} M N X benzoxycarbonyl-L-lysine thiobenzyl ester (BLT), as substrate in 0.2 M Tris-HCL buffer pH 8.1 incorporating the chromogenic capture agent Fast Blue BB salt at 0.2 mg/ml (both Sigma Chemical Co.). After 10-15 min a yellow/brown reaction product develops and the phenotype of the SE^ cells can be determined by APAAP immunocytochemical procedures detailed above (see 2.6.17).
19. **Quantification of serine esterase activity in cell lysates**

In order to quantify SE activity contained within leucocytes or leucocyte SN’s, a modification of the assay described by Coleman and Green (1981) was employed. Briefly 10 - 100 μl cell lysate (cells lysed in 0.1 % TRITON-MES) or cell SN was incubated with 50 μl 0.88 mM 5’5’-dithiobis-2-nitrobenzoic acid and 50 μl BLT both in PBS pH 7.2 for 20-30 min at 37°C. Absorbance was read immediately at 405 nm (Anthos Labtech Instruments). 1 Unit of enzyme activity was defined as an absorbance of 1.0, and BLT esterase activity was expressed as units BLT esterase activity / 1 x 10^6 cells.

20. **Human myelin preparation**

Human myelin (HM) was prepared according to the method of Norton and Poduslo (1973). Briefly fresh or semi-frozen white matter from individuals who had died from non-neurological conditions was homogenised at 4°C in 0.32 M sucrose (10% w/v) using an ultra-turrax T25 homogeniser and layered onto 0.8 M sucrose. The gradient was centrifuged at 40,000 x g for 40 min and the interface layer removed and washed twice in 10 volumes 10mM Tris-HCL, pH 7.4. The protein content was determined and the purity confirmed by SDS-PAGE (Plate 2.1). The myelin had a composition consistent with highly purified myelin and was stored in aliquots in distilled water at -20°C until used. Storage did not affect the stability or total CNPase activity of the myelin preparation.

21. **Mouse myelin preparation**

Myelin was prepared from fresh or frozen whole brain and spinal cord of adult SJL/J mice as above for human myelin (Chapt 2.6.20). The presence and purity of the myelin was confirmed by SDS-PAGE and electron microscopy (kindly undertaken by Dr D Landon, Institute of Neurology). The preparation showed the characteristic appearance of isolated myelin (Plate 2.3) with loose vesicles in the size range of mitochondria or nuclei. Intact multi-lamellar structures were visible although smaller unit membrane fractions were also present.

22. **Isolation of basic protein from guinea pig myelin**

Guinea pig myelin was prepared from fresh or frozen whole brain and spinal cord as for human myelin (Chapt. 2.6.20). The basic protein was then isolated from guinea pig myelin membranes by extracting in 0.1 M HCL / 5% methanol for 40 min at 37°C. The resultant SN was concentrated in an Amicon ultrafiltration stirred cell (Amicon Ltd., Gloucestershire,
U.K.) and run on a Sephadex G50 chromatography column in 10 mM HCL. Purity of the resultant guinea pig myelin basic protein (GPMBP) was assessed by SDS-PAGE. (Plate 2.2).

23. Protein determination
Protein content was determined by a modification of the original Lowry procedure (Lowry et al., 1951) (All reagents BDH). 0.1 ml of sample to be assayed (maximum of 50 µg protein) was added to 1 ml of a mixture of 50 ml 2% (w/v) NaCO₃ in 0.1 M NaOH + 1 ml 0.5% (w/v) CuSO₄·5H₂O in 1% (w/v) sodium citrate. After 10 min at room temperature 0.1 ml 50% (V/V) Folin Cioccatteau reagent in distilled water was added, mixed and left to stand for 30 - 60 min (until a blue colour developed). Absorbance was read at 750 nm on a Beckman DU-6 spectrophotometer.

24. Protein separation by polyacrylamide gel electrophoresis
All samples were separated by electrophoresis on slab gels (0.75 x 125 x 140 mm) of 12.5% acrylamide using the system of Laemmli (1970). Protein samples were mixed with one volume of SDS sample buffer (10% SDS / 10% sucrose / 5 mM dithiothreitol / 0.1% bromophenol blue v/v). Electrophoresis was performed at 20 mA constant current, until the dye front line reached the bottom of the gel. Gels were stained with 0.2% Coomassie Blue in 50% methanol / 10% acetic acid (v/v) and de-stained in 50% methanol / 10% acetic acid (v/v) (all reagents BDH).

25. Myelin degradation assay
A frozen aliquot of human myelin was defrosted and subsequently washed in RPMI tissue culture medium and spun 7,500 x g for 5 min. The myelin pellet was resuspended in RPMI alone and triplicate samples of myelin were incubated with serum (25% v/v) or PBMC in a ratio of 10 µg myelin protein: 2 x 10⁶ PBMC per well in 96 well flat bottomed tissue culture plates (Nunc, Gibco Europe). Triplicate samples of myelin alone in culture medium without serum or PBMC were also incubated as a control at the beginning and end of the plate. After 24 hr incubation at 37°C in a humidified 5% CO₂ / 95% air atmosphere, the myelin with cells or serum was resuspended, transferred to microcentrifuge tubes and centrifuged at 7,500 x g for 5 min. The supernatant was discarded and the myelin / cell or serum pellet was resuspended by vortexing in 400 µl RPMI 1640 and stored at -20°C. 2':3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) activity was assayed within one month of freezing.
CNPase activity was assayed fluorimetrically using cyclic NADP* as substrate (Sogin 1976) according to the method of Weissbarth and colleagues (1980) and modified by Rastogi and Clausen (1985). The optimal conditions for the enzyme assay were determined by varying the myelin protein concentration, substrate concentration and incubation time and the reproducibility of the analytical method was confirmed. The assay conditions are summarised below. The equivalent of 0.5 - 1.0 µg of protein (myelin) was incubated in 280 µg of the reaction mixture (0.2 M MES buffer, pH 6.0; 30 mM MgCl₂; 1mM Na₂ EDTA; 0.025% Triton X-100; 1.2 µg/ml BSA; 1 µM 2',3'-cyclic NADP*; 6 mM glucose-6-phosphate and 5.6 U/ml glucose-6-phosphate dehydrogenase) for 20 min at 30°C. The enzyme reaction was stopped by the addition of 2.52 ml 50 mM sodium-carbonate buffer, pH 10.5, to the incubation mixture. The fluorescence of the end product (NADPH) was measured using 360 nm as excitation and 460nm as emission wavelengths (Perkin Elmer LS-2B filter fluorimeter). Fluorescence of serial dilutions of B-NADPH in sodium carbonate buffer were used to plot a standard curve for each assay. Enzyme activity was expressed as nanomols of end product (NADPH) / 20 min / total volume of sample, as a fixed amount of myelin was used in each set of experiments.

CNPase activity of myelin incubated with culture medium alone did not vary significantly during individual experiments (triplicates of myelin alone were always placed at the beginning and end of the 96 well plate to ensure the reproducibility of the assay). To allow for slight variation between each series of experiments (less than 10%) results are expressed as percentage loss in CNPase activity of myelin after incubation with PBMC or serum and calculated as follows.

\[
\%\text{Loss CNPase Activity} = 100 - \frac{\text{mean CNPase activity in myelin + cells/serum pellet}}{\text{mean CNPase activity in myelin alone pellet}} \times 100
\]

The statistical significance of the loss in CNPase activity of the myelin when incubated with cells or serum as compared to myelin incubated with media alone was calculated using the Students t-test at p < 0.01.
27. Enzyme linked immunosorbant assay (ELISA) for myelin basic protein.

An inhibition enzyme-linked immunosorbent assay (ELISA), using an alkaline phosphatase conjugated monoclonal antibody, clone 12, which is reactive with an epitope of HMBP in the region 86-96 (Groome et al., 1986), was developed in Oxford by Dr. N. Groome. All incubations were carried out at room temperature. Unknowns and standards (serial dilutions of HMBP) were incubated overnight (16-24 h) in microcentrifuge tubes with an appropriate dilution of the MAb. All samples (unknowns, standards and MAb) were diluted in a 0.05 M phosphate buffer, pH 7.0, containing 0.12% (w/v) sodium chloride, 0.05% (w/v) Tween 80, and 0.025% (w/v) calf thymus histones. Duplicate samples (500 µl) of each of the unknowns and standards were then transferred to HMBP-coated Maxisorb tubes and incubated for a further 16-24 h. The tubes were washed three times with ice-cold 1.2% (w/v) sodium chloride, 0.05% (w/v) Tween 80, and 600 µl of a 1 mg/ml p-nitrophenyl phosphate solution, pH 9.8, containing 0.005% (w/v) MgCl and 9.6% (v/v) triethanolamine, was added to each tube. The tubes were then incubated for up to 3 h in the dark, before 200 µl of each sample was plated out on a 96-well microtitre plate and the absorbance read at 405 nm using a Titerpak Multiscan spectrophotometer. A dilution of enzyme-labelled MAb which alone gave an absorbance of approximately 0.7 units was used throughout these experiments. The clone 12 monoclonal antibody employed in this assay was determined to cross-react with mouse MBP with an efficiency of approximately 65% (data not shown). Results are expressed as nanograms of MBP with the proviso that these figures do not distinguish between intact MBP and breakdown products of MBP with intact epitope 86-96 and have not been corrected to account for the efficiency of binding to mouse MBP. Prior to immunoassay an acidified, boiling, extraction step, which removes much lipid and protein material but leaves MBP in solution, as described by Delassalle and colleagues (1980), was included for each of the unknown samples prior to immunoassay.
### TABLE 2.1

SPECIFICITY OF MOUSE MONOCLONAL ANTIBODIES

<table>
<thead>
<tr>
<th>Antibody/clone</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCHT-1</td>
<td>CD3</td>
<td>Serotec Ltd, Oxford, U.K.</td>
</tr>
<tr>
<td>G10 - 1.1</td>
<td>CD8</td>
<td>Dr J A Ledbetter, Genetic Systems Corp. Seattle, U.S.A.</td>
</tr>
<tr>
<td>Tac</td>
<td>CD25</td>
<td>Dr T A Waldmann, Nat. Inst. Health Bethesda, U.S.A.</td>
</tr>
<tr>
<td>DA-2</td>
<td>HLA-DR</td>
<td>Dr J Bodmer, ICRF, London</td>
</tr>
<tr>
<td>UCHM-1</td>
<td>CD14</td>
<td>Serotec</td>
</tr>
<tr>
<td>anti-leu 12</td>
<td>CD19</td>
<td>Serotec</td>
</tr>
</tbody>
</table>

CD, antigen cluster designation  
HLA, human leukocyte antigen  
NK, natural killer cell
### TABLE 2.2

**TITRATION OF MOUSE MONOCLONAL ANTIBODY TO THE HUMAN CD4 ANTIGEN**

<table>
<thead>
<tr>
<th>Dilution of MAb</th>
<th>No. PBL's immunofluorescing</th>
<th>% positive PBL's</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media alone</td>
<td>1/225</td>
<td>&lt;1</td>
</tr>
<tr>
<td>0</td>
<td>2/203</td>
<td>1</td>
</tr>
<tr>
<td>1/2</td>
<td>77/202</td>
<td>38</td>
</tr>
<tr>
<td>1/3</td>
<td>65/211</td>
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<tr>
<td>1/4</td>
<td>102/218</td>
<td>47</td>
</tr>
<tr>
<td>1/5</td>
<td>38/200</td>
<td>19</td>
</tr>
</tbody>
</table>

To determine the optimal dilution of stock anti-leu 3a MAb to use for the identification of cells expressing the CD4 antigen, human PBL's were stained with varying dilutions of MAb by the indirect immunofluorescence technique (see Chapt. 2.6.15). Briefly, stock anti-leu 3a MAb (Becton Dickenson) (25 µg/ml) was diluted in RPMI 1640 medium and added to human PBL's for 30 min at 4°C. The cells were washed three times to remove any unbound first layer antibody before adding fluorescein conjugated rabbit anti-mouse IgG. After a further 30 min incubation at 4°C the cells were washed, mounted on a glass slide with coverslip and counts of at least 200 cells per slide were performed immediately.

The dilution of 1/4 of stock MAb was chosen as the optimal concentration for estimation of the expression of CD4 antigen by lymphocytes.
Resting IL-2 dependent human T cells were incubated in triplicate at $2 \times 10^5$ cells per well with dilutions of recombinant IL-2 or fresh MLA-144 SN (Chapt. 2.6.11 & 2.6.10) at concentrations of 5%, 10%, 25% or 50% (v/v). A proliferative response was measured after 48-72 h incubation at 37°C by pulsing each well with 1 μCi $^3$H-Thymidine for the final 8 h. The cells were harvested and counted on a beta scintillation counter. Results are expressed in mean counts per minute (CPM) ± standard deviation.
IMMUNOFLUORESCENT STAINING OF HUMAN PBL FOR IL-2 RECEPTOR EXPRESSION USING THE MAb anti-TAC

PHA stimulated human lymphocytes examined by phase contrast (A) and by immunofluorescence technique (B) for IL-2 receptor expression (CD25) using the mouse monoclonal antibody anti-TAC (magnification x 630)(see Chapt. 2.6.15).
Myelin proteins separated by SDS polyacrylamide gel electrophoresis (see 2.6.24) and identified by molecular weight markers. (a + e) molecular weight markers, (b) GPMBP, (c) HMBP, (d) human myelin.
PLATE 2.3

ELECTRON MICROGRAPH OF MOUSE MYELIN PREPARATION

Bar = 1 μM.

The smallest proteins (SP) are the most abundant proteins in myelin and account for approximately 20% of the total protein. They are a group of highly charged proteins located at the cytoplasmic face of the myelin sheath and are thought to play a role in forming and maintaining the close junctions of the cytoplasmic faces of the bilayer, which prevent the MDL characteristic of myelin.

The next largest proteins are the hydrolytic enzymes. These proteins include cyclic nucleotide (cNAPase) and MAO cNAPase, which account for nearly 66% of myelin protein and on SDS-PAGE, the activity has been localized to two closely spaced proteins (cNAPase 4, 46 kDa molecular weight and cNAPase II, 46 kDa isoform) to the front of...
CHAPTER THREE

AN IN VITRO ASSAY FOR ASSESSING
MYELIN DEGRADATION

3.1. INTRODUCTION

Myelin is the major constituent of white matter in the brain and is produced as an extension
of the plasma membrane of the oligodendrocyte which wraps spirally around the axon and
thus provides nerve fibres with electrical insulation to facilitate the conduction of nerve
impulses. In adults the myelin sheath is a compact multi-lamellar structure and when viewed
by electron microscopy shows an alternating pattern of thick (major dense, MDL) and thin
(intra period, IDL) lines that represent the apposition of the cytoplasmic and extracellular
membrane surfaces of the oligodendrocyte. During myelination the oligodendrocyte
synthesizes proteins which are inserted into the lipid matrix of the plasma membrane and
which are thought to participate in the initial organization and subsequent maintenance of
the myelin sheath (Gillespie et al., 1990).

Myelin has a low protein content (approximately 30% of dry weight) and a high lipid content.
The major integral membrane protein of the myelin sheath is PLP (approximately 55% of
the total protein content) which contains highly hydrophobic segments that are thought to
be membrane embedded which thus promotes formation and stabilization of the compact
multi-lamellar structure of myelin.

The basic proteins (BP) are the next most abundant proteins in myelin and account for
approximately 30% of the total protein. They are a group of highly charged proteins located
on the cytoplasmic face of the membrane bilayer and are thought to play a role in forming
and maintaining the close apposition of the cytoplasmic faces of the bilayers which produce
the MDL characteristic of myelin.

Two other less abundant proteins are the hydrolytic enzyme 2', 3', cyclic nucleotide 3'
phosphodiesterases (CNPase) and MAG. CNPase represents approximately 5-6% of myelin
protein and on SDS PAGE the activity has been localized to two closely spaced proteins
(CNPase I, 46 kDa molecular weight and CNPase II, 48 kDa) identical to the doublet of
protein previously referred to as the Wolfgram proteins (WP) (Drummond and Dean 1980; Sprinkle et al., 1980). CNPase selectively hydrolyses the 3'-phosphate bond of 2',3' cyclic nucleotides (Drummond et al., 1962) however, the natural substrate for this enzyme and its function has yet to be identified in the brain. It is primarily localised in myelinated regions of the CNS and on the plasma membrane of the oligodendrocyte. The enzyme is thought to participate in the earliest events of myelinogenesis (reviewed by Braun et al., 1987) as it has been detected in rat oligodendrocytes at the start of myelination preceding the deposition of MBP by 1-3 days (Roussel and Nussbaum, 1981). In contrast to other myelin proteins CNPase is not unique to myelin but is also present, although at much lower levels (no more than 3% of the activity of whole brain) in other tissues, most notably thymus, spleen, lymphocytes and platelets, and also the retina (reviewed by Sprinkle, 1989). Within the CNS, biochemical evidence suggests that CNPase is enriched in oligodendrocyte membrane fractions that are distinct from compact myelin fractions (Shapira et al., 1978; McIntyre et al., 1978;) and immunocytochemical and ultrastructural techniques indicate that the hydrolase is preferentially localized near the plasma membrane in the cell body and processes. The enzyme is also found in the non-compacted regions of the myelin sheath such as the paranodal loops, the myelin axonal interface outer tongue processes and incisure like membrane within the myelin sheath (Trapp et al., 1988; Brunner et al., 1989). The exact localisation is still unclear as Brunner and colleagues detected a significant amount of CNPase in compact myelin, whereas Trapp and co-workers report a paucity of the enzyme within this region (see plates 3.1 and 3.2). However, differences in the staining techniques and antibodies used could be responsible for this contentious result and therefore needs further studies to clarify.
Electron micrograph of 60-day old rat peripheral nerve immunostained for CNP (A). Gold particles representing the location of CNP are enriched over periaxonal regions (arrows), along the outer perimeter of the myelin sheath (arrowheads), and in Schmidt-Lanterman incisures (SL). Axons and compact myelin contain few gold particles. Bars = 0.05 μm. (From Trapp BD et al., (1988) J. Neurochem.,51, 859-868).
Quantitative evaluation of antigen distribution in different zones of myelin sheaths and oligodendrocytes as measured by immunostaining. Myelin sheaths and oligodendrocytes were divided into: (1) perinuclear oligodendrocyte cytoplasm, (2) oligodendrocyte membrane-associated zone, (3) axoplasma, (4) myelin/axon interface, (5) compact myelin, and (6) myelin surface-associated zone. The bars represent the ratios between gold grain counts over the respective zones and background counts. The fine-dotted basis zone indicates background counts + 3 standard deviations. MOG, myelin oligodendrocyte glycoprotein; CNP, 2':3''-cyclic nucleotide 3'-phosphodiesterase; MBP, myelin basic protein; NF, neurofilament (From Brunner et al., (1989) J. Neurochem., 52, 296-304).
Apart from this reported difference in localization to compact myelin, there is other evidence showing that CNPase is clearly an integral myelin protein. Its enrichment in oligodendrocyte processes and regions of the myelin sheath that contain oligodendrocyte cytoplasm support a role for CNPase in forming and stabilizing the myelin interface. However, the fact that CNPase is found outside the CNS suggests that its primary metabolic function is not exclusively related to myelin formation. Among the suggestions for a function is a role in cell interactions as it is present in tissues rich in cells whose membrane interactions are important to their function, such as spleen, thymus and bone marrow (reviewed by Sprinkle, 1989). The oligodendrocyte membrane also interacts with both the axon and to a greater extent itself in forming the multiple myelin lamellae thus supporting a possible role in cell interactions (Weissbarth et al., 1981).

Amongst the less abundant myelin constituents, MAG accounts for less than 1% of the total CNS myelin protein and has been shown to be an intrinsic component of myelin where it is responsible for preventing the compaction of cytoplasmic surfaces. MAG also contains an antigenic epitope that is shared by human natural killer cells as well as neural cellular adhesion molecules (N-CAM) leading to the suggestion that MAG might provide an adhesion system for glial-neuronal interactions (reviewed by Quarles, 1984).

In addition to MAG other minor glycoproteins have been detected in myelin. MOG is selectively located on oligodendrocyte surfaces and myelin sheaths (Linington et al., 1988) and seems to be a relatively late differentiation marker for mature oligodendrocytes (Brunner et al., 1989). Its exact function is not known but studies suggest it might be an important antigenic target in antibody-mediated demyelination as acute EAE mediated by BP-specific T cell lines can become a demyelinating disease by intravenous injection of MAb to MOG (8-18C5) (Schluesener et al., 1987).

In MS and CREAE, demyelination occurs with release of myelin protein or peptides. Biochemical studies on white matter lesions in MS show that there is a depletion of MBP, MAG, and WP and an increase in the astrocyte-specific glial fibrillary acidic protein (GFAP) resulting from the proliferation of fibrous astrocytes (Newcombe et al., 1982). The mechanisms of demyelination are still unknown but both humoral and cellular factors have been implicated in MS and EAE. Adoptive transfer of EAE by MBP sensitised T cell lines or clones indicates an essential role for T lymphocytes in the disease and in vitro studies of
demyelination provide evidence for a T cell involvement (Lyman et al., 1986). However, a direct relationship between T cells and myelin destruction has yet to be demonstrated as well as the precise role of accessory cells in the demyelinating process.

The majority of in vitro studies on immune mediated demyelination have relied on relatively imprecise morphological techniques. The demyelinating activity of serum or cells of one species have been tested on explants from developing myelinating CNS cultures of another species with the resulting degree of demyelination assessed by light microscopy.

In this chapter the development and assessment of a simple highly reproducible micromethod for the quantitative assay of myelin degradation is reported. The method is based on the measurement of the myelin specific marker 2':3'-cyclic nucleotide 3'-phosphodiesterase in an isolated syngeneic myelin preparation. Initial model experiments were performed (in conjunction with Carolyn Watson) using mouse myelin (Watson et al., 1988). Once it had been determined that CNPase was a good marker of myelin degradation the model was adapted to a human system so that the role of sera and lymphocytes in initiating in vitro myelin degradation could be studied. Both MBP and CNPase were initially measured to test the validity of the system as a model for assessing myelin degradation.

Isolated myelin has been shown to be vulnerable to proteolytic and lipolytic attack (Banik et al., 1976) and it has been suggested that proteases and phospholipases secreted by inflammatory cells at the lesion site may be instrumental agents in myelin breakdown in vivo (Cammer et al., 1978; Smith et al., 1974; Trotter and Smith, 1986). Lysophosphatidylcholine generated by the action of phospholipases may also contribute to the dissolution of myelin. A combination of trypsin, phospholipase A₂ and lysophosphatidylcholine was therefore used to examine the effects of myelin degrading factors within the model. Cell mediated myelin degradation by both mouse T cells and human PBMC will also be demonstrated.
3.2 RESULTS

3.2.1. Model experiments of myelin degradation

After a 1 h incubation at 37°C approximately 5% of total CNPase activity and 50% of total assayable MBP could be detected in the incubation supernatant (see Table 3.1). Over a 24 h period there was no appreciable change in the level of CNPase activity measured in the residual myelin pellet.

Following incubation with trypsin there was a significant reduction in the levels of both CNPase activity and MBP in the residual myelin pellet with an increase in CNPase activity but not MBP detected in the supernatant. Phospholipase A2 alone had little effect on the recovery of CNPase activity or MBP, however when phospholipase A2 was incubated with trypsin a significant loss of total CNPase activity and assayable MBP was seen. In the presence of lysophosphatidylcholine almost complete solubilisation of the myelin preparation occurred with up to 80% of total CNPase activity being released into the supernatant. The decrease in CNPase activity of the myelin pellet after incubation with the various enzymes correlated well with the decrease in MBP activity of the myelin (r=0.91, p < 0.05).

3.2.2. Mouse myelin degradation by antigen specific cells

Short term murine T cell lines (cultured over a 4-5 week period) specific for MBP or KLH (Tmbp, Tklh) were established from the inguinal and axillary lymph nodes of either MBP or KLH primed SJL mice (Chapt. 2.6.14). Reactivity of the T cells to either MBP or KLH as measured by proliferation is shown in figures 3.1 and 3.2. Both Tmbp and Tklh lymphocytes, which had previously been incubated with antigen and feeder cells for five days and subsequently purified (see chapt. 2.6.14.), when incubated with myelin alone failed to induce any demonstrable loss of CNPase activity or MBP after 24 h co-culture with the mouse myelin preparation (Table 3.2). However, when very low numbers of syngeneic ADH peritoneal macrophages were added to the Tmbp lymphocytes (ratio of 20 T cells : 1 macrophage) significant myelin breakdown was seen as measured by both residual CNPase activity and MBP levels. No myelin degradation was initiated by Tklh lymphocytes either with or without macrophages being present.
3.2.3 Human myelin degradation by MS patients' PBMC

In order to measure the degradation of myelin by human PBMC the assay was adapted to include human myelin instead of mouse myelin. To ensure the reproducibility of the assay system, triplicates of myelin incubated with culture media alone were always placed at the beginning and end of the 96 well plate as controls. The CNPase activity of the myelin alone did not vary significantly during individual experiments however, to allow for a slight variation in CNPase activity between each series of experiments (less than 10%), the degradative activity of the PBMC are expressed as percentage loss in CNPase activity of myelin after incubation with PBMC (see Table 3.3). Cell viability was assessed and there was no observable difference in cell numbers after incubation with myelin for 24 h (data not shown).

The loss of CNPase activity caused by the incubation of PBMC with myelin usually occurred within the first 6 h of incubation, with no significant increase in loss being detectable after 24 h (Fig. 3.3). As there was no significant increase in the loss of CNPase activity from myelin incubated for 24 h with media alone, an incubation time of 24 h was chosen to allow for maximum degradative activity of the PBMC sample. As can be seen from Table 3.3 not all MS PBMC are capable of degrading myelin as only the PBMC from patient 32 and 173 caused a significant loss of CNPase activity. At the time of blood sampling patient 32 and 53 were both in clinical relapse and patient 173 was in remission.

Table 3.4 demonstrates the inter-assay variation of cellular myelin degrading activity as assessed by loss of CNPase activity from myelin. When individual patients' PBMC were incubated with myelin in duplicate experimental assay plates on the same day there was no significant difference between the percentage loss in CNPase activity caused by the PBMC in the two separate experiments (experiment A, r=0.96, p < 0.001). In addition, when cells were incubated with myelin immediately after separation from whole blood and also left overnight on ice and subsequently washed and incubated with myelin at the same ratio of myelin to PBMC as the previous day, an almost identical level of myelin degradation was produced (experiment B, r=0.99, p < 0.001).
TABLE 3.1  *IN VITRO* MYELIN DEGRADATION BY TRYPsin, PHOSPHolipase A<sub>2</sub> AND LYSOPHosphATIDYL CHOLINE AS MEASURED BY THE LOSS IN CNPase ACTIVITY AND MBP FROM MYELIN

<table>
<thead>
<tr>
<th>Myelin incubated with:</th>
<th>CNPase Activity (nmol end product/20min/sample) (n=10)</th>
<th>MBP (ng) (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pellet</td>
<td>Supernatant</td>
</tr>
<tr>
<td>Media alone</td>
<td>20,440 ± 2,505</td>
<td>1,020 ± 147</td>
</tr>
<tr>
<td>Trypsin</td>
<td>15,320 ± 1,449*</td>
<td>3,160 ± 246*</td>
</tr>
<tr>
<td>Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>19,840 ± 2,372</td>
<td>2,080 ± 413*</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>3,480 ± 464*</td>
<td>19,840 ± 1,292*</td>
</tr>
<tr>
<td>Trypsin + Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6,800 ± 1,296*</td>
<td>3,378 ± 290*</td>
</tr>
</tbody>
</table>

* significantly different from myelin incubated with media alone, p<0.001 (Student's t-test).

Each sample consists of 100 µg mouse myelin incubated in 200 µl of RPMI either alone as a control, or with trypsin (25 µg/ml), phospholipase A<sub>2</sub> (80 µg/ml), lysophosphatidyl-choline (1 mg/ml) or a combination of trypsin plus lysophosphatidyl-choline for 1 h at 37°C. At the end of the incubation period trypsin inhibitor, or an equal volume of medium alone was added to each tube. The CNPase activity and MBP content of both supernatant and myelin pellet were determined as described previously (see chapt 2.6.26 & 2.6.27) and results are expressed as mean activity ± standard deviation.
**TABLE 3.2 LOSS OF CNPase ACTIVITY AND MBP FROM MOUSE MYELIN AFTER INCUBATION WITH ANTIGEN SPECIFIC T CELL LINES**

<table>
<thead>
<tr>
<th>Experiment:</th>
<th>CNPase (nmol end product/20min/sample) (n=5)</th>
<th>MBP (ng) (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelin incubated with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media alone</td>
<td>1,315 ± 70</td>
<td>113 ± 17</td>
</tr>
<tr>
<td>T Cells (2x10⁶)</td>
<td>1,302 ± 79</td>
<td>1,484 ± 72</td>
</tr>
<tr>
<td>APC's (1x10⁶)</td>
<td>1,264 ± 0</td>
<td>1,512 ± 39</td>
</tr>
<tr>
<td>T Cells + APC's</td>
<td>904 ± 170*</td>
<td>882 ± 124*</td>
</tr>
</tbody>
</table>

* significantly different from myelin incubated with media alone, p<0.001; # significant at p<0.002 (Student’s t-test)

Myelin (10 μg) was incubated in triplicate either alone or with short term T cell lines specific for MBP and KLH (T<sub>MBP</sub> & T<sub>KLH</sub>) (see Chapt. 2.6.14) with or without syngeneic adherent peritoneal exudate cells (see Chapt. 2.6.7.) acting as antigen presenting cells (APC). After 24 h incubation at 37°C, the resultant CNPase activity and MBP content of the residual myelin pellet was measured as described previously (Chapt. 2.6.26 & 27). Results are expressed as mean activity ± standard deviation. The data from two separate experiments are shown.
TABLE 3.3

LOSS OF CNPase ACTIVITY AFTER INCUBATION OF HUMAN MYELIN WITH PBMC FROM MULTIPLE SCLEROSIS PATIENTS

<table>
<thead>
<tr>
<th>Myelin plus:</th>
<th>CNPase Activity (nmol end product/20min/sample)</th>
<th>Loss CNPase Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EXPT 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media alone</td>
<td>2,955 ± 100</td>
<td>-</td>
</tr>
<tr>
<td>PBMC-patient 32</td>
<td>2,254 ± 161*</td>
<td>24*</td>
</tr>
<tr>
<td>PBMC-patient 53</td>
<td>2,802 ± 57</td>
<td>4</td>
</tr>
<tr>
<td>Media alone</td>
<td>2,928 ± 179</td>
<td>-</td>
</tr>
<tr>
<td><strong>EXPT 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media alone</td>
<td>3,208 ± 133</td>
<td>-</td>
</tr>
<tr>
<td>PBMC-patient 173</td>
<td>2,627 ± 62*</td>
<td>17#</td>
</tr>
<tr>
<td>Media alone</td>
<td>3,087 ± 62</td>
<td>-</td>
</tr>
</tbody>
</table>

* significantly different from myelin incubated with media alone, p<0.005; # p<0.002 (Student’s t-test).

PBMC from three MS patients were incubated at 2×10^5 cells/10 μg myelin (n=3) for 24 h at 37°C. Myelin alone in culture media was incubated at the beginning (n=3) and end (n=3) of each 96 well plate as a control. CNPase activity of the residual myelin pellets are expressed as mean activity ± standard deviation. Loss of CNPase activity after incubation with PBMC is expressed as a % of myelin incubated in media alone subtracted from 100 (see chapt 2.6.26).
TABLE 3.4

INTER-ASSAY VARIATION IN THE PERCENTAGE LOSS OF CNPase ACTIVITY CAUSED BY PBMC INCUBATED WITH MYELIN

<table>
<thead>
<tr>
<th>Patient population</th>
<th>Patient No.</th>
<th>% Loss CNPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plate 1</td>
</tr>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>145</td>
<td>21*</td>
</tr>
<tr>
<td></td>
<td>146</td>
<td>34*</td>
</tr>
<tr>
<td></td>
<td>113</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>532</td>
<td>19*</td>
</tr>
<tr>
<td></td>
<td>591</td>
<td>13</td>
</tr>
<tr>
<td>HEALTHY</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>348</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>349</td>
<td>2</td>
</tr>
<tr>
<td>Experiment B†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>357</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>15*</td>
</tr>
<tr>
<td></td>
<td>156</td>
<td>23*</td>
</tr>
<tr>
<td></td>
<td>408</td>
<td>19*</td>
</tr>
<tr>
<td></td>
<td>556</td>
<td>29*</td>
</tr>
</tbody>
</table>

Significantly different from myelin incubated with media alone, * p <0.01, † p<0.001 (Students t-test).

2 x 10^5 PBMC from MS patients and healthy individuals were incubated with 10 µg myelin (n=3) for 24 h at 37°C (Experiment A, plate 1). In a repeat assay on the same day the PBMC (which had been kept on ice) were again incubated with myelin in exactly the same ratio of cells to myelin as the previous assay (Experiment A, plate 2). In a separate experiment‡ PBMC were incubated immediately with myelin (Experiment B, plate 1) as described above and in addition the remaining PBMC were left overnight on ice and then washed and incubated with myelin at exactly the same ratio of cells to myelin as the assay set 24 h earlier (Experiment B, plate 2). Myelin was always incubated with culture media alone at the beginning and end of each 96 well plate as a control (n=6). The CNPase activity of the residual myelin pellets were determined as previously described (Chapt 2.6.26). Loss of CNPase activity after incubation with PBMC is expressed as a % of myelin incubated in media alone subtracted from 100. Results are from more than one assay.
2 x 10^5 nylon wool enriched T_{MBP} cells plus 1 x 10^4 syngeneic peritoneal macrophages were incubated in triplicate in 96 well plates for 72 h at 37°C with or without antigen (see Chapter 2.6.14). [ ] = cells incubated with media alone, [\|] = guinea pig MBP 1 - 50 μg/ml, [XX] = KLH 1 - 50 μg/ml. In the last 8 h of incubation 1 μCi of ^3H-Thy was added to each well to measure a proliferative response. The cells were harvested and counted on a beta scintillation counter. Results are expressed in mean counts per minute (cpm) ± standard deviation (SD).
FIGURE 3.2
ANTIGEN INDUCED PROLIFERATION OF A KEYHOLE LIMPET HAEMOCYANIN SHORT TERM T CELL LINE

2 x 10^5 nylon wool enriched T<sub>KLH</sub> cells plus 1 x 10^4 syngeneic peritoneal macrophages were incubated in triplicate in 96 well plates for 72 h at 37°C with or without antigen (see Chapter 2.6.14). [ ] = cells incubated with media alone, [] = guinea pig MBP 1 - 50 µg/ml, [XX] = KLH 1 - 50 µg/ml. In the last 8 h of incubation 1 µCi of ^3H-Thy was added to each well to measure a proliferative response. The cells were harvested and counted on a beta scintillation counter. Results are expressed in mean cpm ± SD.
FIGURE 3.3

LOSS OF CNPase ACTIVITY FROM HUMAN MYELIN AFTER INCUBATION WITH PBMC, FROM EITHER AN MS PATIENT OR HEALTHY INDIVIDUAL, OVER 24 HR.

PBMC from an MS patient (patient no. 347) (A) and a healthy control (no. 348) (B) were incubated with human myelin at $2 \times 10^5$ cells/10 μg myelin in triplicate for each time point, myelin in culture media alone was also incubated in triplicate for each time point. At 3, 6, 12, and 24 h the myelin alone (●) and myelin plus PBMC (○) triplicates were harvested and CNPase activity of the pellet assayed as described previously (Chapt. 2.6.27). Each time point represents the mean ± SD of three replicate samples.
3.3 DISCUSSION

Many previous studies of in vitro demyelination have depended on the use of explants of developing CNS tissue (Lyman et al., 1986; Selmaj et al., 1986). However, assessment of the extent of myelin degradation by light and electron microscopy can only be qualitative. For this reason various biochemical markers of demyelination have been studied. These include CNPase (Weissbarth et al., 1980; Roth and Bornstein, 1984), and MBP (Sheffield and Kim, 1977; Nishimura et al., 1986). Due to the differing amounts of myelinated fibres in the cultures, control and experimental values show a relatively wide range of variability. Moreover, addition of control sera to cultures can result in demyelination (Silberberg et al., 1984). To overcome this difficulty suspensions of purified myelin have been used. Such a simple system enables fixed amounts of myelin (determined by protein content) to be used for each assay. Measurements of CNPase and MBP in this system indicate it to be highly reproducible with little intra-assay variation even after 24 h incubation at 37°C (Fig.3.3).

Although the preparation contains a high proportion of compact myelin, split myelin lamellae with exposed cytoplasmic faces are also present. As there is evidence to suggest that MBP is localised at the cytoplasmic surface (MDL) of myelin membranes (Poduslo and Braun, 1975), MBP would therefore be more accessible to proteolytic enzymes than in intact myelin. Earlier research using purified myelin has demonstrated that MBP is readily degraded by trypsin (Banik and Davison, 1974; Banik et al., 1976) and significant losses of MBP from myelin suspensions incubated with trypsin are also reported here. MBP is also susceptible to the action of endogenous proteases present in the purified myelin (Sato et al., 1982; Chantry et al., 1988) which may explain the presence of almost 50% of assayable MBP in the supernatant after incubation in media alone. Also such autolysis could explain the marked reduction in total MBP levels (SN + pellet) detected by ELISA following incubation with the surfactant lysophosphatidylcholine. The possible effect of the lysophosphatidylcholine would be to expose more of the MBP to myelin associated proteases in a manner similar to that described in oedematous CNS tissue (Smith, 1977).

Sprinkle et al. (1980) and Drummond and Dean (1980) have demonstrated that CNPase activity is associated with the W1 component of WP. Topological studies suggest that WP is partially exposed at the cytoplasmic surface of myelin (Delaunoy et al., 1982). Complete solubilisation of CNPase, however, requires the presence of delipidating or chaotropic agents
(reviewed by Sims and Carnegie, 1978); thus CNPase exhibits some of the properties of an intrinsic membrane protein. Although significant release of CNPase activity occurred upon co-incubation with trypsin, proteolysis was greatly enhanced by the addition of phospholipase A$_2$ to the system and extensive solubilisation of CNPase was mediated by lysophosphatidylcholine. Trypsin-related losses of CNPase activity appeared to take place within the first minutes of incubation, especially at high concentrations of trypsin, and reached a maximal level that could not be exceeded even by adding fresh trypsin to the myelin suspension. These losses could represent CNPase that is present in unit membrane of less compact multi-lamellar membrane fragments that are more prone to proteolytic digestion. These findings are consistent with the intrinsic membrane protein properties ascribed to the CNPase enzyme. Thus these experiments would suggest that CNPase is a reliable and sensitive marker of myelin degradation in vitro.

Preliminary experiments (Table 3.2) have been designed to see if mouse T cells sensitised to MBP or to the non-CNS antigen KLH with or without macrophages can induce in vitro myelin degradation as assessed by loss of myelin CNPase activity. T cell lines, specific for MBP or KLH, which were pre-activated (5 days incubation with feeder cells and specific antigen) were added to mouse myelin in the presence or absence of syngeneic macrophages. Nylon wool purified T cells incubated with myelin caused no significant loss of CNPase activity however, significant in vitro myelin degradation could be demonstrated when a limited number of syngeneic elicited macrophages were added to the MBP-sensitised cells. Addition of syngeneic macrophages to the $T_{KLH}$ cells incubated with myelin did not result in significant myelin degradation. However, as there was no KLH added to the myelin culture one cannot conclude that the in vitro myelin degradation demonstrated by the $T_{MBP}$ cells was antigen specific. Future experiments where KLH is added to the $T_{KLH}$ cells incubating with myelin would demonstrate whether T cells sensitised to non-myelin antigens plus syngeneic macrophages were also capable of causing in vitro myelin degradation.

In a similar experiment Lyman and colleagues (1988) demonstrated that both MBP and PPD specific mouse T cells when added to organotypic cultures of syngeneic spinal cord caused decreases in CNPase, with the MBP T cells causing significantly greater and consistent changes. No exogenous antigen (MBP nor PPD) was added to the cultures nevertheless, the T cells had been incubating with antigen for 5 days prior to their addition to the spinal cord cultures and the possibility that some cross-reactivity may exist between PPD and MBP.
cannot be ignored. If lymph node cells freshly isolated from MBP immunised mice were added directly to the organotypic culture then significant loss of CNPase was demonstrated, greater than that seen with T cell lines. They concluded that T cell-mediated CNS demyelination may be comprised of CNS antigen-specific and non-specific components.

Whether this non-specific component is a bystander effect and related to the T<sub>ppd</sub> cells previous stimulation with antigen before addition to the myelinated spinal cord culture could be an alternative explanation to the cross-reactivity of PPD and MBP protein that was proposed by the authors.

By adapting the assay to use human myelin instead of mouse myelin human the role of PBMC and sera in myelin degradation can be investigated. The assay has been shown to give highly reproducible results with little variation in the percentage loss of CNPase activity demonstrated by a PBMC sample incubated with myelin in more than one assay plate on the same day. In addition, when the cellular myelin degrading ability of freshly isolated cells is compared to the myelin degrading ability of the same cells which have been left overnight on ice and subsequently incubated with myelin, there is minimal inter-assay variation.

In conclusion, measurement of the release of CNPase from a myelin preparation provides the basis for a simple, reliable, and precise model for the study of myelin degradation in vitro. Analysis of the effects of trypsin, phospholipase A<sub>2</sub>, and lysophosphatidylcholine in this system has demonstrated the high degree of reproducibility possible with this method. Moreover, in agreement with earlier work in this area (Banik et al., 1976), these agents were found to act cooperatively in degrading myelin; thus under conditions that may be pertinent to demyelination in vivo myelinolysis can be assayed quantitatively.

Three advantages of this micromethod are that (1) relatively low numbers of cells can be studied; (2) it enables the study of cell-mediated demyelination within a syngeneic system as myelin from different species can be substituted quite easily; and (3) it is a much more easily defined system in which the contribution of individual cell types can be examined with minimal interference by contaminating cell populations as is the case with organotypic cell cultures. However, caution needs to be exercised since the conditions in vivo are clearly different and other factors such as antibody, complement and cytokines may operate. Nevertheless, the myelin preparation is a convenient but artificial membrane sample which
remains stable on incubation with media alone.

In the next chapter the \textit{in vitro} myelin degradation assay described above will be used to assess the degradative ability of sera, PBMC and CSF cells obtained from MS patients with different stages of disease activity as it has been previously demonstrated that MS sera and cells can cause \textit{in vitro} demyelination and thus may play a role in the pathogenesis of MS.
CHAPTER FOUR

IN VITRO MYELIN DEGRADATION BY SERUM AND LEUCOCYTES FROM MULTIPLE SCLEROSIS PERIPHERAL BLOOD AND CEREBROSPINAL FLUID

4.1. INTRODUCTION

As early as 1961 Bornstein and Appel demonstrated that the sera of rabbits with EAE caused demyelination when added to myelinated cultures of rat cerebellum. Subsequently it was shown that sera from MS patients were capable of a similar pattern of in vitro demyelination with 63% of sera from patients in exacerbation causing damage to the cultures and only 10% of samples from controls (patients with OND), and sera from MS patients in remission were found to be inactive (Bornstein, 1963). These findings were later confirmed by Hughes and Field (1967) and Lumsden (1971) using similar in vitro assay systems.

It was thought that the demyelinating factor responsible was complement dependent as heating the serum to 56°C destroyed the myelinotoxicity and adding fresh normal human serum restored the activity (Bornstein and Appel, 1965). However, Ulrich and Lardi (1978) could not restore the demyelinating activity by adding new complement and concluded that the factor was thermolabile and more sophisticated methods would have to be used to determine if it was complement dependent. Grundke-Iqbal and Bornstein (1980) showed that depletion of gamma globulin by consecutive treatment with Protein-A and with an immunoabsorbant containing a mixture of antibodies against kappa and lambda light chains caused little or no decrease of in vitro demyelinating activity and that the Ig fractions eluted from the immunoabsorbant did not demyleinate the tissue cultures. It was later confirmed by Bradbury and colleagues (1984) that complement is only involved to a limited extent in MS serum myelinotoxicity as addition of fresh complement to heat inactivated or mercaptoethanol or zymosan treated serum could not completely restore the demyelinating activity. Mercaptoethanol treatment of serum destroys the activity of the complement component C3 and some C2 activity whereas zymosan treatment activates the properdin system and so depletes activity of C3 and C5 leaving C1, C4 and C2 virtually unaffected. He concluded that less than 30% of the in vitro effects of MS sera are complement dependent and that it was unlikely that free circulating IgG is a major component of the factor. However, the possibility that complement may have an antibody-independent role in myelin
and oligodendrocyte injury has been suggested as purified preparations of myelin activate complement in the absence of antibody via the classical pathway (Cyong et al. 1982, Vanguri et al. 1982) with the production of inflammatory mediators (C3a, C3b and C5b) and membrane attack complex (MAC) C5b-9 which can insert itself into the myelin membrane bilayer and directly lead to myelin damage (Silverman et al., 1984). More recently Kerlero de Rosbo and Barnard (1989) have shown that Ig can bind to myelin and activate the myelin neutral protease possibly through release of free calcium from calcium binding sites and that Ig isolated from the NAWM of four MS brains cause significantly more basic protein degradation than Ig isolated from the corresponding regions of four control brains.

Nevertheless, using a different assay system Kerlero de Rosbo and colleagues (1990) have recently demonstrated a role for antibodies in demyelination as they have shown that anti-MOG MAb plus complement can cause the demyelination of aggregating brain cultures. A reduction in the concentration of MBP and CNPase activity of the cell cultures was noted only when anti-MOG antibody was added with complement. Anti-MBP antibodies plus complement or complement alone had no effect. The demyelinating effects were thought to be linked to the generation of MAC's following complement fixation and activation by anti-MOG antibodies. In support of the importance of antibodies in demyelination is the evidence that administration of anti-MOG MAb at the onset of passively transferred EAE augments the severity and duration of clinical signs and results in the formation of large confluent demyelinating plaques (Linington et al., 1988).

The possibility that T cells could be effectors in the immunopathology of the MS has also been investigated. As early as 1964, the effect of mononuclear cells from MS patients on glial cells cultures was investigated (Berg and Kallen). It was found that 11 out of 33 MS PBMC samples heavily degenerated glial cell cultures after 16 - 20 h incubation. Lumsden later showed (1971) that some MS patients' lymphocytes displayed injurious effects towards rat cerebellum myelinated cultures when left in contact for 4 - 6 h. In positive cases, if the cells were 'drained' off, a few lymphocytes remained strongly attached to the cultures which was suggested as indicative of myelin sensitisation. If these cultures were then re-fed with medium the cells would migrate in amongst the satellite cells and myelin sheaths and lead to local demyelination and selective destruction of oligodendrocytes with sparing of astrocytes and neurones. These early experiments provided some suggestion that circulating lymphocytes in MS are myelin sensitised and can in some circumstances be myelinoxic or
myelin aggressors. A role for these cells in the pathogenesis of the disease has therefore been implicated.

Interpretation of these experiments is however, complicated as endogenous macrophages, astrocytes and oligodendrocytes were present in the heterologous tissue culture systems used, where the serum or cells from one species have been applied to tissue cultures of another species. The demyelinating effects seen may therefore have been related to histocompatibility differences and not a direct effect of disease. Quantitative evaluation of the demyelination is also difficult to perform by light microscopy. In order to overcome some of these problems and deduce whether the reported myelinotoxicity is cell or serum mediated I have investigated the effect of both serum and cells on a human cell free myelin preparation which quantitatively measures myelin degradation. Sera, PBMC and cerebrospinal fluid cells from MS patients with different stages of disease activity have been applied to this in vitro assay of myelin degradation.

4.2. RESULTS

4.2.1. Myelin degradation by serum

Fresh samples of serum from 40 MS patients were incubated with human myelin (25% v/v) for 24 h and residual CNPase activity determined as described previously. As a control, myelin was also incubated with heat inactivated pooled normal human serum (NHS). The CNPase activity of the myelin incubated with control NHS did not vary significantly over the 24 h incubation period (data not shown). Table 4.1 is an example of raw data from one experiment where MS serum and matched PBMC samples were incubated with myelin, the appropriate controls are also included (myelin plus media alone for MS PBMC and myelin plus pooled NHS for MS sera).

None of the MS sera analysed caused significant losses of CNPase activity from myelin as compared to the myelin incubated with control pooled heat inactivated NHS (range 0 -21%). Of the 40 serum samples analysed 24 had a matched PBMC specimen, 13 of these PBMC samples caused significant losses of CNPase from myelin (range 13.5 - 46%, all p<0.001). Figure 4.1 demonstrates the in vitro myelin degradation by the matched PBMC and serum
specimens from MS patients. There was a significant difference between the ability of serum
and PBMC to cause in vitro myelin degradation (p=6.2 x 10^-7, Students t-test).

4.2.2. Myelin degradation by PBMC

The percentage loss of CNPase activity from the myelin preparation when incubated with
PBMC was determined with cells from 97 MS patients with different stages of disease
activity. Significant myelin degradation was seen with 42% of samples from MS patients (all
p<0.01) but this degradative activity did not correlate with disease status as there was no
significant difference between the myelin degrading ability of each clinical group as assessed
by mean percentage loss of CNPase activity from myelin (Fig.4.2). Of those patients with
an active disease status, 69% (9/13) caused a significant loss in CNPase activity of the myelin
of between 11-37% and 41% (14/34) of PBMC from patients in relapse also degraded myelin
significantly (range 17-50% loss in CNPase activity). However only 34% (12/35) of
progressive cases and 40% (6/15) of those with a stable disease status caused a significant loss
in residual CNPase activity of the myelin (range 13.5-35% and 13-34% respectively).

The myelin degrading ability of 4 different MS patients' PBMC collected on different days
over a maximum duration of 15 days was assessed. At each assessment an MRI scan with
gadolinium-DPTA enhancement was also performed (Dr's A Thompson and A Kermode,
Institute of Neurology, London). All 4 patients presented in acute relapse over the 2 week
period and two of the patients (represented by symbols □ ■ and ◊ ◊) had evidence of new
lesions on MRI as evidenced by enhancement with gadolinium-DPTA. As can be seen, in
Fig. 4.3., the ability of PBMC to cause a significant loss of myelin CNPase activity (filled
symbols) fluctuated with time regardless of the presence or absence of enhancing lesions.
For example, with respect to the particular patient represented by the symbols "▼ ▼", the
PBMC separated on day 1 were unable to cause significant myelin degradation whereas
PBMC isolated on day 6 from the same patient demonstrated significant myelin degrading
activity causing a 29% loss of CNPase activity (p<0.001). This myelin degrading activity was
still present 7 days later, on day 13, as freshly isolated PBMC caused a significant loss of
myelin CNPase activity of 18% (p<0.001).
4.2.3. Myelin degradation by cerebrospinal fluid leukocytes

Leukocytes from the CSF of seven MS patients with various stages of disease activity were incubated with myelin to assess their myelin degradative activity. CSF cell counts ranged between 3 - 15 x 10^3 cells/mm^3 of CSF. Due to the varying volume of fluid withdrawn from each patient, the total cell yield for each sample ranged between 3 - 18 x 10^4 cells. The specimen was divided into three so that triplicate wells of myelin plus cells could be set up, subsequently the number of CSF cells incubated with myelin was different for each sample (1 - 6 x 10^4 cells/ 6 µg myelin, n=3).

None of the seven MS CSF samples caused a significant loss of CNPase activity of the myelin after incubation for 24 h. When matched PBMC samples (blood and CSF drawn at the same time) were incubated at the same ratio of cells to myelin as the CSF cells, only one of the five PBMC samples caused significant myelin degradation of 18%, p<0.005 (6 x 10^4 cells / 6 µg myelin). This myelin degrading activity as measured by loss in CNPase activity was also present when the same PBMC sample was incubated at the higher ratio of 2 x 10^5 cells / 10 µg myelin (25%, p<0.005) (see Table 4.2).

Whether one would expect to demonstrate significant myelin degradation by CSF cells incubated with myelin at such low cell numbers is questionable. Nevertheless, as indicated above, one of the 7 PBMC specimens incubated with myelin at a low cell concentration, comparable to the matched CSF cell number caused a significant loss of CNPase activity. This was however, the specimen which had the highest CSF cell count and thus the highest ratio of cells to myelin when compared to the other matched PBMC and CSF cell specimens.
### TABLE 4.1

**COMPARISON OF IN VITRO MYELIN DEGRADATION BY MATCHED PBMC & SERUM SAMPLES FROM INDIVIDUAL MULTIPLE SCLEROSIS PATIENTS**

<table>
<thead>
<tr>
<th>Myelin plus:</th>
<th>CNPase Activity (nmol end product/20min/sample)</th>
<th>Loss CNPase Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media alone</td>
<td>2,062 ± 113</td>
<td>-</td>
</tr>
<tr>
<td>PBMC patient 160</td>
<td>1,827 ± 130†</td>
<td>11†</td>
</tr>
<tr>
<td>PBMC patient 145</td>
<td>1,632 ± 189†</td>
<td>21†</td>
</tr>
<tr>
<td>PBMC patient 113</td>
<td>1,445 ± 166‡</td>
<td>30‡</td>
</tr>
<tr>
<td>Heat inactivated</td>
<td>2,005 ± 73</td>
<td>-</td>
</tr>
<tr>
<td>normal human serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERUM patient 160</td>
<td>1,893 ± 198</td>
<td>7</td>
</tr>
<tr>
<td>SERUM patient 145</td>
<td>1,931 ± 221</td>
<td>4</td>
</tr>
<tr>
<td>SERUM patient 113</td>
<td>1,856 ± 224</td>
<td>7.5</td>
</tr>
</tbody>
</table>

† significantly different from myelin incubated with either media alone or NHS p<0.003, ‡ p<0.0002 (Students t-test).

Matched serum and PBMC samples were obtained from the blood of 3 MS patients. 25% (v/v) of fresh serum was incubated in triplicate with myelin (10 μg). In the same assay, cells from the same patient were incubated in triplicate at a concentration of 2 x 10⁵ cells / 10 μg myelin. As a control myelin was also incubated with either media alone (control for PBMC's plus myelin) or 25% (v/v) heat inactivated pooled normal human serum (NHS) (control for MS serum plus myelin) at the beginning and end of the 96 well plate (total n=6). After 24 h incubation at 37°C the CNPase activity of the myelin pellet was determined. CNPase activity of the residual myelin pellets are expressed as mean activity ± SD. Loss of CNPase activity from myelin after incubation with cells or serum is also expressed as a % of the control myelin alone subtracted from 100.
### TABLE 4.2

**IN VITRO MYELIN DEGRADATION BY CEREBROSPINAL FLUID LEUKOCYTES & MATCHED PBMC SAMPLES FROM MS PATIENTS**

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Total CSF cell count (x 10⁶/mm³ CSF)</th>
<th>Number of CSF cells or PBMC incubated with 6 µg myelin³</th>
<th>LOSS OF CNPase ACTIVITY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CSF* CELLS</td>
<td>PBMC†</td>
</tr>
<tr>
<td>515</td>
<td>10</td>
<td>6.0 x 10⁴</td>
<td>6.5</td>
</tr>
<tr>
<td>517</td>
<td>6</td>
<td>2.7 x 10⁴</td>
<td>6</td>
</tr>
<tr>
<td>538</td>
<td>3.5</td>
<td>2.0 x 10⁴</td>
<td>1</td>
</tr>
<tr>
<td>517-2</td>
<td>4</td>
<td>2.7 x 10⁴</td>
<td>0</td>
</tr>
<tr>
<td>535</td>
<td>3</td>
<td>1.0 x 10⁴</td>
<td>5</td>
</tr>
<tr>
<td>536</td>
<td>15</td>
<td>3.0 x 10⁴</td>
<td>5</td>
</tr>
<tr>
<td>508</td>
<td>13</td>
<td>3.7 x 10⁴</td>
<td>11</td>
</tr>
</tbody>
</table>

* Significantly different from myelin incubated with media alone, p<0.005 (Students t-test).

Cerebrospinal fluid (CSF) cells (see Chapt 2.6.3) were incubated with myelin (6 µg/ml,n=3) at the concentration specified above†. PBMC† from the same patients were also incubated with myelin in the identical ratio of cells : myelin as for the CSF lymphocytes (n=3). In the same experiment PBMC‡ were also incubated with myelin at 2 x 10⁵ cells/ 10 µg myelin (n=3). As a control myelin (6 or 10 µg/ml) was also incubated with media alone (n=6). After 24 h incubation at 37°C the CNPase activity of the myelin/cell pellet was determined. Results are expressed as percentage loss of CNPase activity from myelin after incubation with cells as compared to myelin incubated with media alone. The above table includes results from more than one assay.
In vitro myelin degradative activity of matched PBMC and serum samples was determined for 24 MS patients. Each symbol represents the loss in CNPase activity of myelin after incubation for 24 h at 37°C with either an individual patients' serum or PBMC sample as compared to myelin incubated with media alone or myelin incubated with pooled NHS as a control. Filled symbols (●) represent samples where the cells caused a significant loss in myelin CNPase activity as compared to myelin incubated with media alone (p<0.01 - p<8 x 10^-8, Students t-test). Hollow circles (○) represent serum or cell samples where there was no significant loss in CNPase activity. Results are from more than one experiment. Bars indicate overall mean % values ± standard deviation (serum, 3.8 ± 5.2%, PBMC 18.6 ± 11.4%). There was a significant difference between the myelin degrading ability of PBMC and serum when measured by mean % loss of CNPase activity of myelin (p=6.2 x 10^-7).
**FIG. 4.2**

**IN VITRO MYELIN DEGRADATION BY PBMC FROM MULTIPLE SCLEROSIS PATIENTS**

*In vitro* myelin degradative activity of PBMC as assessed by percentage loss in CNPase activity of myelin was determined for 97 MS patients classified as either stable (15), active (13), relapse (34) or progressive (35). Each symbol represents the % loss in CNPase activity of myelin after incubation with PBMC for 24 h at 37°C as compared to myelin incubated with media alone. Filled circles (●) represent samples where the cells caused a significant loss in myelin CNPase activity (*p* ≤ 0.01, Students t-test). Hollow circles (○) represent PBMC samples where there was no significant loss in CNPase activity. Results are from more than one experiment.

Bars indicate overall mean % values ± SD: Stable, 14.2 ± 8%, Active, 18.2 ± 11.8%, Relapse, 16.9 ± 12.6%, Progressive, 13.9 ± 11.2%.
The myelin degrading activity, as measured by the ability to cause a significant loss in CNPase activity of myelin, of PBMC from 4 MS patients in clinical relapse was assessed over a period of 15 days. On each assessment day, blood was drawn and PBMC separated. PBMC were incubated with myelin for 24 h as previously described and loss in CNPase activity of the myelin was determined. Results are expressed as percentage loss of CNPase activity from myelin after incubation with cells as compared to myelin incubated with media alone. Each symbol represents the PBMC from an individual patient. Filled symbols represent PBMC samples that caused a significant loss in myelin CNPase activity as compared to myelin incubated with media alone (p<0.005 students t-test). Hollow symbols represent PBMC samples where there was no significant loss in CNPase activity.
4.3. DISCUSSION

4.3.1. Serum myelin degrading factors

There are numerous reports documenting a toxic effect of MS sera on heterologous myelinating cultures in vitro (Bornstein, 1963; Hughes and Field, 1967; Lumsden, 1971). The mechanisms for the myelinotoxic and cytotoxic effects seen with the MS sera has yet to be elucidated. Both antibody and complement have been suggested as possible mediators of the damage as well as alternative mechanisms such as MAC formation and stimulation of endogenous neutral proteases by release of free calcium from calcium binding sites.

Using a quantitative assay which measures the release of the myelin specific enzyme CNPase from a human cell free myelin preparation, it has been shown that MS sera is devoid of myelin degradative activity. This is in disagreement with the findings of many authors however, a different assay system was used. In what has since become the model system for evaluating the immunological mechanisms responsible for in vitro demyelination by sera, Bornstein (1963) utilising myelinating cultures of rat and mouse cerebellum demonstrated that MS sera caused in vitro demyelination. Approximately 60% of sera were competent in causing partial or total loss of myelin with glial swelling and cell death. These findings were confirmed by others who reported even higher numbers of MS sera that were competent in demyelinating rat cerebellum cultures (Hughes and Field, 1967; Lumsden 1971; Bradbury et al., 1985). In contrast, other groups have reported lower numbers of MS sera capable of in vitro demyelination (Ulrich and Lardi, 1978; Wolfgram et al., 1978).

A possible explanation of these contradictory results is the difference between the culture systems used and the criteria for reporting positive and negative serum results. Usually the in vitro assessment of demyelinating activity using myelinated cultures has depended on a visual scoring method developed by Bornstein and Appel (1965). This method can be susceptible to observer bias and also each published report has different scoring thresholds above which a serum is said to be positive. Considering the vulnerability of cerebella cultures to the composition of the nutrient media and to handling traumas which can cause spontaneous degradation, the position of this background scoring baseline is especially important when considering the degree of non-specific myelinotoxicity. Bradbury and colleagues (1984) also suggest that within cerebellar and spinal cord cultures there may be
a variable disposition of myelin which could lead to unequal exposure of the myelin to test sera and therefore variable results.

Nevertheless, the most striking difference between assays is that a human cell-free myelin preparation has been used in which only direct damage to myelin can be measured, whereas all other investigators have used a heterogeneic system in which human sera is tested on rat or mouse myelinated cerebellum cultures which could lead to problems of incompatibility. Also it could be suggested that the demyelinating factors previously detected in serum are acting on the oligodendrocyte and not primarily on the myelin. Fewster and co-workers (1975) reported the gliotoxic effect of MS and normal sera by measuring ^3Cr release from pre-labelled bovine oligodendrocytes. Sera heated to 56°C did not diminish the oligodendrocyte toxic effects although addition of lymphocytes to the sera greatly increased the release of ^3Cr. They therefore concluded that the toxicity did not involve complement but was in fact antibody-dependant cell-mediated cytotoxicity. In contrast, complement from either normal rat or rabbit serum is bound and activated by rat oligodendrocytes and the oligodendrocyte / type-2 astrocyte (O/2A) progenitor cells, in the absence of anti-myelin antibody, by the classical pathway. This can lead to cellular destruction by MAC mediated lysis due to an intra-cellular rise in calcium (Scolding et al., 1989a; Wren et al., 1989; Scolding et al., 1990b). If sub-lethal concentrations of complement are involved the MAC is shed from the surface of the cell by vesicular extrusions and the cell recovers. However, the capacity for recovery is limited and the cell cannot withstand repeated cycles of sub-lethal complement exposure.

4.3.2. Cellular myelin degrading factors.

Using a quantitative method of assessing in vitro myelinotoxicity by circulating mononuclear cells, it has been shown that 42% of MS PBMC when incubated with human myelin cause significant losses of CNPase activity from the myelin. This is the first quantitative demonstration that MS human PBMC are capable of myelin degradation, as assessment of in vitro demyelination by MS lymphocytes has always been qualitative. Routinely, organotypic CNS tissue cultures have been used where the sera or cells from one species have been applied to myelinated tissue of another. Roth and colleagues (1985) however, were the first to report on an in vitro syngeneic model system that quantitatively assessed myelin.
degradation. Using a model of acute EAE in SJL/J mice and a syngeneic mouse spinal cord organotypic culture system, *in vitro* demyelination as measured by light microscopy and by quantification of CNPase activity of the cultures was demonstrated with both serum and LNC. The difference between the model system presented here and the one described above is that a cell free myelin preparation has been used in which only direct damage to myelin can be measured.

Even though it has been demonstrated that 42% of MS PBMC are myelinotoxic as measured by the release of CNPase activity from myelin no correlation with clinical exacerbations could be shown. As studies using MRI have demonstrated the presence of asymptomatic lesions in the absence of any new clinical signs (Ormerod et al., 1987) an investigation was made into the association between the presence (or absence) of Gd-DPTA MRI enhancing areas of the brain and peripheral cellular myelin degrading activity in four MS patients. Over the two week assessment period there was no clear association between the presence of new lesions on MRI as demonstrated by areas of enhancement with gadolinium-DPTA and the ability of PBMC’s to cause significant *in vitro* myelin degradation. Nevertheless, significant myelin degradative activity was not always present in a patient’s PBMC sample as consecutive assessments over the two week period showed a fluctuating response (Fig 4.3). From the model experiments previously performed (see Chapt. 3, table 3.4.) it can be concluded that this demonstration of fluctuating cellular myelin degrading ability over time is not a reflection of inherent assay variability but is a reflection of a transient effect that is not always present in the peripheral blood of an MS patient. In order to investigate further whether there is any association between *in vitro* myelin degrading activity by PBMC and presence of new lesions as assessed by MRI many more patients would have to be investigated over a longer time period.

It has been reported that in longitudinal studies of peripheral blood T cell subsets no correlation has been found with T cell phenotype and corresponding clinical activity or with T cell phenotype and new MRI lesions as assessed by gadolinium-DPTA enhancing areas in the brain and spinal cord (Capra et al., 1989 & 1992). Nevertheless, it has been shown that changes in cellular immune function correlate with disease activity as recognised by MRI (Oger et al., 1988). In this study 7 relapsing-remitting MS patients were monitored over a period of 6 months for clinical, MRI and cellular immune function changes. There were striking abnormalities in immune function (as measured by reduced Con-A induced
suppression, diminished IgG secretion by PBMC in response to PWM and also in NK cell function) when brain lesions reached their maximal size. These abnormalities were not present one month earlier when lesion size was greatly reduced. Similar changes were not seen in healthy control patients followed in parallel. The changes in immune function were not accompanied by significant changes in lymphocyte phenotype.

Prior to the work of Oger and colleagues (1988) it was demonstrated that *in vitro* demyelination by 30% of MS mononuclear cells on glial cell cultures only occurred during the active phase and chronic progression of the disease but not whilst patients were in stationary phase (Berg and Kallen, 1964). It was suggested that at certain times in the course of the disease, the blood brain barrier becomes damaged and there may be a movement of cells into the CNS with the concomitant disappearance of myelin aggressive cells from the peripheral circulation.

It therefore seems possible that the fluctuating cellular myelin degradative activity found in PBMC that has been demonstrated in the four MS patients could relate to the presence of clinically silent lesions and is dependent on the number of myelinotoxic lymphocytes that may traffic out of the CNS and be present in the blood. However, many more patients would have to be followed serially by MRI with assessment of *in vitro* cellular myelin degrading activity to substantiate this hypothesis.

Failure to detect significant CSF cellular myelin degradative activity in comparison to peripheral blood cellular activity could have been due to the low number of cells incubated with myelin (a consequence of the small volumes of CSF obtained), the ratio of cells to myelin being too small to show significant loss of CNPase activity. This is reflected in the lack of demonstrable PBMC degradative activity at these low cell numbers.

In addition, the lack of degradative activity may have been due to the phenotype/function of the cells isolated from CSF since it has been found that the CSF in acute viral meningoencephalitis does not reflect either functionally or phenotypically the composition of inflammatory cells present at the actual site of CNS pathology (Moench and Griffin, 1984). It has previously been shown that the number of activated T cells (as measured by expression of the IL-2 receptor) in the CSF of MS patients with different disease status fluctuates considerably (0-67% of CSF cells) (Bellamy *et al.*, 1985), and in fact cells that have
passed from the brain into the CSF may have done so after a down regulation in their activation state. In addition it has been suggested that the T cells present in CSF represent a random sample of T cell clones that have been activated by the immune system, not just T cells specific for a CNS located antigen (Hickey et al., 1991). Nevertheless it should also be mentioned that the problem of obtaining fresh CSF samples may influence cell viability and function as the CSF may not be a conducive environment for cell survival. TGFβ, which has been demonstrated to be present in the CSF (Cserr & Knopf, 1992), is a cytokine that has been shown to have many immunosuppressive effects including inhibition of T cell activation and proliferation, down regulation of IFNγ induced class II expression and also a decrease in the generation of cytotoxic lymphocytes (reviewed by Racke et al., 1991). TGFβ could therefore be responsible for a down regulation in the immune activity of cells isolated from the CSF. Thus the CSF may not be an appropriate compartment in which to look for in vitro myelin degrading cells.

In the next chapter the myelin degrading ability of serum and PBMC from control patients has been investigated to determine whether the myelin degrading activity seen with MS PBMC is disease specific. Sera and cells from healthy individuals and patients with OND have been tested. In addition, the myelin degrading capacity of PBMC and sera from RA patients has been examined, since this is an inflammatory disease thought to have an autoimmune basis.
CHAPTER FIVE

IN VITRO MYELIN DEGRADATION BY SERA AND CELLS FROM CONTROL GROUPS

5.1. INTRODUCTION

In addition to MS serum being able to produce demyelination of myelinated rodent organotypic cultures, sera from control patients including healthy individuals has been shown to possess myelinotoxic activity (see Table 5.1). The proportion of sera (both MS and controls) capable of inducing demyelination has varied markedly between studies and differences may be due to slightly different culture conditions and differences in assessment of demyelination which have been criticised as largely subjective (Bradbury et al., 1985). These studies therefore lend support to the uncertainty of the relevance of an MS humoral factor being involved in the pathogenesis of the disease and also to its specificity for in vitro demyelination.

Rheumatoid arthritis is a chronic inflammatory disorder involving the synovial membranes of multiple joints and though its aetiology is unknown it is thought to be an autoimmune disease with T cells playing a major role in its pathogenesis. Based on present knowledge a pathogenic model has been proposed (Kingsley et al., 1991) in which initiating rheumatoid antigen(s) (as yet unknown) is presented in the context of a limited range of MHC class II molecules to CD4+ T cells that become activated. The initial activation of T cells initiates a cascade of activation of various other cells including B and T cells, macrophages, synoviocytes and synovial fibroblasts which release an array of effector molecules such as cytokines (IL-1, IL-6, IL-8, granulocyte macrophage-colony stimulating factor (GM-CSF), IFNy, TNFα/β, TGFβ) antibodies and degradative enzymes. This array of effector molecules leads to synovial inflammation and subsequent joint destruction. Nevertheless the above scenario is only a proposed pathogenic model and it is not known whether the abnormal expression of cytokines that are present in the synovial fluid are a primary event in the disease process or are a secondary consequence.

As has been suggested for MS, there is much evidence to support a role for T cells in RA in the progression of joint destruction. CD4+ cells are the predominant phenotype in pannus, the expression of several T cell activation markers is enhanced (high affinity IL-2R and
transferrin receptor) and T cell targeted therapies such as total lymphoid irradiation, and cyclosporin improve disease status (reviewed by Sewell & Trentham, 1993). Whether the initiating activation of T cells by antigen occurs primarily in the joint after migration of T cells to the synovium is not known but T cell migration is thought to play an important role in generating synovitis.

MS and RA are both chronic inflammatory diseases with a possible autoimmune basis in which T cells are thought to play a central role in the initiation and perpetuation of tissue damage whether it be concentrated in the CNS or joints. For this reason RA was chosen as a suitable disease control along with other neurological diseases (inflammatory and non-inflammatory) and healthy individuals to investigate whether the in vitro myelin degradation observed with MS sera and PBMC is disease specific.

5.2. RESULTS

5.2.1. Myelin degradation by serum

Fresh samples of serum (25% v/v) from control patient groups (OND, RA and healthy individuals) were incubated with human myelin for 24 h and residual CNPase activity determined as described previously. As a control, myelin was also incubated with heat inactivated pooled NHS which caused no residual loss of myelin CNPase activity over the 24 h incubation period. Whenever possible a matched PBMC sample was incubated with myelin at the same time as the incubation of fresh patients serum with myelin.

Of the 13 RA serum samples tested for myelin degrading activity only 2 caused a significant loss of CNPase activity of 32% and 17% (both p<0.01). A matched PBMC sample for the first of these serum samples caused 61% myelin degradation (p<0.001). Nevertheless, 6 of the other 11 matched RA PBMC samples produced myelin degradation (23 - 63%, p<0.002) with no corresponding significant serum induced degradation (0 - 22%, not significant) (Fig. 5.1)

In the OND patient group only 2 serum samples from the 25 tested were myelinotoxic as assessed by a significant release of CNPase activity from the myelin. One of the sera was
from a patient with GBS (17% loss of CNPase, p<0.005) and the other from a patient with myxedema with peripheral neuropathy (18%, p<0.0003). No corresponding activity was seen with matched PBMC samples from these 2 patients. Finally no significant degradative activity was seen with sera or matched PBMC samples from ten healthy individuals (loss of CNPase activity caused by serum, 0 - 8%; matched PBMC, 0 - 11%, not significant) (Fig. 5.1).

5.2.2. Myelin degradation by PBMC

Myelin degradative activity as measured by the loss in CNPase activity of myelin after incubation with PBMC was determined with cells from RA and OND patients as well as from healthy controls (Fig. 5.1). Of the 26 PBMC samples tested in the RA group 58% (15/26) caused significant losses in CNPase activity of the myelin preparation ranging from 20 - 63% (p<0.01). There was a statistically significant difference between the ability of RA PBMC and sera to cause myelin degradation (p <0.01). In the OND group only PBMC from 2 of the 20 samples tested caused a significant loss in CNPase activity of 58% and 28% (both p<0.001) (cerebellar ataxia and Parkinson’s disease respectively). Of the 30 healthy control PBMC’s tested none caused significant \textit{in vitro} myelin degradation (0 - 13%).

There was a statistically significant difference between the ability of RA and OND or healthy individuals PBMC to cause a significant loss of CNPase from the myelin (RA compared to OND, p<0.002; RA compared to healthy individuals’ serum, p<2 x 10^5).

† OND group was compiled of the following disorders; CNS tumour 4, MND 6, cerebellar ataxia 2, Parkinsons disease 1, epilepsy 4, peripheral neuropathy 2, CVA 1, GBS 3, Infected VP shunt 7 and Graves disease 1.
TABLE 5.1 PERCENTAGE OF SERA THAT CAUSED *IN VITRO* DEMYELINATION WHEN ADDED TO ORGANOTYPIC CULTURES AS ASSESSED BY MORPHOLOGICAL CHANGES

<table>
<thead>
<tr>
<th>Researcher</th>
<th>MS</th>
<th>OND</th>
<th>HEALTHY CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bornstein (1963)</td>
<td>63 (100)*</td>
<td>10 (29)</td>
<td>7 (28)</td>
</tr>
<tr>
<td>Hughes &amp; Field (1967)</td>
<td>84 (25)</td>
<td>62 (26)</td>
<td>24 (34)</td>
</tr>
<tr>
<td>Lumsden (1971)</td>
<td>80 (128)</td>
<td>18.5 (93)</td>
<td>0 (55)</td>
</tr>
<tr>
<td>Ulrich &amp; Lardi (1978)</td>
<td>14 (44)</td>
<td>49 (35)</td>
<td>0 (24)</td>
</tr>
<tr>
<td>Wolfgram <em>et al.</em> (1978)</td>
<td>55 (120)</td>
<td>nd</td>
<td>81 (21)</td>
</tr>
<tr>
<td>Silberberg <em>et al.</em> (1984)</td>
<td>nd</td>
<td>nd</td>
<td>100 (20)</td>
</tr>
<tr>
<td>Bradbury <em>et al.</em> (1985)*</td>
<td>74 (53)</td>
<td>68 (53)</td>
<td>22 (20)</td>
</tr>
</tbody>
</table>

* Numbers in parenthesis indicate number of samples tested. nd, not done.
* In addition to visual scoring of myelin damage a quantitative measurement or radiolabel release from the organ cultures was made.
Whenever possible, a matched serum and PBMC sample were obtained from RA (■ □) and OND (● ○) patients or healthy individuals (▲ △). 25% (v/v) of fresh serum was incubated with myelin (10 μg) (n=3). In the same assay cells from the same patient were incubated at 2 x 10⁶ cells with 10 μg myelin (n=3). 10 μg myelin was also incubated with media alone or pooled heat inactivated NHS as a control for the incubation of patients' cells or serum with myelin (n=6). After 24 h incubation at 37°C the CNPase activity of the myelin pellet was determined. Each symbol represents the percentage loss in myelin CNPase activity after incubation with the patients' serum or cells as compared to control myelin. Filled symbols represent samples where the serum or cells caused a significant loss in myelin CNPase activity as compared to control myelin (p<0.01, Students t-test). Hollow circles represent samples where there was no significant loss in CNPase activity. Results are from more than one experiment.

Bars indicate overall mean loss CNPase activity ± standard deviation. RA PBMC 23.7% ± 15.4%, sera 11.2% ± 9.1%. OND PBMC 9.0% ± 14.6%, sera 6% ± 6%. Healthy PBMC 4.4% ± 3.7%, sera 0.5% ± 0.5%.
5.3. DISCUSSION

5.3.1. Serum myelin degrading factors

*In vitro* myelinotoxic sera effects are not just confined to MS as activity has been reported with sera from healthy individuals and other neurological diseases (see Table 5.1). Of the OND patients' sera tested, myelin degradation was only seen with serum from one of the three GBS patients and with one of two sera from patients with peripheral neuropathy. It has previously been reported that sera from patients with GBS cause complement dependent *in vitro* demyelination of both PNS and CNS organotypic cultures (Cook et al., 1971; Dubois-Dalq et al., 1971; Bradbury et al., 1985). Sanders and colleagues (1986) reported the finding of terminal complement C5b-9 complexes in the CSF of 90% of GBS patients tested. In comparison, 75% of MS patients' CSF contained these complexes but at a lower concentration and only 27% of CSF from non-inflammatory OND patients contained the C5b-9 complexes. They suggested that as it has been shown that complement-fixing peripheral nerve myelin antibodies are detected in 100% of patients with acute monophasic GBS which correlates with the clinical course of the disease, then this finding of C5b-9 complexes in the CSF may indicate that terminal complement components are participating in the peripheral myelin damage which occurs in GBS thereby implicating a possible role of serum complement in demyelination. Reduced levels of CSF C9 have been documented in MS patients as compared to OND and this was thought to reflect the possibility that local consumption of the complement terminal component may indicate the involvement of MAC in tissue damage (Compston et al., 1986b).

Demyelinating activity has also been reported with sera from MND patients (Hughes and Field, 1967; Ulrich and Lardi, 1978) although none of the six serum samples tested on isolated myelin caused a significant loss in CNPase activity. Demyelinating activity of OND sera has not only been demonstrated with GBS and MND sera as Ulrich and Lardi (1978) showed that 49% of sera from a wide range of different OND's produced *in vitro* demyelination including sera from patients with Parkinson's disease, cerebral tumours, epilepsy, viral meningitis, amyotrophic lateral sclerosis, headaches and other diagnosis. In comparison only 14% of MS sera had demyelinating properties in the same assay system. Whether serum induced *in vitro* demyelination is an indicator of *in vivo* demyelination, as was previously thought, is probably not so and is more likely to be a reflection of chronic CNS...
disease. In diseases where there is CNS damage such as in stroke patients who have suffered focal CNS damage anti-CNS antibodies may be produced along with toxic products of cellular breakdown which could cause in vitro demyelination (Bradbury et al., 1985).

As a further control RA sera was tested as this is, like MS, an inflammatory condition thought to have an autoimmune basis. A higher percentage of RA sera with degradative activity was seen (15%, 2/13) than with any of the other patient groups as none of the MS sera (n=40) and 8% of the OND sera (2/25) tested caused myelin degradation. Indicating that serum induced myelinotoxicity is not just confined to diseases of the CNS but can also be caused by chronic inflammatory conditions such as RA. Finally in conflict with the reports that healthy individuals sera is capable of in vitro demyelination (Hughes and Field, 1967; Silberberg et al., 1984; Bradbury et al., 1985), none of the sera from ten healthy individuals that were tested on whole myelin preparations caused any significant loss in CNPase activity.

As can be seen with the results presented here and those of others, serum induced in vitro demyelination is not a specific property of MS sera but has been previously shown to be produced by MS and OND and even healthy individuals' sera. Using isolated myelin, in vitro myelin degradation was rarely seen, with a low percentage of OND and RA sera causing a significant loss of myelin CNPase activity and no myelinotoxic effects being produced by MS and healthy individuals sera (see Table 5.1).

The general lack of serum induced myelin degradation in this assay system suggests that the previously reported sera induced demyelinating effects may have been due to its action on the oligodendrocyte rather than directly on myelin. Only direct damage to myelin can be quantified in the assay system described here whereas problems of incompatibility could occur with the heterogeneic systems used by others, as human serum was added to rodent myelinated cultures. Nevertheless, the fact that RA and OND sera can induce significant losses in CNPase activity of myelin in vitro suggests that serum induced myelin degradation may be a non-specific indicator of chronic disease with a variety of different mechanisms being involved (antibody, complement, cytokines or degradative enzymes secreted by circulating leukocytes). This however, does not explain why healthy control sera in some reports has been able to induce demyelination. It has been suggested that serum induced demyelination (all disease states) could be due to activation of the alternate complement
pathway (Silberberg et al., 1984) as myelin can activate complement directly without the involvement of immunoglobulin (Vanguri et al., 1982).

5.3.2. Cellular myelin degrading factors

It has been demonstrated that PBMC from 42% of MS and 58% of RA patients can cause a significant release of CNPase from myelin. In contrast, only 10% of OND cells caused a significant loss in CNPase from the myelin, and healthy individuals’ PBMC samples possessed no myelin degrading ability. Thus the action of cells or their products is not restricted to MS but is probably a non-specific cellular response shown by chronically stimulated immune cells occurring in inflammatory diseases. In the OND control group, of the two GBS samples tested one caused in vitro myelin degradation. It has previously been demonstrated that PBMC’s and a factor synthesized in vitro by these cells had a myelin-destructive effect on cultures of spinal ganglia (Cook et al., 1971). The role of lymphocytes in the pathogenesis of GBS has been implied as lymphocytic infiltrates have been seen associated with myelin destruction in spinal nerve roots and circulating lymphocytes are sensitised to peripheral nerve myelin (Wisniewsky et al., 1969). More recently it has been shown that, as in MS, there are circulating activated T cells in the blood of GBS patients as assessed by augmented expression of HLA-DR antigen, IL-2 receptor and also increased levels of serum IL-2 and the soluble IL-2 receptor have been seen (Hartung and Tokya, 1990). Circulating monocytes have also been shown to be activated in GBS patients which may contribute to the pathogenesis of the disease (Hartung et al., 1991). Therefore as in MS, the demonstration of myelinotoxic activity by circulating mononuclear cells may imply a role for these cells in myelin destruction in vivo.

There are many similarities between the immunological abnormalities seen in MS and RA. Rheumatoid arthritis is a chronic inflammatory disease with autoimmune features which are primarily localised to the synovial joints. The joints are characterised by infiltration of leucocytes many of which show signs of activation (HLA class II expression). There is intra-joint IgG synthesis with production of oligoclonal bands. Oligoclonal anti-measles antibody represent a minor fraction of the oligoclonal IgG however, the production is not compartmentalised to the joint as the antibody bands have identical migration patterns in synovial fluid and serum (Cruz et al., 1991). As well as elevated class II expression on infiltrating macrophages and T cells many resident cells such as endothelial cells and
fibroblasts are activated which is of critical importance for antigen presentation and the perpetuation of an immune response. Both the infiltrating and resident cells are capable of producing large amounts of inflammatory mediators such as prostaglandin E₂ (PGE₂), degradative enzymes, reactive oxygen species and cytokines such as IL-1α and β, TNF-α, IL-6, IL-8 and GM-CSF (Brennan et al., 1990). These cytokines may be responsible for many manifestations of the disease process, including bone and cartilage destruction (Bertolini et al., 1986) as well as the persistence of the inflammatory state by attracting other inflammatory cells to the joint and the augmentation of HLA class II expression (Haworth et al., 1991).

The demonstration of in vitro myelin degradation by both RA and MS PBMC may therefore indicate that activated cells, both lymphocytes and monocytes, which are producing inflammatory mediators that could lead to tissue damage whether it be in the CNS or the joint have entered the circulation and are equally able to cause non-specific myelin damage in vitro.

In the next chapter an examination of the MS and RA PBMC cell type / factor causing in vitro myelin degradation will be performed as this may provide an insight into the pathogenesis of MS and RA.
CHAPTER SIX

ARE ACTIVATED T CELLS RESPONSIBLE FOR IN VITRO MYELIN DEGRADATION

6.1. INTRODUCTION

It has been shown that MS lymphocytes are able to cause in vitro demyelination using various tissue culture techniques (Berg and Kallen, 1964; Lumsden, 1971). In addition, Selmaj and colleagues (1986) demonstrated that SN from both unstimulated and mitogen stimulated lymphocytes from MS patients caused morphological changes to rat cerebellum cultures when incubated for 72 h. No glial cell damage was observed but changes to myelin included sheath destruction and single fibre fragmentation and disintegration. The SN from mitogen activated healthy individuals' lymphocytes also caused myelin damage but it was not as enhanced as the damage caused by MS lymphocytes. As the degrading activity was not complement dependent the authors suggested that a lymphokine(s) was responsible for the damage observed. Further studies showed that unstimulated and mitogen stimulated MS T cells produced gliotoxic as well as myelinotoxic SN for the rat cerebella cultures and it was concluded that MS T cells were preactivated in vivo and produced demyelinating factors, probably cytokines (Selmaj et al., 1988a).

Suggestions for the role of centrally produced cytokines in the development of the demyelinating lesion may involve more than one mechanism. Cytokines may be acting as chemoattractants for mononuclear cells which could then aid the demyelinating process. Alternatively they may directly cause damage to the oligodendrocyte or the myelin sheath. The release of cytokines by antigen specific cells could also cause an upregulation in class II expression thereby increasing antigen presentation and the perpetuation of an immune response with the release of inflammatory mediators and attraction of further immunocompetent cells.

Products of activated T cells have previously been implicated in the demyelinating process by causing the accumulation of a mononuclear cell infiltrate. When SN's of non-specifically activated lymphocytes were injected into the vitreous of rabbit eyes who had previously been immunised with syngeneic spinal cord homogenate, the injected SN initiated a mononuclear cell infiltrate which induced a demyelinating lesion within the retinal fibres. However, if the
rabbit had not been previously immunised, the injected SN caused the accumulation of a mononuclear cell infiltrate which failed to damage myelin (Stoner et al., 1977). Animals previously immunised with non-CNS antigens experienced no demyelination. This implied that only CNS antigen specific lymphocytes were capable of initiating demyelination after being attracted to the site by the products of activated lymphocytes.

More recently Simmons and Willenborg (1990) were able to induce an inflammatory response in the spinal cord of rats similar to that observed during EAE, by direct microinjection of TNFα or IFNγ into the spinal cord (no demyelination was observed). They proposed that the cytokines caused changes in the adhesiveness of CNS endothelial cells thereby increasing the migration of cells into the CNS. Also TNFα and IL-1β have been shown to play an indirect chemotactic role as they have been shown to stimulate the production of a neutrophil chemotactic factor from human endothelial cells (Streiter et al., 1989).

The direct effect of cytokines on the oligodendrocyte and myelin sheath have also been investigated and suggest that TNFα may be directly involved in the pathogenesis of the demyelinating plaque (Selmaj and Raine, 1988). Oligodendrocyte necrosis and myelin dilation were observed when recombinant human TNFα was added to SJL/J mouse spinal cord cultures. Recombinant IL-2 and IFNγ had no effect. In another study a retrospective look at the CNS tissue of EAE animals revealed that similar changes in myelin pathology could be seen in animals which had EAE induced by the passive transfer of activated lymphocytes (Brosnan et al., 1988). It was suggested that the damage was due to the local release of TNFα and TNFβ by activated macrophages and lymphocytes which may cause physiological dysfunction and even expose previously concealed epitopes.

Another role for cytokines in the pathogenesis of MS has been intimated by the following experiment. Rabbit intraocular injections of IFNγ, IL-1β and TNFα were shown to cause delays in nerve conduction. This effect was thought to be mediated by a cytokine induced increase in permeability of the blood brain barrier, which may result in the leakage of neuroelectric blocking factors into the retinal parenchyma (Brosnan et al., 1989). Other contributing mechanisms of action of the cytokines may also be an alteration in regional blood flow due to the inflammatory infiltrate or a direct effect of the cytokines on neuronal tissue or myelin.
In vivo, there have been reports suggesting a direct role for cytokines in the pathogenesis of MS. When IFNγ was given systemically to MS patients it increased the number of exacerbations (Panitch et al., 1987). It was suggested that this could have been due to an augmentation of class II MHC antigen expression on peripherally circulating monocytes and especially on cells within the CNS (microglia, endothelial cells and astrocytes). This augmentation of class II expression might therefore have played a critical role in the generation of an autoimmune response to CNS myelin. The opposite effect was produced when IFNα was used in the treatment of relapsing remitting MS, as fewer clinical attacks were seen which may have been a reflection of the down regulation of MHC Class II antigen expression (Knobler et al., 1984). Due to the mechanisms of action of interferons, antiviral as well as immunomodulatory, they have been investigated as a possible treatment strategy for patients with MS. Other studies using both α and β IFN’s have been encouraging but inconclusive, possibly due to the small numbers of patients as well difficulty in comparing results in different MS patient populations, ie. relapsing remitting compared to chronic progressives (Knobler et al., 1984; Camenga et al., 1986; Panitch et al., 1987; Kinnunen et al., 1993). Nevertheless, promising results have recently been reported on a large two year placebo controlled trial of IFNβ (The IFNβ MS study group, 1993). It was demonstrated that the annual exacerbation rate was significantly reduced in patients receiving IFNβ in comparison to those receiving placebo. In addition MRI results supported the clinical results in showing a significant reduction in disease activity as measured by the number of active scans and appearance of new lesions (Paty et al., 1993). The mechanisms of action of IFNβ as a treatment for MS are unknown but its immunoregulatory properties include inhibition of IFNγ synthesis as well as inhibition of MHC Class II expression induced by IFNγ. In addition it down regulates TNF production. The possibility that IFNβ modified the response to viral infections, that have been suggested as triggering exacerbations in MS, is also conceivable (Sibley et al., 1985; Panitch et al., 1992).

Interestingly it has been shown that the ability of mitogen stimulated MS PBL’s to produce IFNγ and TNFα was increased approximately two weeks preceding clinical symptoms suggesting that these cytokines may trigger off exacerbations (Beck et al., 1988). However, others have been unable to confirm a role for IFN and TNF in the progression and maintenance of demyelination in MS. When direct analysis of relapsing remitting or chronic progressive MS serum for TNFα was performed, levels equivalent to those found in sera from normal control subjects and patients with degenerative neurological disorders was seen,
patients with RA and those with other inflammatory neurological diseases had greatly increased levels of TNFα. (Franciotta et al., 1989).

A failure to detect significant levels (if at all) of TNFα in MS CSF has also been reported (Franciotta et al., 1989) even when extensive demyelination was documented (Gallo et al., 1989). Nevertheless, using a different assay system (radioimmunoassay as compared to an ELISA), Hauser and colleagues (1990) have successfully detected both IL-1β and TNFα in MS CSF. The cytokines were also detectable in OND patients’ CSF but at lower levels than that found in MS active patients’ CSF. There was no correlation between CSF pleocytosis and cytokine levels suggesting that CNS resident cells are the producers of the cytokines rather than the CSF cells. More recently, Sharief and Hentges (1991) found high levels of TNFα in the CSF of 53% of patients with chronic progressive MS and none in the CSF of patients with stable disease. TNFα was only detected in 7% of OND controls. The levels of CSF TNFα found in the chronic progressive group were greater than that found in matching sera. In addition the CSF TNFα levels correlated with the degree of disability experienced by the patient as measured on the EDSS. Whether TNFα is involved directly in the progression and maintenance of demyelination in MS has yet to be determined.

Apart from CNS infiltrating macrophages and lymphocytes being a source of TNFα and β, both microglia and astrocytes can be induced to secrete cytokines such as IL-1, IL-6, TNFα and TGFβ (Giulian et al., 1986; Sawada et al., 1989; Constam et al., 1992). TNFα has been detected in association with astrocytes and macrophages in MS lesions (Hofman et al., 1989). Other cytokines have also been detected in the MS lesion such as IL-1, IL-2 as well as IFNγ and PGE (Hofman et al., 1986; Bellamy et al., 1985; Traugott and Lebon 1988b). Locally these cytokines could have a role in the perpetuation of an immune response due to their ability to amplify immune effector mechanisms. The upregulation of expression of class II antigens and adhesion molecules, enhancement of vascular permeability, stimulation of cytokine production by infiltrating and resident cells and activation of macrophages to produce reactive oxygen species (ROS) (superoxide anion, hydrogen peroxide, hydroxyl radicals) are all properties of cytokines which may be important in the pathogenesis of MS (reviewed by Hartung and Heinenger, 1989).

In the previous two chapters it has been shown that both MS and RA PBMC are capable of in vitro myelin degradation, whereas PBMC from control patients (OND and healthy
individuals) have little or no effect in this assay system. In order to assess the possible role of T cells in causing these significant losses of myelin CNPase activity further investigation of the factors/mechanism responsible was performed. Initially the possibility that activated T lymphocytes, as measured by their expression of the IL-2 receptor (CD25) and MHC class II molecules (HLA-DR), are responsible for causing in vitro myelin degradation was investigated. In addition, the possibility that activated T lymphocytes, whether stimulated non-specifically by mitogens or specifically by a myelin specific antigen such as MBP, may induce in vitro myelin degradation was investigated. The reactivity of MS and RA PBMC to MBP was also analysed to ascertain whether there was any correlation between T_{MBP} and myelin degrading activity. The likelihood that a PBMC secreted factor was responsible for in vitro myelin degradation was also investigated. Finally the role of recombinant cytokines in causing myelin degradation was also investigated as it has been previously shown that TNFα causes the disruption of the myelin sheath in vitro (Selmay and Raine, 1988).

6.2. RESULTS.

6.2.1. Relationship between myelin degradative activity and cell phenotype

Several phenotypic indices were measured on PBMC from MS patients to see whether expression of the IL-2 receptor (CD25) and MHC class II antigen (HLA-DR), which are both expressed by activated T cells, with HLA-DR also being expressed by monocytes, correlated with the resulting myelin degradative activity produced by these cells. IL-2 receptor expression, as measured by indirect immunofluorescence staining with the anti-Tac MAb, ranged from 0 - 6% of the total PBMC population. 44% (11/25) of these samples caused a significant loss in CNPase activity however, no correlation was apparent with IL-2 receptor expression (r=0.29, not significant). Of the 13 PBMC assayed for MHC class II expression as measured by indirect immunofluorescence with the MAb DA-2, expression ranged from 7 - 39% of PBMC staining positive for HLA-DR with 31% (4/13) of these PBMC causing significant in vitro myelin degradation. As with expression of the IL-2 receptor, there was no correlation with myelin degradative activity and HLA-DR expression (r=0.43, not significant) (see Fig. 6.1).
6.2.2. Do $T_{MBP}$ cells initiate myelin degradation in vitro

Proliferative responses of MS and RA PBMC to MBP, the major myelin protein were compared to the myelin degradative activity of the same PBMC. The proliferative response to antigen was expressed as a stimulation index (SI) (proliferation to antigen / background proliferation) where a significant proliferative response was said to have occurred when the proliferation to antigen (MBP) was at least two times greater than that of cells incubated without antigen (SI $\geq 2$). Of 49 MS PBMC samples tested, 14 caused a significant loss of CNPase when incubated with myelin (range 13.5 - 50% loss of CNPase activity, all $p<0.01$). However, only 2 of these PBMC samples proliferated in response to MBP (both SI = 2, resultant loss of CNPase activity 19 and 21% $p<0.01$). Seven other samples proliferated in response to MBP but caused no degradation of myelin (resultant loss of CNPase activity 0-15% $^{a}$). In total 9/49 PBMC samples proliferated in response to MBP with stimulation indices ranging from 2 to 35. There was no correlation between the PBMC's ability to cause a significant loss of myelin CNPase activity and proliferation in response to MBP (as assessed by SI $\geq 2$) ($r=0.006$, not significant) (Fig. 6.2). Figure 6.3 shows a proliferation profile for an MS PBMC sample incubated with HMBP. As can be seen, HMBP at concentrations as low as 0.1 $\mu$g/ml resulted in the significant proliferation of PBMC (SI = 26) as compared to PBMC incubated with culture media alone for 4 days. The mitogens PHA and Con-A were also incubated with PBMC as positive controls. However, neither PHA nor Con-A stimulated a proliferative response comparable to that demonstrated with HMBP (PHA SI = 3; Con-A SI = 9). This was a reflection of the incubation time as the mitogen induced proliferation of PBMC generally peaked day 2 - 3 whereas antigen induced proliferation peaked day 4 - 5.

The proliferative response of RA PBMC to MBP was also compared with the myelin degradative activity of the same cells. Of the 7 samples assayed only 1 proliferated in response to MBP (SI = 2.7) but on incubation with myelin did not cause myelin degradation (8% loss of CNPase activity). In total 4/7 RA PBMC tested caused a significant loss in CNPase activity (range 23 - 37% loss of activity, all $p<0.01$).

Even though there was no correlation with reactivity to MBP and ability to cause a significant loss in CNPase activity of myelin by MS PBMC, it was thought that the frequency of antigen reactive cells in the mixed cell population may be too low and therefore the ability of MBP
reactive T cell lines in causing myelin degradation was tested.

Myelin basic protein reactive T cell lines (T<sub>MBP</sub>) (the kind gift of Dr. M.A. Bach, Institute Pasteur, Paris, France) were raised from the PBMC of a healthy volunteer and an MS patient. Six days after previous activation, by addition of HMBP and autologous Epstein Barr Virus-transformed B cells, T<sub>MBP</sub> were added to myelin for 24 h to assess whether they possessed any myelin degradative activity. Neither cell line was able to cause a significant loss in CNPase activity (Table 6.1) however both proliferated in response HMBP and isolated myelin (Fig. 6.4 & 6.5).

6.23. In vitro myelin degradation by unstimulated, mitogen and antigen stimulated PBMC and their supernatants

The ability of unstimulated and mitogen stimulated cell supernatants to degrade myelin <em>in vitro</em> was assessed with PBMC samples obtained from 10 MS patients (see Table 6.2). Four of the 10 MS PBMC samples when added to myelin caused significant <em>in vitro</em> myelin degradation as assessed by loss of CNPase activity (patient no.s 103, 182, 127 & 507). In a separate experiment, fresh SN's from the same PBMC, which had been incubated for 48 h with or without mitogen were added to myelin for 24 h. The 48 h incubated unstimulated or stimulated cells were also added to myelin in the same assay. None of the original 4 PBMC samples that caused a significant loss in CNPase activity when freshly incubated with myelin retained their demyelinating activity after incubation in culture media alone for 48 h before addition to myelin for 24 h. However 2 samples (patient no.s 484 & 497) that did not demylinate when freshly added to myelin caused significant losses in CNPase after prior incubation in media alone for 48 h. Only one PBMC sample that originally degraded myelin produced a SN (unstimulated) that was capable of myelin degradation (patient 103). Mitogen stimulation (PHA or Con-A) of the PBMC resulted in the loss of myelin degrading activity that was originally demonstrated by four of the ten PBMC samples. In addition mitogen stimulation had no effect on the ability of the other six PBMC samples or supernatants produced to cause a significant loss of myelin CNPase activity.

It was thought that mitogen stimulation was too non-specific and if the PBMC were incubated with HMBP, a myelin specific antigen, it might activate antigen specific cells which could be responsible for the <em>in vitro</em> myelin degradation seen with MS PBMC. Cells were
also stimulated with a non-myelin antigen PPD. As can be seen (Table 6.3), only SN from one patient (556-1) stimulated with PPD for 48 h caused a significant loss of CNPase activity. Prior stimulation with HMBP did not cause either the cells or SN produced to degrade myelin. Proliferation to HMBP and PPD by 3 of the 5 patients’ PBMC samples was also assessed in routine proliferation assay’s at the same time as assessing the myelin degrading ability of the PBMC and their supernatants (patient no.s 555, 556-1 & -2). None of the 3 patients’ PBMC proliferated in response to HMBP (SI’s all <2). However, PBMC from patient 556 proliferated in response to PPD on both occasions tested (556-1 SI = 3.5; 556-2 SI = 6.4) and also produced a supernatant, after stimulation with PPD for 48 h, that was capable of producing a significant loss of myelin CNPase activity (16%, p<0.01).

If PBMC were incubated with PHA at the same time as incubation with myelin then the myelin degrading ability of the cells increased from 4/10 PBMC samples causing a significant loss in CNPase activity when incubated with myelin alone, to 7/10 causing significant myelin degradation when myelin and cells were incubated with 10 μg/ml PHA (Fig. 6.6). This PHA induced increase in the number of PBMC samples causing in vitro myelin degradation was not statistically significant (p<0.5, Students t-test). Incubation of PBMC with LPS or Con-A (both at 10 μg/ml) at the same time as incubation with myelin for 24 h did not significantly alter the ability of the cells to cause myelin degradation (4/10 and 2/8 respectively, causing significant losses in CNPase activity).

6.2.4. In vitro myelin degradation by recombinant cytokines

To assess whether recombinant cytokines were able to degrade myelin in vitro and to also address the possibility that cell SN’s might have lost their myelin degradative activity before addition to the myelin, fresh cytokines were incubated with myelin for 24 h to investigate whether they possessed myelinotoxic activity. Recombinant human TNFα, recombinant rat IFNγ, human recombinant IL-1 and recombinant human IL-2 were all diluted in RPMI and added to myelin at varying concentrations ranging from 100 U/ml - 1000 U/ml (Table 6.4). TNFα and IFNγ were added together at varying concentrations to observe whether there was any synergistic activity between the two cytokines as it has been shown that IFNγ can enhance TNFα mediated cytolysis (Feinman et al., 1986).
After 24 h incubation no loss of CNPase activity of the myelin was observed with any of the cytokines even at the highest concentration. There was no synergism between IFNγ and TNFα in causing myelin degradation. The above experiments were repeated two more times and similar results were obtained in each, with the cytokines exerting no myelin degradative activity.
<table>
<thead>
<tr>
<th>Myelin incubated with:</th>
<th>Multiple Sclerosis T&lt;sub&gt;MBP&lt;/sub&gt; cell line</th>
<th>Healthy control T&lt;sub&gt;MBP&lt;/sub&gt; cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture medium</td>
<td>2760 ± 58</td>
<td>2537 ± 160</td>
</tr>
<tr>
<td>T cells</td>
<td>2655 ± 175</td>
<td>2503 ± 119</td>
</tr>
<tr>
<td>T cells + APC</td>
<td>2778 ± 159</td>
<td>2405 ± 107</td>
</tr>
<tr>
<td>T cells + APC + IL-2</td>
<td>2740 ± 135</td>
<td>2459 ± 87</td>
</tr>
<tr>
<td>APC (5x10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>2620 ± 47</td>
<td>2401 ± 123</td>
</tr>
<tr>
<td>APC (5x10&lt;sup&gt;5&lt;/sup&gt;) + IL-2</td>
<td>2621 ± 106</td>
<td>nd</td>
</tr>
<tr>
<td>Culture medium</td>
<td>2818 ± 109</td>
<td>2547 ± 21</td>
</tr>
</tbody>
</table>

Three days after stimulation with antigen, T<sub>MBP</sub> cell lines were incubated with myelin at a concentration of 2 x 10<sup>5</sup> cells/10 μg myelin. Autologous EBV transformed B cells were added as antigen presenting cells (APC) at 5 x 10<sup>5</sup> cells/ 2 x 10<sup>5</sup> T cells/ 10 μg myelin. Recombinant IL-2 was added at 1/10 dilution. After incubation at 37°C for 24 h the CNPase activity of the myelin was determined as described previously. nd = not done.
TABLE 6.2 IN VITRO MYELIN DEGRADATION BY PBMC AND SUPERNATANTS FROM UNSTIMULATED AND MITOGEN STIMULATED PBMC FROM MULTIPLE SCLEROSIS PATIENTS

Loss CNPase Activity (%)

Supernatant or cells from PBMC incubated +/- mitogen for 48 h prior to addition to myelin

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>PBMC* alone</th>
<th>Media alone</th>
<th>PHA 10 μg/ml</th>
<th>CON-A 10 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SN</td>
<td>cells</td>
<td>SN</td>
<td>cells</td>
</tr>
<tr>
<td>103</td>
<td>15*</td>
<td>3</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>496</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>182</td>
<td>19*</td>
<td>2</td>
<td>5</td>
<td>nd</td>
</tr>
<tr>
<td>360</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>484</td>
<td>7</td>
<td>19*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>127</td>
<td>16*</td>
<td>1</td>
<td>15</td>
<td>nd</td>
</tr>
<tr>
<td>497</td>
<td>9</td>
<td>19*</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>499</td>
<td>4</td>
<td>nd</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>507</td>
<td>17*</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>502</td>
<td>9</td>
<td>13</td>
<td>0</td>
<td>nd</td>
</tr>
</tbody>
</table>

* significant at p<0.007, ** p<0.005 (Student's t-test). nd, not done.

PBMC* were incubated with myelin at 2 x 10^5 cells/10 μg myelin (n=3) for 24 h at 37°C. PBMC (1 x 10^6) from the same patients were also incubated with or without mitogen at 37°C. After 48 h the cells were spun and the cell free supernatant (SN) collected, the cells were washed 3 times and resuspended in culture medium. In a separate experiment the cell free SN and the 48 h incubated cells were immediately incubated with myelin (75% SN/10 μg myelin; 2 x 10^5 cells / 10 μg myelin) for 24 h at 37°C (n=3). Results are expressed as percentage loss of CNPase activity from myelin after incubation with PBMC or SN as compared to myelin incubated with media alone. The above table includes results from more than one assay.
**TABLE 6.3** IN VITRO MYELIN DEGRADATION BY PBMC AND SUPERNATANTS FROM UNSTIMULATED AND ANTIGEN STIMULATED PBMC FROM MULTIPLE SCLEROSIS PATIENTS

Loss CNPase Activity (%)

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>PBMC alone</th>
<th>Media alone</th>
<th>HMBP 50 μg/ml</th>
<th>PPD 20 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SN cells</td>
<td>SN cells</td>
<td>SN cells</td>
<td>SN cells</td>
</tr>
<tr>
<td>591</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>555-1</td>
<td>22**</td>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>556-1</td>
<td>7</td>
<td>0</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>556-2</td>
<td>29**</td>
<td>7</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>590</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>

*Supernatant or cells from PBMC incubated +/- antigen for 48 h prior to addition to myelin*

*PBMC were incubated with myelin at 2 x 10⁵ cells/10 μg myelin (n=3) for 24 h at 37°C. PBMC (1 x 10⁶) from the same patients were also incubated with HMBP, PPD or media alone at 37°C. After 48 h the cells were spun and the cell free SN collected, the cells were washed 3 times and resuspended in culture medium. In a separate experiment the cell free SN or 48 hour incubated cells were immediately incubated with myelin (75% SN / 10 μg myelin; 2 x 10⁵ cells / 10 μg myelin) for 24 h at 37°C (n=3). Results are expressed as percentage loss of CNPase activity from myelin after incubation with PBMC or SN as compared to myelin incubated with media alone. Results from more than one assay are shown above.*

**significant at p<0.008; **p<0.00008 (Student's t-test). nd, not done.
### TABLE 6.4 EFFECT OF RECOMBINANT CYTOKINES ON CNPase ACTIVITY OF HUMAN MYELIN IN VITRO

<table>
<thead>
<tr>
<th>Myelin +/- cytokine (U/ml)</th>
<th>CNPase activity (nmol end product/20 min/sample)</th>
<th>Myelin +/- cytokine (U/ml)</th>
<th>CNPase activity (nmol end product/20 min/sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media alone</td>
<td>3338 ± 299</td>
<td>Media alone</td>
<td>3267 ± 159</td>
</tr>
<tr>
<td>TNFα 100</td>
<td>3492 ± 326</td>
<td>IL-1α 100</td>
<td>3249 ± 191</td>
</tr>
<tr>
<td>500</td>
<td>3235 ± 224</td>
<td>500</td>
<td>3478 ± 112</td>
</tr>
<tr>
<td>1000</td>
<td>3366 ± 389</td>
<td>1000</td>
<td>3334 ± 36</td>
</tr>
<tr>
<td>5000</td>
<td>3199 ± 107</td>
<td>5000</td>
<td>3459 ± 190</td>
</tr>
<tr>
<td>10000</td>
<td></td>
<td>10000</td>
<td>3331 ± 172</td>
</tr>
<tr>
<td>IFNγ 100</td>
<td>3244 ± 231</td>
<td>IL-2 100</td>
<td>3419 ± 211</td>
</tr>
<tr>
<td>500</td>
<td>3104 ± 272</td>
<td>500</td>
<td>3314 ± 187</td>
</tr>
<tr>
<td>1000</td>
<td>3418 ± 468</td>
<td>1000</td>
<td>3104 ± 159</td>
</tr>
<tr>
<td>5000</td>
<td>3573 ± 139</td>
<td>5000</td>
<td>3528 ± 251</td>
</tr>
<tr>
<td>10000</td>
<td>3414 ± 252</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα + IFNγ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>3391 ± 442</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>3480 ± 109</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Myelin (10 μg/ml) was incubated with recombinant cytokines of varying concentrations, diluted in RPMI alone, for 24 h at 37°C (n=3). Myelin alone in RPMI was incubated at the beginning, middle and end of the 96 well plate as a control (total n=9). After incubation at 37°C for 24 h the CNPase activity of the myelin was determined as described previously. The above table shows the results of two separate experiments. The cytokines TNF and IFN were assayed together and IL-1 and IL-2 were assayed in a separate experiment.
PBMC were assayed for the expression of the IL-2 receptor (n = 25) and MHC class II molecules (n = 13), using the MAb's anti-Tac and anti-HLA-DR, as detailed in Chapter 2.6.15. In addition, the PBMC were incubated with myelin, as described previously, and the loss of myelin CNPase activity was assessed (Chapt. 2.6.25 & 26). Graph A shows the relationship between IL-2 receptor expression and in vitro myelin degrading activity as assessed by percentage loss of myelin CNPase activity caused by the PBMC. Graph B shows the relationship between expression of HLA-DR and percentage loss of myelin CNPase activity caused by the PBMC.
PBMC from 49 MS patients were assayed for reactivity to HMBP in a lymphocyte proliferation assay, as detailed in Chapter 2.6.12. A positive response to antigen was said to occur when the stimulation index (SI) (proliferation to antigen / background proliferation) was ≥ 2. In addition, the PBMC were incubated with myelin, as described previously, and the loss of myelin CNPase activity was assessed (Chapt. 2.6.25 & 26).
2 x 10^5 MS PBMC (patient 383) were incubated in triplicate for 96 h at 37°C with either PHA (1 µg/ml), Con-A (10 µg/ml), HMBP (0.1 - 100 µg/ml) or alone (Chapt 2.6.12). To measure a proliferative response, 1 μCi ^3H-thymidine was added to the culture for the final 8 h. Cells were harvested and then counted on a beta scintillation counter. Results are expressed as mean counts per minute (cpm) ± SD.
Resting $T_{\text{HMBP}}$ cells previously isolated from an MS patient (see Chapt 2.6.13) were incubated in triplicate for 72 h at 37°C with or without antigen (GPMBP 5-50 µg/ml; HMBP 50 µg/ml; human myelin (HM) 50 µg/ml; PPD 20 µg/ml) in the presence of autologous irradiated (8000R) EBV transformed B cells ($10^4$ T cells: $2.5 \times 10^4$ EBV cells). To measure a proliferative response, $1 \mu$Ci $^3$H-thymidine was added to the culture for the final 8 h. Cells were harvested and then counted on a beta scintillation counter. Results are expressed as mean counts per minute ± SD.
FIGURE 6.5

PROLIFERATIVE RESPONSE TO MYELIN BASIC PROTEIN OF AN MBP REACTIVE T CELL LINE DERIVED FROM THE BLOOD OF A HEALTHY CONTROL SUBJECT

Resting T<sub>HMBP</sub> cells previously isolated from a healthy volunteer (see Chapt 2.6.13) were incubated in triplicate for 72 h at 37°C with or without antigen (GPMBP 5-50 μg/ml; HMBP 50 μg/ml; PPD 20 μg/ml) in the presence of autologous irradiated (8000R) EBV transformed B cells (10<sup>4</sup> T cells: 2.5 x 10<sup>4</sup> EBV cells). To measure a proliferative response, 1 μCi 3H-thymidine was added to the culture for the final 8 h. Cells were harvested and then counted on a beta scintillation counter. Results are expressed as mean cpm ± SD.
2 x 10^5 MS PBMC were incubated with 10 μg myelin in triplicate with or without 10 μg/ml mitogen (PHA-P, Con-A or LPS) for 24 h at 37°C. Myelin alone was also incubated at the beginning and end of the plate as a control (n=6). The CNPase activity of the myelin / cell pellet was determined as previously described and is expressed as a % of myelin incubated in media alone subtracted from 100. Each line represents a different MS PBMC sample (n=10). Filled symbols (●) represent PBMC samples which caused a significant loss in myelin CNPase activity p<0.01 (Students t-test). Results from more than one assay are shown.

Overall mean percentage loss of myelin CNPase activity, no mitogen 14.2 ± 10.2; PHA-P 22.4 ± 13.3; LPS 10.7 ± 9.6; Con-A 9.6 ± 6.8.
In order to further investigate the factor / mechanism responsible for the \textit{in vitro} myelin degradation produced by MS and RA PBMC as measured by loss in CNPase activity, the role of activated T cells was initially investigated. The involvement of PBMC secreted factors and the degrading ability of recombinant cytokines was also explored.

The question as to whether the T cells of MS patients are in a state of enhanced activation and produce factors which are able to degrade myelin was investigated as it may be possible that a sub-population of \textit{in vivo} activated lymphocytes are responsible for the \textit{in vitro} myelin degradation observed. Activated T cells express surface molecules such as the receptor for IL-2 (CD25) and class II antigens (HLA-DR) (Fredrikson \textit{et al.}, 1979). IL-2 receptor expression is a marker of early T cell activation whereas HLA class II antigens appear later (Cotner \textit{et al.}, 1983). Expression is not restricted to activated T cells as B cells and monocytes also express HLA-DR (Fu \textit{et al.}, 1978). It has been shown that MS peripheral blood lymphocytes express increased levels of IL-2 receptor as compared to PBL from non-inflammatory OND and healthy individuals (Bellamy \textit{et al.}, 1985). HLA-DR expression by PBL from MS patients and patients with the autoimmune diseases SLE and RA has also been shown to be increased as compared to controls (Tournier-Lasserve \textit{et al.}, 1987; Yu \textit{et al.}, 1980) thereby implying that an active immune response is occurring.

No correlation between the expression of activation antigens by MS PBMC and the ability of these cells to degrade myelin \textit{in vitro} was shown. Using a different assay system, Selmaj and colleagues (1988a) showed a positive correlation between IL-2 receptor expression by unstimulated lymphocytes and \textit{in vitro} demyelination produced by SN from these cells. It was suggested that this indicated that MS lymphocytes are activated \textit{in vivo} and may be responsible for producing a demyelinating factor \textit{in vitro}. Since the expression of the IL-2 receptor by T cells is an early activation marker indicating recent antigenic stimulation, it may have been more relevant to have investigated the correlation between \textit{in vitro} myelin degrading ability and expression of other T cell antigens such as CD45R which is the extracellular domain of a T cell membrane protein in the LCA family. It is thought that CD45 plays a critical role in regulating T cell responses as the maturation or differentiation of T cells results in the expression of different CD45 isoforms. CD45RA$^+$ T cells are thought to be 'naive' lymphocytes that do not respond to recall antigens, whereas CD45RO$^+$ T cells
are mature 'memory' cells that have been activated by prior exposure to antigen (Akbar et al., 1988; Sanders et al., 1988). Whether these 'memory' cells are involved in the in vitro myelin degradation observed would therefore have been interesting to investigate.

The role of antigen specific lymphocytes in degrading myelin was subsequently investigated. There is much evidence to suggest that autoimmune responses against neural antigens, especially MBP may be involved in the pathogenesis of demyelination. Acute disseminated encephalomyelitis can be induced after rabies vaccination with inactivated virus prepared from infected rabbit brains, suggesting that myelin antigens are encephalitogenic. It has been shown that these patients have significantly elevated anti-MBP antibody levels which correlates with the presence of major neurological complications. This does not imply that the pathogenesis is wholly dependent on a humoral rather than cell mediated mechanisms as lymphoproliferation to MBP has also been shown in post rabies vaccination patients (Hemachuda et al., 1987). Cellular and humoral responses to MBP have also been demonstrated in post-viral encephalomyelitis which followed measles infection (Johnson et al., 1984). In addition MBP T cell lines have been raised from the CSF of a patient with chronic rubella panencephalitis showing that persistent viral infection can give rise to autoreactive T cells. Rubella is a non-cytopathogenic agent suggesting that the widespread destruction of white matter and loss of myelin that can be seen histopathologically could be due to auto-reactive MBP specific T cells (Martin et al., 1989). Also, further support for MBP's role in the pathogenesis of demyelination and MS is that activated MBP T cells when injected into MHC identical recipient animals induce EAE, an animal model with clinical and histopathological features similar to MS.

The importance of cellular reactivity to MBP is however controversial as it has been shown that both MS and healthy subjects show cellular responses to MBP while others have been unable to confirm this (see Chapt. 1.5.4.), reactivity to MBP has also been demonstrated in patients with juvenile RA (Offner et al., 1981). It has been suggested that to differentiate pathogenic and non-pathogenic autoreactive T cells, the pathogenic cells should be activated in MS patients or should have undergone clonal expansion in vivo (Wucherpfennig et al., 1991). It has been shown that MBP reactive T cell clones isolated from MS peripheral blood are activated in vivo whereas activated MBP reactive cells could not be isolated from the blood of normal subjects. Gene mutation as assessed using a hrpt' mutant cell assay was used to detect activated cells as mutations appear to occur preferentially in dividing cells.
(Allegretta et al., 1990). Therefore, these activated autoreactive cells may play a role in the pathogenesis of MS.

Using a five day standard proliferation assay no correlation was demonstrated between MBP reactivity (by both MS and RA PBMC) and myelin degradative activity. This could suggest that MBP reactive cells are not involved in the PBMC induced loss of CNPase activity of myelin and that reactivity to another myelin antigen is more important. Alternatively, the type of proliferation assay used is a rather crude method that does not indicate the frequency of antigen reactive cells in the cell population being tested which may have been more appropriate to analyse.

The possibility that MBP reactive T cell lines may cause more significant losses in CNPase from myelin than PBMC was therefore investigated. Neither the MS, or healthy individuals’ derived MBP specific T cell lines were able to cause in vitro myelin degradation when incubated with myelin after previous antigenic stimulation. Addition of APC's and IL-2 to the T cells, when incubating with myelin, did not stimulate significant myelin degradation. The 24 h incubation with myelin may not have been sufficient to stimulate the production of a myelinotoxic factor or myelinotoxic mechanism. Alternatively, the possibility exists that even though these cell lines proliferate in response to MBP they have lost their ability to be myelinotoxic. It has been demonstrated that the continuous passage of rodent MBP specific T cell lines results in the concomitant loss of encephalitogenicity although they retain their proliferative response to MBP (McCarron et al., 1991). In addition, the EBV transformed B cells may only be providing the necessary co-stimulatory signals for a certain subpopulation of T cells which may not possess the ability to degrade myelin. Recent studies indicate that different subsets of T cells have differences in their requirements for co-stimulatory signals such as IL-1 (reviewed by Weaver and Unanue, 1990). Another possibility to explain the lack of myelin degradation by MBP specific T cells could be that when a PBMC sample is added to myelin for 24 h, and degrades myelin, the monocytes are acting as antigen presenting cells as well as mediators of myelin damage. Antigen specific lymphocytes become activated by the presentation of processed myelin antigen peptides which leads to the release of cytokines including IFNγ which could further activate and stimulate release of myelinotoxic factors from monocytes within the PBMC population. Therefore, the inability to show a myelin degradative effect with MBP specific T cell lines could be a reflection of the EBV transformed autologous B cells only being able to provide the
necessary co-stimulatory function as an APC but unable to function as an effector cell with
the production of potentially myelinotoxic factors.

The potential role of PBMC secreted demyelinating factors was subsequently investigated.
In contrast to the reports of Selmaj and colleagues (1986 and 1988a), the ability of MS
PBMC SN to cause in vitro myelin degradation of isolated myelin could not be demonstrated.
Even after mitogen stimulation (PHA and Con-A), the SN's produced did not cause any
significant loss in CNPase activity of the myelin. With the neonatal rat cerebellum culture
system used by Selmaj and colleagues mitogen stimulation, especially Con-A, enhanced the
demyelinating activity of the MS PBL SN's. Mitogen stimulation of healthy control PBL also
produced a SN that was capable of some degree of in vitro demyelination, although less than
that reported with MS PBL and requiring a longer culture incubation time (Selmaj et al.,
1988a). Therefore non-specific activation of T cells can stimulate the production of factors
that will demyelinate neonatal rat cerebellum cultures. An explanation for the lack of
degradation of isolated myelin, as assessed by loss in CNPase activity, by mitogen stimulated
SN could be due to an insufficient incubation time of the PBMC with mitogen to stimulate
production of degrading factors. Alternatively, if myelin degradative factors were produced
by mitogen stimulation the resultant SN may lose its activity before it was added to myelin.

Stimulation of the five MS PBMC for 24 h with HMBP did not produce a SN capable of
significantly degrading myelin which is not surprising since there was no reactivity to HMBP
as assessed in lymphocyte proliferation assays. However, when PBMC were stimulated with
PPD, a non-CNS antigen, one of the five PBMC samples produced a myelin degrading SN.
In addition, this one PBMC sample proliferated in response to PPD on both occasions tested.
Thus demonstrating that stimulation with a non-CNS antigen causes the release of a factor
into the cell SN which is capable of producing significant myelin degradation.

Addition of PHA directly to the PBMC incubating with myelin caused an increase in the
number of samples effecting a significant loss in CNPase activity (more samples would have
to be analysed to see whether this was a significant increase). Nevertheless, addition of Con-
A or LPS to the cells when incubating with myelin did not alter the myelin degrading ability
of these cells. LPS is a monocyte mitogen which stimulates the secretion of many
inflammatory mediators (for example; TNFα, IL-1α & β, IL-6 and PGE) within a few hours
of addition to the culture. PHA-P and Con-A are both agglutinating mitogens which may
increase the cell to myelin contact (Bevan and Cohn, 1975). Whether these results suggest that direct cell myelin contact is required for myelin degradation or that certain mitogens can in fact stimulate cells to produce degrading factors remains unclear. Nevertheless, this data indicates that non-specific activation of monocytes/macrophages by LPS is not sufficient for effecting a loss of myelin CNPase activity.

In summary using the isolated myelin in vitro assay system, unstimulated and stimulated (mitogen or HMBP) MS PBMC SN's were generally unable to cause in vitro myelin degradation which may be explained by the following. Firstly, if a secreted factor is responsible for the myelin degradation, the fact that neither stimulated nor unstimulated PBMC SN can reproduce the degradation which occurs when the same PBMC are in direct contact with myelin may indicate that the degrading factor has a short half-life. Secondly, there may be a sub-population of cells which are responsible for the in vitro myelin degradation, that are already activated within the MS PBMC population and further activation with HMBP or mitogen does not stimulate production of the same array of cytokines/degrading factors that myelin does. Alternatively, another myelin antigen such as MAG or MOG may be involved in stimulating in vitro myelin degrading activity rather than HMBP. In fact, preliminary data demonstrates that activation of non-myelin antigen specific cells causes the secretion of a factor/s capable of effecting a significant loss of CNPase activity. Further investigation of activation of antigen specific cells (non-myelin specific) in the vicinity of myelin should be performed to verify that the effect seen with PPD specific cells was reproducible. Finally, the in vitro myelin degradation assay described here quantifies direct damage to the myelin caused by a loss in an integral myelin protein, whereas in other model systems the factors may be acting directly on the oligodendrocyte and not on the myelin. Selmaj and colleagues (1988a) attempted to quantify the demyelination seen with MS PBMC SN by assessing radiolabel release. In virtually all cases (SN from MS, stroke and control patients' PBL) PBL SN caused radiolabel release indicating either that the cultures were extremely sensitive to the addition of exogenous factors or that all lymphocytes produce demyelinating factors.

The direct role of cytokines in effecting in vitro myelin degradation was finally assessed as there is mounting evidence to suggest particularly a role for TNFα in oligodendrocyte and myelin damage (Selmaj and Raine 1988; Brosnan et al., 1988). When recombinant cytokines (IL-1, IL-2, TNFα and IFNγ) were added to isolated human myelin for 24 h no significant
loss in CNPase activity was produced. This is in contrast to that shown by Selmaj and Raine (1988) who demonstrated that when TNFα was applied to mouse spinal cord cultures, myelin sheaths exhibited dilations (described as bubbling and ballooning of the sheath) and there was degeneration of oligodendrocytes. These effects were only seen after the TNFα had been in contact with the spinal cord culture for at least 18 h, with 50% of myelin fibres being affected after 48 h incubation. Recombinant IL-2 and IFNγ had no effect on myelin and oligodendrocyte pathology. The precise mechanism by which TNFα elicited its myelinotoxic effects on mouse spinal cord cultures are unclear. In other systems, a shift of water and electrolytes to the extracellular space has been observed soon after the administration of TNF. The authors, by extrapolation, suggest that there is an interaction between TNF and ion channels either in the axolemma or on the surface of the oligodendrocyte which is responsible for the observed myelinotoxic and cytotoxic effects. In the assay system presented in this thesis when TNFα was added at a very high concentration (5000 U/ml) no significant loss in myelin CNPase activity was demonstrated. In addition LPS stimulation of PBMC, which is known to stimulate the release of TNFα, did not result in significant in vitro myelin degradation. This suggests that the demyelinating effects seen previously with TNFα were probably mediated through its effect on the glial cells in the myelinated spinal cord culture and not through a direct effect on myelin. Alternatively, the possibility exists that in the assay system presented in this thesis the cytokines should have been left in contact with the isolated myelin for longer than 24 h as more myelin damage was noted in the mouse spinal cord cultures when TNFα had been in contact with the cultures for at least 48 h.

In conclusion, the possibility that the myelin degradation produced by 42% of MS PBMC and 58% of RA PBMC (see Chapt 4 and 5) is mediated directly by cytokines does not seem likely as recombinant cytokines have been unable to mimic the degradative ability of PBMC after 24 h contact with myelin. Nevertheless, cytokines could be involved indirectly by activating other cell types within the PBMC population thereby stimulating the release of potentially myelinotoxic factors. It would have been interesting to investigate this possibility further by adding cytokines directly to PBMC incubating with myelin as it is on these cells rather than isolated myelin that cytokines would exert their effect.

In the next chapter a closer look at which cell type within the PBMC population could be responsible for the observed in vitro myelin degradation produced MS and RA PBMC was investigated.
CHAPTER SEVEN

ARE T CELLS RESPONSIBLE FOR IN VITRO MYELIN DEGRADATION

7.1. INTRODUCTION

The mechanisms by which cytotoxic lymphocytes and NK cells kill their targets remains controversial and two hypothesis have been proposed based on recent experimental evidence. One hypothesis proposes that preformed lytic mediators exist within the lymphocyte in secretory granules which are released after stimulation by a target cell and cause cell lysis by pore formation. Alternatively a receptor-triggered disintegration model has been proposed as it has been observed that target cell DNA is released from the nucleus before lysis during lymphocyte-mediated cytotoxicity. In this model the effector cell induces an autolytic mechanism in the target cell resulting in DNA fragmentation. The two mechanisms may in fact exist as mechanisms of cytolysis but apply to different types of effector cell or the same effector cell(s) at different stages of activation (reviewed by Hayes et al., 1989; Berke, 1991).

Within the cytoplasm of cytolytic lymphocytes there is a set of proteins that are thought to be responsible for the cytolytic activity of these cells. Although other mechanisms of killing may exist, the exocytosis of these granules after stimulation by a target cell provides an important mechanism. The major constituents of these secreted granules are perforin (pore forming protein) (Masson & Tschopp, 1985), a family of highly homologous serine esterases (SE) (Pasternack et al., 1986) also known as granzymes (4 in humans, A,B,3,H and 7 in mice A-G) (reviewed by Griffiths & Mueller, 1991) and proteoglycan molecules of the chondroitin sulfate A type (Schmidt et al., 1985).

Target cell lysis caused by purified perforin (similar to complement induced lysis) is characterised by the disintegration of the cellular membrane without nuclear breakdown (Gromkowski et al.,1988). Purified granzyme A alone has no cytolytic effects however, in combination with purified perforin induces DNA degradation of target cells (Hayes et al.,1989). Also, serine protease inhibitors inhibit lysis caused by cloned T cells and isolated granules. However, the role of granzymes are still unclear. It is not known whether SE is released onto a target cell to degrade the cell membrane or cause intracellular DNA fragmentation or alternatively it may transform other endogenous proteins into active
cytolytic configurations.

Serine proteases and perforin have been isolated as proteins and cDNA clones and their expression in vivo may provide markers for (activated) functional cytotoxic lymphocytes that may play a role in autoimmune disease along with transplant rejection and viral diseases (Griffiths & Mueller, 1991). In animal models of viral infection, in situ hybridisation to perforin and granzyme mRNA has been used to detect cytolytic lymphocytes in vivo. Muller and colleagues (1989) demonstrated that when the Armstrong strain of the lymphotropic choriomeningitis virus is injected intracerebrally into mice, perforin and granzyme A expressing CD8* lymphocytes are seen in the CNS infiltrate 6 - 7 days post inoculation. These cells were seen in close apposition to the virally infected cells and there were histological signs of cellular destruction of the infected cells, demonstrating that these perforin and granzyme A expressing cells could be involved in the cytolytic response to virus infected cells.

Perforin and granzyme A expressing lymphocytes have also been detected in autoimmune disease. In the nonobese diabetic (NOD) mouse which is used as an animal model for studying the pathogenesis of type 1 insulin dependent diabetes mellitus, perforin and granzyme A expressing CD8* cells have been detected in the Islets of Langerhans in the pancreas as well as CD4* cells expressing the TNFα gene (Young et al., 1989). In fact adoptive transfer experiments have shown the requirements for both CD4* and CD8* cells in the development of diabetes in these NOD mice (Young et al., 1989; Held et al., 1990). Perforin and granzyme A expressing cells have also been detected in the synovial fluid of RA patients (Griffiths et al., 1992).

Methods of detection of granzyme A include in-situ hybridisation and by utilising its enzymatic activity with the SEspecific substrate N-benzyloxy-carbonyl-L-lysine-thiobenzylester (BLT) (Green & Shaw, 1979). Spectrophotometric assays have been used to detect activity levels in cell lysates, and SDS-PAGE used for characterisation of the enzyme within cell lysates. Serine esterase is present within cytolytic granules as it has been shown to co-sediment with perforin and cytolytic granules in density gradient sedimentation (Masson and Tschopp, 1985; Ferguson et al., 1988). Cell surface staining using a polyclonal antisera to SE has shown that it can also be membrane associated (Masson and Tschopp, 1987). The majority of the work describing the presence of SE positive cells has concentrated on
cytotoxic T cell lines. SE activity has also been described in T cell lines with a phenotype indicative of T helper cells which were capable of killing specific antigen bearing target cells (Pasternack et al., 1986).

Recently, Wagner and colleagues (1991) have reported a rapid cytochemical staining method for SE which when used with MAb to cell surface proteins can identify enzyme activity at a single cell level. Immunocytochemical staining of peripheral blood leucocytes from healthy volunteers showed that SE activity is expressed in 100% of NK cells (CD57+) and 28 ± 7% of CD8+ T cells. B lymphocytes were uniformly negative and only 2 ± 1.5% of CD4+ T cells stained weakly. Only 35 ± 6% of petri dish adherent cells stained positive for SE which is possibly a reflection of their state of activation. This cytochemical method for detection of intracellular serine proteases has recently been have validated as the expression of serine proteases by ex vivo lymphocytes has been correlated with their cytotoxic ability in vitro (Wagner et al., 1993).

In order to deduce what factors are responsible for the observed loss in CNPase activity of isolated myelin after incubation with MS PBMC and RA PBMC it was previously demonstrated that both PBMC SN's as well as a variety of recombinant cytokines including TNFα were generally unable to mimic the in vitro myelin degradation produced by the PBMC (see Chapt. 6). The direct role of T cells, either alone or in combination with monocytes, in the myelin degrading process was therefore investigated. To deduce which cells were responsible for the loss in CNPase, the PBMC T cell population was enriched and the resultant population added to isolated myelin with or without syngeneic adherent cells. Initially T cells were isolated from monocyte depleted PBMC on the basis of their lack of adherence to nylon fibres and added to myelin. The resultant T cell population was not homogeneous as it contained a small percentage of monocytes. The ability of T cells to bind SRBC via CD2 molecule was therefore utilised to obtain a purer T cell population before addition to myelin.

The possible role of cytotoxic T lymphocyte serine esterases in myelin degradation was also preliminary investigated as cell derived cytolytic mediators have been implicated in the demyelinating process (Scolding et al., 1990a).
7.2. RESULTS

7.2.1. Myelin degradation by nylon wool separated MS PBMC

In order to determine what cell type in the mixed PBMC population is responsible/required for degradation of myelin the T cells and monocytes were separated from the mixed cell population. A combination of petri dish adherence to obtain an enriched monocyte population of adherent cells (ADH) followed by passage over nylon wool columns, to capture the B cells as they bind avidly to nylon wool, which gave an effluent non-adherent (NA) population of T lymphocytes was used. PBMC and the different separated cell populations were then added to myelin for 24 h to determine myelin degrading ability.

When the PBMC T cell population was enriched and B cells and monocytes depleted, 6 of the 9 resultant NA cell samples when added to myelin retained the myelin degrading ability of the original PBMC sample and caused a significant loss of CNPase activity. In addition one of the PBMC samples (patient no. 500) that did not cause a significant loss in CNPase activity of myelin, when removal of monocytes and enrichment of the T cell population was performed the resultant NA population caused significant myelin degradation. Also one of the NA samples (patient no. 484) did not retain the demyelinating activity of the original PBMC population. When NA cells were incubated with syngeneic ADH cells (2 x 10^5 NA cells plus 2 x 10^4 ADH cells plus 10 μg myelin) there was generally no difference between the resultant CNPase loss caused by this combination of cells over that produced by NA cells alone or PBMC. Adherent cells, at the approximate concentration found in a PBMC population (10%) when incubated alone with myelin (2 x 10^4 cells / 10 μg myelin) in general did not degrade myelin as only one of the 9 samples tested caused a significant loss of CNPase activity (patient no. 479). However, 4 out of 7 ADH cells samples when incubated at a higher concentration with myelin (2 x 10^5 cells / 10 μg myelin) caused a significant loss in CNPase activity of the myelin (patient nos 479, 117, 546, & 486). Nevertheless, this is an artificially high number of macrophages to incubate with myelin, compared to the concentration in the mixed PBMC population.

Figure 7.1 shows the overall mean CNPase activity of myelin before and after incubation with the different separated cell populations. Pooled data obtained from 9 different PBMC samples and the resultant separated cells after incubation with myelin has been plotted. As
can be seen myelin incubated with either PBMC, NA cells alone or NA plus ADH cells results in an overall significant loss of CNPase activity of the myelin with there being negligible differences between the mean CNPase activity of myelin after incubation with PBMC or the NA cells (+/- ADH cells) (myelin alone, 3315 ± 262; myelin + PBMC, 2556 ± 290; myelin + NA cells, 2527 ± 116; myelin + NA + ADH cells, 2442 ± 158. All significantly different from myelin alone p<<2 x 10^-4 - 0.0004, Students t-test). Only ADH cells at the high concentration caused an overall significant loss in CNPase activity when incubated with myelin (2892 ± 258 p<0.003).

Identification of the cell types in the NA populations using the APAAP method of staining revealed that the number of cells staining positive for CD3 using the MAb UCHT1 averaged 86% (range 77 - 94%), with a mean of 3% monocytes/macrophages identified with the MAb UCHM1 (range 1 - 5%) (Table 7.2).

7.2.2. Myelin degradation by nylon wool separated RA PBMC

Very similar results to those demonstrated with MS cells were obtained when PBMC and the resultant separated cell populations from 6 RA patients were incubated with myelin (Table 7.3). Three of the NA cell populations retained the significant myelin degradative activity of the mixed PBMC (patient no.s 462, 489 & 490). When a low number of ADH cells were incubated with NA cells and myelin, separated cells from 3 of the 4 original PBMC samples that caused a significant loss of CNPase activity retained this myelin degrading activity. When low numbers of ADH cells were incubated with myelin alone one of the six samples assayed caused a significant loss of CNPase activity from the myelin (pt. 490) which was also demonstrated at the higher cell concentration. When the number of ADH cells was increased 5 - 10 times, in addition ADH cells from patient no. 489 caused a significant loss of CNPase activity of the myelin which was not seen at the lower cell concentration.

Figure 7.2 shows the overall mean CNPase activity of myelin after incubation for 24 h alone or with the different cell populations from all 6 RA PBMC samples assayed. PBMC and NA cells plus ADH cells when incubated with myelin produced an overall significant loss in CNPase activity of the myelin (myelin alone, 3482 ± 389; myelin + PBMC, 2836 ± 230; myelin + NA + ADH cells, 2759 ± 351. All significantly different from myelin alone p<0.007, Students t-test). Neither myelin incubated with NA cells, nor with ADH cells
incubated at either the lower or higher cell concentration caused an overall significant loss in CNPase activity (myelin + NA cells, 2883 ± 433; 2 x 10^4 ADH cells, 3162 ± 397; 2 x 10^4 ADH cells, 3030 ± 212; not significantly different from myelin alone).

The number of CD3^+ cells in the NA cell population as estimated by APAAP staining was 79.5% (range 72 - 98%) with 2% (range <1 - 3%) of the total number of cells staining positive with the MAb UCHM1 (results not shown).

7.2.3. Myelin degradation by SRBC separated MS PBMC

To obtain a more enriched T cell population, the ability of T cells to bind SRBC via the CD2 molecule, was used (Table 7.4). As with nylon wool purification, the T cell enriched population (E^+, erythrocyte binding) retained the significant myelin degradative activity of the mixed cell population (PBMC) (patient no.s 558-3 & 570). In addition, one T cell enriched sample caused a significant loss of myelin CNPase activity even though the matched PBMC sample did not (patient no. 558-4). All enriched T cell samples when incubated with myelin and a small number of ADH cells retained their myelin degrading activity of the PBMC with one additional sample acquiring myelin degrading activity that was not present in either the PBMC's or T cell enriched population (patient no. 573). Both the ADH cell population (at low cell numbers) and the B cell enriched population (E^-, non-erythrocyte binding) failed to cause any significant loss of CNPase when incubated with myelin. When the ADH cell concentration was increased 5 - 10 times, only one of the five samples tested (patient no. 591) caused a significant loss in CNPase activity. Figure 7.3 shows the overall mean CNPase activity of myelin after incubation for 24 h with all MS PBMC or separated cell populations (data from 9 separate experiments pooled). (myelin alone, 2859 ± 170; myelin + PBMC, 2565 ± 139 p<0.001; myelin + E^+ cells, 2608 ± 242 not significantly different; myelin + E^+ cells + ADH cells, 2607 ± 216 p<0.01; 2 x 10^4 ADH, 2860 ± 241 not significantly different).

Assessment of the purity of the T cell enriched population by APAAP staining with MAb specific for CD3, CD4, CD8, CD19 and CD14 (Table 7.5) showed that 91% of the E^+ cells were CD3^+ (range 89 - 94%) and 2% (range <1 - 3%) were positive for CD14 (monocyte/macrophage marker).
7.2.4. Serine esterase expression by MS PBMC incubated with myelin assessed by a cytochemical staining method

The expression of SE activity by T lymphocytes was assessed for 12 MS PBMC samples (see chapt 2.6.18) before and after incubation with myelin for 24 h (same experimental conditions as in the myelin degradation assay). The phenotype of the lymphocytes expressing SE was determined with the MAb Leu 3a (CD4⁺) and G10-1.1 (CD8⁺) and the APAAP method of staining. Expression of SE activity was also assessed before and after incubation of the PBMC samples in media alone or with myelin for 24 h. At time 0, 26 ± 14% of MS CD8 lymphocytes expressed SE activity. Incubation in media alone for 24 h did not affect the number of cells expressing SE activity as 28 ± 15% of MS CD8⁺ lymphocytes were positively stained for SE. However when the cells were incubated for 24 h in the presence of myelin the expression of SE by CD8⁺ lymphocytes increased to 41 ± 23%. This increase was not statistically significant (Fig. 7.4).

SE expression by CD4⁺ MS lymphocytes was also assessed at time 0, 24 h and at 24 h after incubation with myelin, and no observable difference in expression was noted (0 h, 5 ± 4%; 24 h 4 ± 3%; incubated with myelin 24 h, 2.5 ± 2%). The total number of SE⁺ cells was always assessed and the increase in cells expressing SE after incubation with myelin was always accounted for by the CD8⁺ population (Fig. 7.4).

SE expression by CD4⁺ and CD8⁺ lymphocytes before and after addition to myelin was also assessed in the PBMC of 4 healthy individuals. The number of CD4⁺ and CD8⁺ lymphocytes at time 0 expressing SE was slightly lower than in the MS samples however, there was no increase in the number of CD8⁺ cells expressing SE after incubation with myelin for 24 h as was demonstrated with MS samples (CD8⁺ cells expressing SE at time 0, 19 ± 11%; after incubation with media for 24 h 20 ± 7%; after incubation with myelin for 24 h 21 ± 8%; CD4⁺ cells expressing SE at time 0, 1 ± 1%; after incubation with media for 24 h 2 ± 0.5%; after incubation with myelin for 24 h 1 ± 1%) (Fig. 7.4).

Myelin degradative activity as assessed by loss in CNPase was also assessed for each PBMC sample (12 MS and 4 healthy individuals PBMC samples). For the 12 MS samples incubated with myelin, 5 caused a significant loss in CNPase activity (range 18 - 34% loss p<0.00001 - 0.002). When loss in CNPase activity was compared to number of CD8⁺ cells expressing SE
after incubation with myelin for 24 h no correlation was seen ($r=0.04$, not significant).
None of the 4 PBMC samples from the healthy individuals caused a significant loss in
CNPase activity (2 - 8% loss, all not significant).

7.2.5. Serine esterase activity content of MS PBMC & SN's after incubation with myelin

Using a sensitive coupled reaction involving BLT and DTNB the amount of SE activity
contained intracellularly was investigated in 8 MS PBMC samples. There was no statistically
significant difference between the amount of intracellular esterase activity found within the
PBMC lysates before or after incubation with myelin for 24 h (time 0 h, 0.55 ± 0.62; time
24 h, 0.46 ± 0.55; time 24 h + myelin, 0.62 ± 0.54; activity is expressed as units of BLT
esterase activity / $1 \times 10^6$ cells, with 1 unit of enzyme activity defined as an absorbance of
1.0). There was also no correlation between percentage loss of CNPase activity from the
myelin and intra cellular SE activity of the 8 MS PBMC samples ($r=0.6$, not significant).

The SE activity of the cell SN's was also assessed at the same time points as the SE activity
of the cells. Time 0 h, 0.3 ± 0.72; time 24 h, 0.84 ± 0.72; time 24 h + myelin, 0.16 ± 0.52.
As can be seen, the results show a wide distribution which may be a reflection of the
instability of SE in tissue culture media. Further experiments would have to be performed
where cell SN's were collected at earlier time points as preliminary data shows that SN SE
activity rapidly declines with time (data not shown).
<table>
<thead>
<tr>
<th>Patient No.</th>
<th>PBMC 10^5</th>
<th>NA 10^5</th>
<th>NA + ADH 10^5</th>
<th>ADH 2 x 10^4</th>
<th>ADH 1-2 x 10^5</th>
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<tr>
<td>479</td>
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<td>30*</td>
<td>27*</td>
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<tr>
<td>485</td>
<td>20*</td>
<td>23*</td>
<td>21*</td>
<td>3</td>
<td>nd</td>
</tr>
<tr>
<td>117</td>
<td>33*</td>
<td>26*</td>
<td>23*</td>
<td>0</td>
<td>15*</td>
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<tr>
<td>484</td>
<td>32*</td>
<td>17</td>
<td>26*</td>
<td>6</td>
<td>nd</td>
</tr>
<tr>
<td>486</td>
<td>16*</td>
<td>32*</td>
<td>59*</td>
<td>0</td>
<td>30*</td>
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<td>4</td>
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<td>0</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

* significantly different from myelin incubated with media alone, *p* < 3 x 10^-4 - 0.007 (Students t-test). nd, not done

Myelin (10 μg) was incubated in triplicate for 24 h at 37°C with either PBMC (2 x 10^5), non adherent cells (NA) (2 x 10^5) eluted from nylon wool columns (see Chapt. 2.6.8.), NA cells (2 x 10^5) plus petri dish adherent cells (ADH) (2 x 10^4) (see Chapt. 2.6.5.), or ADH cells alone (2 x 10^4 or 1 - 2 x 10^5). Loss of CNPase activity of the residual myelin pellet is expressed as % of myelin incubated in media alone subtracted from 100. Results from more than one assay are shown above.
TABLE 7.2

ASSESSMENT OF NYLON WOOL COLUMN ENRICHMENT OF MS T CELLS BY IMMUNOCYTOCHEMICAL STAINING USING MONOCLONAL ANTIBODIES & THE APAAP METHOD OF STAINING

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Percentage non-adherent cells staining positive for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3^+</td>
</tr>
<tr>
<td>479</td>
<td>86</td>
</tr>
<tr>
<td>480</td>
<td>83</td>
</tr>
<tr>
<td>485</td>
<td>91</td>
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<td>117</td>
<td>77</td>
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<tr>
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<td>93</td>
</tr>
<tr>
<td>500</td>
<td>94</td>
</tr>
<tr>
<td>493</td>
<td>nd</td>
</tr>
</tbody>
</table>

Patient samples correspond with those shown in Table 7.1. Adherent (ADH) cells were separated from PBMC by incubation on plastic petri dishes. The T cell population was enriched on the basis of their lack of adherence to nylon fibres (B cells bind avidly to nylon fibres) (see Chapt. 2.6.5 & 8). The NA cells (enriched T cells) were then examined immunocytochemically using the single alkaline phosphatase anti-alkaline phosphatase (APAAP) method of stained (Chapt 2.6.17). The following MAb's were used; UCHT-1 for CD3; anti-leu 3a for CD4; G10 - 1.1 for CD8; DA-2 for HLA-DR; UCHM-1 for CD14 (see Table 2.1).
**TABLE 7.3**

LOSS OF CNPase ACTIVITY FROM MYELIN AFTER INCUBATION WITH MIXED PBMC OR NYLON WOOL SEPARATED PBMC FROM RHEUMATOID ARTHRITIS PATIENTS

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>PBMC</th>
<th>NA</th>
<th>NA + ADH</th>
<th>ADH 2 x 10⁴</th>
<th>ADH 1 - 2 x 10⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>462</td>
<td>17*</td>
<td>28*</td>
<td>14*</td>
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<td>11</td>
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<td>18</td>
<td>9</td>
<td>5</td>
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</table>

* significantly different from myelin incubated with media alone, p<0.0001 - 0.01 (Students t-test). nd, not done.

Myelin (10 µg) was incubated in triplicate for 24 h at 37°C with either PBMC (2 x 10⁵), non adherent (NA) cells (2 x 10⁵) eluted from nylon wool columns after incubation with PBMC (see Chapt. 2.6.8), NA cells (2 x 10⁵) plus petri dish adherent cells (ADH) (2 x 10⁴) (see Chapt. 2.6.5), or ADH cells alone (2 x 10⁴ or 1-2 x 10⁵). Loss of CNPase activity of the residual myelin pellet is expressed as % of myelin incubated in media alone subtracted from 100. Results from more than one assay are shown in the above table.
## TABLE 7.4

**LOSS OF CNPase ACTIVITY FROM MYELIN AFTER INCUBATION WITH MIXED PBMC OR SRBC SEPARATED PBMC FROM MULTIPLE SCLEROSIS PATIENTS**

Loss of CNPase activity (%) from myelin when incubated with:

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>PBMC</th>
<th>E*</th>
<th>E* + ADH</th>
<th>ADH $2 \times 10^4$</th>
<th>ADH $1-2 \times 10^5$</th>
<th>E*</th>
</tr>
</thead>
<tbody>
<tr>
<td>558-3</td>
<td>17*</td>
<td>25*</td>
<td>14*</td>
<td>0</td>
<td>10</td>
<td>nd</td>
</tr>
<tr>
<td>558-4</td>
<td>11</td>
<td>16*</td>
<td>18*</td>
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<td>2</td>
<td>9</td>
</tr>
<tr>
<td>570</td>
<td>17*</td>
<td>13*</td>
<td>9*</td>
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<td>2</td>
<td>3</td>
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<tr>
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</tbody>
</table>

* significantly different from myelin incubated with media alone, $p < 5 \times 10^{-6} - 0.002$ (Students t-test). nd, not done.

Adherent (ADH) cells were separated from PBMC by incubation on plastic petri dishes. The ability of T cells to bind SRBC via CD2 was used to isolate T cells from the non-adherent cell population (see Chapt. 2.6.5 & 2.6.8). The E* cell population are enriched T lymphocytes, E cells are non-T lymphocytes. Human myelin (10 Ìg) was incubated in triplicate for 24 h at 37°C with either PBMC ($2 \times 10^5$), E* or E cells ($2 \times 10^5$), E* ($2 \times 10^5$) plus ADH cells ($2 \times 10^4$) or ADH cells alone ($2 \times 10^4$ or 1-2 $\times 10^5$). Loss of CNPase activity of the residual myelin pellet is expressed as % of myelin incubated in media alone subtracted from 100. Results from more than one experiment are shown.
TABLE 7.5

ASSESSMENT OF SRBC PURIFICATION OF MS T CELLS BY IMMUNOCYTOCHEMICAL STAINING USING MONOCLONAL ANTIBODIES AND THE APAAP METHOD OF STAINING

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CD3⁺</th>
<th>CD4⁺</th>
<th>CD8⁺</th>
<th>CD19⁺</th>
<th>CD14⁺</th>
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</table>

Adherent (ADH) cells were separated from PBMC by incubation on plastic petri dishes. The ability of T cells to bind SRBC via CD2 was used to isolate T cells from the NA cell population (see Chapt 2.6.9.). The E⁺ cells (enriched T cells) were then examined immunocytochemically using the single alkaline phosphatase anti-alkaline phosphatase (APAAP) method of stained (Chapt 2.6.17). The following MAb's were used: UCHT-1, for CD3; anti-leu 3a for CD4; G10 - 1.1 for CD8; anti-leu 12 for CD19; UCHM-1 for CD14 (see Table 2.1).
Human myelin was incubated alone (n=6) or with either PBMC or its different separated cell populations as detailed in Table 7.1; NA cells, NA + ADH cells or ADH cells alone (low or high concentration) (n=3). The CNPase activity of the residual myelin alone or myelin plus cell pellet was determined after 24 h incubation. The data from 9 MS PBMC samples and their separated cell populations when added to myelin are included in the above figure (myelin, n = 54; myelin + cells, n = 27) (data from 9 separate experiments).

* significantly different from myelin incubated with media alone, p < 2 x 10^-6 - 0.003 (Students t-test).
Human myelin was incubated alone (n=6) or with either PBMC or its different separated cell populations as detailed in Table 7.3; NA cells, NA + ADH cells or ADH cells alone (low or high concentration) (n=3). The CNPase activity of the residual myelin alone or myelin plus cell pellet was determined after 24 h incubation. The data from 6 RA PBMC samples and their separated cell populations when added to myelin are included in the above figure (myelin, n = 36; myelin + cells, n = 18) (data from 6 separate experiments).

* significantly different from myelin incubated with media alone, p < 0.007 (Students t-test).
Human myelin was incubated alone (n=6) or with either PBMC or its different separated cell populations as detailed in Table 7.4; E⁺ cells, E⁻ cells, E⁺ + ADH cells or ADH cells alone (low or high concentration) (n=3). The CNPase activity of the residual myelin alone or myelin plus cell pellet was determined after 24 h incubation. The data from 5 MS PBMC samples and their separated cell populations when added to myelin are included in the above figure (myelin, n = 30; myelin + cells, n = 15) (data from 9 separate experiments).

* significantly different from myelin incubated with media alone, p < 0.001 - 0.01 (Students t-test).
2 x 10^5 PBMC were incubated with media alone or myelin (10 μg) (n = 3) for 24 h at 37°C. Cytocentrifuge slides of the PBMC or PBMC plus myelin were prepared and stained by the APAAP method of staining utilising the MAb's anti-leu 3a (CD4+) (O ●) and G10-1.1 (CD8+) (▲ △) as detailed Chapt. 2.6.17. At the same time the cells were also cytochemically stained for the expression of serine esterase (SE) (see Chapt. 2.6.18). SE expression by lymphocytes from 12 MS patients (filled symbols) and 4 healthy individuals (hollow symbols) at the different time points are shown above.
The expression of serine esterase (identified by yellow colouration of cells) by both (a) CD3+ lymphocytes (pink colouration of cells) and (B) CD8+ lymphocytes was determined as described previously (2.6.17 & 18). The MAb’s UCHT-1 (CD3+) and G10 - 1.1 (CD8+), and the APAAP immunocytochemical procedure was used.
When an enriched population of T lymphocytes isolated from either MS or RA PBMC was added to isolated human myelin, significant loss of CNPase activity was often seen comparable to the loss caused by the original mixed PBMC sample. When petri-dish adherent monocytes, isolated from PBMC that possessed myelin degradative activity, were added back to the enriched T cell population and incubated with myelin the resultant significant loss of CNPase activity was similar to that produced by the enriched T cells incubated with myelin alone. There was no augmentation of the loss of CNPase activity induced by NA alone when ADH cells were incubated together with myelin. Due to the inefficiency of the T cell purification procedures employed in successfully depleting the monocyte population, it cannot be concluded that it was the T cell population within the PBMC sample that was wholly responsible for the loss of CNPase activity of myelin. By both methods of T cell isolation from the mixed population, petri-dish adherence followed by nylon wool column enrichment of T cells or petri-dish adherence followed by the rosetting of T cells with SRBC, contaminating monocytes were always present in very low cell numbers in the enriched T cell population. Lack of access to a fluorescence activated cell sorter meant that the more efficient technique of cell separation by way of either positive or negative selection with MAb was not possible. Limited experiments were performed using antibody bound magnetic beads (DYNAL) to isolate T cells, but due to inconclusive preliminary data and insufficient time this was not pursued. Nevertheless, adherent cells representative of monocytes/macrophages, at a comparable concentration to that expected within the PBMC population (10%) were not usually able to reproduce the myelin degradation produced by the original mixed cells when incubated with myelin. However, if the concentration of adherent cells was increased 10 times then a significant loss of CNPase activity from myelin was more frequently detected. This however, is an artificially high concentration of cells to incubate with myelin that does not reflect the mean concentration of monocytes in the PBMC population.

Therefore, is the resultant loss in CNPase activity that certain MS and RA PBMC produce, that is also generated by the enriched T cell population, due to the small number of contaminating monocytes that could facilitate an MHC restricted T cell effect? The extraction of myelin from white matter disrupts the normal topographical arrangement of membrane proteins and thus exposes antigens such as MBP that were previously localised
on the unexposed cytoplasmic face of the myelin membrane. When enriched T cells are added to myelin the presence of contaminating monocytes could allow class II mediated recognition of MBP (or other myelin antigens) by T cells with the resultant production of cytokines. The cytokines could either damage myelin directly or cause the activation of monocytes leading to macrophage mediated damage of the myelin. In vivo, the release of soluble products from both T cells and macrophages could cause oligodendrocyte lysis and myelin injury as has been shown in vitro with TNFα (Selmaj et al., 1988c) which is produced primarily by stimulated macrophages and to a lesser extent by T lymphocytes (Turner et al., 1987). TNFα and IL-1 are mutually inductive and it has been demonstrated that MS blood macrophages spontaneously produce elevated levels of IL-1 (Merrill et al., 1989) which could stimulate TNFα production.

It seems likely that a mixture of monocytes and T cells are required for the myelin degradation that occurs when certain enriched T cell populations (isolated from PBMC samples with myelin degradative ability) are added to myelin. Monocytes alone when added to myelin at an approximate concentration reflective of that within the PBMC population do not cause a significant loss in CNPase activity which may indicate that oligodendrocyte/myelin surface antigen specific antibodies are required to opsonize the oligodendrocyte/myelin unit and thus stimulate Fc receptor attachment and phagocytosis by macrophages. As there is unlikely to be any antibody producing plasma cells in the enriched population and also no extra sources of complement, opsonisation is probably not occurring. Therefore, how is the myelin degradation occurring? It is probably not due to direct phagocytosis but by the activation of macrophages by T cell cytokines such as IFNγ which can stimulate the monocytes to produce myelinotoxic mediators (neutral proteases, lysosomal hydrolases, TNF and ROS). Reactive oxygen species (superoxide anion, hydrogen peroxide and hydroxyl radicals) have been shown to degrade myelin in vitro (Konat and Williams, 1985) and apart from macrophages are released by microglia and possibly by astrocytes thus suggesting a role for these cells in the pathogenesis of demyelination in MS (reviewed by Hartung and Heinenger, 1989). Oligodendrocytes are also very sensitive to the effects of superoxide anion whereas other glial cells are resistant to its effects (Griot et al., 1990).

When monocytes are incubated with myelin at a high concentration (relative to that found within the PBMC population) there is an increase in the number of samples that degrade the myelin, this could be due to the incubation of the monocytes on plastic prior to addition to
myelin which activates the cells, possibly stimulating them to produce myelinotoxic factors at high enough concentrations to have a demonstrable effect on the myelin. Nevertheless, monocytes alone at a low concentration similar to that found in the PBMC population cannot reproduce the loss of CNPase activity from myelin seen with an enriched T cell population.

There is a possibility that released toxic mediators (perforin and serine esterases) from the granules of CD8\(^+\) lymphocytes may also be involved in the \emph{in vitro} loss of CNPase activity which is observed when enriched T lymphocyte samples from a percentage of MS and RA patients are incubated with isolated myelin.

Cell associated proteases have in fact been implicated in the pathogenesis of MS as the proteolytic enzyme activity of circulating blood leukocytes was shown to be increased in MS patients during exacerbation of the disease as compared to control patients. The soluble enzymes were also shown to degrade MBP and CNPase \emph{in vitro} (Cuzner \emph{et al.}, 1978).

Neutral and acid proteases have also been detected in increased amounts within MS and EAE plaques and lesions thus implicating cell derived soluble enzymes in the degradation of myelin and plaque formation (reviewed by Rastogi and Clausen, 1981). The enzymes possibly originate from infiltrating lymphocytes and macrophages, and resident microglia and astrocytes in humans (Allen \emph{et al.}, 1979). Neutral protease has also been detected in the CSF from patients with MS and was elevated in exacerbation of the disease as compared to remission (Inuzuka \emph{et al.}, 1987). Rastogi and Clausen (1980) showed that MS brain specific antigen and not an unrelated antigen caused the release of proteinase activity by leukocytes.

Cammer and colleagues (1978) also showed that stimulated mouse peritoneal macrophages release a neutral protease plasminogen activator that both directly and indirectly degrades myelin proteins especially basic protein. Transposing this to the \emph{in vivo} situation they hypothesised that macrophages and mononuclear cells are attracted by chemotaxis to the site of initial T cell stimulation by antigen. The macrophages are activated and secrete neutral proteases that act directly on myelin which is in close proximity. Also, plasminogen activator may interact with zymogens that are present in serum and tissue spaces to produce plasmin (serine protease) which is also capable of degrading BP in myelin.

Preliminary experiments looking at the expression of SE by CD8\(^+\) lymphocytes before and
after incubation with myelin have shown that there is an overall increase (not significant) in the number of CD8\(^+\) cells expressing SE after 24 h incubation with myelin from the MS population and not the healthy individual population. More cell samples would have to be tested to confirm these results as it is an interesting effect that needs to be pursued. When the myelin degrading ability of the mixed cell samples (MS and healthy individuals) was compared to the increase in CD8\(^+\) cells expressing SE no correlation was evident.

Nevertheless, an increase in the number of CD8\(^+\) cells staining positive for SE after incubation with myelin for 24 h indicates that the cells had previously been activated \textit{in vivo} as SE is usually expressed within cells 4-6 days after primary stimulation with antigen. The genes encoding both perforin and granzyme A are expressed within T cells 1-2 days after primary stimulation with target antigens, the proteins being produced days 3-4, peaking days 6-7. The technique employed for determination of SE expression only allows an enumeration of the cells expressing SE, it does not allow one to estimate the concentration of the proteinase within the specific subpopulation. It is therefore difficult to determine whether SE has already been released and possibly causing myelin degradation. When quantitative intracellular SE activity was assessed, no differences were seen between the SE activity of PBMC incubated with media or with myelin. However, as this reflects total intracellular SE activity of all cells within the PBMC population (T cells, NK cells, monocytes and contaminating granulocytes) a small but important rise in the number of CD8\(^+\) cells expressing SE activity may be masked especially since the myelin could also be causing the release of SE. Preliminary experiments assessing the SE activity within the PBMC SN after incubation with myelin were inconclusive and would have to be investigated more rigorously (data not shown).

It is unclear whether myelin is causing the increased expression of SE directly or indirectly. There is a possibility that myelin could indirectly be causing the upregulation of SE expression (upregulation of gene expression) by stimulating myelin specific T cells to produce IL-2 and other cytokines that in turn are known to induce granzyme and perforin gene expression. IL-2 rapidly induces granzyme and perforin mRNA production and IL-4 has been shown to increase granzyme expression (reviewed by Griffiths \textit{et al.}, 1991).

Investigation into the expression \textit{in vivo} of these putative cytotoxic proteins is therefore very important. \textit{In vivo} upregulation of serine protease in different lymphocyte subpopulations

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has been demonstrated in organ transplantation and viral infection (Sunder-Plassman et al., 1990; Wagner et al., 1993).

Whether intracellular enzymes (either from activated mononuclear cells, polymorphonuclear cells or cytotoxic lymphocytes) are the primary or secondary mediators of demyelination is unclear. However the data showing an increase in the number of CD8\(^+\) cells expressing SE after incubation with myelin suggests that lymphocytes may be involved in mediating myelin damage \textit{in vivo} through the release of a protease.
CHAPTER EIGHT

GENERAL DISCUSSION

The overall aim of this thesis was to investigate the myelin degrading activity of MS T cells and T cell products as there is considerable evidence to suggest an important role for T cells in the pathogenesis of MS. They have been shown histologically to be present in actively demyelinating plaques and thus the question arises are they the effector cells involved in initiating and maintaining demyelination or are they inducing a process which, in turn, leads to demyelination?

In attempts to elucidate the mechanisms of demyelination that are occurring, in MS and the animal model EAE, previous studies have employed in vitro cultures of developing CNS tissue where often serum or cells from one species are added to CNS cell cultures of another species and demyelination assessed qualitatively. Apart from the problems of incompatibility across species there can be varying amounts of myelinating fibres within each culture which could lead to wide ranges in the reported levels of demyelination. In addition these methods are open to observer bias and quantitative evaluation of demyelination is difficult by light microscopy. To overcome these difficulties, suspensions of fixed amounts of purified myelin have been utilised with the loss of an intrinsic myelin protein (CNPase) used as a marker for myelin breakdown. Model experiments showed that CNPase is a reliable and sensitive marker for myelin breakdown that can be used for the quantification of myelin degradation in vitro by different mechanisms. This assay system is by no means a true reflection of in vivo conditions but serves as a useful artificial membrane model system for investigating mechanisms of direct myelin damage by potential mediators of demyelination in vivo.

Using this model system it was shown that 43% of MS PBMC were able to cause a significant loss of the intrinsic myelin protein CNPase indicating that they were capable of in vitro myelinotoxicity. This activity did not correlate with clinical disease status, but considering that in MS clinical status often does not mirror activity within the CNS this is not surprising. Nevertheless, when 4 patients PBMC were followed longitudinally over a period of 2 weeks the myelinotoxicity of the cells fluctuated as measured by ability to cause significant losses in CNPase activity. This suggests that myelinotoxic cells may traffic in or out of the CNS and be present in the blood at various time points in disease. Alternatively it could reflect a
changing level of immune reactivity of the cells. All 4 of the patients who were followed longitudinally were in acute relapse and two of the patients had evidence of new lesions as shown by enhanced MRI.

The ability to degrade myelin in vitro was also shown by PBMC from 58% of RA patients. This disease has similarities to MS in that it is a chronic inflammatory disease of the joints with strong evidence for a role of T cells in the tissue damage. As in MS, it has been suggested that circulating effector T cells could initiate the disease process by triggering an immune reaction in the joint which could lead to cartilage and bone destruction by inflammatory mediators. Chronically stimulated mononuclear cells could potentially migrate out of the joint into the blood and continue to produce inflammatory mediators that are thus capable of myelin degradation in vitro.

Cells from patients with OND were generally devoid of myelin degrading activity and none of the healthy individuals' PBMC assayed caused significant losses of CNPase activity. Therefore indicating that in vitro degradation of myelin may be an indicator of the presence of pre-activated cells in the circulation that are more easily triggered to release effector molecules which can be myelinotoxic or are capable of myelin damage by another mechanism.

Recently Imamura and colleagues (1993) reported that peripheral blood monocytes / macrophages from MS patients in the acute phase of a relapse, when stimulated with LPS, produced significantly higher amounts of TNFα, IL-1α & β and IL-6 than patients with stable disease or from control patients. In addition, Maimone and colleagues (1993) demonstrated that MS patients had circulating monocytes that spontaneously secreted IL-6 or contained detectable cell-associated IL-1β. They suggested that monocytes in MS patients are partially primed or pre-activated during the course of the disease, possibly as a consequence of direct contact with activated T cells in vivo.

Previous investigations into potential mechanisms of demyelination have demonstrated that MS serum contains myelinotoxic factors. However the data is conflicting as sera from OND patients as well as from healthy individuals has also been shown to possess demyelinating properties as assessed by morphological changes to the CNS culture systems used. In contrast it has been shown in this thesis that MS sera was devoid of myelin degrading activity.
when added to an isolated myelin preparation. In general this was also true of OND and RA serum, although slightly more samples were myelinotoxic (8% and 15% respectively of samples being myelinotoxic). Nevertheless, healthy control sera showed no myelin degrading activity. The differences in assay systems employed, isolated myelin compared to organotypic tissue cultures, would explain the discrepancy between the lack of serum myelinotoxicity shown in this thesis and the previously reported demyelination by MS serum.

What is mediating \textit{in vitro} myelin degradation observed with MS and RA PBMC?

Initially the comparison between expression of the IL-2 receptor (CD25) or HLA-DR antigen and myelinotoxicity was investigated as expression of both of these activation antigens are raised in MS, and it may be these cells which are responsible for the myelin degradation seen \textit{in vitro}. No correlation was found but as CD25 is only transiently expressed it may have been more prudent to investigate the expression of other markers of T cell activation such as VLA antigens (cellular integrins) which are expressed several weeks post activation unlike the earlier expressed IL-2 receptor (Pischel \textit{et al.}, 1987). Antibodies against VLA-4 when injected intraperitoneally two days after the transfer of encephalitogenic T cell clones prevented the accumulation of leukocytes within the CNS and the development of EAE (Yednock \textit{et al.}, 1992). Whether one can relate this finding to MS and investigate whether there is a correlation between expression of VLA molecules and myelin degrading ability would have been interesting to investigate. Both MS and RA T cells have been shown to express VLA-1 indicating a prolonged state of activation (Hafler \textit{et al.}, 1985; Hemler \textit{et al.}, 1986).

The possibility that MBP specific lymphocytes are responsible for the \textit{in vitro} loss in CNPase activity observed with MS PBMC was also investigated since EAE, the experimental animal model of demyelination can be induced by T$_{\text{MBP}}$ cells. Whether these myelin antigen specific cells are involved directly in demyelination or are effectors of BBB breakdown allowing the entry of non-myelin specific inflammatory cells and serum products is unclear at present. However, it has been reported that T lymphocytes are observed first at the site of demyelination in EAE with macrophages and other inflammatory cells being recruited afterwards (Sedgewick \textit{et al.}, 1987). Thus the proliferative reactivity of PBMC to MBP was investigated and compared with myelinotoxicity of these cells. With both MS and RA PBMC, although proliferation to MBP and an ability to cause a significant loss in CNPase
activity was observed there was no correlation between these two parameters. It was thought that the concentration of MBP reactive cells may have been too low to achieve significant myelinotoxicity and therefore two MBP specific T cell lines isolated from a healthy individual and an MS patient were added to myelin. Neither cell line was myelinotoxic even with the addition of autologous EBV-transformed B cells acting as APC's. This may be explained by the fact that continuous passage of a T cell line could result in the loss of potential myelinotoxic capability. Alternatively, monocytes / macrophages may be required as both APC's and for effecting the release of CNPase activity from myelin. In addition, autoreactive T cells specific for other myelin antigens such as MAG or MOG may be more important in initiating demyelination in vivo.

In fact the antigen specificity of the T cells is not likely to be relevant since it was demonstrated that circulating cells from a chronic inflammatory disorder of the joints (RA) also caused in vitro myelin degradation in addition to MS cells. This suggests that the activation state of the cells is more important than the antigen specificity. Interestingly it has been shown that any activated T cell can cross the BBB (Hickey 1991) and it is the exposure to self antigen within the CNS that determines whether a cell remains there. The same principle could apply in RA where circulating activated cells cross the synovium and remain there only if they encounter the relevant antigen.

On the premise that circulating activated cells in MS and RA may release cytokines and other inflammatory mediators that in vitro are able to degrade myelin, the role of cell supernatants from stimulated and un-stimulated PBMC was investigated. In general, neither antigen (CNS specific and non-CNS specific) stimulation or mitogen stimulation resulted in the production of a SN that when added to myelin caused a significant loss in CNPase activity. This may have been because the concentration of the released mediators was too low or that they possessed short half-lives plus the possibility that proteases could have been present in the SN thus affecting protein stability. However, in one instance PPD stimulation of an MS PBMC sample, that demonstrated significant proliferation to PPD, produced a SN that was able to cause a significant loss of myelin CNPase activity. Thus suggesting that the antigen specificity of the cells is not as important as the state of activation. Preliminary experiments showed that when MS PBMC were stimulated with PHA whilst incubating with myelin there was an increase in the number PBMC samples that caused a significant loss of CNPase. In this instance PHA is acting both as a non-specific cell activator and as an
agglutinin thereby causing both the clumping of cells with the myelin and release of cytokines.

Due to the previous suggestion that a cytokine, probably TNFα, was responsible for the in vitro demyelination demonstrated when MS PBL SN's were added to demyelinating cultures (Selmaj et al., 1988a). Also, when TNFα was added directly to demyelinating cultures it demonstrated myelinotoxicity as well as oligodendrocyte cytotoxicity (Selmaj and Raine, 1988). Recombinant cytokines (IL-1, IL-2, IFNγ and TNFα) were therefore added directly to isolated myelin to assess whether they possessed myelin degrading activity however, no myelin damage was observed. This is probably a reflection of the fact that cell-myelin contact is required for PBMC's to effect their myelin damage. Addition of different cytokines to the PBMC population whilst in contact with myelin may have been a more appropriate way to investigate whether myelinotoxic factors / mechanisms can be induced by cytokines as they may not be acting directly on myelin but via secretion of various other cytokines which operate within a network depending on concentration and cells type present.

Nevertheless, cytokines especially TNF may contribute to lesion pathogenesis in MS. TNFα, which is produced by stimulated macrophages and some T cells, causes oligodendrocyte necrosis and myelin dilation when added to mouse spinal cord cultures (Selmaj and Raine, 1988). More recently it has been demonstrated that lymphotoxin (TNFB), which is produced by antigen stimulated T cells, is a more potent cytotoxic agent for oligodendrocytes than TNFα and the mechanism of action is one of DNA fragmentation of the target cell (apoptosis) (Selmaj et al., 1991b). In contrast, Zajícek and colleagues (1993) were unable to reproduce TNF induced cytotoxicity when human TNFα and β were added to rat oligodendrocyte cultures which they suggested may be a reflection of non-heterogeneity. They also suggest that cell surface TNF is more effective in killing oligodendrocytes indicating that cell contact is important. These conflicting reports nonetheless illustrate the difficulties encountered when trying to interpret results obtained using cell culture systems for measuring demyelination.

The direct role of T cells in the myelin degrading process was next investigated by enriching the T cell population of the PBMC's and removing monocytes. Using two different methods of T cell enrichment the resultant T cell population almost always retained the myelin degrading activity shown by the mixed cell (PBMC) population. When a small number of
adherent cells were added to the enriched T cell population whilst incubating with myelin the myelin degrading ability of the original PBMC population was retained however, there was no augmentation in the percentage loss of CNPase activity from the myelin. Due to the inefficiency of the T cell purification techniques used contaminating monocytes were always present at a very low concentration. Thus it cannot be concluded that T cells alone were responsible for the observed myelin degradation as further experiments would have to be done with an improved method for purification of T cells and complete removal of monocytes to clarify this. Nevertheless, with a low concentration of monocytes, comparable to that found in the PBMC population, there was no significant loss of CNPase activity in the absence of lymphocytes. Hence it is likely that a combination of T cells and monocytes are required to effect the in vitro myelin degradation observed. Additional experiments utilising anti-class II antibodies to block antigen presentation as well as other T cell specific blockers such as anti-CD4 and CD8 antibodies may help to resolve which cell types are necessary and how the loss of CNPase activity is effected.

Secreted granules from cytotoxic T cells which contain perforin and serine esterases might also contribute to the myelin degradation observed when PBMC are added to myelin. Preliminary experiments showed that incubation with myelin caused an increase in the number of MS CD8+ cells expressing SE, a putative cytotoxic mediator although more experiments would have to be done to confirm whether the increase was significant. Nevertheless, incubation of myelin with a limited number of healthy individuals' PBMC resulted in no change in the number of CD8+ lymphocytes expressing SE. It has been suggested that SE expression in vivo may provide a marker for activated functional cytotoxic lymphocytes that are involved in autoimmune disease (Griffiths & Mueller, 1991). The exact role of SE in lytic granule exocytosis and subsequent cytolysis is in dispute although it has been demonstrated that purified SE alone has no cytolytic effects but in combination with purified perforin induces DNA degradation of target cells (Hayes et al., 1989). Alternatively it has been suggested that lytic granules containing perforin and SE are not necessary for T lymphocyte-mediated cytolysis by mature effector cells but that SE is generated as part of T cell activation in vivo and its primary role may be in the extravasation of responding lymphocytes and secretion of cytokines (Berke & Rosen, 1988). In fact it has been proposed that SE synergises with endoglycosidase in the degradation of endothelial cell matrix and therefore could be crucially involved in cellular migration (Simon et al., 1987). From the preliminary experiments performed it cannot be concluded that the increase in the number
of MS CD8^ cells that express SE after incubation with myelin is related to the myelin
degradation activity demonstrated by the PBMC. However it is an interesting observation
considering cell associated proteases have been detected within MS and EAE demyelinating
lesions thereby implicating them in the pathogenesis of demyelination (Rastogi & Clausen,

Recently it has been demonstrated that both MBP and PPD specific T cell lines produce
soluble factors that are cytotoxic to both syngeneic and non-syngeneic oligodendrocytes but
not astrocytes (Selmaj et al., 1991a). When the authors attempted to neutralise the
cytotoxicity with a variety of antibodies and inhibitors they concluded that the cytotoxicity
could only partially be attributed to TNFα/β activity and was not associated with perforin or
serine protease. They suggested that the state of activation of the lymphocytes was probably
more important than antigen specificity. Similarly it has been demonstrated that pre-
activated cytolytic CD4^ T cell lines specific for either MBP or ovalbumin caused damage
to syngeneic brain vascular endothelial cells in vitro with no requirement for antigen.
However T cell SN had no cytolytic effect for the endothelial cells and it was suggested that
other factors such as cell-cell adhesion and appropriate homing of cells are of overriding
importance. Alternatively pre-activated cells could be secreting endoglycosidase which
degrades endothelial cell matrix (Sedgewick et al., 1990).

If both circulating MS and RA PBMC are capable of in vitro myelin degradation as assessed
by loss of CNPase activity why do RA patients not present with CNS damage as well as joint
tissue damage and MS patients with joint damage? If a sub-population of T cells are
responsible for this damage it could be a reflection of different homing patterns of cells due
to a combination of selective attraction to the joints or CNS by cytokines and the expression
of tissue specific adhesion molecules or cross-reacting antigen yet to be identified.

Recently the heat shock proteins (HSP) have received much attention as potential antigens
in autoimmune diseases through their cross-reactivity with tissue-specific proteins which may
contain similar amino acid sequences to the HSP's (Jones et al., 1993). Studies investigating
the role of T cells bearing the y8 TCR have shown that in general they are potentially
cytotoxic, display NK activity and respond to alloantigens (Smith et al., 1990). Also, their
antigen specificity is biased towards HSP (O'Brian et al., 1989). In fact both y8 T cells and
HSP's have been implicated in the pathogenesis of tissue damage seen in RA and MS.
In RA, endothelial cells of synovial capillaries have increased expression of intercellular adhesion molecule-1 (ICAM-1) which acts as a site of attachment for circulating T cells (Sewell and Trentham, 1993). Distinct populations of T cells have been shown to leave the circulation and migrate into the joint as the majority of T cells found in the joint are of the mature memory subtype (CD45RO / CDw29^) (Cush and Lipsky, 1991). Also T cells expressing \( \gamma \delta \) receptor have been found to be concentrated in the synovial fluid (Holoshitz et al., 1989). The possibility exists that these cells recognised their target in the joint and underwent clonal expansion although the stimulating antigen in this study has not yet been identified. One candidate is the HSP p65 which has been shown to be expressed on cells lining the synovium (Crick & Gatenby, 1992). p65 is thought to be produced by cells of the joint in response to inflammation and destruction which could then lead to the generation of autoreactive T cells which may initiate a chronic cycle of inflammation and thus destruction of the synovial cells. The generation of these autoreactive cells is thought to occur through molecular mimicry as some mycobacterial antigens belong to the group of HSP and exposure during infection to microbial stress proteins may initiate the proliferation of autoreactive T cells. Mycobacterial antigen specific \( \gamma \delta \) T cell clones have been isolated from blood, synovial fluid and synovium thus supporting the concept of molecular mimicry as there are shared epitopes of microbial HSP with HSP generated by cells within the joint which may be important in the pathogenesis of RA (Res et al., 1988; Holoshitz et al., 1989; Crick and Gatenby, 1992)

A possible role for T cells bearing the \( \gamma \delta \) receptor and expression of HSP in the pathogenesis of demyelination in MS has also recently been studied. These cells were frequently found in chronically demyelinated areas where there was co-localisation with HSP's which were expressed on immature oligodendrocytes (Selma et al., 1991d). When CNS tissue from other non-MS CNS inflammatory conditions was investigated \( \gamma \delta \) T cells could not be detected suggesting a unique immunopathologic reaction between these cells and HSP expressed on oligodendrocytes. As these cells were only found in chronic lesions in contrast to T cells expressing the \( \alpha \beta \) TCR which predominate in the more active lesions it may suggest that \( \gamma \delta \) T cells are associated more with the persistence of demyelination rather than the initiation of a lesion. However, more recently Wucherpfennig and colleagues (1992) have demonstrated that \( \gamma \delta \) T cells were found to accumulate in acute demyelinating plaques and seemed to have undergone clonal expansion suggesting recognition of a CNS antigen. The presence of HSP's was also investigated and it was demonstrated that normal CNS tissue
expressed both 60kDa and 90kDa HSP's with acute MS plaques showing over-expression of these antigens. Therefore, reactivity to self HSP could either lead to the initiation or amplification of an autoimmune reaction at the site of tissue injury. It would have been interesting to study the expression of \( \gamma \delta \) by both MS and RA T cells and investigate whether there is any association with myelin degradation.

In MS the tissue damaging cells preferentially migrate to the CNS and cause demyelination, whereas the RA chronically activated cells are preferentially attracted to the joints by the inflammatory mediators in the synovial fluid and the expression of synovial membrane specific adhesion molecules. In EAE it has been demonstrated that all activated T cells can cross the BBB and gain entry to the CNS, and it is thought that only those T cells that encounter their antigen remain and induce further T cell activation and thus initiate or contribute to the demyelination and inflammation that is a hallmark of this disease. Candidates for chemoattractants at the site if tissue damage are IL-8 and RANTES (both members of a superfamily of cytokines). It has been shown \textit{in vitro} that IL-8 is a chemoattractant for neutrophils and all T cell phenotypes whereas RANTES selectively attracts T cells of the 'memory' phenotype (CD45RO) and monocytes (Schall \textit{et al.}, 1990). Memory T cells are more potent effectors than naive cells as they are more able to produce certain multipotent cytokines on antigenic restimulation and are thought to be involved in chronic autoimmune disorders mediated by T cells.

Within MS and RA PBMC there may be a sub-population of T cells that have been pre-activated \textit{in vivo} that are easily triggered \textit{in vitro} to produce cytokines/inflammatory mediators that are either directly capable of tissue damage or produce cytokines that stimulate the production of these potentially myelinotoxic mediators. Recently, Brod and colleagues (1991) showed that the CSF and peripheral blood of MS patients contained a functionally distinct sub-population of T cells on the basis of selective cytokine secretion of IL-2 and IFN\( \gamma \) which were not found in patients without systemic inflammation, therefore suggesting that T cell clones with distinct cytokine profiles may be functionally involved in inflammatory responses. There is strong evidence to suggest a role for cytokines particularly TNF in the pathogenesis of demyelination. \textit{In vivo} both TNF\( \alpha \) and \( \beta \) have been identified \textit{in vivo} in acute and chronic active MS lesions, TNF\( \alpha \) in association with astrocytes and microglia and foamy macrophages and TNF\( \beta \) in association with T cells and microglia (Hofman \textit{et al.}, 1989; Selmaj \textit{et al.}, 1991c). In addition the CSF from MS patients with chronic progressive disease has been
shown to contain elevated levels of TNFα which can be correlated with the degree of disability in these patients (Sharief and Hentges, 1991). The CSF levels of TNFα were greater than the matched serum levels suggesting intrathecal synthesis, as there was a lack of correlation between cell pleiocytosis and TNF concentration indicating that it may have been derived from CNS cells (macrophages, microglia, astrocytes or T cells) and not CSF cells. In addition mitogen-stimulated TNFα production by monocytes isolated from the blood of MS patients has been shown to be greater in patients in clinical exacerbation as compared to those in remission (Beck et al., 1988). Merrill and colleagues (1989) also showed that in 40% of MS patients tested their peripheral blood macrophages spontaneously produced elevated levels of IL-1 and all mediators of inflammation were produced in increased amounts by MS CSF leukocytes after stimulation. In EAE the level of TNFβ cytotoxic activity expressed by TMBP has been shown to correlate with the ability to transfer disease (Powell et al., 1990) and anti-TNF antibodies have been successful in preventing the transfer of T cell clone mediated EAE (Ruddle et al., 1990). Nevertheless, it is becoming clear that measurement of cytokines in human substances (blood, CSF or directly in tissue) is unreliable and dependent on cytokine half-life, the presence of inhibitory proteins such as proteases and TGFβ and also the specific assay used. Therefore the role of cytokines in MS is still controversial although in vitro models of demyelination can at least give some information as to which cytokines might be relevant to disease pathogenesis.

In summary, the work included in this thesis has raised many questions as to the mechanisms of myelin breakdown occurring in MS which is clearly a complicated process involving a variety of cell types and inflammatory mediators that act together in causing myelin degradation.
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# Appendix 1

PBMC, serum or CSF specimens from MS patients

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**Note:** Tab. 4.1 etc. & Fig. 5.1: ✓ PBM, ✓✓ PBM & matched serum, ✓✓✓ PBM & matched serum sample. Fig. 6.1: ✓ IL-2R expression, ✓✓ IL-2R & HLA-DR expression.
| Patient number | Date       | Tab. 3.3 | Tab. 3.4 | Tab. 3.4 | Tab. 4.1 | Tab. 4.2 | Fig. 4.3 | Tab. 6.2 | Tab. 6.3 | Fig. 6.1 | Fig. 6.2 | Fig. 6.5 | Tab. 7.1 | Tab. 7.2 | Tab. 7.4 | Tab. 7.5 | Tab. 7.6 | Fig. 7.1 | Fig. 7.2 | Fig. 7.3 |
|----------------|------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 455            | 25.04.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 461            |            |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 466            | 10.05.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 468            |            |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 469            |            |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 469            | 19.05.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 471            | 20.05.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 146            | 23.05.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 472            | 09.06.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 473            | 16.06.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 512            | 15.12.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 474            | 21.06.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 479            | 26.07.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 480            | 26.07.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 483            | 27.07.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 484            | 02.08.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 485            |            |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 486            | 15.08.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 493            | 09.09.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 504            | 12.10.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 507            | 27.10.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 509            | 04.11.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 500            | 10.11.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 501            |            |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 484            | 24.11.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 496            |            |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 497            |            |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 499            | 30.11.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 502            | 30.11.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 500            | 06.12.88   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 513            | 18.01.89   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 507            | 16.01.89   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 508            |            |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 474            | 27.01.89   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| 514            | 30.01.89   | ✓        |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |

Note: Tab. 4.1 etc. & Fig. 5.1: ✓ PBMC, ✓/s serum, ✓/✓ PBMC & matched serum sample. Fig. 6.1: ✓ IL-2R expression, ✓/✓ IL-2R & IIa DR expression
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Note: Tab. 4.1 etc. & Fig. 5.1: ✓ PBMC, ✓'s serum, ✓✓ PBMC & matched serum sample.
Fig. 6.1: ✓ IL-2R expression, ✓✓ IL-2R & HLA-DR expression
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Appendix 2

PBMC and serum specimens from RA & OND patients and healthy individuals

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Note: Tab. 4.1 etc. & Fig. 5.1: ✓ PBM C, ✓'s serum, ✓✓'s PBM C & matched serum sample. Fig. 6.1: ✓ II-2R expression, ✓✓ II-2R & IIL-A-DR expression.
The transplantation was done on Feb 9, 1988. 42 000 units of wet-heat-treated FVIII concentrate were given before, during, and 24 h after the operation. 13 units of red blood cells and 8 units of fresh frozen plasma were transfused during the operation. During the operation the finding of a positive crossmatch was transmitted to a plasmapheresis was done; this induced severe disseminated intravascular coagulation and was successfully treated with anti-thrombin-III. The substitution of FVIII concentrate could be stopped 24 h after the transplantation because the new liver was synthesising FVIII in normal levels. The patient’s serum α-fetoprotein dropped to 0.8 ng/ml after transplantation. On the 12th day after the operation the patient had mild rejection crisis successfully treated with corticosteroids. He returned to employment in good health 8 weeks after the operation. 10 weeks after transplantation he was HBsAg negative and anti-HBs positive. He is now on cyclosporin.

7 months after liver transplantation there are no traces of coagulation defect or bleeding tendency, and he remains free of HBsAg and liver cancer. Liver transplantation may prevent impending death in haemophilia patients with end-stage liver disease and it can cure the phenotypic coagulation defect and the bleeding tendency.

MULTIPLE SCLEROSIS: A T-CELL DISEASE?

Sir,—The role of immune cells and soluble factors in the serum of patients with multiple sclerosis (MS) is uncertain. To address this question we devised a simple method for measuring the in vitro degradation of human myelin by fluorimetric assay of a myelin-specific enzyme, 2'-3'-cyclic nucleotide 3'-phosphodiesterase (CNPase). Previous workers have used tissue culture explants of human myelini preparation can be used. This may explain why we have come to recognise most of the problems that clinicians had arrived at the correct diagnosis in almost 90% of cases before necropsy. This is to put the cart before the horse; it is by comparing the clinical diagnosis with necropsy findings that we have come to recognise most of the problems that beset newborn infants and, in many cases, to treat rationally or prevent them. Moreover, our knowledge of necroscopy is not yet complete and new conditions are likely to continue to turn up as we get better at managing old ones.

Sir Peter Tizard once said that in trying to pick the best Indian graduates for posts on his unit at the Hammersmith Hospital, the necropsy rate in the institution in which they were educated was the best guide to competence. If the provision of a morbid anatomical

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I. SCHARRER A. ENCKE C. HOTTENROTT


Mycylin degradation by PBMC from patients with active MS.

<table>
<thead>
<tr>
<th>Group</th>
<th>Samples showing significant* CNPase loss from myelin for myelin incubated with:</th>
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<tr>
<td></td>
<td>Serum</td>
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<tr>
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<td>Rheumatoid arthritis</td>
<td>1/12</td>
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<td>Healthy controls</td>
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</table>

*p < 0.05.

PBMC ADH T CELL ADH + T CELL

Myelinn degradation by PBMC from patients with active MS.

2 x 10^6 PBMC, 2 x 10^6 adherent cells (ADH), 2 x 10^6 T cells, or 2 x 10^6 ADH plus 2 x 10^6 T cells were incubated in triplicate with 10 µg human myelin for 24 h at 37°C. Concentration of CNPase remaining in myelin pellet was measured fluorometrically, and % loss of CNPase activity from myelin calculated as 100 minus activity (myelin alone) as a percentage of activity (myelin plus cells).

Data as mean (SD) for 5 relapsing-remitting cases of MS. All losses significantly different from control myelin (p < 0.05) except for ADH alone.

NEONATAL NECROPSY IN DEVELOPING COUNTRIES

Sir,—Professor Singh and his colleagues (Aug 27, p 502) belittle the importance of the neonatal necropsy in developing countries on the grounds that clinicians had arrived at the correct diagnosis in almost 90% of cases before necropsy. This is to put the cart before the horse; it is by comparing the clinical diagnosis with necropsy findings that we have come to recognise most of the problems that beset newborn infants and, in many cases, to treat rationally or prevent them. Moreover, our knowledge of neonatology is not yet complete and new conditions are likely to continue to turn up as we get better at managing old ones.

Sir Peter Tizard once said that in trying to pick the best Indian graduates for posts on his unit at the Hammersmith Hospital, the necropsy rate in the institution in which they were educated was the best guide to competence. If the provision of a morbid anatomical
MS: a localized immune disease of the central nervous system

Virginia Calder, Samantha Owen, Carolyn Watson, Marc Feldmann and Alan Davison

The precise role of T cells in multiple sclerosis (MS) remains to be defined. No MS-specific antigen has been found. The autoimmune hypothesis for MS rests on immune changes seen in the spinal fluid and brain and on the demonstration, in an experimental animal model, that T cells raised to myelin basic protein transfer demyelination. In this review, Virginia Calder and colleagues focus on recent studies suggesting that in MS, the initial T-cell response occurs within the central nervous system and that the blood poorly reflects this immune activity. This contrasts with the animal model, experimental allergic encephalomyelitis, where the initial immune response is peripheral.

MS is a relapsing–remitting disease affecting the mature central nervous system (CNS). The disease is characterized by widespread lesions confined to the myelin. Primary demyelination interferes with nervous conduction in the brain and spinal cord. Epidemiological evidence suggests that MS involves an infectious agent, probably acquired between the ages of five and fifteen years. The higher concordance rate for MS in monozygotic compared with dizygotic twins provides strong evidence for a genetically-determined susceptibility to the disease. The major histocompatibility complex (MHC) antigens have been found to be important predisposing genes to both experimental allergic encephalomyelitis (EAE) and MS. EAE in mice, rats or guinea pigs is associated with certain MHC class II alleles and MS is more common in HLA-DR2 individuals. DR2 may not be the sole HLA susceptibility allele as the HLA-B7, -CW1, -DR2 and -DQW1 haplotypes are more common in MS. HLA class II association is also noted in many autoimmune diseases (apart from those associated with HLA-B27) and indicates that CD4+ T cells recognizing...
antigen in association with class II products are involved in the disease pathogenesis. However, the role of lymphocytes and the autoimmune hypothesis are still controversial since, in contrast to other autoimmune diseases, no clear auto-antigenic response has been identified.

In this review, we address the current hypotheses of the role of immune cells in MS and present data suggesting that MS is an autoimmune disease that is initiated within the CNS. Among the problems discussed here are: (1) The study of the peripheral immune system — is it relevant to an inflammatory CNS disease? (2) Are T cells of major importance in the pathogenesis of MS? (3) How is demyelination initiated within the CNS? (4) Are there specific MS antigens? (5) Are attempts at immunotherapy in MS justifiable?

Immune abnormalities in multiple sclerosis

Plasma cells are present in MS plaques and, as the disease progresses, are also found in increased numbers in normal appearing white matter. Plasma cells may secrete antibody for a long time after the antigenic stimulation has ceased, and the stimulation of small numbers of plasma cells may explain the presence, and continual synthesis, of oligoclonal bands in the cerebrospinal fluid (CSF) of MS patients. However, the antigens related to these restricted bands of antibody have not yet been identified. It is possible that most of the antibody produced is directed against antigens that are no longer present in the brain, or that the IgG is the product of a nonspecific response by nonspecific B cells attracted into the CNS.

In addition to B cells, T cells and macrophages are found among the infiltrating mononuclear cells in the initial acute lesions of the disease. There is a corresponding increase in the number of cells in the CSF during the acute phase of MS. While macrophages and occasional polymorphs are present, T cells predominate. Phenotypic analysis shows an increased ratio of helper/inducer (CD4+) to suppressor/cytotoxic (CD8+) T cells in the CSF during this phase. This does not appear to be due to cells sequestered from the blood, as the number of CD8+ cells also diminishes in peripheral blood during an exacerbation. Defective activated CD8+ suppressor T-cell function has been suggested in both progressive and stable MS. This may be related to recent studies showing that CD45+ suppressor-inducer cells are selectively lost from the circulation of progressive MS patients. The interpretation of such changes is complicated by the considerable fluctuation of peripheral blood T-cell subset concentrations seen in longitudinal studies of both patients and their close relatives. Indeed, the correlation between clinical manifestations of disease and the underlying pathological activity is notoriously poor. Using magnetic resonance imaging (MRI), many 'silent' lesions can be identified that often fail to correlate with any clinical signs (Fig. 1). This questions the relevance of the common patient-classification into relapsing-remitting or progressive categories. Nevertheless, MRI is the most sensitive technique for establishing the diagnosis and course of the disease. The variable nature of MS, with unpredictable exacerbations and sometimes long periods of remission, is one of the major hindrances to scientific research on the disease and, more especially, to therapy.

A role for T lymphocytes in the central nervous system

Contrary to the earlier view that the brain is an immunologically privileged site, it is now clear that a small number of lymphocytes are always found throughout the normal appearing white matter in MS. Several cell types bearing MHC class I and class II antigens are present in higher concentrations throughout the CNS in patients with active MS than in control subjects, and even than in patients in remission. MHC antigen expression would permit localized antigen presentation at sites well removed from areas of previous acute lesions and may thus facilitate progression. The presence of a high proportion of Tα1+ cells (a marker of post-differentiated T cells) in the CSF of MS patients indicates the availability of memory cells within the CSF. In addition, it has been suggested that CSF T cells are selectively refractory to stimulation via the CD2 but not the CD3 pathway — unlike T cells in the blood — although this observation needs to be confirmed.

Activated lymphocytes bearing interleukin 2 (IL-2) receptors (detected by anti-Tac monoclonal antibody (mAb)) and lymphocytes with IL-2 localized on their membrane and within the cytoplasm (detected by the 3.9C2 mAb) are found in the perivascular areas of the brain of some patients with acute MS. These changes are reflected in the CSF where T lymphoblastoid cells are seen by flow cytometric techniques and approximately 50% of the helper T cells are activated. In addition expression of IL-2 receptors is considerably greater in lymphocytes from the CSF compared with those from peripheral blood. In a study of patients with acute MS there has been some indication that T cells can migrate from the blood into the CNS. Movement of T cells may
Experimental allergic encephalomyelitis (EAE)

EAE can be induced in susceptible animals following active sensitization with suitable adjuvants and spinal cord or CNS antigen, such as myelin basic protein (MBP). A monophasic, acute or spontaneous relapsing–remitting chronic form of the disease may develop, according to the mode of sensitization, genetic background and age of the animal. While acute EAE presents a pathology similar to acute human inflammatory CNS diseases, the neuropathological changes and disease course observed in chronic relapsing EAE (CREAE) are more typical of MS (reviewed by Lassmann19).

Thus, similar immunopathological mechanisms may be operating in both CREAE and MS. There is abundant data to suggest that EAE is primarily a T-cell-mediated disease and that immune reactivity to MBP is an important factor in its pathogenesis. MBP-reactive lymphocytes have been recovered from the spinal cord and draining lymph nodes of Lewis rats 12 days after inoculation with MBP20. Trafficking of MBP-sensitized T cells into the CNS from peripheral blood in animals with EAE has also been shown21. Recent studies on the T-cell receptors of MBP-specific T-cell clones in EAE have shown that the expression of T-cell receptor genes may be important in the development of the disease. A very limited range of diversity in the T-cell receptors of MBP-specific clones in mice have been found: 83% of the clones used the same V gene segments, and J gene usage and junctional diversity were severely restricted22. At present, there is no evidence that this is also true in MS.

The most compelling evidence that MBP-reactive T cells mediate experimental demyelination, however, comes from adoptive transfer experiments. The chronic relapsing form of EAE can be induced in susceptible naive recipients by MBP-specific T-cell lines or clones of the helper/inducer phenotype (Lyt-1+2~/L3T4+)23-25. The resulting demyelination is more pronounced in syngeneic or MHC class II (Ia)-compatible animals and in X-irradiated or congenitally athymic animals26. Further evidence that T cells mediate the disease is shown by the demonstration that demyelination can be prevented by peripheral immunosuppressants or by inhibiting antigen presentation with anti-MHC-class-II mAbs27.

Reactivity to MBP in MS

Since MBP-sensitized mouse T cells can initiate demyelination on adoptive transfer into rodents, there have been many attempts to elucidate the role of these
MBP-reactive cells. In many neurodegenerative disorders higher concentrations of MBP, or its fragmented peptide, are found in the CSF compared to the blood\(^9\). In MS, levels of MBP and its antibody in the CSF correlate with the course of demyelination, suggesting a localized sensitization of lymphocytes to this antigen within the CNS compartment\(^9\). T-cell reactivity to CNS antigens has been examined by several groups\(^3\)\(^0\)\(^\text{-}^3\). Lisak and Zweiman\(^2\) reported significant responses to MBP by CSF lymphocytes in patients in relapse but not those in remission, and MBP-reactive T-cell clones and lines have been raised from CSF of MS patients\(^3\)\(^3\)\(^4\). However, whether or not the MBP-specific T cells are a secondary phenomenon that result from myelin breakdown products being released, remains unclear.

**Mechanism of demyelination**

According to the autoimmune hypothesis, sensitized T cells with or without antibody or complement may cause demyelination by their direct action on myelin, or possibly on oligodendrocytes (the myelin-producing cells within the CNS). In one study specialized contact has been seen between lymphocytes and macrophages adjacent to areas of demyelination in MS\(^1\). In addition, the presence of receptor-rich areas (coated pits) on the macrophage surface in contact with superficial myelin lamellae has been reported\(^3\). Due to technical difficulties, further studies of lymphocyte-macrophage interaction in the human CNS have not yet been carried out and quantitative studies are needed.

Selmay and his colleagues\(^3\) have shown significant release of IL-1 from peripheral blood adherent cells from MS patients on the addition of MBP (50 \(\mu\)g ml\(^{-1}\)). This response is much less in peripheral mononuclear cells from other neurological diseases and from healthy controls. Monocytes from MS patients show increased spontaneous chemiluminescence activity and more cytotoxic oxygen metabolites when exposed to inflammatory stimuli\(^3\)\(^6\)\(^7\). There is also evidence that supernatants from peripheral blood lymphocytes from MS patients contain a lymphokine capable of inducing demyelination in rat cerebellum cultures\(^3\). However, the lymphokine involved has not yet been identified. In addition, tumour necrosis factor\(^3\)\(^8\), proteolytic enzymes and phospholipases have been shown to have high myelinolytic activity\(^3\). It is interesting that some serine proteinases, as well as perforin, are released by exocytosis of cytoplasmic granules in cytolytic T lymphocytes\(^3\). Demyelinating activity by MBP-specific T-cell lines has been shown in vitro using myelinated spinal cord explants\(^3\). This suggests that purified populations of MBP-specific T cells alone can initiate demyelination. However, in this culture system, putative accessory glial cells may be present and therefore the mechanism of myelin damage remains to be fully defined. It is possible that lymphokines such as macrophage-activating factors (which are not characterized) are released from the T cells and that the activated phagocytes participate in demyelination. Macrophages are involved at an early stage in plaque-formation in CREA. Large mononuclear cells invade the parenchyma from the perivenous space in this condition. Ultrastructural studies show that myelin-stripping is initiated by an intimate contact with macrophage cell membrane\(^3\). This is indicative of receptor-mediated endocytosis and may explain the specific effect on myelin and sparing of the axon\(^3\).

When peripheral blood lymphocytes from MS patients in relapse or with progressive disease are incubated with isolated human myelin, degradation of the myelin can be shown in about 40% of cases\(^4\). In contrast, this in-vitro demyelinating activity is not seen in samples from healthy individuals or in other neurological disease cases, but is also present in blood from patients with rheumatoid arthritis (RA). It is interesting that the latter is an autoimmune disorder with typical relapses and remission, in which the inflammatory reaction is localized to the joints, whereas in MS only the CNS is affected. Degradation of myelin is thus likely to be due to a nonspecific effect of soluble factors produced by chronically-stimulated immune cells occurring in RA and MS. It is probably relevant that in peripheral blood lymphocytes from both RA and MS patients, there is increased expression of the IL-2-receptor (Tac) and HLA-DR antigens compared to normal cells\(^1\)\(^7\)\(^8\).

**Immunosuppressants**

Various forms of immunotherapy have been used in treatment of MS, but mainly with relatively little success. For example, chronic administration of azathioprine has proved of limited value since it affects only those with a mild, or recent-onset disease\(^4\)\(^6\). We propose that, since modulation of the peripheral immune system alone in MS patients appears to have little clinical benefit\(^7\), effective therapy requires access of the immunosuppressant to the site of ongoing immune activity within the CNS. This is supported by the finding that cyclophosphamide penetrates the blood–brain barrier and, despite its toxicity, is arguably one of the most effective immunosuppressants in MS patients, although the value of the treatment is not yet established\(^4\). One possible therapy for MS might be cyclosporin A (CsA), a potent immunosuppressive known to act by inhibiting IL-2-receptor expression. However, CsA did not affect the level of IL-2-receptor-bearing cells in the CSF, although it caused a decrease of IL-2-receptors in the blood\(^9\). Perhaps even at relatively high concentrations it fails to penetrate the CNS and so does not reach the site of ongoing disease activity.

Steroids are widely used as anti-inflammatory drugs for treatment of exacerbations in MS when immune activity within the CNS is presumably maximal. Recent observations show that glucocorticoids have distinct in-vitro immunosuppressive activity (e.g. inhibiting lymphokine release and suppressing lymphocyte activation)\(^10\). Indeed methylprednisolone is as active as other immunosuppressants tested in various in-vitro proliferation assays to mitogens (ConA and anti-CD3) and alloantigens (mixed lymphocyte response). Temporary clinical improvement is seen in MS patients in relapse when they are treated with high intravenous doses of methylprednisolone. The problem here is the nonspecific mode(s) of action – immunosuppression, dispersal of oedema, alteration of electrolyte composition. Dispersal of oedema is certainly possible and there is evidence from MRI studies that suggest dehydration of the brain by methylprednisolone\(^1\). The effect on electrolytes is not known. Methylprednisolone treatment has been shown to lead to a significant reduction in intrathecal IgG synthesis and to a fall in the concentration of the autoantigen MBP and antibody to \(^1\)\(^2\). One problem with
objective assessment of patient response is measurement of the effect of the drug within the CNS. MRI scanning allows quantitative measurements within the brain before and after treatment. However the interpretation of this data is complicated by a lack of knowledge of the drug concentrations within the CNS and the stage of disease most susceptible to immunotherapy.  

**Conclusion**

Our discussion, therefore, leads us to the following conclusions. First, that there is a need to focus research on the immunological events within the CNS rather than the peripheral blood. Second, we have argued for a pivotal role for T cells sensitized to MBP and, perhaps, to other myelin antigens within the CNS. The presence of activated T cells responsive to MBP in the CSF suggests that activation and expansion of MBP-specific cells occurs in the CNS. Third, demyelination may be initiated by memory cells triggered into expansion by as yet unidentified factors such as intercurrent infection or stress. Homology between sequences in MBP and a stress. Homology between sequences in MBP and a...
An In Vitro Micromethod for the Quantitative Assessment of Central Demyelination

C. M. Watson, J. Najbauer, S. J. Owen, and A. N. Davison

Department of Neurochemistry, Institute of Neurology, Queen Square, London, England

Abstract: We report the development of a simple and reliable method for the study of demyelination in vitro based on the measurement of 2':3'-cyclic nucleotide 3'-phosphodiesterase in isolated myelin. Using only small quantities of myelin (equivalent to 100 μg of myelin protein) the system was tested under conditions that are believed to approximate those found at the site of an inflammatory demyelinating lesion. Treatment with a combination of trypsin, phospholipase A₂, and lysophosphatidylcholine was used to evaluate the method. This microsystem has the potential not only for testing the myelinotoxicity of soluble factors but also for investigating the involvement of inflammatory cells in the demyelinating process. Myelin degradation by elicited peritoneal macrophages could be demonstrated at relatively high densities of these cells. Nylon wool purified lymph node T cells from myelin basic protein-primed SJL/J mice, after selective expansion with antigen and interleukin 2, failed to induce any significant myelin breakdown unless a limited number of syngeneic activated macrophages were also present. T cells from mice that had been inoculated with keyhole limpet haemocyanin failed to show any effect. The advantages of this technique over other in vitro systems are that it enables the study of demyelination using syngeneic sources of myelin and defined cell populations. Key Words: Demyelination—2':3'-Cyclic nucleotide 3'-phosphodiesterase—Myelin basic protein—Trypsin—Phospholipase A₂—Lysophosphatidylcholine. Watson C. M. et al. An in vitro micromethod for the quantitative assessment of central demyelination. J. Neurochem. 50, 1469-1477 (1988).
appears to be lost early in the development of MS plaques (Newcombe et al., 1982). MBP is also readily digested by endogenous proteases present in myelin plaques (Newcombe et al., 1982). MBP is also readily phosphate in aqueous solution (sp act 1,158 Ci/nmol) was purified as described previously by Groome et al. (1985). The MLA 144 cell line was a gift from Dr. P. C. Beverley, University College Hospital, London, U.K. Sulphur-35, as sulphate (0.3 Ci/g) at 12 days (4–5 g) and on weaning at 21 days (7–10 g) postpartum. The mice were killed 1 month after the second injection of [35S]sulphate. The brains and spinal cords were dissected out and myelin was prepared and stored as described above. The specific activity of the myelin was determined to be 680 cpm/mg myelin protein. After lipid extraction (Folch et al., 1957) at least 70% of the radiolabel was associated with the sulphatide fraction as identified by TLC (Banik and Davison, 1971).

**MATERIALS AND METHODS**

**Materials**

2:3-Cyclic NADP+, β-NADPH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucose-6-phosphate, calf thymus histones (type 2s), Tween-80, diethanolamine, and egg phosphatidylcholine generated by the action of phospholipases may also contribute to the dissolution of myelin (Thompson, 1964). Thus a combination of trypsin, phospholipase A₂, and lysophosphatidylcholine was chosen to examine the effects of positive demyelinating effects within the model. The applicability of the system to the study of cell-mediated demyelination will also be demonstrated using lymph node T cells from mice primed with the encephalitogenic antigen, MBP, or a non-myelin-related antigen, keyhole limpet haemocyanin (KLH), and peritoneal macrophages.

**Preparation of radiolabelled myelin**

SIL mice were injected intraperitoneally with [35S]sulphate (0.3 Ci/g) at 12 days (4–5 g) and on weaning at 21 days (7–10 g) postpartum. The mice were killed 1 month after the second injection of [35S]sulphate. The brains and spinal cords were dissected out and myelin was prepared and stored as described above. The specific activity of the myelin was determined to be 680 cpm/mg myelin protein. After lipid extraction (Folch et al., 1957) at least 70% of the radiolabel was associated with the sulphatide fraction as identified by TLC (Banik and Davison, 1971).

**CNPase assay**

CNPase activity was assayed fluorimetrically using cyclic NADP⁺ as substrate (Sogin, 1976) according to Weissbarth et al. (1980) and modified by Rastogi and Clausen (1985).

In summary, the equivalent of 0.5–1.0 µg of protein (myelin) was incubated in 280 µl of the reaction mixture (0.2 M MES buffer, pH 6.0; 30 mM MgCl₂; 1 mM Na₂EDTA; 0.025% Triton X-100; 1.2 µg ml⁻¹ of bovine serum albumin; 1 µM 2:3-cyclic NADP⁺; 6 mM glucose-6-phosphate; and 5.6 U ml⁻¹ glucose-6-phosphate dehydrogenase) for 20 min at 30°C. The enzyme reaction was stopped by the addition of 2.52 ml of 50 mM sodium carbonate buffer, pH 10.5, to the incubation mixture. The fluorescence of the end product (NADPH) was measured using 360 nm as excitation and 460 nm as emission wavelengths. Fluorescence of serial dilutions of β-NADPH in sodium carbonate buffer was used to plot a standard curve for each assay. Enzyme activity was expressed as nanomoles of end product (NADPH)/20 min/total volume of sample, as a fixed amount of myelin was used in each set of experiments.

**MBP assay**

An inhibition enzyme-linked immunosorbent assay (ELISA), using an alkaline phosphatase conjugated monoclonal antibody, clone 12, which is reactive with an epitope of HMBP in the region 86–96 (Groome et al., 1986), was developed in Oxford by Dr. N. Groome. The assay was conducted over 3 days. All incubations were carried out at room temperature. At day 1, unknowns and standards (serial dilutions of HMBP) were incubated overnight (16–24 h) in microcentrifuge tubes with an appropriate dilution of the monoclonal antibody. The unknowns, standards, and monoclonal antibody were diluted in a 0.05 M phosphate buffer, pH 7.0, containing 0.12% (wt/vol) sodium chloride, 0.05% (wt/vol) Tween 80, and 0.025% (wt/vol) calf thymus histones. At day 2, duplicate samples (500 µl) of each of the unknowns and standards were transferred to HMBP-coated Maxisorb tubes and incubated for a further 16–24 h. At day 3, the tubes were washed three times with ice-cold 1.2% (wt/vol) sodium chloride, 0.05% (wt/vol) Tween 80, and 600 µl of a 1 mg ml⁻¹ p-nitrophenyl phosphate solution, pH 9.8, containing 0.005% (wt/vol) MgCl₂ and 9.6% (vol/vol) triethanolamine, was added to each tube. The tubes were then incubated for up to 3 h, in the dark, before 200 µl of each sample was plated out on a 96-well microtitre plate and the absorbance read at 405 nm using a Titertek Multiscan. A dilution of enzyme-labelled monoclonal antibody which, alone, gave an absorbance of approximately 0.7 was used throughout these experiments. The clone 12 monoclonal antibody employed in this assay was determined to cross-react with mouse MBP with an efficiency of approximately 65% (data not shown). Results are expressed as nanograms of MBP with the proviso that these figures do not distinguish between intact MBP and breakdown prod-
products of MBP with intact epitope 86–96 and have not been corrected to account for the efficiency of binding to mouse MBP. MBP results are usually referred to in the text as assayable MBP to make this distinction clear. An acidified, boiling, extraction step, which removes much lipid and protein material but leaves MBP in solution, was also included for each of the unknown samples prior to immunoassay.

**Incubation of myelin with trypsin, phospholipase A₂, and lysophosphatidylcholine**

Samples of myelin were washed once with RPMI containing penicillin and streptomycin (50 U of penicillin and 50 μg of streptomycin ml⁻¹), gentamycin (50 μg ml⁻¹), and nystatin (50 U ml⁻¹), and resuspended in fresh medium. Aliquots were then incubated for 1 h with either trypsin (25 μg ml⁻¹), phospholipase A₂ (80 μg ml⁻¹), or lysophosphatidylcholine (1 mg ml⁻¹), or a combination thereof, in a 1.5-ml capacity microcentrifuge tubes in a humidified 5% CO₂/95% air atmosphere at 37°C. Approximately 100 μg of myelin protein in a total volume of 200 μl was added to each tube. Myelin incubated in medium only was used as a control for each experiment. At the end of the incubation trypsin inhibitor, or an equivalent volume of medium only, was added to each tube, as appropriate, and the myelin was resuspended and then centrifuged at 7,500–8,000 g for 5 min in an Eppendorf centrifuge. The supernatant was retained for analysis and the myelin pellet was resuspended in 200 μl of PBS containing 10% FCS and anti-KLH cell lines

**Incubation of myelin with short-term anti-MBP and anti-KLH cell lines**

SJL/J mice, 6–8 weeks old, were injected subcutaneously on each abdominal flank with a total of 0.1 ml of inoculum consisting of either 100 μg of KLH in PBS emulsified 1:1 (vol/vol) with complete Freund's adjuvant (CFA) or 200 μg of guinea pig myelin basic protein (GPMBP) in PBS emulsified 1:3 (vol/vol) with CFA supplemented with 50 μg Mycobacterium tuberculosis H37Ra. Bacillus peritissus was administered intravenously via the tail vein at 24 h and 48 h postinoculation with GPMBP.

At 7–10 days postinoculation the inguinal and axillary lymph nodes were excised and the tissue disrupted through a 0.5-mm mesh sieve in RPMI without FCS. The resulting cell suspension was washed three times with RPMI plus 10% FCS (vol/vol) and the cells plated out at 2 × 10⁵ cells ml⁻¹ in 24-well tissue culture plates with the appropriate antigen (10 μg ml⁻¹ KLH or 5–25 μg ml⁻¹ GPMBP). The cells were then fed at intervals of approximately 7 days with syngeneic irradiated (3,000 rads) spleen cells, at a ratio of 1:15-1:20 (lymph node cells/feeder cells), and antigen (Kimoto and Fathman, 1983). At 7 days into culture and subsequently at 3 to 4-day intervals half of the culture supernatant was replaced with IL-2 containing supernatant from the Gibbon cell line MLA 144 (Rabin et al., 1981). After 4–5 weeks in culture, and at least 7 days after the last addition of feeder cells, and antigen, the lymph node cells were purified by double passage over nylon wool columns at 37°C in the presence of serum (20% FCS). The effluent cell population, enriched for T-lymphocytes, was washed once with RPMI plus 10% FCS (vol/vol) and the cell density was adjusted as required. Cell viability at this stage was >95% as assessed by Trypan Blue exclusion. These cells were then incubated for 24 h with aliquots of washed myelin suspension (2 × 10⁵ cells/10 μg myelin protein) in 96-well tissue culture plates. Syngeneic peritoneal exudate adherent cells (1 × 10⁴ cells) were added to some wells. At the end of the incubation the myelin and cells were harvested following the procedure outlined above.

**RESULTS**

**Purity of the myelin preparation**

The myelin sample showed the characteristic appearance for such preparations by electron microscopy (Fig. 1) with loose vesicles in the size range of mitochondria or nuclei. Intact multilamellar structures were clearly visible although smaller unit membrane fragments were also present. No recognizable derivatives of other subcellular fractions were apparent. On sodium dodecyl sulphate-gel electrophoresis the preparation had a protein composition consistent with highly purified myelin.

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Model experiments of demyelination

After a 1-h incubation at 37°C approximately 5% of total CNPase activity and approximately 50% of total assayable MBP could be detected in the incubation supernatant (Table 1). Over 24-h incubation at 37°C there was no appreciable change in the level of CNPase activity measured in the residual myelin pellet (Fig. 3). Electrophoretic analysis of protein composition revealed that almost all the Wolfram protein (CNPase) could be recovered in the myelin pellet after 24 h of incubation at 37°C but that there was almost complete loss of intact MBP. The MBP detected in the residual myelin pellet by ELISA probably represents peptide fragments with intact epitope 86–96.

Following incubation with trypsin there was significant reduction in the levels of both CNPase activity and MBP, determined by ELISA, in the residual myelin pellet with a concomitant increase in CNPase activity, but not MBP, detected in the supernatant. Loss of total CNPase activity and assayable MBP occurred in the presence of both trypsin and phospholipase A2 together, but phospholipase A2 alone had little effect on the recovery of CNPase activity or MBP. Almost complete solubilization of the myelin preparation appeared to take place in the presence of lysophosphatidylcholine, with up to 80% release of total CNPase activity into the incubation supernatant. There was a similar reduction in MBP levels detected in the residual myelin pellet, although this reflected instead a loss of total assayable MBP.

At high concentrations of trypsin ( >25 μg ml⁻¹) release of CNPase activity from the isolated myelin occurs quite rapidly, with maximal loss of CNPase detected within the first 30 min of incubation. The addition of fresh media containing trypsin under these conditions does not lead to further release of the CNPase enzyme. Gradual breakdown of the myelin in suspension was observed with lower concentrations of trypsin (<25 μg ml⁻¹) with an increase in CNPase release with length of time and less than maximal loss of CNPase activity from the residual myelin pellet over a 24-h incubation period. The maximal loss of CNPase that could be induced with trypsin alone was 45% regardless of the ratio of trypsin to myelin protein employed or length of incubation (data not shown). In contrast almost total loss of CNPase activity or assayable MBP could be induced with high concentrations of lysophosphatidylcholine (1–2 mg ml⁻¹) after a relatively short incubation (1 h).

<table>
<thead>
<tr>
<th>Treatment: Myelin incubated with</th>
<th>CNPase (nmol end product/20 min/sample) (n = 10)</th>
<th>MBP (ng) (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Water supernatant</td>
</tr>
<tr>
<td>Media only (control)</td>
<td>1,020 ± 147</td>
<td>311 ± 41</td>
</tr>
<tr>
<td>Trypsin (25 μg ml⁻¹)</td>
<td>3,160 ± 246*</td>
<td>460 ± 6*</td>
</tr>
<tr>
<td>Phospholipase A2 (80 μg ml⁻¹)</td>
<td>2,080 ± 413*</td>
<td>301 ± 41</td>
</tr>
<tr>
<td>Trypsin (25 μg ml⁻¹) + phospholipase A2 (80 μg ml⁻¹)</td>
<td>3,378 ± 290*</td>
<td>363 ± 30</td>
</tr>
<tr>
<td>Lysophosphatidylcholine (1 mg ml⁻¹)</td>
<td>19,840 ± 1,292*</td>
<td>413 ± 19*</td>
</tr>
</tbody>
</table>

Each sample consists of myelin (=100 μg protein) incubated in 200 μl of RPMI containing trypsin and/or phospholipase A2 or lysophosphatidylcholine for 1 h at 37°C. After centrifugation the incubation supernatant (supernatant) was removed, and the residual myelin pellet washed with 200 μl of ice-cold distilled water (water supernatant) and resuspended in a final volume of 200 μl of RPMI (pellet). Results are expressed as mean ± SD, n, number of replicate samples per group.

* Significant at p < 0.001 (calculated by Student's t test).

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TABLE 2. CNPase activity in the supernatants from intact myelin preparations after incubation with trypsin

<table>
<thead>
<tr>
<th></th>
<th>CNPase (nmol end product/20 min/sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optic nerve (n = 3)</td>
</tr>
<tr>
<td>Media only</td>
<td>307 ± 92</td>
</tr>
<tr>
<td>Trypsin (25 μg ml⁻¹)</td>
<td>1,187 ± 220</td>
</tr>
</tbody>
</table>

Whole optic nerves or 4 x 1 mm diameter discs of corpus callosum, from adult Balb/c mice, were incubated in 1 ml of RPMI containing trypsin (control, medium only) for 1 h at 37°C. After centrifugation to remove residual tissue the supernatants were assayed for CNPase activity (as described in text). Results are expressed as means ± SD.

This effect could be mimicked only when a combination of both trypsin and phospholipase A₂ was used (data not shown).

In other experiments release of ³⁵S-labelled sulphatide from myelin treated with trypsin (25 μg ml⁻¹) could be demonstrated only on subsequent incubation with lysophosphatidylcholine to solubilize released sulphatide. Following this protocol a 30% greater loss of the radiolabel was seen from myelin preincubated with trypsin than could be accounted for by the effect of lysophosphatidylcholine alone (data not shown).

When tissues with intact myelinated tracts, such as optic nerve and corpus callosum, were incubated with trypsin a three- to fourfold increase in CNPase activity above background enzyme release into the incubation supernatant was noted (Table 2).

Phagocytosis of myelin

Incubation of myelin for 24 h with different densities of adherent and nonadherent populations of peri-

toneal exudate cells resulted in significant loss of CNPase activity and assayable MBP only in the presence of relatively high numbers of cells (Table 3). Such macrophage-enriched or macrophage-depleted cell populations at a ratio of 5 x 10⁴ cells/μg myelin protein caused a reduction of CNPase activity of approximately 60% and 40%, respectively. The data indicate that significant loss of CNPase can be demonstrated with lower numbers of the elicited macrophage population than the nonmacrophage population (Table 3). The MBP results concur with the CNPase data. Similar results were obtained when this experiment was repeated using resident peritoneal cells (data not shown). Active phagocytosis of the myelin by the macrophages was confirmed by electron microscopy which revealed numerous phagosomes containing mostly multilamellar myelin structures (Fig. 2). Under optimal conditions (5 x 10⁴ adherent peritoneal exudate cells/μg myelin protein) the first detectable loss of CNPase occurs after approximately 6 h of coculture with myelin (Fig. 3). After this there was continued loss of CNPase activity with length of culture with macrophages, resulting in a 60% loss at the end of 24 h (Fig. 3). There was little variation in the amount of CNPase lost from the myelin suspension with length of incubation at 37°C in the absence of cells.

Demyelination by sensitized cells

Cells from the lymph nodes of MBP-primed mice after selective expansion with antigen and IL-2 were purified over nylon wool columns. These enriched T cells failed to induce any demonstrable loss of CNPase or MBP after 24 h coculture with the mouse myelin preparation. When low numbers of syngeneic elicited peritoneal macrophages were added to these cells (at a ratio of 20 T cells:1 macrophage), however, significant myelin breakdown was seen (Table 4). Allogeneic elicited or resident, or even syngeneic resi-

TABLE 3. Loss of CNPase activity and MBP after 24-h incubation of purified myelin with adherent or nonadherent populations of peritoneal exudate cells

<table>
<thead>
<tr>
<th></th>
<th>CNPase (n = 3)</th>
<th>MBP (ng) (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol end product/20 min/sample)</td>
<td>Adherent</td>
</tr>
<tr>
<td></td>
<td>Adherent</td>
<td>Nonadherent</td>
</tr>
<tr>
<td>Medium only</td>
<td>1,558 ± 70</td>
<td>72</td>
</tr>
<tr>
<td>5 x 10⁴</td>
<td>1,384 ± 44*</td>
<td>1,493 ± 32</td>
</tr>
<tr>
<td>1 x 10⁴</td>
<td>1,467 ± 89</td>
<td>1,515 ± 175</td>
</tr>
<tr>
<td>5 x 10⁴</td>
<td>1,141 ± 96*</td>
<td>1,384 ± 68</td>
</tr>
<tr>
<td>1 x 10⁴</td>
<td>1,083 ± 258*</td>
<td>1,216 ± 215</td>
</tr>
<tr>
<td>5 x 10³</td>
<td>680 ± 180*</td>
<td>989 ± 116*</td>
</tr>
</tbody>
</table>

Figures represent CNPase activity or MBP (single determination) in residual myelin pellet after 24-h incubation at 37°C. A total of 10 μg myelin protein/200 μl medium ± cells was used for each replicate. At the end of the incubation the myelin pellet (± cells) was washed with 200 μl of ice-cold distilled water and resuspended in a final volume of 200 μl RPMI. Results are expressed as means ± SD. n, number of replicate samples. Medium, RPMI + FCS (10% vol/vol).

* Significant at p < 0.005, †significant at p < 0.001 (calculated by Student's t test).
dent, peritoneal macrophages were not effective in this respect (data not shown). At the density of macrophages employed no effect was recorded in the absence of T cells. KLH-sensitised T cells did not cause any significant myelin degradation either with or without syngeneic elicited peritoneal macrophages.

DISCUSSION

Many previous studies of in vitro demyelination have depended on the use of organotypic CNS tissue cultures (Lyman et al., 1986; Selmaj et al., 1987). However, assessment of the extent of myelin degradation by light and electron microscopy can only be qualitative. For this reason various biochemical markers of demyelination have been studied. These include CNPase (Weissbarth et al., 1980; Roth et al., 1985), cerebrosides (Roth et al., 1983; Roth and Bornstein, 1984), and MBP (Sheffield and Kim, 1977; Shin et al., 1984; Nishimura et al., 1986). Due to the differing amounts of myelinated fibres in the cultures, control and experimental values show a relatively wide range of variability. To overcome this difficulty we have used suspensions of purified myelin. Such a simple system enables fixed amounts of myelin (determined by protein content) to be used for each assay. Measurements of CNPase and MBP in this system indicate it to be highly reproducible with little interassay variation even after 24-h incubation at 37°C (Fig. 3).

Although the preparation contains a high proportion of compact myelin, split myelin lamellae with exposed cytoplasmic faces are also present. As there is evidence to suggest that MBP is localised at the cytoplasmic surface (major dense line) of myelin membranes (Poduslo and Braun, 1975) MBP would therefore be more accessible to proteolytic enzymes than in intact myelin. Earlier research using purified myelin has demonstrated that MBP is readily degraded by trypsin (Banik and Davison, 1974; Banik et al., 1976), and significant losses of MBP from myelin suspensions incubated with trypsin are also reported here. MBP is also susceptible to the action of endogenous proteases present in the purified myelin (Sato et al., 1982; Berlet et al., 1984). Such autolysis could explain the marked reduction in total MBP levels (supernatants + pellet) detected by ELISA following incubation with the surfactant lysophosphatidylcholine. The possible effect of the lysophosphatidylcholine would be to expose more of the MBP to myelin.
associated proteases in a manner similar to that described in oedematous CNS tissue (Smith, 1977).

Sprinkle et al. (1980) and Drummond and Dean (1980) have demonstrated that CNPase activity is associated with the W1 component of Wolfram protein (WP). Topological studies suggest that WP is partially exposed at the cytoplasmic surface of myelin (Delaunoy et al., 1982). Complete solubilisation of CNPase, however, requires the presence of delipidating or chaotropic agents (reviewed by Sims and Carnegie, 1978); thus CNPase exhibits some of the properties of an intrinsic membrane protein. Although significant release of CNPase activity occurred upon coincubation with trypsin, proteolysis was greatly enhanced by the addition of phospholipase A2 to the system and extensive solubilization of CNPase was mediated by lysophosphatidylcholine. Trypsin-related losses of CNPase activity appeared to take place within the first minutes of incubation, especially at high concentrations of trypsin, and reached a maximal level that could not be exceeded even by adding fresh trypsin to the myelin suspension. These losses could represent CNPase that is present in unit membrane or less compact multilamellar membrane fragments that are more prone to proteolytic digestion. Our findings are consistent with the intrinsic membrane protein properties ascribed to the CNPase enzyme. Thus these experiments would suggest that CNPase is a reliable and sensitive marker of myelin degradation in vitro. The enzyme is an intrinsic component of myelin membranes and in the system we describe the loss of MBP with intact region 86–96, and the release of sulphatide, which is located in the bimolecular lipid leaflet of the intact membrane, can also be demonstrated.

The in vitro myelin model can be used to assess myelin breakdown by different cell populations. For example (see Table 3), the effect of different densities

![FIG. 3. Loss of CNPase activity from mouse myelin suspension (=10 µg protein) in medium (200 µl RPMI + FCS) alone (•—•) or in the presence of 5 X 10⁵ syngeneic elicited peritoneal macrophages (•—•) over a 24-h incubation period at 37°C. Each point represents the mean ± SD of three replicate samples.](image)

<table>
<thead>
<tr>
<th>Table 4. Loss of CNPase activity and MBP after 24-h incubation of purified myelin with lymph node T cells from MBP- or KLH-primed SJL mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment:</strong></td>
</tr>
<tr>
<td>Myelin incubated with</td>
</tr>
<tr>
<td>Media only</td>
</tr>
<tr>
<td>2 X 10⁶ T cells</td>
</tr>
<tr>
<td>2 X 10⁶ T cells + 1 X 10⁶ adh PECs</td>
</tr>
<tr>
<td>1 X 10⁶ adh PECs</td>
</tr>
</tbody>
</table>

Figures represent CNPase activity or MBP in residual myelin pellet following 24-h incubation at 37°C. A total of 10 µg of myelin protein/200 µl medium ± cells was used for each replicate. At the end of the incubation the myelin pellet (± cells) was washed with 200 µl of ice-cold distilled water and resuspended in a final volume of 200 µl RPMI. The numbers (1) and (2) represent data from two separate experiments. (1) T cells from MBP-primed mice only were used. (2) Comparison of T cells from MBP- or KLH-primed mice. Cells used were nylon wool purified T cells from lymph node cell suspensions cultured over a 4- to 5-week period with appropriate antigen and IL2. The adherent peritoneal exudate cells (adh PECs) used were prepared from syngeneic mice. Results are expressed as means ± SD, n, number of replicate samples per group. Medium, RPMI + FCS (10% vol/vol).

* Significant at p = 0.01; ** significant at p < 0.002 (calculated by Student's t test).
of adherent and nonadherent peritoneal exudate cells can be studied. Concomitant release of CNPase and MBP can be demonstrated at lower densities of macrophage-enriched cells compared to nonadherent cell populations. Preliminary experiments (Table 4) have been designed to see if sensitised T-cells with or without macrophages can induce in vitro demyelination. Lymph node cell preparations from mice primed with MBP or KLH in the presence or absence of added macrophages were incubated with myelin. Nylon wool purified T-cells by themselves had no significant effect on incubated myelin but demyelination could be demonstrated when a limited number of syngeneically elicited macrophages were added to MBP-sensitized cells. A similar requirement for adherent accessory cells in the adoptive transfer of EAE by GPMBP-sensitised lymph node cells has recently been described by Mannie et al. (1987). Further dissection of the synergistic interaction of T-cells sensitised to specific myelin antigens and activated macrophages leading to the breakdown of myelin observed with our in vitro system may provide valuable insights into the mechanisms of demyelination in vivo.

In conclusion, measurement of the release of CNPase from a myelin preparation provides the basis for a simple, reliable, and precise model for the study of demyelination in vitro. Analysis of the effects of trypsin, phospholipase A₂, and lysophosphatidylcholine in this system has demonstrated the high degree of reproducibility possible with this method. Moreover, in concord with earlier work in this area (Banik et al., 1976), these agents were found to act cooperatively in degrading myelin; thus under conditions that may be pertinent to demyelination in vivo myelinolysis can be assayed quantitatively. Three advantages of this micromethod are that (1) relatively low numbers of cells can be studied; (2) it enables the study of cell-mediated demyelination within a syngeneic system as myelin from different species can be substituted quite easily; and (3) it is a much more easily defined system in which the contribution of individual cell types can be examined with minimal interference by contaminating cell populations as is the case with organotypic cell cultures.

Acknowledgment: We gratefully acknowledge the assistance of Dr. N. P. Groome, who developed the MBP ELISA for our use, and Dr. D. N. Landon, who prepared the electron micrographs. This work was supported by the Medical Research Council and the Multiple Sclerosis Society of Great Britain. Joseph Najbauer was the 1985-1986 recipient of the du Pre Fellowship awarded by the International Federation of Multiple Sclerosis Societies.

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Degradation of human myelin in vitro by leucocytes from patients with multiple sclerosis

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(Accepted for publication 16 May 1990)

SUMMARY

In order to study the possible autoimmune basis of multiple sclerosis (MS) a quantitative method has been used to investigate breakdown of human myelin in vitro. We found that serum from MS patients and controls was generally devoid of any myelin degradative activity. However, isolated peripheral blood mononuclear cells from 43% of MS patients showed significant myelin degradative activity as did those from 61-5% of patients with rheumatoid arthritis (RA). Myelin degradation by cells was found in only 13% of patients with other neurological diseases and in no healthy controls. It is proposed that this non-specific peripheral cellular immune degradative activity originates from cells activated within the central nervous system of MS patients or the joints of individuals with RA. As a result, activity in the blood only indirectly reflects the ongoing inflammatory response at the primary site, accounting for the lack of correlation between changes in the blood and the clinical status of the MS patient. We further propose that the lack of in vitro myelin degradative activity in cells recovered from the cerebrospinal fluid is due to autoaggressive cells being sequestered to the brain.

Keywords multiple sclerosis CNPase, myelin degradation assay autoaggressive cells

INTRODUCTION

Various proposals have been made implicating cellular and humoral factors in the pathogenesis of multiple sclerosis (MS) suggesting an autoimmune basis for the disease. Perivascular cuffs containing activated T lymphocytes expressing interleukin-2 (IL-2) receptors and producing IL-2 are found close to or within acute lesions (Bellamy et al., 1985). Increased numbers of activated T cells have also been detected in cerebrospinal fluid (CSF) in MS in comparison with peripheral blood as measured by RNA and DNA content (Noronha, Richman & Arnason, 1980) and IL-2 receptor expression (Bellamy et al., 1985; Tournier-Lasserve et al., 1987). There is also increased intrathecal synthesis of oligoclonal IgG and persistence of IgM. This and other evidence (reviewed by Calder et al., 1989) suggest that the ongoing immune activity is localized within the central nervous system (CNS). However, there is some evidence that a relapse may be initiated peripherally, for recent studies on lymphocyte migration have found CD2-positive cells crossing the blood-brain barrier from the blood into the CSF in progressive MS (Hafler & Weiner, 1987). It has also been suggested that cells may circulate from the CNS to the blood (Oehmichen et al., 1979; Wekerle et al., 1986). Therefore, the blood may be useful in monitoring the immunological activity during disease.

Based on studies using developing rodent CNS explants, it has been claimed that demyelinating activity present in the serum of some MS patients is due to a toxic factor (Seil, 1977), complement or possibly to antibody directed against myelin or oligodendrocytes, the myelin-producing cells within the CNS. However, this activity is not specific to MS, since demyelination has been reported with sera from healthy controls as well as from other neurological diseases (Bornstein & Hummelgard, 1976; Ulrich & Lardi, 1978; Silberberg, Manning & Schreiber, 1984; Bradbury, 1987). In addition, peripheral T lymphocyte supernatants from MS patients in relapse can induce demyelination in cerebellar organ cultures (Selmaj et al., 1987). Interpretation of all these experiments is complicated because endogenous macrophages and myelin-forming cells are present in the heterologous tissue culture systems, quantitative evaluation of the demyelination is difficult by reverse-phase microscopy.

In order to overcome some of these problems, exposure of a human cell-free myelin preparation to serum or peripheral blood mononuclear cells (PBMC) has been examined using a biochemical method to quantitatively assess myelin degradation (Watson et al., 1988). The assay measures the release of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), a component of the Wolfgram protein fraction which has been shown to be
localized mainly at the myelin/axon interface and found in low concentrations throughout the compact myelin (Brunner et al., 1989). The enzyme's activity correlates well with the amount and integrity of myelin, suggesting its use as a quantitative measure of demyelination in vitro (Kurihara, Nussbaum & Mandel, 1970; Roth et al., 1985). Sera and PBMC from MS patients at different stages of disease activity as well as from controls (healthy individuals and patients with other neurological diseases) have been studied. In addition, the demyelinating capacity of PBMC and sera from rheumatoid arthritis (RA) patients has been examined, since this is an inflammatory disease thought to have an autoimmune basis. A preliminary report of some of this work has been published (Owen, Watson & Davison, 1988).

**SUBJECTS AND METHODS**

**Patients**

PBMC were obtained from 81 patients identified as having clinically probable MS by the criteria of McDonald & Halliday (1977). Patients were classified as in relapse when a new clinical sign had developed within the 3 weeks previous to examination; those with active disease had a clear previous history of new signs or symptoms within the past 3 months; patients with stable disease had no new sign or symptom for 6 months or more. Progressive cases had evidence of two or more separate CNS lesions and a history of progressive paraplegia. In the other neurological disease group with inflammatory changes there were two with Guillain–Barré syndrome; four with CNS postoperative infection; one with infected ventricular shunt; and one with viral meningoencephalitis. Non-inflammatory other neurological disease cases included three with epilepsy; two with non-demyelinating peripheral neuropathy; four with tumours; five with motor neuron disease; three with cerebrovascular disease; and one patient with cerebellar ataxia. None of the healthy volunteers or above patients were on immunosuppressive therapy. In the RA group all but three of the 26 patients were on some form of immunosuppressive therapy.

**PBMC and serum preparation**

PBMC were isolated from whole heparinized blood by Ficoll–Hypaque density gradient centrifugation (Pharmacia, Milton Keynes, UK). The cells were harvested from the interface layer and washed three times in RPMI 1640 supplemented with 50 U penicillin/50 μg streptomycin ml⁻¹, 2 mM glutamine, 25 mM HEPES buffer (all from Flow Labs., Rickmansworth, UK) and 10% heat-inactivated fetal calf serum (Gibco, Paisley, UK). The PBMC preparation contained not more than 3% polymorphonuclear granulocytes. Fresh serum was collected from blood that had been allowed to clot at room temperature and then spun for 5 min at 200 g. The serum was always assayed within a few hours of the blood being taken (frozen serum was never used).

**Myelin preparation**

Human myelin was prepared according to the method of Norton & Poduslo (1973). The purity of the myelin preparation was confirmed on SDS-PAGE and had a composition consistent with highly purified myelin. The myelin was stored in aliquots in distilled water at −20°C until used. Storage did not affect the stability or total enzyme activity of the myelin preparation.

**Myelin degradation assay**

Triplicate samples of myelin were incubated with serum (25% v/v) or PBMC in a ratio of 10 μg myelin protein:2 × 10⁶ PBMC in 96-well, flat-bottomed tissue culture plates (Nunc, Uxbridge, UK) for 24 h at 37°C in a humidified 5% CO₂/95% air atmosphere. Triplicate samples of myelin alone in culture medium without serum or PBMC were also incubated for 24 h as a control at the beginning and end of the plate. The myelin plus cells or serum was resuspended and transferred to microcentrifuge tubes and centrifuged at 7500 g for 5 min. The supernatant was discarded and the myelin/cell or serum pellet was resuspended by vortexing in 200 μl RPMI 1640 and stored at −20°C. CNPase activity was assayed within 1 month of freezing.

CNPase activity was assayed fluorimetrically using cyclic NADP⁺ as substrate (Sogin, 1976) according to the method of Weissbarth, Maker & Lehner (1980) modified by Rastogi & Clausen (1985). The optimal conditions for the enzyme assay were determined by varying the myelin protein concentration, substrate concentration and incubation time and the reproducibility of the analytical method was confirmed. The assay conditions are summarized below. The equivalent of 0.5–1.0 μg of protein (myelin) was incubated in 280 μl of the reaction mixture (0.2 mM MES buffer, pH 6.0; 30 mM MgCl₂; 1 mM Na₂EDTA; 0.025% Triton X-100; 1 μg/ml bovine serum albumin; 1 μM 2′,3′-cyclic NADP⁺; 6 mM glucose-6-phosphate; and 5.6 U/ml glucose-6-phosphate dehydrogenase) for 20 min at 30°C. The enzyme reaction was stopped by the addition of 2:52 ml 50 mM sodium-carbonate buffer, pH 10.5, to the incubation mixture. The fluorescence of the end product (NADPH) was measured using 360 nm as excitation and 460 nm as emission wavelengths. Fluorescence of serial dilutions of B-NADPH in sodium-carbonate buffer were used to plot a standard curve for each assay. Enzyme activity was expressed as nanomols of end

<table>
<thead>
<tr>
<th>Myelin incubated with</th>
<th>CNPase activity of myelin/cell pellet (nmol end product/20 min/sample)</th>
<th>Loss CNPase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture medium</td>
<td>2955 ± 100</td>
<td>—</td>
</tr>
<tr>
<td>PBMC patient 1</td>
<td>2254 ± 161</td>
<td>24*</td>
</tr>
<tr>
<td>PBMC patient 2</td>
<td>2802 ± 57</td>
<td>4</td>
</tr>
<tr>
<td>Culture medium</td>
<td>2928 ± 179</td>
<td>—</td>
</tr>
</tbody>
</table>

PBMC from two MS patients in relapse were incubated at a concentration of 2 × 10⁶ cells/10 μg myelin (n = 3) for 24 h at 37°C. Myelin alone in culture medium was incubated at the beginning and end of plate as a control (n = 3). CNPase activity of the residual myelin pellet was expressed as mean ± s.d.

Loss of CNPase activity after incubation with PBMC is expressed as a percentage of myelin incubated in culture medium alone subtracted from 100.

* Significant at P < 0.005, Student’s t-test.
Table 2. Comparison of in vitro myelin degradation by peripheral blood mononuclear cells (PBMC) and serum from individual patients

<table>
<thead>
<tr>
<th>Clinical group</th>
<th>Loss of CNPase (%) from myelin after incubation with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myelin degradation by Serum and cells Serum and cells Cells alone Neither</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Serum and cells 13* (1) 18* (1) Serum and cells 21† (1) 23* (1) Cells alone NS (9) 13-5.46† (9) Neither NS (9) NS (9)</td>
</tr>
<tr>
<td>Other neurological diseases</td>
<td></td>
</tr>
<tr>
<td>Inflammatory</td>
<td></td>
</tr>
<tr>
<td>Infected ventricular shunt (1)</td>
<td>Neither NS (3) NS (3)</td>
</tr>
<tr>
<td>GBS (2)</td>
<td></td>
</tr>
<tr>
<td>Post-operative infection (4)</td>
<td></td>
</tr>
<tr>
<td>Non-inflammatory</td>
<td></td>
</tr>
<tr>
<td>CVD</td>
<td>Cells alone 0 (1) 28* (1) Serum alone 18* (1) 6 (1)</td>
</tr>
<tr>
<td>PN</td>
<td></td>
</tr>
<tr>
<td>CVD (1), PN (1), epilepsy (2), MND (5)</td>
<td>Neither NS (9) NS (9)</td>
</tr>
<tr>
<td>Post-operative VP shunt (3)</td>
<td></td>
</tr>
<tr>
<td>Tumour (3)</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td></td>
</tr>
</tbody>
</table>

The number of patient samples assayed is shown in parentheses. NS, no significant loss of CNPase from myelin after incubation with PBMC or serum. GBS, Guillain-Barré syndrome; CVD, cerebrovascular disease; PN, peripheral neuropathy; MND, motor neurone disease; VP, ventriculo-peritoneal.

* P < 0.05; † P < 0.001; ‡ P < 0.001 - P < 0.05 (Student’s t-test).

product (NADPH)/20 min per total volume of sample, as a fixed amount of myelin was used in each set of experiments.

CNPase activity of myelin incubated with culture medium alone did not vary significantly during individual experiments (triplicates of myelin alone were always placed at the beginning and end of the 96-well plate to ensure the reproducibility of the assay). To allow for slight variation between each series of experiments (less than 10%) results are expressed as percentage loss in CNPase activity of myelin after incubation with PBMC or serum and calculated as follows (see Table 1 for typical myelin CNPase values before and after incubation with PBMC).

\[
\text{% Loss CNPase activity} = \left[ 100 - \frac{\text{Mean CNPase activity in myelin + cells/serum pellet}}{\text{Mean CNPase activity in myelin alone pellet}} \right] \times 100.
\]

The statistical significance of the loss in CNPase activity of the myelin when incubated with cells or serum was calculated using the Student’s t-test.

\[CSF\]

CSF collected from seven patients with MS and two with other neurological diseases was taken (after obtaining informed consent) and always used primarily for routine laboratory investigations. The CSF was immediately spun at 200 g for 10 min, the supernatant removed and the cell pellet resuspended in RPMI 1640+10% FCS+supplements. The sample was split into three and incubated with 6 µg myelin/well (triplicate). For comparison PBMC were set up in the same ratio of cell/myelin as CSF and also as previously described at 2 x 10^6+10 µg myelin/well. Triplicate samples of 6 µg and 10 µg myelin alone in culture medium were also incubated for 24 h as a control at the beginning and end of the plate. Total CNPase activity of the myelin was assayed as above.

Immunofluorescent staining of lymphocytes

PBMC (2 x 10⁶), in RPMI 1640 medium containing 5% newborn bovine serum (Gibco) were incubated in flexible plastic 96-well plates for 20 min at 4°C. The cells were washed and incubated with 40 µl of monoclonal antibody used at optimal concentrations, as determined by previous titration experiments, for 30 min at 4°C. After two washes with cold medium, 40 µl of fluorescein-conjugated rabbit anti-mouse IgG (Nordic Immunological Laboratories, Maidenhead, UK) was diluted 1/20 and added for 30 min at 4°C. The cells were then washed three times and resuspended in 10 µl of medium and mounted on
Table 3. In vitro myelin degradation by peripheral blood mononuclear cells (PBMC) from patients with multiple sclerosis and other patients

<table>
<thead>
<tr>
<th>Clinical group</th>
<th>PBMC causing significant CNPase loss from myelin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple sclerosis</td>
<td></td>
</tr>
<tr>
<td>Stable</td>
<td>5/13 (38.5%)</td>
</tr>
<tr>
<td>Active</td>
<td>8/13 (61.5%)</td>
</tr>
<tr>
<td>Relapse</td>
<td>11/24 (46%)</td>
</tr>
<tr>
<td>Progressive</td>
<td>11/31 (35%)</td>
</tr>
<tr>
<td>Total</td>
<td>35/81 (43%)</td>
</tr>
<tr>
<td>Other neurological disease</td>
<td></td>
</tr>
<tr>
<td>(inflammatory)</td>
<td>0/4</td>
</tr>
<tr>
<td>(non-inflammatory)</td>
<td>2/15 (13%)</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>16/26 (61.5%)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0/22</td>
</tr>
</tbody>
</table>

* \( P < 0.001 - P < 0.05 \) (Student’s \( t \)-test).

Myelin degradation in multiple sclerosis

Myelin degradation by serum

Fresh samples of serum were incubated with the human myelin preparations for 24 h and residual CNPase activity determined as described above. In contrast to the findings of other investigators, only two out of 20 (10%) sera from MS patients showed significant degradation of myelin in vitro; PBMC from these two patients also caused significant myelin degradation. However, serum from nine patients with degradative activity in the PBMC fraction caused no significant loss of CNPase (Table 2). Similar results were obtained with RA samples, as only one of the 13 (8%) sera tested caused a significant loss of CNPase. Apart from one non-inflammatory sample (from a patient with myxoedema with peripheral neuropathy) there was no serum myelin degradative activity in the group of patients with non-inflammatory or inflammatory other neurological disease or healthy controls.

Myelin degradation by PBMC

The percentage loss of CNPase activity from the myelin preparation when incubated with PBMC was determined with cells from patients with MS, RA and other neurological disease patients as well as from healthy controls. Significant myelin degradation was seen with 43% of samples from MS patients \( (n=81) \) but this degradative activity did not correlate with disease status (Table 3).

Myelin degradative activity measured by the loss in CNPase activity of myelin after incubation with PBMC was also assessed in other disease groups. Of the 26 PBMC samples tested in the RA group 61.5% (16/26) caused significant losses in CNPase activity of the myelin preparation ranging from 18 to 63%. There was no indication that treatment with immunosuppressants affected these results. However, in the patients with other, non-inflammatory neurological diseases, only PBMC from two patients with cerebrovascular disease out of the 15 samples tested caused a significant loss in CNPase activity of 38% \( (P < 0.001) \) and 28% \( (P < 0.05) \). Both the PBMC from patients with other, inflammatory neurological diseases \( (n = 4) \) and from the 22 healthy control caused no evidence of significant in vitro myelin degradation (Table 3).

Myelin degradation by CSF cells

In a limited number of experiments CSF cells were incubated with myelin and loss of CNPase was measured. In no case was...
marked myelin degradative activity observed even when significant activity was present in PBMC at an equivalent low cell number (Table 4).

The relation between demyelinating activity and cell phenotype
Several phenotypic indices were measured in PBMC from 35 MS patients to see whether expression of surface molecules such as receptor for IL-2 (CD25) and MHC class II antigens (HLA-DR) which are both expressed by activated T cells correlated with the resulting myelin degradative activity shown by these cells. No correlation was apparent in any respect with cell phenotype and percentage losses in CNPase (data not shown).

DISCUSSION
Although it has been shown that in multiple sclerosis perivascular cuffs of inflammatory cells are frequently associated with areas of demyelination, the immune mechanisms involved are not clear. Macrophages containing myelin debris have previously been identified (Prince & Wright, 1978) but these phagocytes do not appear to initiate demyelination. In order to study the possibility that lymphocytes (or their soluble products) are primarily involved, we have devised a simple in vitro method to assess human myelin degradation.

Serum demyelinating factors
It has been suggested that soluble factors in MS may be implicated in primary demyelination. Silverman et al. (1984) showed that complement could be activated via the classical pathway by isolated rat myelin with the subsequent formation of membrane attack complexes in the membrane bi-layer of myelin leading either directly to myelin damage or to antibody-mediated demyelination. Myelotoxic complement-dependent antibody has also been reported in the CSF and sera of MS patients and in some controls, but complement depletion studies (Bradbury et al., 1984a) and removal of serum immunoglobulins (Grundke-Iqbal & Bornstein, 1980) both show that complement and anti-myelin antibodies play a minor role in in vitro demyelination. More recently in has been postulated that membrane-bound proteinase may be implicated in the pathogenesis of MS. Kerlero de Rosbo & Barnard (1989) have shown that immunoglobulins bind to myelin and activate the myelin neutral protease possibly through release of free calcium from calcium-binding sites and that MS immunoglobulins cause significantly more basic protein degradation than control immunoglobulin. Our results are in conflict with previous studies as myelin degradation was seen in only two out of 20 sera tested. In both of these the cellular degradative activity was high (Table 2), suggesting that the in vitro demyelinating factor may be secreted by the circulating leucocytes. Grundke-Iqbal & Bornstein (1980) speculated that the demyelinating factor found in MS sera may be a proteolytic enzyme secreted by activated leucocytes in the blood or locally in the perivascular cuffs in the brain. However, cellular demyelinating activity was not usually accompanied by serum myelin degradative activity (Table 2). Our data also show that apart from one case of peripheral neuropathy in vitro myelin degradative activity was absent from the sera of patients with other neurological diseases including motor neurone disease, Guillain-Barré syndrome, inflammatory conditions and from all healthy controls. A possible explanation of these contradictory results is that demyelinating factors previously detected in serum may be acting on the cultured oligodendrocyte and not primarily on myelin. Bradbury et al. (1984b) have developed a new technique of assessing sera-induced myelinotoxicity and cellular toxicity by using a combination of visual assessment of myelin damage and measuring radiolabel release from damaged glia and myelin in CNS tissue culture. In their studies (Bradbury et al., 1985) the sera from the MS group induced the greatest radiolabel release, which they suggested may be associated with cytotoxicity towards the oligodendrocyte, in addition to a direct effect on myelin. However, 22% of healthy controls and 68% other neurological diseases scored positively compared with 74% of MS sera.

Cell-mediated myelin degradation
We have demonstrated that PBMC from 43% of MS and 61-5% of RA patients can cause significant release of CNPase from the myelin preparation. Thus the action of the cells (lymphocytes or macrophages) or possibly their products is not restricted to MS, but is probably a non-specific cellular response shown by chronically stimulated immune cells occurring in inflammatory autoimmune diseases. Initially in the first phase of the disease we propose that there will be an antigen-specific response within the CNS in MS and in the synovial joints in RA. Cytokines will be released which will serve to expand the immune response by recruitment of further inflammatory cells from the peripheral blood. Subsequently the immune cells will become chronically stimulated and may migrate back into the peripheral blood. Thus it is these chronically activated cells or their products (cytokines) that we find in both RA and MS that can non-specifically degrade myelin in vitro. It has been proposed that tumour necrosis factor or other cytokines may participate in myelin damage in early lesion development in MS (Brosnan, Selmaje & Raine, 1988).

In MS there is evidence that the immune response is concentrated in the CNS (Calder et al., 1989). However, we have been unable to show a direct correlation with clinical exacerbations and in vitro PBMC-mediated myelin degradation. This is not surprising, since recent studies using magnetic resonance imaging (MRI) have demonstrated the presence of 'silent' lesions in the absence of any new clinical signs (Ormerod et al., 1987). Moreover, in longitudinal studies of peripheral blood T lymphocyte subsets no correlation has been found between phenotype and clinical activity (Capra et al., 1989). However, it has been shown that changes in immune function correlate with disease activity as recognized by MRI (Oger et al., 1988). It therefore seems probable that the cellular myelin degradative activity found in PBMC relates to the presence of these clinically silent lesions and depends on the number of activated lymphocytes that migrate into the blood. Failure to detect significant degradative activity in the CSF is not surprising, since it has been found that the CSF in acute viral meningoencephalitis does not reflect either functionally or phenotypically the composition of inflammatory cells present at the actual site of CNS pathology (Moench & Griffin, 1984).

It would appear probable that the myelin degradative activity involves T lymphocytes (with or without macrophages); however, it remains to be answered whether some of these cells have been sensitized to specific antigens (e.g. myelin basic protein, myelin-associated glycolipid, gangliosides or cerebroside) or whether some homology occurs in the epitopes of...
Myelin degradation in multiple sclerosis

myelin basic protein in MS and epitopes in the causative agent in RA. An alternative possibility is that sensitization of cells occurs peripherally and activated lymphocytes migrate from blood into the target organ (Hafer & Weiner, 1989). In a preliminary study we have found that in MS and RA the activity is present in the T-lymphocyte fraction of PBMC (Owen et al., 1988). This is consistent with the hypothesis that in MS these T lymphocytes have migrated from the CNS where activation by antigen-presenting cells has already occurred, since long-term treatment with cyclosporin A has relatively little effect on the progress of multiple sclerosis (Rudge et al., 1989).

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