The Role Of Macrophages In The Formation And Repair Of Myelin

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By

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ABSTRACT

Foetal rat brain aggregate cultures resemble the developing brain providing a unique system to investigate myelinogenesis, demyelination and repair. Supplementing aggregate cultures with macrophages accelerated cellular organisation and increased myelin deposition over time without affecting activity of the oligodendrocyte marker 2',3'-cyclic nucleotide 3'- phosphodiesterase (CNP).

Pro-inflammatory cytokines and anti-myelin oligodendrocyte glycoprotein (MOG) antibodies induced demyelination in myelinated aggregate cultures while oligodendrocytes were spared. Demyelination was associated with increased levels of a myelin basic protein (MBP) degradation peptide indicating proteolysis of myelin. MBP continued to accumulate following removal of demyelinating agents while peptide levels declined.

Myelinogenesis in the aggregates was associated with patterns of growth factor mRNA expression comparable with those of the developing brain. The mRNA levels of platelet-derived growth factor-A (PDGF-A), a potent mitogen for oligodendrocyte progenitors, rose rapidly while fibroblast growth factor-2 (FGF-2) and ciliary neurotrophic factor (CNTF) mRNA increased gradually as MBP accumulated. The peak of transforming growth factor-β1 (TGF-β1) and neurotrophin-3 (NT-3) mRNA expression coincided with the appearance of MBP mRNA, while that of insulin-like growth factor-I (IGF-I) was more closely associated with the detection of MBP protein. Enhanced myelination in macrophage-enriched cultures was associated with reduced expression of CNTF and increased levels of TGF-β1 and FGF-2 mRNA both of which promote oligodendrocyte development in vitro.

Demyelination induced a distinct pattern of expression of many myelination-associated growth factors. A rapid rise in CNTF mRNA in standard cultures closely followed by increases in FGF-2 and IGF-I was in contrast to the delayed induction of PDGF-A mRNA. In macrophage-enriched aggregates the rise in IGF-I mRNA
following demyelination preceded that in standard cultures suggesting that macrophage-enrichment instigates a faster IGF-I response during remyelination.

Since macrophage-rich demyelinating multiple sclerosis lesions also display signs of remyelination, macrophages, as a source of growth factors, have the potential to promote myelination and repair.
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</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<tr>
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</tr>
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<tr>
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<td>Dulbecco's modified Eagle's medium</td>
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<td>dTTP</td>
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<tr>
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<td>experimental allergic encephalomyelitis</td>
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<td>Earle's balanced salt solution</td>
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<tr>
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<td>epidermal growth factor</td>
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<tr>
<td>EM</td>
<td>electron microscope</td>
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</tr>
<tr>
<td>Fc</td>
<td>constant fragment of immunoglobulins</td>
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<tr>
<td>FcR</td>
<td>receptor for the Fc portion of immunoglobulins</td>
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<tr>
<td>FGF-2</td>
<td>fibroblast growth factor-2</td>
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<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
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</tr>
<tr>
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<td>glial fibrillary acidic protein</td>
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<tr>
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<tr>
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</table>
LAP latency associated protein
LTBP latent TGF-β1 binding protein
LIF leukaemia inhibitory factor
LPS lipopolysaccharide
M molar
Mϕ macrophage
MAC membrane attack complex
MAG myelin-associated glycoprotein
MBP myelin basic protein
MCM macrophage-conditioned medium
MHC major histocompatibility complex
M-MLV Moloney murine leukaemia virus
MOG myelin oligodendrocyte glycoprotein
MRI magnetic resonance imaging
mRNA messenger ribonucleic acid
MS multiple sclerosis
MW molecular weight
MOSP myelin oligodendrocyte specific protein
n number of experiments
NF-H neurofilament heavy
NF-M neurofilament medium
NGF nerve growth factor
NS nervous system
NSB non specific binding
NT-3 neurotrophin-3
NT-4/5 neurotrophin-4/5
O-2A oligodendrocyte-type 2 astrocyte progenitor cell
OMgp oligodendrocyte myelin glycoprotein
P postnatal
PBS phosphate buffered saline
PDGF platelet-derived growth factor
PDGF-Rα platelet-derived growth factor-α receptor
PKC protein kinase C
PLP proteolipid protein
PNS peripheral nervous system
RIA radioimmunoassay
RNase ribonuclease
rRNA ribosomal ribonucleic acid
RT-PCR reverse transcriptase polymerase chain reaction
SEM standard error of the sample mean
SFM serum free medium
TGF-β1 transforming growth factor-β1
Th cell T helper cell
TNF-α tumour necrosis factor-alpha
trk tyrosine kinase receptor
uPA urokinase plasminogen activator
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Above all, my sincerest thanks go to my parents, Melanie, my grandma and to Jonathan for their never failing understanding, support and encouragement.

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Dedicated to my parents.
CHAPTER 1

INTRODUCTION

1.1 The central nervous system

The central nervous system (CNS), consisting of the brain and spinal cord, communicates with other body systems to continually receive, elaborate and interpret information enabling it to determine many aspects of behaviour and participate in a vast complexity of direct and indirect control actions on the rest of the body. The complex and diverse functions of the mature CNS, including perception, motor co-ordination, motivation and memory, depend on the precise interactions of many thousands of neural cells. Hence, the individual nerve cell, the neuron, is a key unit of the CNS which operates by generating and propagating electrical signals down the length of an axon. Axons are covered by a high resistance, complex multi-layered membranous sheath called myelin which, in the CNS, is formed by glial cells called oligodendrocytes.

1.2 Oligodendrocyte development and myelination

1.2.1 The oligodendrocyte lineage

Oligodendrocytes are derived from pluripotent stem cell precursors located in the subventricular zones of the developing CNS. Their development and the subsequent process of myelination occurs relatively late during development after the formation of most neurons and astrocytes (Norton, 1981). The earliest precursors of rat spinal cord oligodendrocytes are evident from embryonic day 12 (E12) to E14 in the ventral portion of the neural tube (Warf et al., 1991; Yu et al., 1994). Insights into the stages of oligodendrocyte differentiation have been gained from both tissue culture and in vivo studies and have revealed that these cells undergo a process of extensive maturation through a number of stages defined by the sequential expression of specific markers and responses to distinct growth factors (Figure 1.1).
Figure 1.1. Schematic representation of oligodendrocyte maturation in vitro. The expression of specific cell surface antigens is shown along with the growth factors that influence each stage of development. Dotted lines represent possible alternative pathways of differentiation.
The first cell identified in the oligodendrocyte lineage is the pre-progenitor giving rise to the bipotential oligodendrocyte-type 2 astrocyte progenitor cell (O-2A). This cell, named after its two types of progeny, has been shown to differentiate \textit{in vitro} into oligodendrocytes, unless modulated by growth factors to differentiate into A2B5 positive type 2 astrocytes (Raff \textit{et al.}, 1983) although differentiation into astrocytes \textit{in vivo} has not been consistently observed (Franklin \textit{et al.}, 1995; Sawamura \textit{et al.}, 1995). These progenitors are small, proliferative, bipolar cells that are highly motile \textit{in vitro} and characterised by the expression of the ganglioside GD$_3$ (Curtis \textit{et al.}, 1988; LeVine & Goldman, 1988; Hardy & Reynolds, 1991; Levine \textit{et al.}, 1993), the tetra-3-sialo-gangliosides recognised by A2B5 monoclonal antibody (Raff \textit{et al.}, 1983), the NG2 chondroitin sulphate proteoglycan (LeVine & Goldman, 1988; Nishiyama \textit{et al.}, 1996; Levine \textit{et al.}, 1993), the cytoplasmic intermediate filament vimentin (Raff \textit{et al.}, 1984) and the platelet-derived growth factor-α receptor subunit (PDGF-Rα) (Pringle \textit{et al.}, 1992). These precursor cells leave the subventricular zones and continue to proliferate as they migrate towards their targets (Reynolds & Wilkin, 1988; Hardy & Reynolds, 1991; Levison \textit{et al.}, 1993). From O-2A progenitor cells, oligodendrocytes continue to mature by beginning to extend numerous processes and although the oligodendrocyte is still bipotential and continues to proliferate at this stage it ceases to be mobile (Warrington \textit{et al.}, 1993). Furthermore, this pro-oligodendrocyte acquires cell surface antigens that are recognised by the monoclonal antibody O4 which reacts with an, as yet, unidentified protein named pro-oligodendrocyte antigen on immature proliferating cells, sulphatide in mature cells and several other lipids (Bansal \textit{et al.}, 1989; Bansal \textit{et al.}, 1992) with a corresponding loss of vimentin filament staining (Warrington & Pfeiffer, 1992; Hardy & Reynolds, 1993). As differentiation proceeds oligodendrocytes extend an elaborate network of branched processes and begin to express galactocerebroside (GC) which indicates terminal oligodendrocyte differentiation with the combined loss of proliferative and migratory
potential (Raff et al., 1978). During the final stages of maturation there is a sequential expression of myelin specific proteins with DM-20, the first oligodendrocyte specific protein detected, followed by the expression of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), then myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG). Ultrastructurally oligodendrocyte differentiation is associated with a decrease in cell size and an increase in organelle density. The growth, differentiation and survival of oligodendrocytes and their progeny, as well as the synthesis of myelin, is extensively influenced by multiple growth factors including platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-I) and 2, neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF), glial growth factor (GGF), retinoic acid, interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF). Growth factor induced proliferation may be mediated by protein kinases with protein kinase C implicated in the signal transduction pathways of oligodendrocytes (Bhat & Zhang, 1996).

It has been suggested that rather than follow this pattern of differentiation, some cells persist as adult oligodendrocyte progenitors (Wren et al., 1992). Evidence now increasingly favours the view that once cells have differentiated they are not able to dedifferentiate (Lee et al., 2000). Hence, the normal adult CNS contains cells that are positive for the proteoglycan NG2 and express the PDGF-α receptor on their surface and are therefore, probable adult oligodendrocyte progenitor cells (Levine, 1994). Adult O-2A progenitor cells (O-2A adult progenitor) have been described in both animals (ffrench Constant & Raff, 1986; Wolswijk & Noble, 1989; Reynolds & Hardy, 1997), and humans (Raine et al., 1981; Scolding et al., 1995; Scolding et al., 1999). While these cells have the potential to differentiate into oligodendrocytes they are different from their perinatal counterparts in that they are unipolar and vimentin negative (Wolswijk & Noble, 1989). Furthermore, these progenitors are
considerably less mobile than their perinatal equivalents and have a longer cell cycle time 
(Wolswijk et al., 1990; Scolding et al., 1995).

1.3 Myelin

Myelin accounts for approximately 70% of the dry weight of the CNS with a single 
oligodendrocyte able to myelinate between 30 and 50 internodal segments of neighbouring 
axons (Butt & Ransom, 1989). During myelination an oligodendrocyte is able to produce up 
to 3 times its weight of myelin membrane per day (McLaurin & Yong, 1995).

1.3.1 Myelination

Myelin is laid down and maintained around axons in the CNS by oligodendrocytes 
and accounts for 50% of the mammalian CNS white matter, about 35% of the adult human 
brain and 20-25% of the adult rat brain (Norton & Cammer, 1984). Myelination is a tightly 
controlled process that begins in the peripheral nervous system (PNS) and follows in the 
CNS in a caudal to rostral sequence. It is initiated in the CNS when the axons to be 
myelinated reach a diameter of approximately 1 micron. In the human spinal cord the 
myelin sheath protein, MBP, can be detected in the myelin forming cell, the 
oligodendrocyte, at nine to ten weeks gestation indicating the onset of myelination 
(Weidenheim et al., 1993). Hence, MBP can first be detected in the cell body of 
oligodendrocytes prior to the appearance of myelin and is only later detectable in cellular 
processes when the intensity of oligodendrocyte MBP staining is diminished, after the onset of 
myelination (Sternberger, 1978). The human spinal cord is myelinated between mid to late 
term and three months post natal, while brain myelin is laid down in the months and years 
following birth (Folch-Pi, 1955). Myelin is first seen in the rat at about P9 and while the
maximal rate of myelin deposition occurs at P20, myelination continues for at least another 6 months (Norton & Cammer, 1984).

Oligodendrocytes extend multiple processes that locate axons prior to myelination. Processes are extended which, after tentative contact, anchor to the axon and extend longitudinally before surrounding the target axon (Ludwin & Szuchet, 1993; Hardy & Friedrich, 1996). Further extension of each edge of the oligodendrocyte process results in one edge, the future inner tongue, passing beneath the other. Subsequent development of the myelin sheath is believed to involve this oligodendrocyte inner tongue elongating and rotating around the axon to form multiple concentric layers or lamellae (Raine, 1984b). As myelin is formed it compacts whereby the cytoplasm is extruded so that the two cytoplasmic (inner) surfaces of the membrane become opposed and fuse to form a single major dense line (dark line) while the two extracellular surfaces of adjacent layers of the membrane intimately abut to form the intraperiod line although these two surfaces do not fuse (Raine, 1984b). CNS myelin displays a periodicity of 11.5nm with each lipid bilayer approximately 4-5nm thick. Ultimately only the inner and outer tongues do not compact so that intracellularly myelin contains very little cytoplasm, although larger amounts are visible at the node of Ranvier.

Oligodendrocyte maturation and myelination is influenced by a combination of growth factors as well as cell surface and extracellular matrix molecules. Multiple proteoglycan proteins including chondroitin sulphate proteoglycans are particularly abundant in CNS white matter suggesting a role in myelination (Ruoslahti, 1996). The extracellular matrix proteins vitronectin, laminin, thrombospondin and tenascin-C are expressed in the developing CNS at a time when oligodendrocyte precursor cells are migrating (McLoon et al., 1988; O'Shea et al., 1990; Sheppard et al., 1991; Bartsch et al., 1992). Accordingly, thrombospondin-1, fibronectin and the laminin family member merosin promote oligodendrocyte precursor cell migration in vitro (Frost et al., 1996; Scott-Drew & ffrench-
Constant, 1997). Furthermore, laminin-2 enhances myelin membrane process formation and extension in vitro (Buttery & ffrench-Constant, 1999). However, unlike the thrombospondins, neither fibronectin or laminin are widely expressed in white matter tracts during postnatal development (O'Shea et al., 1990; Scott-Drew & ffrench-Constant, 1997).

Tenascin-C inhibits oligodendrocyte precursor cell adhesion and migration thereby defining the pattern of myelination (Bartsch et al., 1992; Frost et al., 1996; Kieman et al., 1996). In contrast, despite a number of behavioural abnormalities in tenascin-C null mice, the distribution of oligodendrocyte precursor cells and myelination appeared unaffected (Kieman et al., 1999). Tenascin-R expression is restricted to the nervous system where it is produced by type-2 astrocytes, a small subset of neurons and oligodendrocytes during CNS myelination (Pesheva et al., 1989; Wintergerst et al., 1993). Its expression is subject to regulation by cytokines and growth factors with, for example, PDGF able to up-regulate tenascin-R expression on O-2A progenitor cells (Jung et al., 1993). In the developing CNS white matter tracts, tenascin-R mRNA and protein are present both within the pathways of oligodendrocyte precursor cell migration and during myelination (Pesheva et al., 1989). Accordingly, tenascin-R interacts with cell surface gangliosides and inhibits GD3+ oligodendrocyte precursor cell adhesion and migration (Probstmeier et al., 1999). However, as oligodendrocytes mature tenascin-R promotes the stable adhesion, process formation and terminal differentiation of O4+ oligodendrocytes by a sulphatide mediated mechanism in vitro (Pesheva et al., 1997). This implies that oligodendrocytes may respond differently to the same extracellular signal as their molecular repertoire changes with ongoing maturation. Furthermore, growth factors may influence the expression of tenascin-R and other extracellular matrix molecules thereby affecting the myelinating capacity of these cells.
1.3.2 Myelin composition

As with other biological membranes myelin is composed of lipids and proteins, however, it is unusual in that the lipids comprise more than 70% of its dry weight (Rumsby, 1978) compared to 40% in the oligodendrocyte. Since a high lipid concentration effectively excludes water and any substances soluble in water such as sodium and potassium ions the high lipid content contributes to myelin’s function as an insulator. As observed in other membranes these lipids are asymmetrically distributed across the bilayer. Hence, the highly compacted myelin lipid bilayer consists of 70 to 85% lipid, including 40-45% phospholipid, 25-30% glycolipid and approximately 25% cholesterol (Table 1.1). The phospholipids are made up of diminishing concentrations of ethanolamine phosphatides, phosphatidyl choline, sphingomyelin, phosphatidyl serine and three inositol phosphatides (Cuzner & Norton, 1996). The glycolipids, all of which are specific to oligodendrocytes and myelin, include galactosyl ceramide (galactocerebroside, GC), sulphatide, galactosyl diglyceride, digalactosyl diglyceride and fatty acid esters of cerebroside with GC and sulphatide accounting for 24% and 3.5-7% respectively of the total lipids while the remainder account for 1% (Cuzner & Norton, 1996). Finally, gangliosides comprise a minor component of the myelin sheath accounting for 0.1 to 0.3% of the lipid content of myelin.

Although less abundant than lipids proteins, which account for 25-30% of the myelin sheath, contribute to stabilising the membrane. In fact, myelin contains many fewer proteins than other surface membranes with, for example, proteins that facilitate the passage of ions through the lipid bilayer absent therefore contributing to the ionic impermeability of the myelin sheath. The two main polypeptides present which comprise 80% of the total myelin protein are PLP and MBP. PLP is a 30kDa lipophilic integral membrane polypeptide, and together with its alternatively spliced isoform DM-20 contributes 50% of the total protein. MBP is the major extrinsic membrane polypeptide (30-40%) which is hydrophilic and exposed primarily
<table>
<thead>
<tr>
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<th>Myelin</th>
<th>Liver</th>
<th>Erythrocyte</th>
<th>Endoplasmic Reticulum</th>
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<td>13</td>
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Table 1.1. Comparison of the lipid compositions of myelin membrane with other cellular plasma membranes.

on the cytoplasmic membrane surface. It exists in multiple isoforms encoded by a single gene that is alternatively spliced. In humans there appear to be 4 main isoforms, a 21.5kDa protein, encoded by all 7 exons, a 20kDa protein where exon 5 is missing, the major 18.5kDa protein, where exon 2 is deleted, and a 17.2kDa protein, where exon 2 and 5 are removed (Kamholz et al., 1986; Roth et al., 1987). Rat CNS myelin comprises of at least 4 major isoforms including 21.5 and 18.5kDa proteins in addition to a 17kDa protein where exon 6 is deleted and a 14kDa protein where both exon 2 and 6 are spliced (Campagnoni et al., 1987). MBP and PLP through their adhesive properties are both implicated in myelin compaction (Boison et al., 1995; Staugaitis et al., 1996) while DM-20 may be involved in myelinogenesis (Ludwin, 1997). Shiverer mouse mutants that have no myelin basic protein consequently display abnormal CNS myelination and are severely myelin deficient.
CNP, an enzyme of 46 and 50kDa isoforms, is primarily present in oligodendrocytes and their processes as opposed to compacted myelin. These two CNP isoforms occur in approximately equal amounts and account for about 4% of the total myelin protein. CNP is a guanosine triphosphate binding protein and may be important in oligodendrocyte differentiation (Ludwin, 1997). Glycoproteins such as myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), 105kDa oligodendrocyte myelin glycoprotein (OMgp) and myelin oligodendrocyte specific protein (MOSP) are also present at very low levels (Cuzner & Norton, 1996) while there are probably others that have yet to be characterised. MAG comprises approximately 1% of the total myelin protein existing as two isoforms, L-MAG and S-MAG, both of about 100kDa. In rodents the ratio of these isoforms varies with development with the larger L-MAG isoform predominating during myelination while S-MAG is the major isoform in the adult (Cuzner & Norton, 1996). A role for MAG has been suggested in axon-myelin contact and spacing. The 26kDa MOG which appears late in development and only constitutes 0.05% of the total protein may have a role in myelin maintenance (Ludwin, 1997). The 48kDa MOSP present on the oligodendrocyte surface may have a role in membrane-cytoskeletal interactions (Dyer et al., 1991; Dyer, 1993). Additionally, tubulin, which is not specific to myelin, is also present.

Myelin components do turnover, although usually at slower rates than other brain membranes. Most of the lipids in myelin have half lives of several weeks although this may be longer with cholesterol, cerebroside and sulphatide having half lives of a few months (Morell et al., 1994). Myelin proteins have a fast phase half life of 2 to 3 weeks in addition to a slow phase (Morell et al., 1994).
1.3.3 Molecular organisation of myelin

Myelin is considered to be a typical example of a fluid mosaic membrane with an extracellular surface rich in carbohydrate residues from glycoproteins and glycolipids. Cerebroside and other glycolipids are located on the extracellular surface of the lipid bilayer, hence on the intraperiod line (Braun, 1984). Similarly, cholesterol appears to be distributed in the outer half of the membrane bilayer (Braun, 1984). This half of the bilayer contains nearly all of the cholesterol and glycolipids while phospholipids including phosphatidyl ethanolamine (plasmalogen) and the phosphatidyl inositols predominate on the cytoplasmic half of the membrane (Kirschner & Ganser, 1982).

Of the myelin proteins, it seems that PLP loops the bilayer three or four times leaving large polypeptide chains exposed on the extracellular surface that may stabilise the intraperiod line (Figure 1.2). MBP is extrinsic and restricted to the cytoplasmic domain (Braun, 1984) where it appears to complex to itself possibly acting to compact and stabilise the apposed cytoplasmic surfaces at the major dense line (Cuzner & Norton, 1996). MAG is situated in the periaxonal membrane with the carbohydrates of one transmembrane domain exposed on the external surface of the membrane bilayer just adjacent to the axon (Cuzner & Norton, 1996). Like CNP, it is believed to be absent from the majority of compact myelin although it has been suggested that it could reside on the external surface of the intraperiod region in compact myelin (Sato et al., 1982).

1.3.4 Myelin function

Myelin ensheaths and insulates axons while also facilitating rapid saltatory conduction of action potentials. The high resistance, low capacitance myelin sheath prevents the local current generated by depolarisation from passing to the axolemma. This promotes saltatory conduction of action potentials from node to node enhancing the rate of impulse transmission.
Figure 1.2. Schematic representation of the molecular organisation of central nervous system myelin. Cross-sectional view illustrating the possible positions of myelin proteins. The diagonal stripes of the lipid bilayer represent the polar groups while the dots represent the hydrocarbon region. Extracellular apposition forms the intraperiod line while the cytoplasmic surfaces fuse to become the major dense line.
In non-myelinated axons the speed of excitation is proportional to the axon diameter. Hence, in myelinated axons, where the signal is propagated 100 times faster, the diameter of the fibre can be significantly reduced while maintaining the same performance. Thus, in addition to increasing the speed of impulse transmission, myelin enables many more smaller axons to be present thereby contributing to the extremely compact organisation of the CNS. Moreover, signal conduction is more energy efficient in myelinated axons as the flow of sodium and potassium ions necessary to depolarise the small area of exposed axonal membrane at the node is modest in comparison to non-myelinated fibres where the whole axon membrane must depolarise and subsequently repolarise.

1.4 Multiple sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory and progressively demyelinating disease of the mature CNS. The disease primarily affects young adults with onset most common among people between twenty and thirty years of age with a female to male preponderance of 2:1 (MacDonald et al., 2000). It is estimated that 85 000 people in the United Kingdom are affected with around 2400 new cases diagnosed each year.

The course of MS varies considerably between people. However, in approximately 66-85% of all cases the disease initially follows a relapsing-remitting course (Weinshenker et al., 1989a; Weinshenker et al., 1989b) with symptoms experienced for periods of days to months during recurrent relapses and interspersed with periods of remission where a partial or complete recovery is evident. Relapsing-remitting MS accounts for approximately 45% of all diagnosed cases which, in most instances (30-40%), gradually changes over time into a secondary progressive phase resulting in irreversible disability (Weinshenker et al., 1989a; Weinshenker et al., 1989b). In up to 20% of cases the course of MS may be essentially benign with no exacerbations experienced after initial diagnosis while
approximately 10% of individuals experience a primary progressive course where the condition develops with no alleviation.

Demyelinating lesions develop irregularly over several decades and clinical features, which occur as a result of neurological dysfunction, are followed by complete or partial recovery. These neurological deficits are highly variable among individuals and are closely associated with the pathology of the disease. A range of primary symptoms arising initially as a consequence of impaired nervous conduction due to inflammation and myelin loss include, weakness, pain and fatigue, optic neuritis, bladder and bowel instability, spasticity, gait disorders and sexual dysfunction. Persistent deficits arise as a consequence of progressive demyelination with permanent interruptions to axonal conduction while in more extreme circumstances chronic disabilities may be associated with axonal degeneration.

1.4.1. Cause of multiple sclerosis

The cause of MS remains unclear although evidence suggests that both genetic and environmental factors may be important. Among environmental factors that may influence disease onset infectious agents have been implicated, particularly viruses. It has been suggested that molecular mimicry, similarity between a microbial antigen and another tissue, could trigger the immune system to damage the CNS. Hence, exposure outside the nervous system to a virus, which has a molecular similarity to a component of the myelin sheath, could activate T cells to cross the blood-brain barrier (BBB). Therefore, in genetically pre-disposed people, myelin antigens may be mis-identified as virus thereby resulting in an autoimmune tissue attack. Furthermore some key components of myelin are expressed outside of the CNS with, for example, MBP expressed in the thymus during development (Pribyl et al., 1993; Zelenika et al., 1993) and key myelin lipids found in other plasma membranes.
A genetic pre-disposition to MS has been suggested (Dyment et al., 1997) and associated with certain major histocompatibility complex (MHC) genes. However, no single gene has been identified suggesting that multiple genes interact to increase susceptibility (Sawcer et al., 1996; Haines et al., 1996; Ebers et al., 1996). Furthermore, the concordance rate of MS in monozygotic twins is only 30% implying a multifactorial aetiology (Noseworthy, 1999).

1.4.2 Mechanisms and mediators of demyelination in multiple sclerosis

The healthy CNS is generally considered to be an immunologically privileged site (Barker & Billingham, 1977) shielded from the immune system by the selectively permeable BBB which restricts the passage of large molecules. CNS injury which involves damage to the BBB results in the recruitment of inflammatory cells from the circulation thereby destroying the previously immunologically privileged status. The events that initiate the inflammatory process leading to BBB damage and eventual myelin destruction in MS remain unclear. In normal physiological circumstances the BBB is not entirely impermeable to lymphocytes and small numbers of these cells are detectable in the brain and cerebrospinal fluid (CSF). It is possible that in inflammatory demyelinating diseases T cells are activated by an unknown peptide, either in the periphery or the CNS, which along with peripheral blood macrophages cross the BBB into the CNS where microglia and astrocytes are activated. Whatever the trigger, BBB disruption would escalate the immune response by allowing further inflammatory mediators access to the brain. Oligodendrocytes and myelin are subsequently damaged by toxic substances produced by lymphocytes and activated macrophages including pro-inflammatory cytokines, antibody, complement components, proteolytic and lipolytic enzymes, reactive oxygen intermediates and nitric oxide before the damaged myelin is phagocytosed by macrophages and microglia (Cuzner & Norton, 1996).
1.4.2.1 T-lymphocytes

It is thought that CD4+ Th1 T lymphocytes are pivotal in disease progression and extension of the inflammatory lesion by secreting cytotoxic cytokines such as interferon-gamma (IFN-γ) and enhancing macrophage activity. The release of local cytokines and chemotactic factors may facilitate the recruitment and activation of macrophages, microglia and additional lymphocytes. IFN-γ, which up-regulates MHC class II molecules and activates macrophages, is thought to be produced exclusively by T lymphocytes and has been shown to play a major role in MS exacerbation (Panitch et al., 1987) while it is also associated with peak expression of experimental allergic encephalomyelitis (EAE) (Issazadeh et al., 1995a; Issazadeh et al., 1995b). Activated CD4+ T cells and microglia may also lyse oligodendrocytes by binding via the Fas ligand to the Fas receptor expressed on a variety of glial cells in the CNS including oligodendrocytes thereby activating the Fas signalling pathway (Hahn et al., 1995; Yagita et al., 1995).

Oligodendrocytes in vivo do not express MHC class II molecules and therefore, CD4+ T cells are unlikely to be directly responsible for their death. Cytotoxic CD8+ autoreactive T cells may predominate in some MS lesions where they may bind to MHC class I antigens on oligodendrocytes causing cell injury or contributing to demyelination by producing inflammatory cytokines (Tsuchida et al., 1994). Similarly, γδ T cells, also present in MS lesions, possess potent cytotoxic activity in vitro and are capable of damaging oligodendrocytes (Freedman et al., 1991). These cells are known to respond to heat shock proteins which may be increased in MS (van Noort et al., 1995) and furthermore, depletion of this subset of T cells reduced EAE severity (Rajan et al., 1996). However, although it seems that a T cell response is crucial for disease induction, T cell mediated inflammation is often only associated with minimal demyelination.
1.4.2.2 Macrophages in demyelination

The main functions of macrophages in the body include phagocytosis, processing and presentation of foreign antigens to T lymphocytes via an MHC Class II molecule and secretion of cytokines and a variety of growth factors in response to inflammation, infection and injury. Macrophages and microglia have been implicated in the destruction and phagocytosis of myelin in inflammatory demyelinating diseases. Microglia are considered to be the main cell type found in early active MS plaques while haematogenous monocytes and macrophages predominate in later more advanced lesions where BBB breakdown is apparent (Li et al., 1996). Evidence suggests that macrophages may be involved in the initial attack as myelin disruption in very early MS lesions was detectable in close proximity to macrophages prior to any signs of phagocytosis (Li et al., 1993). Furthermore, ultrastructural studies in MS and EAE have determined that macrophages attach to and penetrate between myelin lamellae and are subsequently responsible for stripping myelin from the axon prior to receptor-mediated phagocytosis (Lampert, 1965; Prineas & Connell, 1978). Macrophages containing myelin debris within coated pits and vesicles have been identified in active demyelinating MS lesions suggesting that these cells are responsible for receptor-mediated phagocytosis of myelin (Prineas & Connell, 1978; Epstein et al., 1983).

Accordingly, macrophages and microglia have been shown to possess a wide variety of receptors which are implicated in myelin uptake in vitro including the Fc, lectin, scavenger and complement type 3 (CR3) receptors (Mosley & Cuzner, 1996). Furthermore, macrophage depletion in EAE protects against the manifestation of clinical symptoms (Brosnan et al., 1981; Huitinga et al., 1990) implying that recruitment and activation of macrophages are central to the disease process.

The functional properties of macrophages and microglia are dependent on their state of activation which is subject to regulation by cytokines with, for example, the key
macrophage activator IFN-γ able to up-regulate MHC class II expression as well as the Fc, CR3 and scavenger receptors reflecting an enhanced phagocytic capacity. Similarly, tumour necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) up-regulate the expression of the Fc receptor (Loughlin et al., 1992). Activated macrophages and microglia, also secrete a variety of potentially cytotoxic soluble substances thought to further contribute to demyelination including pro-inflammatory cytokines, complement components, proteolytic and lipolytic enzymes and reactive oxygen intermediates which are volatile and reactive oxidants capable of causing potent tissue damage (Hartung et al., 1992).

1.4.2.3 Cytokine mediated demyelination

Cytokines produced by activated T lymphocytes and macrophages as well as resident CNS cells such as astrocytes, microglia and neurons have been implicated in the initiation and maintenance of inflammatory demyelination. A multitude of cytokines associated with clinical disease activity have been detected in human MS tissue. In situ hybridisation studies have shown that mRNA encoding the cytokines IL-6, TNF-α and IFN-γ predominate in inflammatory perivascular lesions while, in more hypercellular areas with out myelin loss, increased levels of T cell derived IL-2 and IFN-γ mRNA were apparent (Woodroofe & Cuzner, 1993). Perivascular macrophages would be the major source of IL-6 and TNF-α although T cells are also capable of producing these cytokines. Accordingly, the presence of IL-1, IL-6 and TNF-α in macrophages, microglia and astrocytes has been demonstrated (Hofman et al., 1989; Selmaj et al., 1991a).

IFN-γ enhances T cell cytotoxicity, B cell proliferation and antibody production as well as activating macrophages and up-regulating MHC II expression, Fc receptor and complement receptor expression (Hartung et al., 1992). In situ hybridisation and

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immunohistochemical studies have identified this cytokine in both chronic and active MS lesions (Woodroofe & Cuzner, 1993). The major cytokines expressed by activated macrophages are TNF-α and IL-1 and both of these are elevated in MS lesions as well as in macrophages located in the peripheral blood and CSF (Hofman et al., 1986; Hofman et al., 1989; Merrill et al., 1989). Additionally, both TNF-α and IL-1 increase the expression of the Fc receptor on macrophages and microglia (Loughlin et al., 1992). TNF-α mediates immune injury and demyelination and has been shown to be toxic for oligodendrocytes in vitro and to induce myelin swelling and loss (Selmaï & Raine, 1988; Selmaï et al., 1992). Accordingly, oligodendrocytes express TNF-α receptors 1 and 2 (Wilt et al., 1995) while intra-peritoneal injection of anti-TNF-α antibody has been shown to inhibit the clinical signs of EAE (Selmaï et al., 1991b). IL-1 may be involved in activating astrocytes as well as inducing the production of other cytotoxic cytokines such as TNF-α and IL-6 (Lee et al., 1995). IL-1 is expressed by macrophages and microglia in MS lesions (Brosnan et al., 1995; Cannella & Raine, 1995) while it has also been found to exacerbate EAE (Jacobs et al., 1991). However, not all cytokines are pro-inflammatory, in fact, IFN-α and β are thought to contribute to down-regulating the inflammatory process as are cytokines characteristic of a Th2 response such as IL-4 and IL-10 (Miller & Karpus, 1994; Olsson, 1995) and may subsequently be important in the recovery process.

1.4.2.4 Antibody-mediated demyelination

Demyelinating activity has also been attributed to antibodies particularly as they are found in the sera of MS patients (Walsh & Tourtellotte, 1983; Raine, 1984a). Any antigen present on the myelin sheath, particularly on the external surface, is a potential target for a demyelinating auto-antibody response however, the particular antigen responsible for
demyelination in MS has not been identified. Polyclonal antibodies directed against the myelin membrane proteins PLP, MBP and MAG did not produce demyelination in CNS cultures (Seil & Agrawal, 1980; Seil et al., 1981), however, it has been suggested that the CNS specific external minor membrane protein MOG is a good target for antibody induced demyelination due to it's location and specificity (Lassmann & Linington, 1987). Accordingly, MOG specific mouse monoclonal antibodies have been found to induce demyelination in rats with EAE (Lassmann et al., 1988; Linington et al., 1988) and antibodies directed against MOG have shown demyelinating activity in aggregating foetal rat brain cultures (Kerlero de Rosbo et al., 1990).

Antibodies may opsonise myelin enabling receptor-mediated phagocytosis via a number of cell surface receptors located on macrophages and microglia including Fc receptors, complement receptors and scavenger receptors (Mosley & Cuzner, 1996). Immunoglobulin deposition is associated with macrophages and disintegrating myelin sheaths at the edge of MS plaques indicating a humoral contribution to lesion formation (Prineas & Graham, 1981). In EAE immunoglobulin has been identified between clathrin-coated pits and associated myelin debris suggesting that immunoglobulins may opsonise myelin and subsequently act as a ligand facilitating Fc receptor-mediated phagocytosis of myelin (Raine & Scheinberg, 1988). Furthermore, that complement deposition is associated with these areas (Storch et al., 1998) suggests that antibodies may further contribute to demyelination and injury in MS by activating the antibody-dependent classical complement cascade. The presence of activated complement products and terminal membrane attack complexes (MAC) have been observed in plaques and CSF of patients with MS (Compston et al., 1989). The monoclonal anti-MOG antibody, Z12, fixes complement in vitro and has been identified as a potent demyelinator (Piddlesden et al., 1993) while complement appears to be activated in areas of myelin breakdown (Compston et al., 1989).
1.5 Pathology of multiple sclerosis lesion

The predominant pathological features that characterise MS are widespread inflammatory cell infiltration and multifocal patches of demyelination in the CNS white matter which are known as plaques. These plaques occur at irregular intervals over several decades and are largely responsible for the disruption in saltatory nerve conduction leading to neurological deficits. Generally, MS lesions can be divided into 3 categories active, chronic active and chronic inactive (Lassmann et al., 1998).

1.5.1 The active lesion

Magnetic resonance imaging (MRI) data has shown that one of the earliest detectable pathological signs in new lesion development is an increase in BBB permeability which is associated with inflammation (McDonald, 1994). Therefore, in this early active stage of classical MS plaque development immune cells can penetrate the BBB and cause inflammatory oedema as well as characteristic perivascular cuffs which are found predominantly around postcapillary venules, particularly in the periventricular white matter, optic nerve and tract, corpus callosum and brain stem (Brosnan & Raine, 1996). Perivascular infiltrates in these small early active lesions are thought to initially comprise all major lymphocyte classes, particularly T lymphocytes and to a lesser extent B lymphocytes, but macrophages become a major component of these cuffs as the lesion progresses and myelin breakdown products become abundant (Woodroffe et al., 1986; Esiri & Reading, 1987). The extent of perivascular lymphocytic infiltration has been shown to correlate with disease activity (Adams, 1975; Guseo & Jellinger, 1975). Active lesions have abundant and evenly distributed MHC class II positive cells which are mainly macrophages (Bo et al., 1994) although may also be associated with both astrocytes and endothelial cells within active plaques. Hence, there is a hypercellular interface between...
normal and degenerating myelin in active MS plaques due primarily to infiltrating immune
cells, macrophages and proliferating glia. In very early MS lesions macrophages
containing MBP or oil red O staining are evident but there are no obvious signs of myelin
loss (Li et al., 1993). These lesions may only be 2 to 3 weeks old as myelin proteins are
rapidly degraded while in active plaques that are 2 to 3 months old lipid laden macrophages
are evident. Histochemical studies have shown that many myelin components are lost
during demyelination, including MBP, MAG, sphingolipids, cerebroside and cholesterol
(Hallpike et al., 1970) while an increase in cholesterol esters is evident.

Early active lesions are therefore small and hypercellular, they undergo a variable
degree of oligodendrocyte loss and up to 40% of plaques display signs of attempted
remyelination, particularly in the early phase of the disease (Prineas et al., 1993a). In some
instances there is also profound gliosis and even neuronal and axonal loss. As the plaque
develops CD8+ lymphocytes are found located towards the edge of the lesion while CD4+
lymphocytes are concentrated within the core. B lymphocytes and antibody can also be
detected which is consistent with the finding of oligoclonal banding in the CSF of MS
individuals.

1.5.2. The chronic lesion

Chronic active plaques are characterised by MHC class II positive lipid containing
macrophages at the lesion edge although these cells may also contain myelin proteins
indicative of more recent myelin phagocytosis (Bo et al., 1994). Macrophages in the centre
of these chronic active plaques may contain lipids but not myelin protein degradation
products.

Older more chronic lesions are hypocellular and become immunologically silent
with no apparent lymphocytic infiltration or myelin filled macrophages. Particularly,
CD4+ T lymphocytes are reduced while T cells expressing the γδ T cell receptor become the predominant T cell (Selmaj et al., 1991). Instead, the inflammatory cell infiltrate is replaced by astrocytic gliosis and widespread regions of myelin loss (Raine & Bornstein, 1970; Brosnan & Raine, 1996). While axons are initially thought to be preserved it seems that there is variable axonal loss in more chronic lesions (Trapp et al., 1998) causing irreparable neurological deficits.

1.5.3 Oligodendrocyte preservation

The extent to which the myelin forming oligodendrocyte is preserved during demyelination remains unclear. While it seems that oligodendrocytes are largely absent from chronic MS plaques (Prineas, 1985) there appears to be a variable degree of cell preservation in actively demyelinating lesions (Raine et al., 1981; Prineas et al., 1989; Rodriguez et al., 1993; Bruck et al., 1994; Ozawa et al., 1994; Lucchinetti et al., 1996). It has been suggested that oligodendrocytes survive at the edge of early lesions and sometimes proliferate there in partial or abortive attempts at remyelination. Thus, the preservation and proliferation of oligodendrocytes in active MS plaques, particularly in the early stages of the disease, has been observed (Prineas et al., 1989) with evidence of shadow plaques indicating attempts at remyelination (Prineas et al., 1993a). Lucchinetti et al. (1999) determined that there was a variable degree of oligodendrocyte loss in MS lesions ranging from a complete absence of these cells to numbers that were comparable with or even greater than those in periplaque white matter. Furthermore, it was determined that different MS plaques from the same patient exhibited uniform patterns of inflammation, demyelination and oligodendrocyte pathology (Lucchinetti et al., 1996). However, the pattern of lesion pathology and the extent of oligodendrocyte preservation between MS patients was highly variable. Therefore, while the mechanisms of
oligodendrocyte injury are uniform in the individual MS patient, the pathology in different MS patients is heterogeneous which may be attributed to distinct pathogenetic mechanisms (Lucchinetti et al., 1996; Lucchinetti et al., 1999). Thus in some instances, myelin may be the primary object of the immune attack while in other cases oligodendrocytes may be targeted.

Subsequently, it has been found that an increased macrophage presence within an MS lesion is associated with reduced oligodendrocyte preservation and impaired remyelination (Lucchinetti et al., 1999). Hence, macrophages and their multiple secretory products are likely to play a major role in oligodendrocyte injury. Paradoxically, in addition to secreting cytotoxic factors, macrophages also produce a multitude of growth factors and cytokines which have the potential to promote oligodendrocyte maturation and myelination (Diemel et al., 1998).

1.6 Remyelination in multiple sclerosis

1.6.1 Morphological evidence for central nervous system remyelination

It is now recognised that attempts at remyelination in CNS demyelinated lesions do occur in both acute nascent lesions (Prineas et al., 1993a; Raine & Wu, 1993) and chronic lesions (Prineas & Connell, 1979) with the appearance of shadow plaques indicating remyelinated areas containing thinly myelinated fibres and shorter internodes than would normally occur. These thin myelin sheaths were also found to be associated with oligodendrocytes that contained myelin protein mRNA in their cytoplasm. Most evidence implies that there is a progressive loss of oligodendrocytes associated with disease chronicity and continual repeated demyelination in MS although a high density of these cells can be identified in plaques during the early stages of the disease (Raine et al., 1981; Prineas et al., 1993b; Ozawa et al., 1994; Bruck et al., 1994). Generally MS plaques
containing a high density of oligodendrocytes do show signs of spontaneous remyelination (Bruck et al., 1994). However, the ongoing nature of MS and continual myelin attacks cause progressive injury and render any natural repair mechanisms incapable of counteracting damage.

While either adult oligodendrocyte progenitors or pre-existing mature oligodendrocytes that were not damaged during demyelination may be responsible for remyelinating demyelinated areas evidence increasingly suggests that remyelination is carried out by adult oligodendrocyte progenitor cells which have been identified in both the rat and human adult CNS (ffrench Constant & Raff, 1986; Wolswijk et al., 1990; Armstrong et al., 1992; Scolding et al., 1995) and can be recruited into demyelinated areas (Blakemore & Keirstead, 1999). Oligodendrocytes have been identified in early MS lesions and in areas that have undergone extensive demyelination (Raine et al., 1981; Prineas et al., 1989) which implies that surviving mature oligodendrocytes may be able to remyelinate areas which have been demyelinated. Accordingly, reduced myelination was observed in cocultures following antibody lysis of mature oligodendrocytes while myelination was preserved after enrichment with mature oligodendrocytes supporting a role for these cells in remyelination (Wood & Bunge, 1991). However, while there is some in vitro and in vivo evidence for the involvement of surviving mature oligodendrocytes in remyelination (Wood & Bunge, 1986; Ludwin, 1984; Ludwin & Bakker, 1988) studies on demyelinating lesions in the adult rat spinal cord induced by exposure to anti-galactocerebrosidase antibodies plus complement show that approximately 50% of oligodendrocytes survive within an area of demyelination but they do not undergo cell division and although they extend myelin processes towards axons they are unable to form myelin sheaths and therefore do not contribute to remyelination (Blakemore & Keirstead, 1999). Furthermore, tissue culture preparations of adult human oligodendrocytes had little or no mitotic potential (Armstrong et al., 1992; Gogate et al., 1994) and were not able to form
myelin sheaths when transferred into demyelinating lesions of immunosuppressed rats despite axon engagement (Targett et al., 1996) supporting a role for adult oligodendrocyte progenitor cells in remyelination. Moreover, the density of cells positive for NG2, a chondroitin sulphate proteoglycan found on oligodendrocyte progenitor cells, increased locally in normal white matter following demyelination (Keirstead et al., 1998). Furthermore, Gensert et al., (1997) has shown that endogenous proliferating oligodendrocyte progenitor cells located in adult rat white matter, which probably correspond to these NG2 positive precursors, differentiated into myelinating oligodendrocytes after initiation of lysolecithin-induced demyelination and remyelinated axons (Gensert & Goldman, 1997).

However, Franklin et al., (1997) showed that there is limited migration of remyelinating cells into the area of demyelination and furthermore, recruitment depletes the available but limited population of NG2 positive dividing progenitor cells present in the white matter surrounding demyelination (Keirstead et al., 1998; Blakemore & Keirstead, 1999). Similarly, in chronic and repeated cases of demyelination depletion of these cells could occur resulting in a failure to remyelinate (Blakemore & Keirstead, 1999).

1.6.2 Macrophages and remyelination

Macrophages and microglia, with their wide range of effector properties, are not only implicated in CNS damage but also play a fundamental role in CNS development and the maintenance, restoration and defence of any damaged tissue (Figure 1.3). Phagocytosis of cellular debris from an injury site and the secretion of growth factors may enhance remyelination and repair (Diemel et al., 1998). Accordingly, in the developing CNS macrophages are involved in phagocytosing dying and apoptotic cells which facilitates growth and myelination (Perry & Gordon, 1991). Similarly, microglia were found to
Figure 1.3. Schematic representation of the contribution of macrophage-derived cytokines and growth factors to myelin synthesis and degradation. * denotes in the presence of astrocytes.
stimulate oligodendrocyte myelinogenesis in monolayer CNS cell cultures (Hamilton & Rome, 1994) and we have shown that macrophages enhance myelination in foetal brain aggregate cultures (Loughlin et al., 1994; Loughlin et al., 1997). Furthermore, macrophages are associated with the microscopic hallmarks of remyelination in MS (Prineas et al., 1993a) and glial precursor cells were found to accumulate following ethidium bromide induced demyelination at a time when microglia were actively removing myelin debris (Reynolds & Wilkin, 1991).

Inflammation in itself is a necessary response to injury and can promote regeneration. For example, neuronal death is increased following ischaemic injury in knockout mice where microglial recruitment or activation was reduced (Bruce et al., 1996; Fedoroff et al., 1997) suggesting a neuroprotective role. Similarly, eliminating macrophages from a wound site by injection of anti-leukocyte serum slows the process of healing (Rappolee & Werb, 1992) while microglia implanted into spinal cord lesions have been shown to stimulate CNS axon regeneration (Rabchevsky & Streit, 1997). Studies have shown that the capacity for remyelination is reduced in the absence of extensive inflammatory episodes following ethidium bromide induced demyelination (Graca & Blakemore, 1986). Furthermore, morphological studies have confirmed that the efficiency of remyelination is compromised when demyelinated axons are in the continued presence of myelin debris (Graca & Blakemore, 1986; Gilson & Blakemore, 1993; Shields et al., 1999) indicating that there is a relationship between the efficient removal of myelin debris by macrophages and enhanced remyelination.

In addition to their phagocytic capacity macrophages and microglia produce a variety of secretory products including cytokines and growth factors that are implicated in the proliferation, differentiation and survival of various cell types including myelin-producing oligodendrocytes during both development and repair. Hence, macrophages in
vitro have been shown to release platelet-derived growth factor (PDGF) (Bonner et al., 1991) as well as fibroblast growth factor-2 (FGF-2) (Shimojo et al., 1991; Araujo & Cotman, 1992), transforming growth factor-β1 (TGF–β1) (Wahl et al., 1990), insulin like growth factor-I (IGF-I) (Nagaoka et al., 1991), epidermal growth factor (EGF) (Plata-Salaman, 1991) and the neurotrophins, nerve growth factor (NGF) (Mallat et al., 1989; Rappolee & Werb, 1990), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Elkabes et al., 1996). Furthermore, oligodendrocytes are known to express receptors and respond to these factors (Bansal et al., 1996; Miyake et al., 1996; Pringle et al., 1992; Otero & Merrill, 1994; Masters et al., 1991; Simpson et al., 1982; Barres et al., 1994c; Wetmore & Olson, 1995; Cohen et al., 1996). Mammalian optic nerve extended neurites in the presence of macrophages or macrophage-conditioned medium further supporting a role for these cells in creating a permissive substrate for axonal regrowth (David et al., 1990). Furthermore, wound fluid conditioned by macrophages in vivo is a rich source of mitogenic and angiogenic activity (Rappolee et al., 1988). Embryonic rat brain macrophage-conditioned medium promoted neurite outgrowth and regeneration of cultured CNS neurons following injury by secreting the extracellular matrix glycoprotein thrombospondin (Chamak et al., 1994) suggesting that, in addition to growth factors, other macrophage-derived proteins may have the potential to promote repair. Thus it would appear that macrophages are capable of enhancing both oligodendrocyte survival and their ability to synthesise myelin and, with their wide range of effector mediators, may not only be associated with myelin breakdown, but may also be crucial in promoting remyelination in acute MS lesions (Diemel et al., 1998).
1.7 Growth factors in myelination and remyelination

Growth factors are multifunctional proteins that affect a broad range of fundamental cell processes by acting in both an autocrine and paracrine fashion usually by binding to high affinity plasma membrane receptors that transduce signals via an intracellular cascade. Regulation of their actions is controlled at many levels including gene transcription, translation, receptor activation and intracellular signalling. Furthermore, the method of secretion has the potential to influence their actions. For example, neuregulins, such as glial growth factor-2 (GGF-2), are expressed by neurons in the CNS (Marchionni et al., 1993; Chen et al., 1994) where they are thought to be largely membrane associated at the axon surface (Morrisey et al., 1995) due to the presence of a transmembrane domain (Lemke, 1996). In addition, while neuregulin may eventually be secreted or cleaved, it has heparin binding properties which bind it to cell surface extracellular matrix molecules, such as heparan sulphate proteoglycan (Wen et al., 1992; Sudhalter et al., 1996). On the other hand, PDGF is synthesised and secreted by astrocytes and neurons in a diffusible form (Richardson et al., 1988; Yeh et al., 1991). Hence, in the developing white matter tract, oligodendrocyte precursors may migrate in response to PDGF and proliferate in response to both PDGF and GGF-2 (Milner et al., 1997). In close proximity to axons, these progenitor cells will cease migrating and continue to divide in response to axonally associated GGF-2 thereby influencing the number of cells that differentiate into mature myelinating oligodendrocytes (Milner et al., 1997).

Furthermore, many growth factors, such as IGF and TGF-β, are secreted in inactive latent forms and are complexed to binding proteins which have to be removed prior to receptor binding (Matsumoto et al., 1996; Nunes et al., 1996) while PDGF and FGF, for example, bind to the extracellular matrix in a manner that affects their availability (LaRochelle et al., 1991; Flaumenhaft et al., 1989). Therefore, a number of growth factors are implicated in
oligodendrocyte development and myelination and it has been suggested that they may also promote myelin regeneration in MS. Furthermore, macrophages and microglia have been shown to express a wide variety of growth factors and cytokines that influence oligodendrocyte proliferation, differentiation, survival and myelinating capacity (Table 1.2).

1.7.1 Platelet-derived growth factor

PDGF, a growth promoting factor that is released from blood platelets, is a dimeric protein of A and B chains which link by disulphide bonds to form either PDGF-AA, BB or AB. The predominant PDGF isoform in the CNS is PDGF-AA which is synthesised by both astrocytes and neurons within the white matter tracts (Richardson et al., 1988; Yeh et al., 1991) in a diffusible form. Two types of PDGF receptor exist, PDGF-Rα and β, with the former able to bind both A and B chains while the latter only binds the B chain isoform (Heldin et al., 1988). The PDGF receptor is localised to a number of cell types including fibroblasts, vascular smooth muscle and glial cells. Binding of PDGF to the receptor results in dimerisation, autophosphorylation of tyrosine residues and activation of second messenger pathways.

PDGF is mitogenic for brain and optic nerve derived oligodendrocyte progenitor cells in vitro (Noble et al., 1988) although only for a limited number of divisions whereupon these cells cease to respond (Raff et al., 1988). Additionally, PDGF enhances the survival of immature oligodendrocytes in vitro and in vivo (Barres et al., 1992b). PDGF has also been found to enhance myelin gene expression in cerebral white matter cultures (Grinspan et al., 1993a) while the finding that it is chemotactic for oligodendrocyte precursors (Armstrong et al., 1990) suggests it may be involved in recruiting progenitor cells to demyelinated areas.
<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Cellular expression</th>
<th>Receptor distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF</td>
<td>Neurons, endothelial cells, astrocytes, macrophages</td>
<td>Oligodendrocytes -PDGF-Rα</td>
</tr>
<tr>
<td>FGF-2</td>
<td>As PDGF</td>
<td>Neurons, astrocytes, oligodendrocytes</td>
</tr>
<tr>
<td>CNTF</td>
<td>Neurons, astrocytes,</td>
<td>Neurons</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Most nucleated cells eg. endothelial cells, T cells, oligodendrocytes, macrophages</td>
<td>Most cells including oligodendrocytes</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Neurons, astrocytes, oligodendrocytes, macrophages</td>
<td>Oligodendrocytes</td>
</tr>
<tr>
<td>EGF</td>
<td>As IGF-I</td>
<td>Astrocytes, oligodendrocytes</td>
</tr>
<tr>
<td>NGF</td>
<td>Neurons, astrocytes, macrophages</td>
<td>Sensory &amp; sympathetic neurons, astrocytes, oligodendrocytes, macrophages, monocytes, B lymphocytes</td>
</tr>
<tr>
<td>NT-3</td>
<td>As NGF</td>
<td>Astrocytes, oligodendrocytes, macrophages</td>
</tr>
</tbody>
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Table 1.2. Central nervous system cellular distribution of growth factors and their receptors in the rodent.
1.7.2 Fibroblast growth factor-2

Acidic fibroblast growth factor (aFGF) or FGF-1 and basic FGF or FGF-2 are two highly homogenous forms of FGF which are present in the developing and adult CNS and have potent effects on the growth and differentiation of many cell types including fibroblasts, endothelial, muscle and glial cells (Gonzalez et al., 1990; Kalcheim & Neufeld, 1990) although FGF-2 is 10 to 100 fold more potent at inducing proliferation than FGF-1 (Kahn & de Vellis, 1995).

FGF-2 is synthesised by astrocytes, neurons and microglia in the peripheral and central nervous systems (Pettmann et al., 1986; Ferrara et al., 1988) as a single chain 18kDa polypeptide that lacks a signal sequence for secretion (Burgess & Maciag, 1989) and therefore, the mechanism by which it is released remains controversial (Jaye et al., 1986). Two classes of FGF receptor have been identified which are high and low affinity. A heparan sulphate proteoglycan present on the cell surface and the extracellular matrix represents a low affinity, high capacity binding site and is involved in modulating the interaction of FGF-2 with its tyrosine kinase receptors (Yayon et al., 1991) while it may also mediate the internalisation of FGF into the cell (Roghani & Moscatelli, 1992; Rusnati et al., 1993). Tyrosine kinase transmembrane receptors represent high affinity low capacity binding sites that mediate intracellular signal transduction and biological responses within the cell (Mohammadi et al., 1992; Peters et al., 1992). These high affinity FGF receptors (FGFR) of 125-165kDa comprise four members FGFR-1 (Flg), FGFR-2 (Bek), FGFR-3 and FGFR-4 with both FGF-1 and FGF-2 known to bind with high affinity to FGFR-1 and 2 (Dionne et al., 1990). Transcripts encoding FGFR-1 have been localised to adult neurons while FGFR-2 and 3 have been found in glia (Yazaki et al., 1994). Both progenitor and mature oligodendrocytes have been shown to express FGF receptors in vivo and in vitro (Gonzalez et al., 1995; Bansal et al., 1996). Cultured oligodendrocytes express mRNAs for 3 of the 4 FGF-2 receptors at different
stages of their development (Bansal et al., 1996) with FGFR-1 progressively expressed in progenitors and mature oligodendrocytes unlike FGFR-2 which is primarily found in mature oligodendrocytes. FGF-2 may act by increasing diacylglycerol through the hydrolysis of phosphatidyl inositol bisphosphate which in turn stimulates protein kinase C (PKC) (Oh et al., 1997). Hence, FGF-2 is believed to influence the proliferation and differentiation of oligodendrocytes by increasing PKC activity. The high level of FGF-2 in the developing brain suggests that it is involved in normal development. Accordingly, FGF-2 has been shown to enhance the proliferation of oligodendrocyte progenitor cells in vitro (Bogler et al., 1990; McKinnon et al., 1990).

1.7.3 Ciliary neurotrophic factor

Ciliary neurotrophic factor (CNTF) is a cytosolic helical protein of about 25kDa which is found at high levels in the CNS optic nerves, olfactory bulbs and spinal cord. In the unlesioned rat CNS it is thought to be produced primarily by astrocytes during the first postnatal week and increases progressively thereafter (Stockli et al., 1991; Dobrea et al., 1992). CNTF has not yet been shown to be synthesised by microglia or macrophages. CNTF binds to a multicomponent receptor which shares subunits with the receptor complexes for IL-6 and LIF. This CNTF receptor complex, known as CNTF-receptor α, exists in both membrane-bound and soluble forms (Davis & Yancopoulos, 1993).

CNTF induces oligodendrocyte progenitor cells to express astrocyte-specific glial fibrillary acidic protein thereby driving these cells towards an astrocytic phenotype (Hughes et al., 1988). Additionally, CNTF is implicated in enhancing PDGF-AA stimulated oligodendrocyte precursor cell proliferation both in vitro and in vivo (Barres et al., 1996). CNTF is also a potent survival factor for neurons and oligodendrocytes (Ip et
al., 1992; Barres et al., 1993; Barres & Raff, 1994b) while it has been shown to protect oligodendrocytes from cytotoxic cytokine induced cell death in vitro (Louis et al., 1993).

1.7.4 Transforming growth factor-β1

TGF-β1 is a multifunctional growth factor secreted by a wide variety of cells including T cells and activated macrophages primarily in its latent form which must be activated in order to mediate any biological effect (Massague, 1990). O-2A progenitor cells and oligodendrocytes were also found to be a source of this factor in vitro (McKinnon et al., 1993) with mature oligodendrocytes also producing TGF-β2 and 3 mRNA and protein. Murine TGF-β1 is synthesised as a large 390 amino acid precursor polypeptide containing a hydrophobic signal sequence, a latency associated protein (LAP; 30-278 amino acids) and a mature carboxy terminal subunit of 112 amino acids (Derynck et al., 1985). Interchain disulphide bonding between two proTGF-β peptides occurs before these precursors undergo proteolytic digestion to generate the biologically active mature TGF-β domain disulphide linked 25kDa homodimer consisting of two chains of 112 amino acids (Derynck et al., 1985; Daopin et al., 1992). However, the mature TGF-β dimer remains non-covalently associated with the LAP to form a 75kDa latent complex which is secreted by most cells in vitro (Sporn & Roberts, 1992) and renders it inactive and unable to interact with its receptor. This entire latent precursor molecule can be covalently linked to a 135kDa latent TGF-β1 binding protein (LTBP) which is transcribed from a separate gene but becomes associated with the latent protein before it is secreted from the cell (Kanzaki et al., 1990). Thus a key regulatory step between the secretion of latent TGF and its biological function is protein activation. Biologically active mature TGF-β1 can be released from its latent protein in vitro by extremes of pH, high temperatures, (Brown et al., 1990), thrombospondin (Schultz et al., 1994) and
proteases such as plasmin and cathepsin D (Lyons et al., 1988). However, the in vivo mechanism of activation has not yet been elucidated but is likely to involve proteolytic cleavage. A plasmin-dependent mechanism requiring urokinase plasminogen activator (uPA) to cleave plasminogen to generate the protease plasmin is a possible mechanism (Nunes et al., 1995) although cathepsin or other proteases may also be involved implicating macrophages as mediators of TGF-β1 activation (Sato & Rifkin, 1989; Wallick et al., 1990). Microglia, astrocytes, oligodendrocytes and neurons all synthesise TGF-β isoforms, which are differentially expressed during development, while also expressing the TGF-β receptor. Thus, in the intact CNS there is widespread expression of TGF-β2 and 3 preferentially by astrocytes and neurons during CNS development and persisting into adulthood while TGF-β1 is virtually absent (Lindholm et al., 1992; Unsicker et al., 1991). However, within hours of injury in many models of neurodegeneration and neuroinflammation TGF-β1 mRNA is induced with microglia and macrophages the principal cellular source (Lindholm et al., 1992; Lehrmann et al., 1998; McTigue et al., 2000).

Most cells express 3 classes of TGF receptor including a 53kDa type I receptor, a 70-85kDa type II receptor and a 250-350 kDa type III receptor (Kingsley, 1994). It is currently thought that TGF-β1 binds the type II receptor with recruitment of the type I receptor to form a type I/II heterodimer transmembrane receptor complex. This results in transphosphorylation of receptor I by the intracellular serine/threonine kinase domain on receptor II thereby initiating the signal transduction pathway and biological responses (Chen et al., 1993; Wrana et al., 1994). Mammalian proteins called Smads are thought to be phosphorylated on activation of the type I/II receptor before they are translocated to the nucleus to act as transcriptional activators (de Caestecker et al., 1997). Under normal conditions the widely expressed TGF-β2 and TGF-β3 isoforms bind to this receptor complex while in the injured
CNS activation is predominantly by microglia-macrophage-derived TGF-β1 (Unsicker et al., 1991). The type III receptor, a chondroitin/heparin sulphate and beta-glycan receptor, is apparently not capable of signal transduction but may present TGF-β to the functional type II receptor (Wrana et al., 1994).

This growth factor is implicated in a variety of biological functions acting in a context dependent autocrine, paracrine or endocrine fashion via cell surface receptors. In the CNS TGF-β1 has been shown to promote neuronal survival and enhance neurite outgrowth (Krieglstein & Unsicker, 1994; Ishihara et al., 1994), reduce neuronal damage in models of cerebral ischaemia (Flanders et al., 1998) and protect against EAE development (Johns et al., 1991). Elevated blood levels of TGF-β1 were demonstrated in secondary progressive and relapsing-remitting MS where augmentation of this factor was associated with the relapse phase (Nicoletti et al., 1998). Similarly, recovery from EAE is associated with increased TGF-β1 expression (Issazadeh et al., 1998) while anti-TGF-β1 antibodies exacerbate the disease (Racke et al., 1992). Additionally, TGF-β1 promotes oligodendrocyte differentiation (McKinnon et al., 1993) and protects oligodendrocytes from microglial cytotoxicity in vitro (Merrill & Zimmerman, 1991).

1.7.5 Insulin-like growth factor-I

IGF-I is a 7.5kDa basic polypeptide which is secreted by astrocytes, neurons, oligodendrocytes and macrophages. The activity of IGF-I is modulated by a family of 6 IGF binding proteins (IGFBP) with IGFBP-2 present in the CNS and CSF (Webster, 1997). The IGF-I receptor is a tyrosine kinase membrane bound heterotetrameric protein consisting of two alpha and two beta subunits. In addition to binding IGF-I it binds IGF-II and has extensive homology to the insulin receptor which IGF-I is also able to bind. IGF-I receptors were
identified on A2B5-positive progenitors as well as GC-positive oligodendrocytes in vitro suggesting that IGF can influence multiple stages of oligodendrocyte development (McMorris et al., 1990; McMorris et al., 1993). IGF-I and its receptor are also synthesised in the CNS in vivo with high levels of expression during development.

IGF-I is implicated in the proliferation, differentiation and survival of oligodendrocytes while it has also been shown to enhance the expression of myelin basic protein in vitro (McMorris & Dubois Dalcq, 1988; Saneto et al., 1988; Mozell & McMorris, 1991). Furthermore, in organotypic cultures IGF-I not only enhances myelin production, but it also inhibits anti-white matter serum plus complement induced demyelination (Roth et al., 1995).

1.7.6 Neurotrophins

The neurotrophins, a family of growth factors which include NGF, BDNF, NT-3 and NT-4/5 are thought to regulate aspects of neuronal and glial development and function (Barde, 1994). NGF binds to a low affinity p75 receptor and preferentially binds to tyrosine kinase receptor A (trkA) (Chao et al., 1986) while BDNF binds trkB and NT-3 and NT-4/5 bind trkC although this specificity is not absolute (Kumar et al., 1990; Maisonpierre et al., 1990b). Oligodendrocytes express p75 receptor, however despite expressing trkB mRNA transcripts no protein was found in vitro, while no trkA receptor mRNA or protein were detected (Kumar et al., 1993). NT-3, but not NGF has been found to enhance oligodendrocyte survival (Barres et al., 1993). Additionally, NT-3 has proliferative effects on oligodendrocyte precursors both in vitro and in vivo (Barres et al., 1994c) suggesting that regulation of oligodendrocyte development is at least in part regulated by NT-3.
1.7.7 Epidermal growth factor

EGF is composed of 53 amino acids and has a molecular weight of approximately 6kDa (Carpenter, 1985). EGF acts in an autocrine, paracrine or endocrine manner through specific cell surface receptors. Various cell types within the CNS produce EGF including macrophages and microglia, glial cells and neurons (Fallon et al., 1984; Rappolee et al., 1988; Schaudies et al., 1989). The EGF receptor is a transmembrane glycoprotein of 170kDa which has tyrosine kinase activity (Gill et al., 1988) and widespread distribution in the mammalian CNS. While the majority of EGF receptors are associated with astrocytes (Leutz & Schachner, 1982) there are some present on oligodendrocytes (Simpson et al., 1982). EGF is present in the developing nervous system (Fallon et al., 1984) and is mitogenic for glial cells while also acting as a neurotrophic and survival factor (Plata-Salaman, 1991). EGF has also been shown to stimulate oligodendrocyte differentiation and maturation in aggregating brain cultures where it provokes increased expression of MBP (Honegger & Guentert Lauber, 1983; Almazan et al., 1985).

1.8 Three dimensional foetal rat brain aggregate cultures

Dissociated foetal telencephalon cells maintained under constant rotation meet in the culture medium and re-assemble spontaneously within one hour following cell to cell contact and recognition to form three-dimensional aggregates. With subsequent cell migration and maturation these cultures form a homogeneous population of aggregates containing fully differentiated neurons, astrocytes and oligodendrocytes. Foetal tissue is used to both minimise cell damage during dissociation and to obtain a high proportion of cells which are undifferentiated and have maximal ability to grow and differentiate in vitro (Honegger, 1985). Morphological studies have revealed that these cells undergo extensive morphological differentiation including synaptogenesis and myelination (Honegger, 1985). Furthermore, the
chemical composition of the cultured myelin closely resembles that found in normal brain tissue (Matthieu et al., 1979).

Recent studies have shown that the pattern of major MBP protein isoforms in the aggregate cultures was similar to that which occurs in developing rat brain during myelinogenesis with exon-2 containing MBP mRNA transcripts peaking prior to exon-1 containing total MBP mRNA (Kruger et al., 1999). Thus, three-dimensional aggregating brain cultures provide a system that incorporates a mixed cell population which accurately models myelinogenesis in the developing CNS in vivo (Honegger, 1985; Loughlin et al., 1997; Kruger et al., 1999). This model, therefore, serves as a link between single cell in vitro systems and the in vivo situation in which modulation such as macrophage-enrichment and addition of potentially cytotoxic cytokines and antibodies cannot be easily undertaken and is ideal to study the processes of myelinogenesis, demyelination and remyelination.

1.9 Proposed investigation for this study

Macrophages appear to influence myelination and may also be associated with remyelination in demyelinating MS lesions where they also have a well documented role in inflammation and myelin degradation. In view of this the aims of this project were to assess myelination and remyelination in three-dimensional aggregate cultures and then to ascertain the effect that addition of peritoneal macrophages had on these processes.

The effect of macrophages, the demyelinating potential of various cytokines and antibodies along with the capacity for remyelination following demyelinating treatment is assessed using a reproducible radioimmunoassay (RIA). This was established to allow the quantitative analysis of MBP in control and treated aggregate cultures since the MBP content correlates with the extent of myelination (Shine et al., 1992). Furthermore assessment of
growth factor expression in both standard and macrophage-enriched cultures was determined during these processes to ascertain the effects of macrophage-derived growth factors on the processes of myelination, demyelination and remyelination in the three-dimensional aggregating culture system. Promoting myelination and remyelination would provide a potential strategy for therapeutic intervention in CNS demyelinating diseases such as MS.
CHAPTER 2

MATERIALS AND METHODS

2.1 Myelinating foetal rat brain aggregate culture system

2.1.1 Media

During the mechanical dissociation and washes of the foetal telencephalon cells D1 solution was used (Honegger, 1985), containing 138mM NaCl, 5.4mM KCl, 0.17mM Na₂HPO₄, 0.22mM KH₂PO₄, 5.55mM D-glucose and 58.43mM sucrose supplemented with phenol red (5mg/l; Life Technologies Limited, Paisley, UK). Telencephalon cells were cultured using high glucose serum-free Dulbecco's Modified Eagle's medium without sodium pyruvate (DMEM; Life Technologies Limited, Paisley, UK) supplemented with nutrients, vitamins, hormones and trace elements as described in Table 2.1. Resident peritoneal macrophages were collected in Earle's balanced salt solution, without calcium and magnesium (EBSS-Ca/Mg; Life Technologies Limited, Paisley, UK). Penicillin (100U/ml) and streptomycin solution (100μg/ml; Life Technologies Limited, Paisley, UK) were added to all of the above solutions as an antibiotic.

2.1.2 Preparation of foetal rat brain aggregate culture system

The preparation of mechanically dissociated foetal rat brain telencephalon cells was performed under sterile conditions on ice. Foetuses were removed from Sprague-Dawley rat mothers after 16 days gestation and placed in D1 solution. The frontal telencephalon cells of each brain were then dissected out and pooled into a 50ml centrifuge tube containing D1 solution. Immature foetal tissue was used to minimise cell damage during
High glucose DMEM, without sodium pyruvate supplemented with:

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Final Concentration</th>
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<tbody>
<tr>
<td>Insulin (bovine)</td>
<td>800 nM</td>
</tr>
<tr>
<td>Triiodo-L-thyronine (T₃)</td>
<td>30 nM</td>
</tr>
<tr>
<td>Hydrocortisone-21-phosphate</td>
<td>20 nM</td>
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<tr>
<td>Transferrin (human)</td>
<td>13 nM</td>
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Vitamins

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<tr>
<td>Choline chloride</td>
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</tr>
<tr>
<td>L-Carnitine.HCl</td>
<td>10.1 µM</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>Retinol (vitamin A)</td>
<td>trace*</td>
</tr>
<tr>
<td>DL-α-Tocopherol (vitamin E)</td>
<td>trace*</td>
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Basal Medium Eagle Vitamins:

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<tbody>
<tr>
<td>i-Inositol</td>
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</tr>
<tr>
<td>Nicotinamide</td>
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</tr>
<tr>
<td>Choline chloride</td>
<td>7.1 µM</td>
</tr>
<tr>
<td>Pyridoxal.HCl (vitamin B₆)</td>
<td>4.8 µM</td>
</tr>
<tr>
<td>D-biotin (vitamin H)</td>
<td>4.0 µM</td>
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<tr>
<td>Thiamine. HCl</td>
<td>2.9 µM</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2.2 µM</td>
</tr>
<tr>
<td>D-calcium pantothenate</td>
<td>2.0 µM</td>
</tr>
<tr>
<td>Riboflavin (vitamin B₂)</td>
<td>0.2 µM</td>
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Trace Elements

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<tbody>
<tr>
<td>Sodium metasilicate</td>
<td>Na₂SiO₃.5H₂O</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>ZnSO₄.7H₂O</td>
</tr>
<tr>
<td>Sodium selenite</td>
<td>Na₂SeO₃</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>CuSO₄.5H₂O</td>
</tr>
<tr>
<td>Manganese chloride</td>
<td>MnCl₂.4H₂O</td>
</tr>
<tr>
<td>Cadmium sulphate</td>
<td>CD₉₇SO₄.8H₂O</td>
</tr>
<tr>
<td>Molybdcic acid</td>
<td>(NH₄)₆Mo₇O₂₄.4H₂O</td>
</tr>
<tr>
<td>Nickel sulphate</td>
<td>NiSO₆H₂O</td>
</tr>
<tr>
<td>Tin (II) chloride dihydrate</td>
<td>SnCl₂.2H₂O</td>
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Others

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<tr>
<td>Lipoic acid</td>
<td>1 µM</td>
</tr>
<tr>
<td>Albumax II</td>
<td>1 %</td>
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**Table 2.1. Constituents of complete chemically defined serum free medium.**

*Retinol (5mg/l) and DL-α-tocopherol (10mg/l) are added after sonication and sterile filtration, therefore, due to a considerable loss during filtration concentrations remain theoretical.*
the mechanical dissociation step and to obtain a high proportion of undifferentiated cells which have a high capacity for growth and maturation.

2.1.3 Mechanical dissociation of telencephalon cells

The brain tissue was washed twice by carefully decanting the existing D1 and replacing it with fresh medium before sieving through a coarse 200μm porosity nylon mesh (Nybolt, Zurich, Switzerland) submerged in D1 with the help of a sterile pipette held outside the sieve to gently mash the tissue. The filtrate was then poured through a 115μm porosity nylon mesh (Nybolt, Zurich, Switzerland), under gravity. The resulting cell suspension was made up to 50ml in D1 solution before it was centrifuged in a Sorvall RT6000B centrifuge (Dupont Limited, Stevenage, UK) at 300g for 15 minutes at 4°C with the brake off. The supernatant was removed by pipette and the pellet was fully resuspended in a further 50ml of D1 solution. Prior to recentrifugation, a sample of this suspension was diluted in 0.1% trypan blue/1X phosphate-buffered saline (PBS; 1.84mM KH₂PO₄, 10mM K₂HPO₄·3H₂O, 154mM NaCl, pH 7.2-7.4) to enable total viable cells to be counted using trypan blue exclusion. Once centrifuged the supernatant was removed and the pellet was resuspended in an appropriate volume of complete serum-free DMEM to give a cell suspension of 10⁷ viable cells per ml. Then, 4ml aliquots (4x10⁷ cells) of standard cultures were seeded into 25ml De Long flasks (Aimer Products, Enfield, UK) and a 19mm diameter Clark Fin culture cap (Merck Limited, Lutterworth, UK) was placed onto each flask to reduce the risk of contamination. The cultures were incubated at 37°C in a humidified atmosphere of 10% CO₂/90% air (Heraeus Incubator, Philip Harris Scientific, Park Royal, UK) under constant rotation (Kuhner Shaker, Philip Harris Scientific, Park
Royal, UK). A proportion of this telencephalon cell suspension was kept on ice to await macrophage enrichment. This is termed day in vitro zero (DIV=0). The rotations per minute (rpm) were increased gradually from 68 to 80rpm over a period of 5 days to ensure optimal cell aggregation.

2.1.4 Macrophage enrichment of aggregate cultures

Peritoneal macrophages were obtained from 10 to 12 week old Sprague-Dawley rats by washing the peritoneal cavity with 50ml of ice cold EBSS-Ca/Mg. The cells were centrifuged for 10 minutes, 300g, 4°C before they were resuspended in 50ml EBSS-Ca/Mg and spun again as above. Cell viability was assessed using trypan blue exclusion, as already described, before they were resuspended in EBSS-Ca/Mg and centrifuged for a final time. Once completed the pellet was resuspended in 2ml of serum-free complete DMEM and an appropriate volume of the macrophage cell suspension was added to the remaining telencephalon cell suspension to achieve 5 or 10% macrophage enrichment i.e. a concentration of 10⁶ cells per ml for 10% macrophage enrichment. Then, 4ml aliquots of this macrophage-enriched telencephalon cell suspension were seeded as described above.

2.1.5 Culture maintenance

On DIV=2 the aggregates were transferred to 50ml De Long flasks (Aimer Products, Enfield, UK) already containing 4ml of pre-warmed complete serum-free DMEM making a total volume of 8ml per flask. Without disturbing the aggregates on DIV=5, 8, 11, 14 and every other day thereafter 5ml of the complete DMEM was replenished with fresh pre-warmed medium.
On DIV=21, the cultures were split into two by gently agitating the aggregates before transferring 4ml from each flask to a fresh 50ml De Long flask already containing 4ml of pre-warmed medium. 4ml of pre-warmed medium was then added to the original flasks maintaining a volume of 8ml per flask. By taking these measures to meet the nutritional needs of the cultures the aggregates can be maintained for up to DIV=48.

2.1.6 Collection of macrophage-conditioned medium and addition to aggregate cultures

Peritoneal macrophages from 10 to 12 week old Sprague-Dawley rats were prepared as described in section 2.1.4. Once cell viability had been assessed and the cells had been centrifuged for a final time the pellet was resuspended in the appropriate volume of serum-free complete DMEM containing 10% foetal calf serum to achieve a cell concentration of $10^6$ cells per ml. Then, up to 30ml of this macrophage cell suspension was transferred to each sterile 80cm² tissue culture flask (Life Technologies Limited, Paisley, UK) and the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂/90% air (Heraeus Incubator, Philip Harris Scientific, Park Royal, UK). One day later the medium was replenished with fresh pre-warmed serum-free complete DMEM containing 10% foetal calf serum. On DIV=4 the medium was exchanged for pre-warmed serum-free complete DMEM with a further medium change 24 hours later. On DIV=8 the macrophage-conditioned medium (MCM) was collected and filter sterilised before it was aliquoted and frozen at -20°C. MCM was collected in the same manner every 3 days as desired. In some instances lipopolysaccharide (LPS) (1μg/ml; Sigma-Aldrich Company Limited, Poole, UK) was added to the serum-free complete DMEM prior to its addition to the macrophages for a period of 24 hours after which time the treatment was washed out and replaced with serum-
free complete DMEM. This medium was left for a further 24 hours before it was collected, filter sterilised and stored as above until required to add to aggregate cultures.

On pre-determined days during the culture period the MCM was thawed and diluted 1:1 with serum-free complete DMEM. The mixture was filter sterilised and pre-warmed to 37°C before it was exchanged for 6ml of the complete DMEM. The MCM was replenished with fresh pre-warmed MCM every other day and aggregate samples were removed for harvesting and further analysis as desired.

2.2 Demyelinating treatment of aggregate cultures

Aggregate cultures were treated with the demyelinating cytokines interleukin-1 alpha (IL-1α), interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) or anti-myelin oligodendrocyte glycoprotein (anti-MOG) antibodies.

2.2.1 Addition of cytokines to the cultures

Stock solutions of the following cytokines were aliquoted and stored as described to avoid repeated freeze-thaw cycles. Rat recombinant IFN-γ (10^6 U/ml in sterile distilled water; Life Technologies Limited, Paisley, UK) and murine recombinant TNF-α (4x10^7 U/ml in sterile PBS; Life Technologies Limited, Paisley, UK) were stored at -20°C. Recombinant mouse IL-1α (2x10^5 U/ml in 0.1% bovine serum albumin (BSA)/PBS; Genzyme, Kent, UK) was stored at -70°C.

Aggregate cultures were exposed to the demyelinating cytokines IFN-γ (25 and 50U/ml), IL-1α (200U/ml) and TNF-α (5 and 25ng/ml) for a period of four days usually on DIV=25 and again on DIV=27 or one week later on DIV=32 and DIV=34 corresponding to
times when myelin accumulation is high. Dose ranges were chosen on the basis of previous
*in vitro* cell studies. Stock solutions of all the cytokines were diluted under sterile
conditions to 16 times their final concentration in complete serum-free DMEM solution
containing filter sterilised 1.6% BSA which stabilises the cytokines (Sigma-Aldrich
Company Limited, Poole, UK). All solutions were filter sterilised prior to adding them to
the cultures. Then, 5ml of medium was removed from each flask and replaced with 4.5ml
of pre-warmed complete DMEM and 0.5ml of the desired cytokine. Addition of 0.5ml of
the 16 times cytokine solution containing 1.6% BSA into a total flask volume of 8ml results
in the desired final concentration of cytokine and 0.1% BSA. Control cultures received
either medium alone or medium plus 0.1% BSA.

2.2.2 Addition of myelin oligodendrocyte glycoprotein antibody to the cultures

Aggregate cultures were exposed to two different clones of the monoclonal
antibody directed against MOG which is located at the surface of the CNS myelin sheath.
An IgG1, kappa (IgG1,κ) mouse monoclonal antibody derived from clone 8-18C5
(Linington *et al.*, 1984) was used as was the IgG2a, kappa (IgG2a,κ) MOG specific
(5mg/ml) were aliquoted and stored at -20°C while the Z12 monoclonal antibody
(880μg/ml) was stored in 1ml aliquots at -70°C. Both of the antibodies were added to the
culture in the presence of complement (guinea pig serum; Serotec, Oxford, UK) which was
used fresh each time, frozen at -20°C immediately on arrival and not re-used.

Control flasks received the corresponding purified immunoglobulin mouse IgG1,κ
or IgG2a,κ (Sigma-Aldrich Company Limited, Poole, UK) in the presence of complement
or complement alone. Prior to their use the purified mouse immunoglobulins IgG1,κ and IgG2a,κ were dialysed with 1X PBS at 4°C to remove azide ions.

Antibody treatments were added to the cultures on DIV=34 for a period of forty eight hours. On the day of treatment aliquots of the desired antibody and respective controls were thawed under sterile conditions. The anti-MOG antibodies were made up to the desired concentration in sterile PBS before all solutions were filter sterilised. From each flask 5ml of complete DMEM was removed and replaced with 4.5ml of pre-equilibrated medium. Then 250μl of anti-MOG or corresponding purified immunoglobulin and 200μl of guinea pig serum were added as desired to give final concentrations of 31.3μg/ml antibody and 25μl/ml of guinea pig serum. Further control flasks received guinea pig serum or medium alone.

Complement, in the form of guinea pig serum, was heat inactivated for 30 minutes at 56°C on a shaker prior to adding it to the aggregate cultures to determine the effect of antibody addition in the absence of complement. The solution was filter sterilised before it was added to the cultures as desired.

2.2.3 Removal of any treatment and further maintenance of cultures

Aggregate cell cultures were treated with cytokines for a period of four days, while treatment with anti-MOG lasted for forty eight hours. After these times the cytokine or antibody was diluted out and the cultures were maintained until the desired day, usually DIV=48.

To dilute out any treatment 4ml of medium per flask was exchanged with fresh pre-warmed complete serum-free DMEM. Six hours later a further 6ml of medium was
exchanged for fresh pre-warmed serum-free DMEM and this procedure was repeated again the following day. Thereafter, 5ml of medium was exchanged every other day up to DIV=48 when the aggregates were harvested.

2.3 Harvesting the cultures

Samples of aggregate cultures were removed from flasks and harvested for subsequent biochemical, morphological or molecular biological analyses at desired time points throughout the culture period. The days on which harvesting occurred varied according to individual experimental design.

Aggregate samples destined for time course studies were taken throughout the culture period, from as early as DIV=1, up to and including DIV=48. Samples of cultures used in demyelination experiments were usually harvested immediately prior to addition of treatment and again at the end of the treatment period. These aggregates were also sampled a further one or two times before the end of the culture such that if the demyelinating treatment was administered between DIV=34 and DIV=36 aggregate samples would be harvested immediately prior to addition on DIV=34 and then again on DIV=36, before the treatment was washed out. Further sampling could occur on DIV=39 and DIV=48.

Every effort was made to ensure that harvesting coincided with aggregate feeding to minimalise disturbance to the aggregates. Similarly, while enough aggregate material was harvested for the various analyses it was ensured that the program was not too exhaustive to disrupt the microenvironment of the aggregates. The harvesting
protocol was modified according to whether biochemical, immunocytochemical, electron microscopy or molecular biology samples were required.

2.3.1 Harvesting procedure for biochemical analyses

The harvesting procedure was performed on ice. Aggregates were gently agitated using a sterile 5ml pipette (Sarstedt Limited, Leicester, UK) to ensure that they were evenly distributed throughout the culture medium. The desired volume of complete DMEM containing aggregates was then transferred to sterile 10ml conical based tubes (Fisher Scientific UK, Loughborough, UK). The aggregates were allowed to settle before the medium was aspirated off and the undisturbed aggregate pellet was washed twice in 4 to 5ml of cold 1X PBS. Then the aggregates were transferred in a small amount of PBS to 1.5ml microcentrifuge tubes where, once they had settled, as much PBS as possible was aspirated off and the tubes were frozen on dry ice for 30 minutes before they were stored at -70°C.

2.3.2 Harvesting procedure for mRNA analyses

The aggregate cultures were harvested as described above, however, precautions were taken to minimise RNA degradation including the use of gloves and RNase free equipment throughout the procedure. The aggregates were washed once only in RNase free 1X PBS treated with diethyl pyrocarbonate (DEPC; Sigma-Aldrich Company Limited, Poole, UK). They were then transferred in a small amount of PBS to RNase free 1.5ml microcentrifuge tubes. After the aggregates had settled and as much PBS as possible
was aspirated off the aggregates were snap frozen in liquid nitrogen prior to being placed on dry ice for 30 minutes before they were stored at -70°C.

2.3.3 Harvesting procedure for immunocytochemical analysis

The harvesting of aggregates for immunocytochemical staining was performed at room temperature. Aggregates were transferred to sterile 10ml conical based tubes and allowed to settle before the medium was aspirated off. The undisturbed aggregate pellets were then washed twice in 4 to 5ml of 1X PBS at 20°C before being transferred in a small amount of PBS to one half of a gelatin capsule (Capsugel, South Carolina, US). Once the aggregates had settled under gravity as much PBS as possible was pipetted off before the capsule became too soft. The gelatin capsule was then gently filled with O.C.T compound (Merck Limited, Lutterworth, UK) without dispersing the aggregate pellet and snap frozen in isopentane cooled on liquid nitrogen to preserve cell integrity before they were stored immediately at -70°C.

2.3.4 Harvesting procedure for electron microscopy analysis

The protocol for harvesting aggregates for electron microscopy was as described above for immunocytochemistry. However, once the aggregates have been washed twice in 1X PBS at 20°C they were fixed at 4°C for at least 2 hours in 3% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4 before being transferred to the electron microscopy unit where they were processed further.
2.3.5 Harvesting procedure for determination of aggregate diameter

The protocol for harvesting aggregates to assess their diameters was as described above for electron microscopy, however, the aggregates were fixed overnight at 4°C in 3% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4. The supernatant containing some aggregates was removed and placed into a fresh 10ml tube before both the pellet and supernatant containing tubes were gently centrifuged at 100g for 2 minutes at 4°C to pellet the aggregates. The glutaraldehyde was aspirated off and the aggregates were washed in cold 1X PBS before being transferred to Dr N.A. Gregson (UMDS, London, UK) where their diameters were assessed.

2.4 Homogenisation of aggregate cultures

Aggregate samples were thawed on ice before 500µl of homogenisation buffer (2mM K$_2$HPO$_4$.3H$_2$O, 1mM EDTA-Na$_2$ adjusted to pH 6.8 using 2mM KH$_2$PO$_4$, 1mM EDTA-Na$_2$) was added to each tube. The aggregates were homogenised using a high intensity ultrasonic processor (Jencons Scientific Limited, Leighton Buzzard, UK) for 10 seconds at 3.7 watts and then sonicated for 10 seconds at 3.2 watts. Homogenised samples were placed immediately on ice before a further 500µl of homogenisation buffer was added to them. Homogenates were vigorously vortex mixed before being aliquoted as follows to avoid repeated freeze-thaw cycles. For future use in MBP radioimmunoassays (RIAs) 200µl of homogenate was transferred to two 1.5ml microcentrifuge tubes while 40µl of homogenate was added to both 1000µl of 0.05M NaOH and 80µl of 1% Triton-X-100 (Sigma-Aldrich Company Limited, Poole, UK) for total protein concentration and 2',3'-
Cyclic nucleotide 3'-phosphodiesterase assays respectively. All aliquoted homogenate samples were stored at -70°C until they were assayed.

2.5 Measurement of total protein concentration

The total protein concentration of aggregate homogenates was assessed using the Folin phenol method (Lowry et al., 1951) which is based on a two step colour reaction. In an alkali solution copper associates with protein and subsequently reduces the Folin phenol reagent (phosphomolybdic-phosphotungstic) to form a coloured product. To maximise the colour resulting from the reduction sodium hydroxide is present to neutralise any acid released on addition of the Folins solution and sodium carbonate is added to buffer the mixture to an optimal pH 10. The optical density which is read at 750nm is proportional to the copper bound protein.

All standards and samples were assayed in duplicate. Standard tubes were prepared using 0.2mg/ml BSA (Sigma-Aldrich Company Limited, Poole, UK) as shown in Table 2.2.

<table>
<thead>
<tr>
<th>Total BSA (µg)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water (µl)</td>
<td>150</td>
<td>125</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>BSA (0.2mg/ml) (µl)</td>
<td>0</td>
<td>25</td>
<td>50</td>
<td>75</td>
<td>100</td>
<td>150</td>
</tr>
</tbody>
</table>

Table 2.2. Preparation of standards for a protein assay.

Samples (40µl homogenate plus 1000µl 0.05M NaOH) were thawed on ice and vortex mixed vigorously before transferring 50µl to a 10ml tube (Fisher Scientific UK,
Loughborough, UK) already containing 100µl of distilled water. Then, 50µl of 0.4M NaOH was added to all of the tubes followed by 1ml of freshly prepared solution X (X = 2% Na₂CO₃, 0.02% K₂ tartrate in 0.1M NaOH added to 1% CuSO₄·5H₂O in a ratio of 99:1). All tubes were vortex mixed and left for a total of 15 minutes beginning from when solution X was added to the first standard tube. After this time, 100µl of Folin and Ciocalteau's reagent (diluted 1:1 with distilled water; Merck Limited, Lutterworth, UK) was added and once the tubes had been vortex mixed they were incubated in the dark for 30 minutes before an ultrospec 2000 ultra-violet/visible spectrophotometer (Amersham Pharmacia Biotech UK Limited, Little Chalfont, UK) was used to read the absorbance of each sample at 750nm. A standard curve (0-20µg BSA) was generated to convert optical density absorbance readings into µg of protein per sample.

Then:

Protein concentration in homogenate (µg/µl or mg/ml) = \frac{M \times D}{V}

where, M is the mean µg protein/sample, D is the dilution of homogenate in 0.05M NaOH (e.g. 40µl homogenate plus 1000µl NaOH, D = 26) and V is the volume of sample used per assay (e.g. 50µl).

2.6 Measurement of myelin basic protein content by radioimmunoassay

Myelin basic protein (MBP) content correlates with the extent of myelination in the CNS as assessed by electron microscopy (Shine et al., 1992). MBP content was therefore assessed using a RIA which provides an accurate means of evaluating the effect of various
treatments on myelination and demyelination in aggregate cultures. Peptides resulting from MBP degradation were measured in the cultures using a modified RIA.

2.6.1 Principle of the radioimmunoassay

A RIA is a limited reagent assay which provides a quantitative measure of ligand e.g. MBP, using a specific antibody exhibiting high affinity for that ligand. By binding a fixed concentration of antibody to a fixed concentration of radiolabelled ligand, in the presence of different amounts of unlabelled ligand a saturable system is obtained whereby the labelled and unlabelled ligand compete for the limited amount of available antibody. Once equilibrium has been reached there should be no further net change between bound and free fractions so they can be separated and the radioactivity counted.

2.6.2 Radiolabelling the myelin basic protein

$^{125}$I was used to radiolabel the purified human MBP (supplied by N. Groome, Oxford Brookes University, Oxford, UK) using Iodogen Iodination Reagent (Pierce and Warriner UK Limited, Chester, UK). Iodine needs to be in its oxidised reactive form to be successfully substituted into aromatic ring structures such as tyrosine. Sodium $^{125}$Iodide can be enzymatically or chemically oxidised into reactive $^{125}$Iodine by various methods including lactoperoxidase (Thorell, 1972), chloramine T (Hunter & Greenwood, 1964), and Iodogen Iodinating Reagent (Fraker & Speck, 1978). Conjugation labelling techniques (Bolton & Hunter, 1973), where an iodinated carrier is covalently coupled to the amino groups of the antigen, can be used as an alternative.
Prior to iodination Iodogen Iodinating Reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril), a water insoluble oxidising agent, was coated onto an iodination vessel providing a reproducible, gentle and simple technique for the iodination of proteins and peptides without interfering with the antigenic binding of the antibody (Figure 2.1).

\[
\text{Sodium Iodide} \xrightarrow{\text{Chemical Oxidation, e.g. Iodogen}} \text{Iodine} + \text{rest of protein} \xrightarrow{\text{iodine incorporates into tyrosine amino acid side-chain}} \text{iodine incorporated into tyrosine amino acid side-chain}
\]

Figure 2.1. Radioiodination of proteins by oxidation of sodium $^{125}$Iodide in the presence of Iodogen Iodinating Reagent and subsequent substitution of iodine into tyrosine aromatic side-chains.

2.6.2.1 Preparation of Iodogen-coated tubes

A 2mg/ml solution of Iodogen was diluted in the organic solvent chloroform. In a fume cupboard 20μl of this solution was added to clean 5ml Pyrex glass test tubes (Bibby Sterilin Limited, Stone, UK) and then allowed to evaporate slowly overnight leaving a
coating of Iodogen at the bottom of each tube. One tube is required as a reaction vessel for each iodination so the remaining Iodogen-coated tubes were stored indefinitely in a desiccator at room temperature.

2.6.2.2 Iodination of MBP

Purified human MBP (1mg/ml in 0.05M sodium phosphate buffer, pH 7) was aliquoted and stored at -20°C to avoid multiple freeze-thaw cycles. An aliquot of this MBP was thawed and 10μl was added to an Iodogen-coated tube already containing 10μl of 1M sodium phosphate buffer, pH 7.5. In the fume cupboard of a room designated for radioactive work, 5μl of sodium ¹²⁵Iodide (approximately 18.5MBq) in dilute sodium hydroxide, pH 7-11 (74MBq/20μl; Amersham Pharmacia Biotech UK Limited, Little Chalfont, UK) was added to the tube. After tapping gently the tube was left for 30 minutes at room temperature.

Meanwhile a PD10 column containing Sephadex G-25 M swollen in distilled water containing 0.15% Kathon CG/ICP Biocide (Amersham Pharmacia Biotech UK Limited, Little Chalfont, UK) was equilibrated with 0.01M HCl containing 0.5mg/ml calf thymus histones type II-S (positively charged; Sigma-Aldrich Company Limited, Poole, UK).

The iodination was stopped by adding 0.5ml of 1M sodium phosphate buffer, pH 7.5 containing tyrosine (1mg/ml) and 1mg of blue dextran to the reaction vessel. The contents of the tube were then pipetted on to the Sephadex column to enable the bound label to be collected. Sufficient time was allowed to enable the contents to run into the column before adding 2ml of 0.01M HCl containing 0.5mg/ml calf thymus histones. The blue coloured fraction containing the iodinated MBP was collected in a screw top plastic
bijou vial and stored in a lead pot at 4°C until required. To ascertain the success of the iodination 5 and 10μl aliquots of the radiolabelled $^{125}$I-MBP were added to LP3 tubes (Elkay Laboratory Products Limited, Basingstoke, UK) and the activity in counts per minute (cpm) was determined using an LKB-Wallac 1275 mini gamma counter (EG & G Wallac, Milton Keynes, UK). The iodinated MBP was used in RIAs at 20,000cpm per 50μl for 3 to 4 weeks since $^{125}$Iodine has a half life of 60 days.

2.6.3 Titration of anti-myelin basic protein antibody

The antibody is another critical component of a RIA and its specificity, titre and avidity need to be considered. The polyclonal antibody used (donated by N. Groome, Oxford Brookes University, Oxford, UK) was raised in rabbit and is directed against a fifteen amino acid (64-78) peptide sequence of human MBP. Specificity of the antibody is important and this peptide sequence was chosen due to the homology exhibited between human and rat in this region (Figure 2.2). The antibody which was supplied in 0.1% azide was aliquoted and stored at -20°C with a working sample kept at 4°C.

The titre of the antibody is the concentration that binds to a given percentage (usually 40-60%) of the radiolabelled ligand, however, this percentage often varies with the experimental conditions used in the assay. In this case an antibody titration was performed to determine the concentration of antibody needed to bind 20-30% of the radiolabelled MBP. Initially all the dilutions made during the titration were performed in two buffers
Alanine Arginine Threonine Alanine Histidine Tyrosine Glycine Serine Leucine Proline Glutamine Lysine Serine Histidine Glycine

Human  64 A  R  T  A  H  Y  G  S  L  P  Q  K  S  H  G -  78

Rat  T  R  T  T  H  Y  G  S  L  P  Q  K  S  Q  -

Threonine Arginine Threonine Threonine Histidine Tyrosine Glycine Serine Leucine Proline Glutamine Lysine Serine Glutamine -

Figure 2.2. MBP amino acid peptide sequences from residue 64 to 78 illustrating homology between rat and human species in this region. The amino acid residue number corresponds to the position in the 18.5kDa human MBP protein sequence. Residues that do not correspond are shown in red while those that are deleted are depicted by a (-).
containing alternative detergents. RIA buffer 1 was prepared using 0.05M sodium phosphate buffer, pH 7, containing 1.2% w/v sodium chloride, 0.5mg/ml calf thymus histones type II-S and 0.05% w/v Tween 80 (polyoxyethylenesorbitan monooleate; Sigma-Aldrich Company Limited, Poole, UK), while in RIA buffer 2 Tween 80 was replaced with 0.05% w/v hexadecyltrimethyl ammonium bromide (CTMB; Sigma-Aldrich Company Limited, Poole, UK). With Tween 80 as a constituent of the RIA buffer it was necessary to boil the samples to ensure complete solubilisation of MBP while no additional boiling was necessary when CTMB was used as a possible alternative.

A series of doubling dilutions of anti-MBP antiserum (anti-MBP) or control rabbit serum were prepared in separate 1.5ml microcentrifuge tubes on ice covering a range from 1/1000 to 1/32,000 using either the CTMB or Tween 80 containing buffers. In duplicate, 50μl of each antibody dilution was transferred to LP3 tubes. The stock \(^{125}\text{I}\)-labelled MBP was diluted in both RIA buffers to give approximately 20,000cpm per 50μl and this amount was added to each tube. A non-specific binding (NSB) tube (to determine the percentage counts in the absence of antibody) containing 50μl \(^{125}\text{I}\)-MBP and 50μl RIA buffer and a total (T) tube containing 50μl \(^{125}\text{I}\)-MBP alone (to measure total radioactive counts) were also prepared in duplicate before each tube was vortex mixed and incubated at 4°C overnight to allow the components to reach equilibrium.

The final stage of the RIA involves the separation of bound and free ligand and many procedures have been devised to achieve this, including adsorption of free ligand, fractional precipitation of bound fraction and the use of second antibodies. After the
overnight incubation the Sac-Cel method (IDS Ltd, Tyne and Wear, UK) of separation was used. This involves the use of a second anti-rabbit antibody covalently coupled to a cellulose suspension (in 0.02M sodium phosphate, 0.01M EDTA, 0.145M sodium chloride, 0.1% w/v sodium azide, 0.5% w/v BSA, 0.05% v/v Tween 20, pH 7.4) which will bind to the primary anti-MBP antibody to form a complex which can be precipitated. The coupling of a second antibody to a solid phase eg. cellulose enhances the specificity of separation (Figure 2.3).

**Figure 2.3.** Separation technique involving the use of a secondary antibody bound to a solid phase.
The Sac-Cel was kept in suspension by mixing continuously on a magnetic stirrer while 100μl was added to each assay tube except the totals. The tubes were vortex mixed and left at room temperature for 30 minutes during which time the cellulose began to settle. Then, 1ml of distilled water was added to all assay tubes, except the totals before they were centrifuged in a Sorvall RT6000B centrifuge for 2 minutes, 18°C, 1000g. The supernatant was discarded and the ¹²⁵I-MBP radioactivity (cpm) retained in the bound fraction within the pellets was counted as were the total tubes using an LKB-Wallac 1275 mini gamma counter.

2.6.3.1 Calculation of results

The values (cpm) obtained for each duplicate sample were meaned and the results were calculated as follows:

For the blank: \[ NSB = \frac{P}{T} \times 100 \]

where, \( P \) is counts per minute in pellet and \( T \) is counts per minute in total. NSB is non-specific binding expressed as a percentage of total counts per minute

For the sample: \[ X = \left( \frac{P}{T} \times 100 \right) - NSB \]

where \( X \) is expressed as percentage counts in pellet bound specifically

A graph was plotted of percentage counts in pellet against log concentration anti-MBP/rabbit serum and this was used to determine the antibody dilution required to bind 20-30% of total counts. An antibody dilution of 1/3000 was selected for use in all future RIAs when using either Tween 80 or CTMB buffers (Figure 2.4).
Figure 2.4. Antibody titration curve. Values were obtained using \((P/T \times 100) - \text{NSB}\). NSB values were obtained using \((P/T \times 100)\). NSB = 10.5 for Tween 80 buffer and 5.1 for CTMB buffer.
2.6.4 Determining the range of concentrations necessary to produce a standard curve

A standard curve is generated in each assay and should cover a range of points from maximum binding ($B_0$) in the absence of unlabelled ligand to NSB. RIA buffer was prepared using Tween 80 and CTMB and all dilutions were performed in both buffers. An aliquot of stock purified human MBP was thawed on ice and then diluted in 1.5ml microcentrifuge tubes to cover a range of MBP concentrations from 1 to 400ng/ml before 50μl of each concentration was transferred to duplicate LP3 tubes.

A 1/3000 dilution of anti-MBP antiserum was prepared in both CTMB and Tween 80 buffers and 50μl of this was transferred to the LP3 tubes. NSB tubes containing 100μl buffer and $B_0$ tubes with 50μl buffer and 50μl antibody were also set up at this stage. All of the tubes were vortex mixed before they were covered and placed at 4°C overnight. After this time 50μl $^{125}$I-MBP with an activity of 20,000cpm was added to all tubes and totals before they were vortex mixed and incubated again overnight at 4°C. Sac-Cel was then used to separate bound and free fractions for counting as described in section 2.6.3.

2.6.4.1 Calculation of results

The percentage counts in the pellet were determined as described for the antibody titration. A standard curve of percentage counts in the pellet against the log concentration of MBP (ng/ml) was plotted for the assays using Tween 80 and CTMB buffers in parallel (Figure 2.5) and the range of concentrations necessary to produce a standard curve was determined as 1 to 100ng of MBP per ml.
Figure 2.5. **Standard curve optimisation.** Values were obtained using \((P/T \times 100) - NSB\). NSB values were obtained using \((P/T \times 100)\). NSB = 7.57 for Tween 80 buffer and 4.35 for CTMB buffer.
2.6.5 Radioimmunoassay for myelin basic protein

A RIA for MBP in aggregate culture homogenates was established by optimising the working conditions of the assay including the buffer constituents and the length and temperature of incubations. The various stages involved in the optimisation of this procedure are detailed in the following sections.

2.6.5.1 Optimisation of the buffer detergents

RIA buffer containing Tween 80 or CTMB detergents was prepared as described in section 2.6.3. An aliquot of stock purified human MBP was thawed on ice and then diluted in 1.5ml microcentrifuge tubes to cover a range of concentrations from 1 to 100ng MBP/ml.

Aggregate cell culture homogenates thawed on ice were sonicated at 2.7 watts for ten seconds using a high intensity Ultrasonic Processor. They were then vortex mixed and diluted in 1.5ml microcentrifuge tubes to concentrations that would fall within the working range of the standard curve.

Samples diluted in Tween 80 buffer were boiled for 10 minutes to destroy proteases and release MBP peptides before they were microcentrifuged for 2 minutes. Pre-treatment of samples diluted in CTMB buffer was not necessary. Each duplicate LP3 tube then received 50μl of either standard or sample along with 50μl of anti-MBP antibody already diluted to 1/3000. B₀ and NSB tubes were also set up at this stage (Table 2.3). All of the tubes were vortex mixed and left to incubate overnight at 4°C.
Maximum binding ($B_0$)  | 50μl buffer | 50μl anti-MBP antibody | 50μl $^{125}$I-MBP  
Non-specific binding (NSB) | 50μl buffer | 50μl buffer | 50μl $^{125}$I-MBP  
Totals (T) | - | - | 50μl $^{125}$I-MBP  
Standards | 50μl standard | 50μl anti-MBP antibody | 50μl $^{125}$I-MBP  
Samples | 50μl sample | 50μl anti-MBP antibody | 50μl $^{125}$I-MBP  

Table 2.3. Tubes necessary for a RIA. All tubes were prepared using Tween 80 and CTMB buffers in parallel assays.

After this time the stock $^{125}$I-MBP was diluted in both RIA buffers to give 20,000 cpm per 50μl before this amount was added to each tube and a set of totals (Table 2.3). All of the tubes were then vortex mixed before they were incubated overnight at 4°C. After this incubation the bound and free fractions were separated using the Sac-Cel method as already described. The supernatant was discarded and the pellets and totals were counted.

2.6.5.1.1 Calculation of results

The percentage counts in the pellet were determined as described for the antibody titration. A standard curve of percentage counts in the pellet against the log concentration of MBP (ng/ml) was plotted for the assays using Tween 80 and CTMB buffers in parallel. The results were also analysed using the computer program RIA Calc LM version 2.25 (EG & G Wallac, Milton Keynes, UK) which converts the cpm values to concentration of MBP (ng/ml).
The MBP content of the homogenates can be expressed further as μg MBP/mg protein:

So:  \[ \text{μg MBP/mg protein} = \frac{\text{MBP (ng/ml)} \times \text{dilution factor}}{\text{protein content (mg/ml)}} \]

(mg/ml of protein values were obtained by the Lowry method discussed in section 2.5.1).

The assay was successful using both Tween and CTMB buffers, therefore, since boiling of the samples can be eliminated when CTMB is a constituent of the buffer it was used routinely for all further assays.

2.6.5.2 Optimising the incubation stages of the assay

A series of experiments were performed to determine whether the RIA would continue to provide valid results if the incubation times were reduced. RIA buffer containing 0.05% CTMB was prepared as already described and used to dilute MBP to concentrations ranging from 1-100ng/ml for preparation of the standard curve. Samples were thawed on ice, sonicated and diluted as described in section 2.6.5.1. The antibody was diluted to 1/3000 and the \(^{125}\text{I}-\text{MBP}\) was diluted to give a specific activity of 20,000cpm per 50μl. Three variations of the initial RIA method were followed, as shown in Table 2.4.
<table>
<thead>
<tr>
<th>RIA Method</th>
<th>a.m.</th>
<th>p.m.</th>
<th>DAY</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50μl antibody + 50μl standard/sample</td>
<td>incubate overnight 4°C</td>
<td>50μl ¹²⁵I-MBP</td>
<td>incubate overnight 4°C</td>
<td>Sac-Cel separation &amp; count</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>50μl antibody + 50μl standard/sample</td>
<td>incubate 4 hours 37°C</td>
<td>50μl ¹²⁵I-MBP</td>
<td>incubate overnight 4°C</td>
<td>Sac-Cel separation &amp; count</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>50μl antibody + 50μl standard/sample + 50μl ¹²⁵I-MBP</td>
<td>incubate overnight 4°C</td>
<td>Sac-Cel separation &amp; count</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4. Summary of the three alternative methods used to optimise the RIA procedure.
RIA method A describes the methodology used up until this point, that is, a 3 day experiment whereby the antibody and standard/samples are pre-incubated overnight at 4°C before $^{125}\text{I}}$-MBP is added and finally after a further overnight incubation at 4°C the Sac-Cel method of separation is employed. Method B reduced the assay to 2 days by pre-incubating the antibody and standard/samples at 37°C in a water bath for 4 hours prior to addition of $^{125}\text{I}}$-MBP and method C also involved a 2 day assay by adding the $^{125}\text{I}}$-MBP with the antibody and standard/samples prior to the overnight incubation at 4°C and separation with Sac-Cel.

2.6.5.2.1 Calculation of results

The results were calculated as described previously and Figure 2.6 shows the graph obtained of percentage specific counts in sample pellet against concentration MBP (ng/ml) for each of the three methods. It was observed that the percentage binding of labelled MBP was lower when the antibody and unlabelled MBP were left overnight at 4°C compared to when the pre-incubation time for the antibody and unlabelled MBP was reduced by adding the labelled MBP after a 4 hour incubation at 37°C or when all three components were added at the same time. The percentage counts in the pellet when labelled and unlabelled MBP were added simultaneously was higher at all time points compared with adding labelled MBP after a 4 hour incubation at 37°C, however, the difference was slight.

The same aggregate samples were assayed using each of the 3 alternative RIA methods and for a given sample the concentration of MBP present per mg of protein...
Figure 2.6. The three standard curves obtained when using three alternative RIA methods. The letters A, B and C refer to the methods described in Table 2.4.
was similar. Since it is important to consistently use the same assay method to minimise inter-experimental error it was decided to pre-incubate the antibody and unlabelled MBP at 37°C for approximately 4 hours before labelled MBP was added in all future RIAs. The 3 components were then incubated overnight at 4°C before Sac-Cel was used to separate the bound and free fractions. This method has the advantage of reducing the assay to 2 days while optimising the binding of unlabelled MBP to the antibody.

2.6.6 Determining the avidity of the antibody using Scatchard analysis

The avidity of the anti-MBP antibody was determined using Scatchard analysis. The association constant \( K_{\text{ass}} \) is an expression for the avidity of the binding of an antibody to its ligand at equilibrium with the reaction represented by:

\[
K_{\text{ass}} \text{ (l/mol)} = \frac{\text{[AbL]}}{\text{[Ab][L]}}
\]

where \([\text{AbL}]\) is the concentration of the bound complex and \([\text{Ab}], [\text{L}]\) represents the concentration of free antibody and ligand respectively.

Given a constant amount of antibody the ratio of bound to free ligand \((B/F)\) at equilibrium is quantitatively related to the concentration of bound ligand (moles/litre) and this can be plotted according to the method of Scatchard to generate a straight line. The \( K_{\text{ass}} \) value can be determined from the slope of the line (slope equals \(-K_{\text{ass}}\)) while the intercept with the \( X \) axis represents the absolute concentration of the antibody. \( K_{\text{ass}} \)
values for antibodies generally range from $1 \times 10^7$ l/mol (low affinity) to $1 \times 10^{12}$ l/mol (high affinity) where the higher the avidity the more ligand will bind.

RIA buffer containing CTMB was prepared and used for all necessary dilutions. Stock MBP (1mg/ml) was initially diluted in 1.5ml microcentrifuge tubes to generate a standard curve with concentrations ranging from 1-100ng/ml. In duplicate, 50µl of each standard was transferred to an LP3 tube before 50µl of the anti-MBP antibody (1/3000) was added. Four sets of $B_0$ tubes containing 50µl of antibody and 50µl of RIA buffer were prepared as were four duplicate NSB tubes containing 100µl of RIA buffer alone. All tubes were covered and placed in a water bath at 37°C for 4 hours. After this time, 20,000cpm $^{125}$I-MBP per 50µl was added to all of the standards, and one set of NSB, $B_0$ and total tubes. The remaining three sets of NSB, $B_0$ and total tubes received 50,000, 250,000 and 500,000cpm $^{125}$I-MBP in 50µl. Each LP3 tube was vortex mixed and incubated overnight at 4°C before Sac-Cel was used to separate the bound and free fractions for counting.

2.6.6.1 Calculation of results

All duplicate values were meaned and the percentage counts in the pellets were determined as described in section 2.6.3.1. A standard curve of percentage specific counts in the pellet against concentration of MBP (ng/ml) was plotted and used to convert the specific percentage counts in the pellet of the $B_0$ tubes (50,000, 250,000 and 500,000cpm) to concentrations of MBP (ng/ml). Choosing one of the $B_0$ values which fell within the working range of the curve enabled the amount of MBP added (ng) equivalent to 20,000cpm to be calculated as follows:
Convert: \[
\text{total cpm/50\mu l to total (T) cpm/ml}
\] where cpm/50\mu l is the actual total read from the gamma counter.

Then: \[
T \text{ (cpm/ml)} = N \text{ (ng MBP/ml)} \quad (1)
\] where, \(N\) is the concentration of MBP (ng/ml) in the labelled fraction of the chosen \(B_0\) read from the standard curve.

Now: \[
T \text{ (cpm/ml)} \text{ can be converted to } \mu \text{Ci, using }
\]
\[
1 \mu \text{Ci} = 1.76 \times 10^6 \text{ cpm} \quad (2)
\]
So: \[
X \text{ (\mu Ci)} = N \text{ (ng MBP)}
\]

If \(Y\) is expressed as the number of \(\mu \text{Ci}\) present in 1\mu g of MBP then:
\[
Y \text{ (\mu Ci/\mu g)} = \frac{X \text{ (\mu Ci)}}{N \text{ (ng MBP)} \times 10^{-3}} \quad (3)
\]

Then, to give the \(\mu \text{g MBP}\) present in 20,000\text{cpm}

Determine from (2) that 20,000\text{cpm} = 0.01136\mu \text{Ci}

So: \[
\mu \text{g MBP/20,000cpm} = \frac{0.01136}{Y}
\]

The value obtained for \(\mu \text{g MBP}\) present in 20,000\text{cpm} was substituted into line 2 of Table 2.5 which describes the steps necessary to construct a Scatchard plot of bound/free (B/F) ligand (line 7) against bound ligand (moles/litre; line 6) from which
the affinity constant or avidity of the antibody and the total antibody concentration (moles/litre) can be determined (Figure 2.7).

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>COLD ANTIGEN/TUBE (ng)</td>
</tr>
<tr>
<td>2.</td>
<td>TOTAL ANTIGEN PRESENT (ng)</td>
</tr>
<tr>
<td>3.</td>
<td>MBP BOUND</td>
</tr>
<tr>
<td>4.</td>
<td>MBP BOUND (ng) / TUBE (B)</td>
</tr>
<tr>
<td>5.</td>
<td>MOLES BOUND/TUBE</td>
</tr>
<tr>
<td>6.</td>
<td>MOLES BOUND/LITRE</td>
</tr>
<tr>
<td>7.</td>
<td>BOUND/FREE (B/F)</td>
</tr>
</tbody>
</table>

Table 2.5. The steps necessary to construct a Scatchard plot of B/F against B (moles/litre).

The affinity constant of the anti-MBP antibody was found to be 4.70 x 10^9 litres/mole while the total concentration of antibody within a RIA experiment was 6.54 x 10^{-11} moles/litre.
Figure 2.7. Scatchard plot illustrating the avidity and total concentration of anti-MBP antibody. The affinity constant or avidity is determined from the slope of the line while the intercept with the X-axis is equivalent to the total concentration of antibody.
2.7 Determination of myelin basic protein degradation peptide content within aggregate cultures using a modified radioimmunoassay

The content of MBP degradation peptides within the aggregates was assessed using a modified RIA. A synthetic MBP peptide (34T) (supplied by N. Groome, Oxford Brookes University, Oxford, UK) was used as the standard and radioligand based on the human amino acid MBP sequence 75-89. However, an NH₂ terminal tyrosine was added to allow iodination and 2 amino acid substitutions occurred to restrict antibody recognition to the epitopes 80 to 89 such that a serine residue was switched to a threonine at position 76 and an arginine was replaced with a lysine residue at position 79 (Figure 2.8).

2.7.1 Radiolabelling the 34T peptide fragment

The 34T peptide fragment (0.1mg/ml in 0.05M sodium phosphate buffer, pH 7 supplied by Nigel Groome, Oxford Brookes University, Oxford, UK) was aliquoted and stored at -20°C to avoid multiple freeze-thaw cycles. The 34T peptide was iodinated using the same procedure as described for human MBP except that the iodinated peptide was separated from free iodine using a PD-10 column containing Sephadex G-10 medium rather than Sephadex G-25M.
Figure 2.8. Amino acid sequence of the synthetic peptide 34T compared to human MBP between the residues 75 and 89. A tyrosine residue has been inserted at the amino terminal to aid iodination and two amino acid residues have been substituted, serine has been substituted for threonine at position 76 and arginine is switched to lysine at position 79 (shown in red).
2.7.2 Titration of anti-myelin basic protein peptide antibody

The polyclonal antibody used (donated by N. Groome, Oxford Brookes University, Oxford, UK) was raised in rabbit and directed against a fifteen amino acid (75-89) peptide sequence of human MBP with operational specificity from 80-89 (refer to Figure 2.8). The antiserum detects peptides with a carboxyl terminus of phenylalanine 89, while reacting poorly with peptides extending beyond this residue and negligibly with intact human MBP. The antibody which was supplied in 0.1% azide was aliquoted and stored at -20°C with a working sample kept at 4°C.

An antibody titration was performed to determine the concentration of antibody needed to bind 20-30% of the radiolabelled MBP peptide as described in section 2.6.3. A graph was plotted of percentage specific counts in pellet against log concentration anti-MBP/rabbit serum and this was used to determine that an antibody dilution of 1/4000 bound to 20-30% of total counts with this dilution used in all further assays.

2.7.3 Determining the range of concentrations necessary to produce a standard curve

RIA buffer was prepared using CTMB, as described previously. An aliquot of the synthetic peptide 34T was thawed on ice and then diluted in 1.5ml microcentrifuge tubes to cover a range of concentrations from 0.005 to 200ng/ml. A standard curve of human purified MBP ranging from 1 to 100ng/ml was set up in parallel to assess the specificity of the antibody. Once diluted 50μl of each concentration was transferred to duplicate LP3 tubes before 50μl of anti-MBP peptide antiserum diluted to 1 in 4000 was added. NSB and B₀ tubes were also set up at this stage. All of the tubes were vortex mixed before they were covered and placed at 37°C for 4 hours.
After this time 50μl \( {^{125}}I\)-MBP (20,000cpm) was added to all tubes and totals before they were vortex mixed and incubated overnight at 4°C. Sac-Cel was then used to separate bound and free fractions for counting as already described. A standard curve of percentage counts in the pellet bound specifically against the log concentration of 34T (ng/ml) was plotted as described for the antibody titration. From the graph it was found that MBP did not compete with 34T peptide at any concentration, with values comparable to those of \( B_o \). The range of concentrations necessary to produce a standard curve for 34T was 0.01 to 50ng per ml.

The RIA for 34T was therefore performed as described for MBP except that the antibody was diluted to 1 in 4000 and the standard curve concentrations ranged from 0.01 to 50ng/ml. Aggregate culture samples were diluted as required so that they fell within the working range of the curve and the concentration of 34T (ng/ml) in each sample was determined using the computer program RIA Calc LM. The 34T peptide content of the homogenates were expressed as ng 34T/μg total MBP.

2.7.4 Determining the avidity of the antibody using Scatchard analysis

The avidity of the anti-MBP peptide antibody was determined using Scatchard analysis as already described in section 2.6.6 with the stock 34T (0.1mg/ml) diluted to generate a standard curve ranging from 0.01-50ng/ml and the anti-MBP peptide antibody diluted to 1/4000.

The Scatchard plot for 34T produced a curved line, due to heterogeneity of antibody binding sites, and was resolved into 2 straight lines. The slope of the steeper upper straight line would be little influenced by the slope of the lower shallow line.
since it defines the most avid receptor and gives the best indication of assay sensitivity hence it was used to determine the avidity of the 34T antibody. The affinity constant of the anti-MBP peptide antibody was therefore found to be $2.64 \times 10^{10}$ litres/mole while the total concentration of antibody within a RIA experiment was $19.72 \times 10^{-12}$ moles/litre.

2.8 Determination of 2',3'-Cyclic nucleotide 3'-phosphodiesterase activity

2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP) activity of aggregate homogenates was assessed as an index of mature oligodendrocyte number using the coupled enzyme assay described by Sogin (1976). The assay works on the principle that β-Nicotinamide adenine dinucleotide 2',3'-cyclic monophosphate (2',3'-Cyclic NADP) is quantitatively hydrolysed to NADP⁺ in the presence of CNP. Subsequently, in the presence of the enzyme glucose-6-phosphate dehydrogenase (G-6-PDH) which is highly specific for NADP⁺, glucose-6-phosphate is dehydrogenated while NADP⁺ is reduced to NADPH (Figure 2.9). The absorbance of the NADPH formed can then be measured spectrophotometrically at 340nm.

All standards and samples were assayed in duplicate. Standard tubes were prepared using CNP (Sigma-Aldrich Company Limited, Poole, UK) diluted in 1% Triton X-100 (aq) to generate a standard curve ranging from 0 to 7.5U/ml. Samples (40μl homogenate plus 80μl 1% Triton X-100) were thawed on ice and vortex mixed vigorously before 10μl of either standard or sample was transferred, in duplicate, to a 96 well plate on ice (Life Technologies Limited, Paisley, UK). A blank containing 10μl of 1% Triton X-100 was also set up at this stage.
A substrate mix was prepared containing Sogin solution (0.274M MES (2-[N-morpholino]ethanesulphonic acid), 41.12mM MgCl$_2$.6H$_2$O, pH 6), 55.5mM glucose-6-phosphate, 11.1M 2',3'-Cyclic NADP, 100U G-6-PDH in Sogin solution containing 0.2% BSA and water in a ratio of 8:1:1:0.1:0.6 (Sigma-Aldrich Company Limited, Poole, UK). Subsequently 200μl of this substrate mix pre-warmed to 37°C was added to each standard, blank or sample well with a multi-channel dispenser. The 96 well plate was immediately placed into an Anthos HTII fully automated microplate reader (Anthos Labtec Instruments, Salzburg, Austria) and the samples were read at 340nm.
2.8.1 Calculation of results

A standard curve (1.25-7.5U/ml CNP) was generated to convert absorbance readings at 340nm into CNP μmol/min per mg of total protein per sample such that:

\[
\text{CNP activity in homogenate} = \frac{\text{Mean CNP (U/ml)} \times 3^*}{\text{Protein concentration (mg/ml)}}
\]

where, * is the dilution of homogenate in 1% Triton X-100 (e.g. 40μl homogenate plus 80μl 1% Triton X-100, = 3)

Then: One unit of CNP converts 1μmol of NADP to NADPH per min so:

\[
= \text{CNP activity in homogenate (μmol/min/mg total protein)}
\]

2.9 Total RNA extraction using a modified Chomczynski and Sacchi procedure

Total RNA was extracted from aggregate cultures using a modified Chomczynski and Sacchi Procedure (Chomczynski & Sacchi, 1987) where the protein denaturants and RNase inhibitors guanidine thiocyanate and guanidine hydrochloride were used.

The entire extraction was performed on ice to slow the rate of RNA degradation unless otherwise stated and all equipment was RNase free with gloves worn throughout. Aggregate cultures in RNase free 1.5ml microcentrifuge tubes were thawed on ice. In a fume cupboard 750μl of filter sterilised guanidine thiocyanate (4.215M guanidine thiocyanate, 25mM sodium citrate pH 7, 0.5% N-lauroyl sarcosine, 0.1M β-mercaptoethanol, 12.5ml DEPC-treated water) was added to each tube containing aggregates. Each sample was triturated by passing it through a 23 gauge needle and syringe several times before transferring it to a 2ml RNase free Eppendorf tube. Then,
to extract the RNA sequentially, 75μl of sodium acetate (2M sodium acetate, 2M glacial acetic acid, pH 4), 750μl of saturated phenol containing 100mg/ml hydroxyquinoline (Life Technologies Limited, Paisley, UK) and 150μl of chloroform:isoamyl alcohol (24:1) were added ensuring that each tube was inverted to mix the contents before the next addition was made. The samples were vortex mixed for 10 seconds before being left on ice for 15 minutes. The tubes were then centrifuged at 8000g for 15 minutes, 4°C to separate the RNA containing upper aqueous phase from the lower organic phase containing DNA and proteins. The aqueous phase was transferred to a fresh 1.5ml Eppendorf tube and 750μl of isopropanol was added before the tubes were vortex mixed for 10 seconds and then incubated overnight at -20 °C to precipitate the RNA.

The following day the samples were centrifuged as described previously to pellet the RNA. Once spun the supernatant was carefully poured off without disturbing the pellet and then 750μl of filter sterilised guanidine hydrochloride (6M guanidine hydrochloride, 0.1M sodium acetate pH 5, 5mM dithiothreitol, 5.1ml DEPC-treated water) was added to redissolve the RNA. The samples were vortex mixed gently and then following a 15 minute incubation on ice 375μl of absolute ethanol was added before the tubes were inverted to mix the contents and left overnight at -20°C.

After this time the samples were centrifuged as described previously before the supernatant was discarded and the pellet was resuspended in 375μl of 70% ethanol. The RNA samples were further dehydrated by repeating this process with sequential washes in 375μl of 80% and 100% ethanol. After this final wash the supernatant was removed and the samples were air dried to ensure that all traces of ethanol had
evaporated. The dried pellets were then resuspended in 40μl of RNase free water before they were vortex mixed and heated at 60°C for 10 minutes to ensure that the RNA pellet was fully resuspended. Prior to freezing at -20°C 3μl of the RNA sample was removed and suspended in 397μl of water to determine the amount of RNA in the sample by spectrophotometric measurement.

2.9.1 Spectrophotometric determination of RNA concentration

Readings of the amount of ultraviolet irradiation absorbed by the samples at wavelengths of 260 and 280nm were taken on an ultrospec 2000 ultra-violet/visible spectrophotometer (Amersham Pharmacia Biotech UK Limited, Little Chalfont, UK). An optical density (O.D.) of 1 corresponds to 40μg per ml of single stranded RNA so to convert optical density absorbance readings into μg of RNA per 40μl of sample:

\[
\text{RNA concentration (μg/ml)} = \frac{260\text{nm reading} \times 40 \times D}{400}\]

where D is the dilution factor (e.g. 3μl sample in a total volume of 400μl, D =400/3= 133.33).

Then for RNA concentration per 40μl the value obtained for RNA concentration (μg/ml) was divided by 25.
The ratio of the absorbancy at 260nm to the absorbancy at 280nm indicates the purity of the RNA sample with a value of 2 obtained for pure RNA and ratios below 2 indicating the presence of protein and phenol contamination.

2.10 mRNA expression by gel electrophoresis and Northern blot analysis

The expression of genes of interest such as MBP were assessed by size fractionating total RNA isolated from aggregate cultures by Northern gel electrophoresis and subsequently transferring the RNA to a nylon membrane before quantifying the mRNA transcript of interest by hybridisation with a digoxigenin labelled cDNA probe.

2.10.1 Preparation of total RNA samples for Northern gel electrophoresis

The appropriate amount of RNA (usually 5-10μg) was placed in a 1.5ml RNase free Eppendorf tube. DEPC-treated water was added to each tube to make the volume up to 100μl. One tenth the volume (10μl) of 3M sodium acetate was added followed by two volumes (220μl) of absolute ethanol. All of the RNA samples were vortex mixed before they were incubated at -20°C until they were required for the running of the agarose formaldehyde gel.

2.10.2 Agarose formaldehyde gel electrophoresis of RNA

The electrophoresis tank, the gel tray and combs were washed sequentially in a mild detergent, distilled water and then absolute ethanol to minimise RNase contamination. After allowing the tank to air dry it was soaked for 10 minutes in 3%
hydrogen peroxide before it was rinsed thoroughly in DEPC-treated water and then levelled inside a fume hood.

In a baked conical flask a 1% agarose (Sigma-Aldrich Company Limited, Poole, UK) gel in DEPC-treated water was melted by heating for 3 minutes in a microwave. The gel was then allowed to cool to 60°C before 1X MOPS-EDTA-sodium acetate buffer (40mM 3-(N-morpholino)propanesulphonic acid (MOPS), 10mM sodium acetate, 1mM EDTA, pH 7; Sigma-Aldrich Company Limited, Poole, UK) and 2.2M formaldehyde (Sigma-Aldrich Company Limited, Poole, UK) were added and mixed by swirling to avoid air bubbles. The gel was then poured into the level gel tank, the comb was inserted and any air bubbles were dispersed before it was allowed to set.

Meanwhile the samples which had been prepared as described previously were centrifuged at 8500g for 30 minutes at 4°C. After this time as much ethanol as possible was removed with a glass pipette and the RNA pellets were left to air dry. Then sequentially to each sample tube 3.5µl of 12.3M formaldehyde was added followed by 10µl of formamide, 2µl of 10X MOPS and 4.5µl of DEPC-treated water to give a final volume of 20µl. After vortex mixing the RNA samples were incubated at 65°C for 10 minutes (with further mixing after 5 minutes) and then quenched on ice for a further 10 minutes. Finally, 2µl of gel loading buffer (1mM EDTA pH 8, 0.25% bromophenol blue in DEPC-treated water) was added before the samples were pulse spun. The gel box was filled with 1X MOPS, ensuring at this point that the gel was not covered, and then an equal volume of each sample was dry loaded into the wells. The gel was pre-run at 3 volts (Bio-Rad model 200 power supply; Bio-Rad Laboratories Limited, Hemel Hempstead, UK) per cm (measured as the distance between the electrodes) until the
samples had run out of the wells and then the gel was covered with the remaining 1X MOPS and run at 3 to 4 volts per cm. After one to two hours the buffer was mixed and the gel was left until the samples had migrated approximately 8cm after which time the electrophoresed RNA was transferred to a nylon membrane by capillary action.

2.10.3 Capillary transfer of RNA to a Nylon membrane

The gel containing the electrophoresed RNA was transferred to a glass dish and trimmed to size using a scalpel blade. The top left hand corner of the gel was cut to aid orientation before it was washed for 2 minutes in DEPC-treated water and then equilibrated in 2X SSC (0.3M sodium chloride, 30mM sodium citrate, pH 7; National Diagnostics, Hessle, UK) for 30 minutes with the solution changed after 15 minutes. While the gel was equilibrating a piece of Nytran-plus positively charged polyamide membrane (Schleicher and Schuell, London, UK) was cut slightly smaller than the gel with a scalpel blade and pre-wet in DEPC-treated water for 5 minutes ensuring that the membrane was wet evenly. The membrane was then washed for 5 minutes in 10X SSC.

The RNA was transferred to the membrane by capillary transfer (Figure 2.10). A piece of Whatman 3MM chromatography paper (Whatman International Limited, Maidstone, UK) was placed over a glass plate ensuring that there was excess paper at both ends. The plate, to be used as a support, was then rested on top of a dish which was subsequently filled with 20X SSC ensuring that the 3MM paper wick reaching into the dish was fully immersed. The glass plate and support were also fully soaked with 20X SSC before any air bubbles were removed with a pipette. The gel was inverted...
Figure 2.10. Capillary transfer of RNA to a nylon membrane. 20X SSC buffer is drawn through the gel towards the blotting paper. The RNA is eluted by the 20X SSC from the gel on to the nylon membrane.

and placed on to the support and again any air bubbles were removed. The pre-wet membrane was carefully lowered onto the gel using autoclaved filter forceps before the left hand corner was cut to enable orientation. The membrane was surrounded by parafilm to prevent short circuiting before it was flooded with 20X SSC. Five sheets of Whatman 3MM chromatography paper and 5 sheets of blotting paper (Sigma-Aldrich Company Limited, Poole, UK), cut no bigger than the membrane, were stacked on top of the membrane and then a glass plate was lowered on to the stack and weighed down with a 500g lead block. The capillary transfer was allowed to proceed overnight at 4°C.

After this time the paper and the parafilm were removed and the gel and the membrane were flipped over and placed, gel side up, on to fresh 3MM chromatography paper. A pencil was used to mark the position of the wells onto the membrane. The gel
was then peeled away from the membrane and stained in 0.5μg/ml ethidium bromide (Sigma-Aldrich Company Limited, Poole, UK) for 5 minutes. After destaining the gel in DEPC-treated water for 20 minutes it was observed under ultraviolet light to ascertain the efficiency of transfer.

Meanwhile the membrane was rinsed in 2X SSC to remove any pieces of agarose stuck to it and then dried on a piece of 3MM paper, RNA side up. Then it was wrapped in cling film and to cross-link the RNA to the membrane both sides of it were exposed to ultraviolet radiation for 3 minutes. The membrane was stored between 3MM paper at room temperature in the dark until required for hybridisation.

2.10.3.1 Methylene blue staining of Northern membrane to detect ribosomal RNA

The membrane was placed in 5% acetic acid for 2 minutes and then stained in 0.04% methylene blue in 0.5M sodium acetate pH 5.2 for 5 to 10 minutes. The membrane was rinsed thoroughly in DEPC-treated water to visualise the 18S (2000 nucleotides) and 28S (5000 nucleotides) ribosomal RNA bands and the membrane was cut to mark their position before a Canon T70 camera fitted with a macro lens was used to take a photograph. The stained gel was stored between 2 pieces of 3MM paper at room temperature in the dark until required for hybridisation.

The photographic negative was scanned using a Bio-Rad model GS-690 imaging densitometer (Bio-Rad Laboratories Limited, Hemel Hempstead, UK) and the density of the 18S rRNA bands were determined using Molecular Analyst software, version 1.5 (Bio-Rad Laboratories Limited, Hemel Hempstead, UK).
2.10.4 Nonradioactive digoxigenin labelled cDNA probes and hybridisation

The nonradioactive digoxigenin (DIG) system (Boehringer Mannheim Limited, Lewes, UK) was used for labelling cDNA probes prior to hybridisation to the Northern blot and subsequent chemiluminescent detection.

2.10.4.1 Probes

The expression of MBP encoding mRNA was assessed using a purified rat 1.45kb MBP cDNA fragment subcloned into the Eco R1 site of pBR322 to generate the clone pMBP-1 and recognise a 2.1 kb brain specific MBP transcript (Roach et al., 1983). A human 1.1kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech Laboratories Limited, Basingstoke, UK) which cross reacts with a 1.4kb rat GAPDH mRNA transcript was used as a positive control to verify equal loading of the RNA samples.

2.10.4.2 Random primed DNA-labelling with digoxigenin

The cDNA probes were denatured and each strand was used as a template. The Klenow enzyme incorporates the deoxyribonucleotides, including digoxigenin-11-dUTP, to synthesise a complementary strand to each template, with one DIG molecule becoming incorporated every 20-25 nucleotides.

An aliquot of cDNA probe (100ng) was placed in a fresh 1.5ml microcentrifuge tube as was 200ng of unlabelled control DNA (pBR328 DNA linearised with Bam H1; Boehringer Mannheim Limited, Lewes, UK). The DNA was denatured by boiling in a water bath for 10 minutes before quenching immediately on ice with sodium chloride.
Then, on ice, 1X hexanucleotide mix (0.125 A<sub>260</sub> units random hexanucleotides in 50mM Tris-HCl, 10mM MgCl<sub>2</sub>, 0.1mM dithioerythritol, 0.2mg/ml BSA, pH 7.2) was added to each tube followed by 1X deoxyribonucleoside triphosphate (dNTP) mix (100μM dATP, 100μM dCTP, 100μM dGTP, 65μM dTTP, 35μM DIG-11-dUTP, alkali labile, pH 7.5) and the necessary amount of DEPC-treated water to give a volume of 19μl. The contents of each tube were microcentrifuged before 2 units of Klenow enzyme were added and following recentrifugation the tubes were incubated overnight at 37°C.

The reaction was stopped by addition of 20mM EDTA, pH 8 and then the labelled DNA was precipitated with 0.1 volume of 4M lithium chloride and 3 volumes of pre-chilled absolute ethanol. After mixing well the labelled DNA was left to incubate at -70°C for 30 minutes before the tubes were centrifuged for 15 minutes at 12000g, 4°C. The supernatant was removed and the DNA pellet was washed in 75% cold ethanol prior to centrifugation for 5 minutes at 12000g, 4°C. Then as much ethanol was removed as possible and the DNA pellets were air dried for 30 minutes at room temperature before they were resuspended in 50μl of Tris-EDTA buffer (10mM tris-HCl, 1mM EDTA, pH 8). DIG-labelled probes were stored at -20°C for at least one year without loss of activity.

2.10.4.3 Estimating the yield of DIG-labelled DNA

Serial dilutions ranging from 1/10 to 1/10,000 in distilled water were set up for the cDNA samples (100ng unlabelled DNA), control DNA (200ng unlabelled DNA), and DIG-labelled control DNA (5ng/μl of DIG-labelled pBR328 DNA). Then 1μl of
each dilution was spotted on to a piece of nylon membrane and allowed to air dry. The DNA was cross-linked to the membrane by 3 minutes exposure to ultraviolet radiation before it was rinsed in maleic acid buffer (150mM NaCl, 100mM maleic acid, pH 7.5, 0.1% (v/v) DEPC, autoclaved). The membrane was then placed in blocking reagent (1% (w/v) blocking reagent in maleic acid buffer, 0.1% (v/v) DEPC, autoclaved; Boehringer Mannheim Limited, Lewes, UK) on a shaker at room temperature for 30 minutes. After this time the membrane was incubated for a further 30 minutes at room temperature in polyclonal anti-DIG antibody conjugated to alkaline phosphatase (150mU/ml in block; Boehringer Mannheim Limited, Lewes, UK). The membrane was then washed twice in maleic acid buffer at room temperature allowing 15 minutes per wash before it was transferred to a fresh dish and rinsed in detection buffer (100mM Tris-HCl pH 9.5, 100mM NaCl) for 3 minutes. Colour substrate (0.34mg/ml nitroblue tetrazolium salt, 0.18mg/ml 5-bromo-4-chloro-3-indolyl phosphate in detection buffer) was then poured over the membrane and allowed to develop overnight at 37°C to enable the spots to be seen with sufficient intensity. The reaction was stopped by washing the membrane in water before the intensity of the DIG-labelled control DNA spots were used to estimate the concentration of the DIG-labelled cDNA probes.

2.10.4.4 Hybridisation

A stock solution of high sodium dodecyl sulphate (SDS; lauryl sulphate) hybridisation buffer (7% SDS, 50% deionised formamide, 5X SSC, 50mM sodium phosphate pH 7, 2% blocking solution, 0.1% N-lauroylsarcosine) was autoclaved
before it was aliquoted and stored at -20°C. Prior to use the hybridisation solution was thawed and pre-heated to 68°C until the components were fully dissolved.

The nylon membranes containing the electrophoresed RNA were rinsed in 2X SSC until they were fully wet. Each membrane was then placed between 2 mesh sheets (Hybaid, Ashford, UK) and placed in a hybridisation bottle (Hybaid, Ashford, UK). Prehybridisation solution was added at 20ml per 100cm² of membrane surface and then the hybridisation tube was placed into a hybridisation oven (Hybaid, Ashford, UK) pre-heated to 50°C and rotated for at least one hour.

An aliquot containing 10 to 20ng of DIG-labelled cDNA probe per ml of hybridisation solution was denatured in a boiling water bath for 10 minutes before it was quenched on ice for 10 minutes and then diluted in hybridisation solution. The prehybridisation solution was discarded and exchanged for the hybridisation solution containing the probe. The hybridisation was allowed to proceed overnight at 50°C.

After this time the hybridisation solution containing the probe was poured into a 50ml centrifuge tube and stored at -20°C until it was required for future use. A small amount of 2X SSC containing 0.1% SDS was added to the hybridisation tube to rinse the membrane before it was transferred to an RNase free dish and washed twice in 2X SSC containing 0.1% SDS for 5 minutes at room temperature on a shaker. The membrane was then washed twice in 0.1X SSC containing 0.1% SDS for 15 minutes at 68°C on a shaker to remove any unbound probe.

On transferring to a clean RNase free dish the membrane was rinsed in maleic acid buffer (as described in section 2.10.4.3) for 3 minutes before it was incubated in blocking reagent (as described previously) on a shaker at room temperature for 30
minutes to prevent non-specific antibody binding. After this time the membrane was incubated for a further 30 minutes at room temperature in polyclonal anti-DIG antibody conjugated to alkaline phosphatase (75mU/ml in block; Boehringer Mannheim Limited, Lewes, UK). The membrane was then washed twice for 15 minutes in maleic acid buffer containing 0.3% (v/v) Tween 20 at room temperature. The membrane was rinsed in detection buffer (as described in section 2.10.4.3) for 3 minutes before it was placed into a hybridisation bag (Life Technologies Limited, Paisley, UK). The chemiluminescent substrate CDP-Star (0.25mM disodium 4-chloro-3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1\(^{3,7}\)]decan}-4-yl)-1-phenyl phosphate; Boehringer Mannheim Limited, Lewes, UK) was then pipetted onto the surface of the membrane (2ml/100cm\(^2\) membrane) and any air bubbles were removed before the bag was sealed, wrapped in foil and left to incubate for 5 minutes. After this time the CDP-Star was recovered and stored in the dark at 4°C for a month for future experiments. The membrane was blotted briefly on 3MM chromatography paper to remove any excess CDP-Star and then sealed in a fresh hybridisation bag. This was placed in a stainless steel film exposure cassette and exposed to Kodak X-Omat AR film (Sigma-Aldrich Company Limited, Poole, UK) for up to one hour. The chemiluminescent signal was developed by placing the film in Kodak GBX developer (1 part developer: 3 parts water; Sigma-Aldrich Company Limited, Poole, UK) for 2 minutes and then Kodak GBX fixer (1 part fixer: 4 parts water; Sigma-Aldrich Company Limited, Poole, UK) for 5 minutes before it was washed thoroughly in cold water and allowed to dry.
The density of the chemiluminescent signal was determined using a Bio-Rad model GS-690 imaging densitometer and Molecular Analyst software, version 1.5 as described in section 2.10.3.1. The arbitrary densitometry values obtained for the MBP and GAPDH signals were expressed over the methylene blue stained 18S ribosomal RNA band values to allow for variations in RNA loading and subsequent transfer to the nylon membrane. Hybridised membranes, which could be reprobed for up to a month, were stored at 4°C in hybridisation bags to prevent them from drying out.

2.10.4.5 Stripping membranes for reprobing

Membranes were rinsed in DEPC-treated water before they were incubated on a shaker in 0.1% SDS pre-heated to 95°C for 10 minutes. The membranes were then washed for a further 5 minutes in maleic acid buffer containing 0.3% Tween 20. After stripping the membranes were either stored in a sealed hybridisation bag containing 2X SSC or immediately prehybridised.

2.11 Reverse transcription and polymerase chain reaction

Growth factor mRNA expression in the aggregate cultures was assessed by reverse transcriptase polymerase chain reaction (RT-PCR).

2.11.1 Reverse transcription

Moloney Murine Leukaemia virus (M-MLV) reverse transcriptase is an RNA-dependent DNA polymerase which uses template single stranded RNA in the presence of an oligo(dT)12-18 primer to catalyse the synthesis of the first strand of
complementary DNA (cDNA). The oligo(dT)$_{12-18}$ primer binds to the poly A tail at the 3' end of the entire population of cellular mRNA to initiate first strand cDNA synthesis (Figure 2.11).

Ten units of ribonuclease inhibitor (10U/µl in 20mM HEPES-KOH pH 7.6, 50mM KCl, 8mM dithiothreitol, 50% glycerol; Life Technologies Limited, Paisley, UK) was added to each 1.5ml RNase free Eppendorf tube. Then, 3µg of total RNA per aggregate sample was added along with DEPC-treated water to bring the volume of RNA plus water to 12.5µl. Two hundred and fifty nanograms of oligo(dT)$_{12-18}$ primer was added to each tube to bind to the poly (A) tail and act as a primer to enable the reverse transcriptase to synthesise first strand cDNA. Kanamycin RNA (1ng) was also added to each tube as a positive control for both cDNA synthesis and subsequent PCR reactions.

The samples were vortex mixed and microcentrifuged before they were incubated in a water bath at 70°C for 10 minutes to denature the RNA and then quenched on ice for 5 minutes. A stock reaction mix was prepared containing 10mM dithiothreitol, 10U ribonuclease inhibitor, 1X first strand buffer (50mM Tris-HCl pH 8.3, 75mM KCl, 3mM MgCl$_2$), 0.5mM dNTP mix (dATP, dCTP, dGTP, dTTP) and 600U M-MLV reverse transcriptase per tube and then the desired amount of this reaction mix was added to each tube. A negative control containing reaction mix without reverse transcriptase was also set up for each reverse transcription experiment. After vortex mixing and a pulse spin the samples were incubated at 37°C for 80 minutes to enable first strand cDNA synthesis.
Thereafter the tubes were incubated at 70°C for 10 minutes to heat inactivate the reverse transcriptase and then quenched on ice for 5 minutes. The cDNA was subsequently diluted in an appropriate volume of DEPC-treated water to give an equivalent total RNA starting concentration of 20ng per μl. Prior to freezing at -20°C
the mRNA:cDNA hybrids were denatured by boiling for 5 minutes and then quenched on ice for 5 minutes.

2.11.2 Polymerase chain reaction

PCR involves the *in vitro* amplification of specific nucleic acid transcripts by repeated thermal cycling. Two oligonucleotide primers anneal to specific nucleotide sequence-defined regions on opposite denatured single cDNA template strands. In a cycling reaction catalysed by a thermostable DNA polymerase such as the bacterium *Thermus aquaticus* (*Taq*) 5’ to 3’ DNA polymerase (which survives extended incubation at 95°C and has a high temperature optimum) the primers, in the presence of dNTPs and buffer, direct the synthesis of DNA towards each other amplifying the selected region in an essentially exponential fashion (Figure 2.12).

2.11.2.1 Primers

The specificity and efficiency of the PCR reaction is maximised when oligonucleotide primers have been carefully designed taking into account parameters such as their length, typically 18 to 30 nucleotides, and their base composition, ensuring that the guanine (G) and cytosine (C) content is 40 to 60% to ensure similar optimal annealing temperatures for both primers. To prevent self annealing and to minimise secondary structure formation primers should not be complementary to each other or to themselves. The cDNA template location is also important as it determines the length of the PCR product which should be between 300 and 2000 base pairs and furthermore primers chosen from alternative exons
Figure 2.12. A PCR thermal cycle. By using primer pairs Taq DNA polymerase synthesises two new DNA strands.
can be used to distinguish PCR products derived from cDNA or contaminating genomic DNA.

Rat primer pairs were selected for platelet-derived growth factor-A (PDGF-A), transforming growth factor-β1 (TGF-β1), insulin-like growth factor-I (IGF-I), ciliary neurotrophic factor (CNTF), fibroblast growth factor-2 (FGF-2), nerve growth factor (NGF), epidermal growth factor (EGF) and neurotrophin-3 (NT-3) all of which have a documented role in the regulation of oligodendrocyte development. Primer pairs were also selected for the house-keeping gene GAPDH and kanamycin to serve as positive controls for determination of sample loading consistency and the efficiency of cDNA synthesis and subsequent PCR reactions.

The human genome mapping project GenBank database was searched for growth factor nucleotide sequences. Primer sequences were selected with the help of the PrimerSelect option from Lasergene biocomputing software for windows (DNASTAR Incorporated, Madison, Wisconsin, USA) as listed below in Table 2.6. Synthesised PCR primer sequences (synthesised by Dr Phil Marsh, Randall Institute, Kings College, London, UK) were aliquoted and stored at -20°C.
<table>
<thead>
<tr>
<th>Product</th>
<th>Nucleotide sequence</th>
<th>cDNA template</th>
<th>Annealing temperature</th>
<th>MgCl₂ concentration</th>
<th>Cycle number</th>
<th>Product size</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>5'-GCC ATT CTC ACC GGA TTC AGT CGT C-3' 5'-AGC CGC CGT CCC GTC AAG TCA G -3'</td>
<td>100ng</td>
<td>60°C</td>
<td>1.5 mM</td>
<td>20</td>
<td>323bp</td>
<td>N/A</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TGG TGC CAA AAG GGT CAT CAT CTC C -3' 5'-GCC AGC CCC AGC ATC AAA GGT G -3'</td>
<td>100ng</td>
<td>60°C</td>
<td>2 mM</td>
<td>20</td>
<td>559bp</td>
<td>M17701</td>
</tr>
<tr>
<td>FGF-2</td>
<td>5'-GGA GAA GAG CGA CCC ACA CG -3' 5'-TGC CAG CCT TCC TCA CAT ACT TG -3'</td>
<td>100ng</td>
<td>57°C</td>
<td>1 mM</td>
<td>25</td>
<td>607bp</td>
<td>X61697</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>5'-CGA CTG GCT CGA AGT CA -3' 5'-GCC AGC TCA TCT CAC CTC A -3'</td>
<td>100ng</td>
<td>60°C</td>
<td>1 mM</td>
<td>25</td>
<td>509bp</td>
<td>D10106</td>
</tr>
<tr>
<td>CNTF</td>
<td>5'-CAC CGC CGG GAC CTC TGT AGC -3' 5'-AGG ACC TTC AAG CCC CAT AGC -3'</td>
<td>100ng</td>
<td>60°C</td>
<td>1 mM</td>
<td>25</td>
<td>452bp</td>
<td>X17457</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5'-TGG ACC GCA ACA ACG CAA TCT A -3' 5'-GTT ACC CAT GAG CAG GAA G -3'</td>
<td>100ng</td>
<td>68°C</td>
<td>1.5 mM</td>
<td>25</td>
<td>449bp</td>
<td>X52498</td>
</tr>
<tr>
<td>IGF-1</td>
<td>5'-TTG CGG GCC TGA GTG GGT GGA C -3' 5'-GCG GTG AGC TGG CAT TTT CTG TCT -3'</td>
<td>100ng</td>
<td>68°C</td>
<td>1 mM</td>
<td>25</td>
<td>395bp</td>
<td>M15480</td>
</tr>
<tr>
<td>NT-3</td>
<td>5'-TTA CTT CTG CCA CGA TCT TAC AG -3' 5'-GGT TGC CCA CAT AAT CTT CC -3'</td>
<td>200ng</td>
<td>55°C</td>
<td>1 mM</td>
<td>30</td>
<td>414bp</td>
<td>M34643</td>
</tr>
<tr>
<td>EGF</td>
<td>5'-TGG GTA CTG CCT CAA TGG TG -3' 5'-TGC TGT CAG GCC CGT TAC T -3'</td>
<td>200ng</td>
<td>60°C</td>
<td>1.5 mM</td>
<td>30</td>
<td>314bp</td>
<td>X12748</td>
</tr>
<tr>
<td>NGF</td>
<td>5'-TTT TGA TCG GCC TAC AGG CAG AAC -3' 5'-ATT TGG GCC TCG GCA CTT GGT C -3'</td>
<td>200ng</td>
<td>62°C</td>
<td>1.25 mM</td>
<td>30</td>
<td>515bp</td>
<td>M36589</td>
</tr>
</tbody>
</table>

Table 2.6. Growth factor PCR primer sequences and conditions.
2.11.2.2 Optimisation of polymerase chain reaction

Each PCR amplification consists of a number of time and temperature specific incubations which need to be optimised for each individual primer pair as do the concentrations of the reaction components including starting cDNA template, dNTPs, thermostable DNA polymerase and the reaction buffer. The presence of divalent cations is critical and the concentration of magnesium (Mg$^{2+}$) can severely affect PCR efficiency. High concentrations of chelating agents within the DNA template can reduce free Mg$^{2+}$ as can dNTPs which are a source of negatively charged phosphate groups which complex Mg$^{2+}$. Low Mg$^{2+}$ impairs *Taq* DNA polymerase extension as it is a required co-factor for enzymatic activity while excess Mg$^{2+}$ reduces enzyme fidelity (accuracy and precision) and may cause non-specific amplification. High concentrations of Mg$^{2+}$ also stabilise double stranded DNA preventing complete denaturation and stabilising primer annealing to incorrect template nucleotides, hence increasing undesired products and lowering specificity.

PCR magnesium chloride titrations were carried out for each primer pair at predetermined optimal annealing temperatures for 25 cycles. The reverse transcribed cDNA aggregate samples and the PCR reaction components were thawed thoroughly on ice and vortex mixed before a PCR stock reaction mix was prepared to give a final tube concentration as shown in Table 2.7.
<table>
<thead>
<tr>
<th>Components</th>
<th>Final concentration per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcribed cDNA</td>
<td>100ng</td>
</tr>
<tr>
<td>10X thermophilic buffer&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1X buffer (50mM KCl, 10mM Tris-HCl pH 9, 0.1% Triton X-100)</td>
</tr>
<tr>
<td>2mM dNTP mix&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2mM dNTPs (dATP, dCTP, dGTP, dTTP)</td>
</tr>
<tr>
<td>20µM Forward primer</td>
<td>1µM Forward primer</td>
</tr>
<tr>
<td>20µM Reverse primer</td>
<td>1µM Reverse primer</td>
</tr>
<tr>
<td>Taq polymerase (5U/µl)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5U Taq polymerase in 0.5% glycerol, 0.5mM Tris-HCl pH 8.1mM NaCl, 1µM EDTA, 50µM DTT, 0.01% Triton X-100</td>
</tr>
</tbody>
</table>

Table 2.7. Final concentrations of components in the PCR amplification mix. <sup>a</sup> purchased from Promega, Southampton, UK. <sup>b</sup> purchased from Life Technologies Limited, Paisley, UK.

After vortex mixing and microcentrifugation the desired amount of amplification mix was added to RNase-free 0.2ml MicroAmp reaction tubes (Perkin Elmer Applied Biosystems, Warrington, UK). Then in duplicate, the desired amount of 25mM magnesium chloride (Promega, Southampton, UK) was pipetted into each tube to give final concentrations of 0.5 to 2.0mM per reaction. The desired amount of DEPC-treated water was then added to each tube to bring the final reaction volume to 50µl. A negative control containing PCR amplification mix without reverse transcribed cDNA template was included for each PCR reaction. MicroAmp caps (Perkin Elmer Applied Biosystems, Warrington, UK) were added to seal the tubes before they were placed into an automated GeneAmp PCR system 9700 (Perkin Elmer Applied Biosystems, Warrington, UK) and the heated cover was pulled forward and sealed. The initial thermal cycle profile was programmed as described in Table 2.8. The amplified PCR products were stored at 4°C until they were analysed on a 1% agarose gel.
<table>
<thead>
<tr>
<th>Step</th>
<th>Details</th>
<th>Time</th>
<th>Temperature (°C)</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>Ensure full denaturation of the template before thermal cycling</td>
<td>5 minutes</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>Denature DNA preparation</td>
<td>45 seconds</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Primer annealing</td>
<td>Anneal primers to single stranded DNA template. Probably most critical step</td>
<td>45 seconds</td>
<td>primer specific</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>Thermostable Taq DNA polymerase synthesises a single strand from 3’-OH end of each primer. Extends at 2-4kb per minute</td>
<td>90 seconds</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>A final extension incubation ensures completion of double stranded DNA</td>
<td>7 minutes</td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>Store</td>
<td></td>
<td>indefinite</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.8. Thermal cycling conditions for PCR amplification.
2.11.2.2.1 Analysis of amplified PCR products by agarose gel electrophoresis

Once amplified the PCR product can be analysed by size separation using ethidium bromide stained agarose gel electrophoresis and subsequent visualisation under ultraviolet light. Ethidium bromide intercalates between the bases of double stranded DNA helices and these complexes fluoresce under ultraviolet light.

In a baked conical flask a 1% agarose (Sigma-Aldrich Company Limited, Poole, UK) gel in 1X Tris-borate-EDTA buffer (TBE; 0.089M Tris base, 0.089M boric acid, 1mM EDTA pH 8) was melted by heating for 3 minutes in a microwave. Once cooled 0.5μg/ml ethidium bromide (Sigma-Aldrich Company Limited, Poole, UK) was added and mixed by swirling before the contents were poured into a level electrophoresis gel running tray containing a comb and any air bubbles were dispersed. The agarose gel was allowed to set before the comb was removed and the tray was placed into an electrophoresis tank containing enough 1X TBE with 0.5μg/ml ethidium bromide to cover the gel.

Equivalent volumes of each PCR product, initially 10μl, were mixed with 1X orange G gel loading buffer (5% sucrose, 0.06% orange G dye; Sigma-Aldrich Company Limited, Poole, UK) and then microcentrifuged to bring the contents to the bottom of the tube before they were loaded into the wells along with a 100 base pair DNA size ladder (Promega, Southampton, UK). The gel was run at 3V/cm (measured as the distance between the electrodes) (Bio-Rad model 200 power supply; Bio-Rad Laboratories Limited, Hemel Hempstead, UK) until the samples had migrated approximately 5cm after which time the gel was destained in DEPC-treated water for 15 minutes at room temperature. The gel was then exposed to ultraviolet light and the
fluorescing DNA bands photographed with a Polaroid MP4 land camera (Polaroid corporation, Cambridge, Massachusetts, USA) using Polaroid black and white instant sheet film, type 55 (Sigma-Aldrich Company Limited, Poole, UK).

The PCR products were analysed to verify the product size and to determine which, if any, magnesium chloride concentration was optimal. Magnesium chloride concentration titrations for PDGF-A, CNTF, GAPDH and FGF-2 are shown in Figure 2.13. If no product was obtained or many bands could be seen the PCR reaction was repeated using an alternative annealing temperature until satisfactory specific bands of the correct product size were visualised. For some growth factors it was necessary to increase the concentration of starting template to 200ng to detect a product (refer to Table 2.6).

2.11.2.3 Determination of PCR amplification efficiency

With total efficiency each PCR cycle essentially doubles the amount of desired DNA product hence, PCR amplification is exponential, with product accumulation proportional to the amount of starting DNA such that:

\[ N = N_0 2^n \]

where, \( N \) is the number of amplified molecules, \( N_0 \) is the initial number of molecules and \( n \) is the number of amplification cycles.
Figure 2.13. Representative ethidium bromide stained gels of magnesium chloride PCR amplification titrations for PDGF-A, CNTF, GAPDH and FGF-2. Numbers 1, 2, 3 and 4 on the figure represent final magnesium chloride concentrations of 0.5, 1.0, 1.5 and 2.0mM per reaction respectively. Equivalent amounts of each PCR product were size fractionated on 1% agarose gels. M denotes a 100bp DNA ladder marker with the brightest band indicating a 500bp product.
The efficiency of amplification decreases towards the latter stages to reach a plateau phase due to depletion of reaction components, diminished enzyme activity and product accumulation, therefore, to quantitate mRNA levels analysis must occur during the exponential phase of amplification. The number of cycles that occur before the plateau phase is reached varies with each primer pair and the amount of mRNA target, therefore, the amplification efficiency of each growth factor primer pair needed to be determined.

The reverse transcribed cDNA aggregate samples and PCR reaction components were thawed on ice and vortex mixed before a PCR stock reaction mix was prepared to give a final tube concentration of each component as shown in Table 2.9.

<table>
<thead>
<tr>
<th>Components</th>
<th>Final concentration per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcribed cDNA</td>
<td>Typically 100-200ng</td>
</tr>
<tr>
<td>10X thermophilic buffer</td>
<td>1X buffer (50mM KCl, 10mM Tris-HCl pH 9, 0.1% Triton X-100)</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>0.5-2.0mM MgCl₂</td>
</tr>
<tr>
<td>2mM dNTP mix</td>
<td>0.2mM dNTPs (dATP, dCTP, dGTP, dTTP)</td>
</tr>
<tr>
<td>20μM Forward primer</td>
<td>1μM Forward primer</td>
</tr>
<tr>
<td>20μM Reverse primer</td>
<td>1μM Reverse primer</td>
</tr>
<tr>
<td>Taq polymerase (5U/μl)</td>
<td>2.5U Taq polymerase in 0.5% glycerol, 0.5mM Tris-HCl pH 8.1mM NaCl, 1μM EDTA, 50μM DTT, 0.01% Triton X-100</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>to bring final volume to 50μl</td>
</tr>
</tbody>
</table>

Table 2.9. Final concentrations of components in the PCR amplification mix.
After vortex mixing and microcentrifugation 50µl of amplification mix was added to duplicate RNase-free 0.2ml MicroAmp reaction tubes and the caps were closed securely before the tubes were transferred to an automated GeneAmp PCR system 9700 and the heated cover was pulled forward and sealed. The thermal cycle profile was programmed specifically for each primer pair for 35 cycles. The amplification was allowed to proceed through the primary denaturation and then for 5 full thermal cycles of denaturation, annealing and extension before the machine was paused and 5µl was removed from each tube. The PCR reaction was resumed and subsequently paused once 10, 15, 20, 25, 30 and 35 cycles had been completed with the removal of a 5µl sample after each pause. A further sample was removed after the final extension step to ascertain the efficiency of the final extension.

The amplified PCR products were stored at 4°C until 5µl of each PCR product with an equivalent volume of DEPC-treated water and 1X orange G gel loading buffer were size separated and analysed on a 1% agarose gel as described in section 2.11.2.2.1. Under ultraviolet light the fluorescing gel-separated DNA bands were photographed (Figure 2.14A) before the negative was scanned using a Bio-Rad model GS-690 imaging densitometer. The density of the DNA bands were determined using Molecular Analyst software, version 1.5.

2.11.2.3.1 Results

The growth factor PCR signals expressed as arbitrary densitometry units were plotted against the number of PCR amplification cycles and graphs such as the one for GAPDH illustrated in Figure 2.14B were obtained for each growth factor.
Figure 2.14. **PCR amplification characteristics for GAPDH.** The equivalent of 100ng of reverse transcribed cDNA was amplified for 35 cycles with the reaction paused and a 5μl sample removed once 5, 10, 15, 20, 25, 30 and 35 cycles had been completed. **A.** An ethidium bromide stained gel of PCR product. **B.** PCR product yield, assessed using image analysis, to determine the density of the electrophoresed DNA bands expressed as arbitrary densitometry units. The number of amplification cycles optimal for GAPDH was 20, with further reactions performed to determine the optimal cycle number for each alternative primer pair. * represents 35 cycles and 7 minutes extension phase.
The optimal cycle number for each primer pair was therefore chosen by selecting a clearly detectable PCR band that was in the exponential range of the curve. The number of amplification cycles optimal for GAPDH was found to be 20, while those for each alternative growth factor primer pair are shown in Table 2.6.

The importance of the final extension incubation on completion of the thermal cycles was illustrated by assessing the PCR product yield before and after this period. This final seven minutes extension period resulted in a 20% increase in PCR product accumulation as depicted in Figure 2.15.

2.11.2.4 Optimised polymerase chain reaction

Once the above steps had been taken to optimise each primer specific PCR it was used routinely to determine growth factor mRNA expression in standard and macrophage-enriched cultures.

The reverse transcribed cDNA aggregate samples and PCR reaction components were thawed on ice and vortex mixed before a PCR stock reaction mix was prepared as already described in Table 2.9 with the exception that the reverse transcribed cDNA aggregate samples were added separately. The desired amount of stock amplification mix was added to RNase-free 0.2ml MicroAmp reaction tubes before the reverse transcribed cDNA samples (typically 100-200ng reverse transcribed cDNA per reaction, refer to Table 2.6 for each growth factor) were added individually. A negative control tube containing all the reaction components except the template DNA was included for every PCR reaction. MicroAmp caps were added to seal the tubes before they were placed into an automated GeneAmp PCR system 9700 and the
Figure 2.15. GAPDH PCR product accumulation during the final extension incubation. The equivalent of 100ng of reverse transcribed cDNA was amplified with a 5μl sample removed on completion of the 35 cycles and after the seven minutes final extension step. PCR product yield, determined by the density of the electrophoresed DNA bands using image analysis, was found to increase by 20% during the final 7 minutes extension period.
heated cover was pulled forward and sealed. The thermal cycle profile followed the pattern described in Table 2.8 but the annealing temperature and cycle number was optimal for each primer pair. The amplified PCR products were stored at 4°C until they were analysed on a 1% agarose gel and photographed as already discussed in section 2.11.2.2.1. Representative ethidium bromide stained gels of growth factor RT-PCR products derived from standard aggregate cultures at DIV=1, 5, 8, 14, 21 and 48 with verification of their product size are illustrated in Figure 2.16.

All aggregate samples from individual preparations underwent PCR amplification with a given primer pair at the same time, in the same PCR machine and with stock reaction mixtures to reduce experimental errors. When possible the resulting PCR products were analysed on the same agarose gel, however, when more than one gel was run at least three identical samples were run on each gel to eliminate any experimental differences.

2.11.2.4.1 Calculation of results

Photographic negatives of the growth factor PCR signals were scanned using a Bio-Rad model GS-690 imaging densitometer and the density of the DNA bands were obtained in arbitrary densitometry units using Molecular Analyst software, version 1.5.

Calculations were made to standardise the semi-quantitative PCR product yields to 25 amplification cycles and 100ng starting cDNA template. Once standardised each PCR sample was expressed over the constitutively expressed GAPDH gene to allow for variations in sample loading and control for subsequent variations during the multiple experimental stages.
Figure 2.16. Representative ethidium bromide stained gels of growth factor RT-PCR products derived from standard cultures at DIV=1, 5, 8, 14, 21 and 48 where n = 3 for DIV=1 and 5 for the remaining time points. Equivalent amounts of each PCR product were size fractionated on 1% agarose gels. M denotes a 100bp DNA ladder marker with the brightest band indicating a 500bp product. Kanamycin was amplified as a positive control for RT-PCR and GAPDH was amplified as an internal standard.
2.12 Immunocytochemical staining of aggregate cultures

Immunocytochemical analysis was performed to assess the spatial distribution and morphology of cells including astrocytes, oligodendrocytes and neurons as well as other antigens of interest within standard and macrophage-enriched aggregates as they matured. To study the cellular composition aggregates were harvested, snap frozen, cryostat sectioned and then blocked to reduce non-specific binding. Aggregates were then incubated with a selection of primary antibodies raised against antigens of interest before a secondary antibody labelled with a fluorescent dye or an enzyme was used to visualise the antigen-primary antibody complex.

2.12.1 Antibodies

The primary antibodies employed to study the cellular composition and spatial distribution within standard and macrophage-enriched aggregates as they mature in culture are shown in Table 2.10.

2.12.2 Cryostat sectioning and avidin-biotin horse radish peroxidase staining of aggregate cultures

The avidin-biotin immunoperoxidase technique was used to localise a variety of immunologically significant antigens. A primary antibody raised against the antigen of interest was added to a tissue section. Then a biotinylated secondary antibody was added which introduces many biotins, a small water-soluble vitamin, to the section at the location of the primary antibody. When the preformed Avidin:Biotinylated enzyme Complex (ABC) is applied to the sections it binds to the biotinylated secondary
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Fixative</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX-42</td>
<td>Anti CD11b, probably against rat complement receptor 3 on most macrophages &amp; microglia</td>
<td>Mouse monoclonal, IgG2a</td>
<td>Ethanol, +20°C</td>
<td>1:750</td>
<td>Serotec Limited, Oxford, UK</td>
</tr>
<tr>
<td>ED1</td>
<td>Glycoprotein on lysosomal membrane expressed by majority of rat monocytes/macrophages</td>
<td>Mouse monoclonal, IgG1</td>
<td>Methanol, -20°C</td>
<td>1:200</td>
<td>Serotec Limited, Oxford, UK</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Against CNS glial astrocytes &amp; ependymal cells in many mammals</td>
<td>Rabbit polyclonal antisera</td>
<td>Acetone, 4°C</td>
<td>1:200</td>
<td>Dako Limited, Cambridge, UK</td>
</tr>
<tr>
<td>14E</td>
<td>Detects the cell body of oligodendrocytes in normal human and rat white matter</td>
<td>Mouse monoclonal, IgM</td>
<td>Ethanol, +20°C</td>
<td>1:10</td>
<td>J. Newcombe, Institute of Neurology, London, UK</td>
</tr>
<tr>
<td>Hu</td>
<td>Against neuronal nuclear mRNA proteins. Stains post-mitotic neurons in multiple species.</td>
<td>Human polyclonal antisera</td>
<td>Acetone, 4°C</td>
<td>1:500</td>
<td>N.A. Gregson, UMDS, London, UK</td>
</tr>
<tr>
<td>pan Axonal marker</td>
<td>Cocktail of antibodies to phosphorylated neurofilaments. Stains axons of most mammals</td>
<td>Mouse monoclonal, IgG1</td>
<td>Methanol, -20°C</td>
<td>1:1500</td>
<td>Affiniti Research Products Limited, Exeter, UK</td>
</tr>
<tr>
<td>Myelin basic protein (MBP)</td>
<td>Against synthetic peptide human MBP 131-136 detecting mammalian MBP</td>
<td>Mouse monoclonal, IgG2b</td>
<td>Acetone, 4°C</td>
<td>1:500</td>
<td>Affiniti Research Products Limited, Exeter, UK</td>
</tr>
<tr>
<td>Proteolipid protein (PLP)</td>
<td>Raised against purified rat PLP and DM20</td>
<td>Rabbit polyclonal antisera</td>
<td>Ethanol, +20°C</td>
<td>1:100</td>
<td>N.A. Gregson, UMDS, London, UK</td>
</tr>
</tbody>
</table>

Table 2.10. Primary antibodies used for immunocytochemical analysis of aggregate cultures.
antibody. Finally, the antigen is located by an enzyme reaction as in the presence of an electron donor substrate such as the chromagen 3,3'-diaminobenzidine tetrahydrochloride (DAB), horseradish peroxidase (40kD) forms an enzyme-substrate complex and then oxidises DAB to produce a brown coloured product with the reduction of hydrogen peroxide to water.

Aggregate samples previously harvested and snap frozen as described in section 2.3.3 were placed in dry ice to ensure that they did not thaw. Using a blade, the gelatin capsule was peeled away from the frozen aggregate and O.C.T. compound without disturbing the aggregate pellet. The sample was then mounted on to a pre-cooled tissue block using O.C.T compound and once frozen clamped into the cryostat (Slee Technik GmbH, Mainz, Germany) specimen holder. The cryostat, set at an optimal cutting temperature of -23 to -24°C, was used to cut 8 to 10 micrometer thick aggregate sections which were subsequently adhered to vectabond coated slides. Uncut aggregate blocks were placed into 1.5ml microcentrifuge tubes and stored at -70°C until they were required for further immunocytochemical processing.

Sections were ringed with a Dako pen (Dako Limited, Cambridge, UK) prior to immediate fixation for 3 minutes in the appropriate fixative at the desired temperature, (see Table 2.10) with the exception of acetone fixed sections which were circled following fixation. Some fixatives preserve the native antigen structure more effectively than others with structural changes affecting antibody recognition. Therefore, when primary antibodies were used for the first time multiple fixatives at different temperatures were routinely tested including acetone, ethanol and methanol at 4°C, 20°C and -20°C before one was chosen that produced optimal cellular staining with minimal
background without disrupting aggregate section integrity. Fixed slides were transferred to cold 1X PBS and washed twice for at least 5 minutes prior to blocking.

The excess PBS was wiped away from each slide before it was placed into a humidified staining tray. To ensure that the sections did not dry out the slides were placed into a humidified staining tray for all incubations and whenever any addition was made to a section each slide was always dealt with one at a time. To block non-specific binding of the secondary biotinylated antibodies 50μl of blocking solution, consisting of 2.5% of normal horse serum (determined by the species in which the secondary antibody was raised; Vector Laboratories Limited, Peterborough, UK) diluted in 1% BSA/1X PBS was added immediately to each section ensuring that it was completely covered. A lid was placed onto the tray and the slides were incubated in block at room temperature for 1 hour. After this time excess blocking solution was drained from the slides before they were placed in a glass trough on a magnetic stirrer and washed three times, each for 5 minutes, in 1X PBS at room temperature. All subsequent washes were done in the same manner.

After the third wash the slides were dried and placed one at a time back into the humidified slide tray to enable the primary antibody to be applied. Each section was covered in 50μl of the desired antibody diluted in 1% BSA/1X PBS to a pre-determined optimal concentration and incubated overnight at 4°C. Antibody titrations, usually at 4 dilutions ranging from 1/100 to 1/2000, were always performed if the antibody had not been used before to stain aggregate culture sections with the dilution chosen that produced optimal specific cellular staining with minimal background signal. To assess the specificity of binding controls were routinely used during each staining run with the
addition of an immunoglobulin isotype specific control for monoclonal antibodies (Sigma-Aldrich Company Limited, Poole, UK) and rabbit serum (Dako Limited, Cambridge, UK) for polyclonal antisera both used at a dilution equivalent to that of the most concentrated primary antibody. Omission of the primary antibody was used as a negative control.

Following the antibody incubation the slides were washed three times in 1X PBS as described above. A stock solution of the secondary antibody, a horse biotinylated anti-mouse rat adsorbed immunoglobulin (0.5mg/ml in 10mM phosphate pH 7.8, 0.15M NaCl, 0.08% sodium azide; Vector Laboratories Limited, Peterborough, UK) was diluted in 1X PBS to give a final concentration of 2.5μg/ml and following the three five minute washes the aggregate sections were incubated for one hour in 50μl of the secondary antibody at room temperature.

The avidin-biotin horse radish peroxidase complex (ABC; Vector Laboratories Limited, Peterborough, UK) was prepared 30 minutes prior to its use to allow the avidin and biotinylated peroxidase to form a complex. A 1% (v/v) dilution of reagent A (Avidin DH) in 1X PBS was vortex mixed before an equivalent volume of solution B (biotinylated horse radish peroxidase H) was diluted in the PBS already containing solution A. After vortex mixing the solution was allowed to stand at room temperature for 30 minutes. Once the secondary antibody had been washed off 50μl of pre-complexed ABC solution was added to each aggregate section and left for 30 minutes.

During the three 5 minute washes to remove excess ABC solution the peroxidase substrate was prepared in 1X PBS by adding sequentially 0.05% 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Company Limited, Poole, UK) followed by
0.01% hydrogen peroxide. The sections were incubated in the peroxidase substrate for 5 to 10 minutes according to the intensity of the staining. The slides were then washed in running tap water. When nuclear localisation was desired the sections were counterstained in Mayer’s haematoxylin (HD Supplies, Aylesbury, UK) for 30 seconds and then washed in running tap water for 5 minutes. Sections were dehydrated by passing them sequentially through graded alcohol solutions of 95%, 100%, and 100% each for a minute and then cleared twice in xylene first for a minute and then again for at least 3 minutes before they were mounted in DPX (Merck Limited, Lutterworth, UK) and sealed with a coverslip.

2.12.2.1 Haematoxylin staining of aggregate cell nuclei

Aggregate cultures were cut as described above before they were fixed in methanol for 3 minutes and then washed in 1X PBS. To visualise cellular nuclei to ascertain total cell number per aggregate section slides were stained in Mayer’s haematoxylin for 30 seconds and then washed in running tap water for 5 minutes before they were dehydrated and DPX mounted as described above.

2.12.3 Immunofluorescent staining of aggregate cultures

Aggregate sections were cut, adhered to vectabond reagent coated slides and fixed as described previously. Since the serum used in the blocking solution is determined by the species in which the secondary antibody was raised, for immunofluorescent staining, the blocking solution consisted of 2.5% normal goat serum (Vector Laboratories Limited, Peterborough, UK) diluted in 1% BSA/1X PBS. The
subsequent incubation in block at room temperature and application of the desired primary antibody were as described in section 2.12.2 except that the primary antibody was diluted in blocking solution not 1% BSA/1X PBS alone.

Following the primary antibody incubation the slides were washed 3 times in 1X PBS before the desired secondary antibody was added. A stock solution of goat anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (FITC; 3.6mg/ml in 0.01M PBS pH 7.4, 0.01% thimerosal; Sigma-Aldrich Company Limited, Poole, UK) was diluted 1/25 in 1X PBS before 50μl was added to sections that had been previously incubated with a mouse monoclonal antibody. Where a rabbit polyclonal antiserum was applied the secondary antibody, a biotinylated goat anti-rabbit immunoglobulin (1.5mg/ml in 10mM phosphate pH 7.8, 0.15M NaCl, 0.08% sodium azide; Vector Laboratories Limited, Peterborough, UK) was diluted 1/200 in 1X PBS to give a final concentration of 7.5μg/ml before 50μl was added to each section and left for 1 hour at room temperature.

Sections incubated in the biotinylated secondary antibody were washed and further incubated in rhodamine avidin DCS (cell sorting grade; Vector Laboratories Limited, Peterborough, UK). A stock solution of rhodamine avidin DCS (2mg/ml in 10mM HEPES, 0.5M NaCl, pH 8.0, 0.08% sodium azide) was diluted in 0.1M sodium bicarbonate buffer (0.1M NaHCO₃, 0.15M NaCl adjusted to pH 8.5 with 0.1M Na₂CO₃) to 10μg/ml before 50μl was added to each section and left for 1 hour at room temperature.

All the slides then received three five minute washes before the sections were mounted in one drop of Citifluor anti-fade reagent (University of Kent, Canterbury, UK).
and a coverslip was lowered on top. Excess Citifluor reagent was removed and the coverslips were sealed around the edges with nail varnish before the slides were stored in the dark at 4°C until they were viewed under a microscope.

2.12.3.1 Double immunofluorescent staining of aggregate cultures

The procedure used for double staining aggregate cultures was as described in 2.12.3 except that the sections were exposed concurrently to 2 primary antibodies that had been raised in different species. For our purposes a mouse monoclonal antibody was used in conjunction with rabbit polyclonal antisera at the desired concentrations. Application of a combination of FITC conjugated anti-mouse and biotinylated goat anti-rabbit secondary antibodies was also necessary with the remaining steps completed as described above.

2.12.4 Immunocytochemical analysis

Avidin-biotin horse radish peroxidase stained sections were viewed under a Leitz Laborlux K light microscope (E. Leitz limited, Luton, UK) while single and double fluorescent stained sections were viewed under a under a Leitz Dialux 20 microscope (E. Leitz limited, Luton, UK) equipped with fluorescent optics. Photographs were taken using a Fujichrome ASA 400 film as desired.

2.12.4.1 Determination of total cell number per aggregate section

Aggregate sections stained in Mayer’s haematoxylin to visualise cellular nuclei were viewed under a light microscope. The number of stained nuclei were counted in
aggregate sections selected on the basis of their diameters, approximately 400 ± 10μM which ensured that comparable areas of each aggregate were being studied.

2.12.4.2 Determination of OX-42 and ED1 positive cells per aggregate section

Aggregate sections stained with OX-42 and ED1 monoclonal antibodies were visualised under a light microscope. The number of positively stained cells were counted per aggregate section selected on the basis of their diameters, approximately 400 ± 10μM and expressed as a percentage of the total cell number.

2.13 Statistical analysis

Results are expressed as means ± 1 standard error of the sample mean (SEM). Comparisons within and between experimental groups were made using paired and unpaired t-tests with a p-value of less than 0.05 indicating statistical significance.
CHAPTER 3

CHARACTERISATION OF STANDARD AND MACROPHAGE-ENRICHED AGGREGATE CULTURES

3.1 Introduction

Mechanically dissociated foetal rat brain telencephalon cells, maintained under constant rotation, re-assemble spontaneously to form highly organised three-dimensional aggregates (Honegger, 1985) which mature on a time scale and in a manner that closely resembles rat brain development in vivo in terms of cell to cell interactions and morphological maturation including myelination (Honegger, 1985; Loughlin et al., 1997; Kruger et al., 1999). Furthermore, electron microscopy studies have confirmed that myelin specific mRNA expression and myelin basic protein (MBP) accumulation in these cultures represents myelin deposition around axons (Loughlin et al., 1997). This culture technique can produce large quantities of uniform cultures which provide an efficient and reproducible test system for studying the processes of myelination, demyelination and remyelination. MBP content correlates with the extent of myelination as assessed by electron microscopy (Shine et al., 1992) and provides a reliable means of evaluating the effects of various treatments on myelin while the activity of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), which is localised predominantly in oligodendrocytes as opposed to myelin, acts as a marker for mature oligodendrocytes.

Macrophages and resident brain microglia have a role in the maintenance, defence and restoration of any damaged tissue and are implicated in the phagocytosis of dying and apoptotic cells. In demyelinating multiple sclerosis (MS) brain lesions they are responsible for the destruction and phagocytosis of myelin and debris (Prineas & Wright, 1978) while they may further contribute to the demyelinating process and
oligodendrocyte toxicity by secreting soluble factors including pro-inflammatory cytokines, leukotrienes, complement components, proteolytic and lipolytic enzymes and reactive oxygen intermediates (Brosnan & Raine, 1996). Paradoxically macrophages are implicated in angiogenesis, gliogenesis and central nervous system (CNS) modelling during development (Giulian et al., 1988a) while they have also been associated with the microscopic hallmarks of remyelination in MS (Prineas et al., 1993a). Hence, the phagocytic clearance of myelin debris may be a necessary prerequisite for successful remyelination (Perry & Gordon, 1991) while as a documented source of growth factors and other mediators which influence the proliferation, migration, differentiation, survival and myelin synthesising capacity of oligodendrocytes, macrophages, have the potential to promote myelin formation and repair (Diemel et al., 1998). In fact, supplementing aggregate cultures with peritoneal macrophages at the beginning of the culture period increased myelin deposition over time compared to standard cultures (Loughlin et al., 1994).

Therefore in the present study the effect that exogenous peritoneal macrophage addition has on the developmental maturation of aggregate cultures was explored. Initially, the ability of peritoneal macrophages, added to the aggregates at the time of seeding, to successfully incorporate and remain integrated throughout the culture period was investigated. The spatial distribution and morphological maturation of cell types within standard mixed-glial cell and macrophage-enriched aggregate cultures as they mature over time was fully investigated and compared. Furthermore, a comprehensive assessment of developmental myelination within the aggregates was performed while the influence that 5 and 10% macrophage addition has on this process was further elucidated.
3.2 RESULTS

3.2.1 Ultrastructural analysis of foetal rat brain aggregate cultures

Foetal rat brain aggregate cultures were harvested at pre-determined time points and processed for electron microscope (EM) analysis as described in Chapter 2, section 2.3.4. Standard mixed cell aggregates were cut into semi-thin sections and stained for light microscopy before thin sections, stained with methanolic uranyl acetate and aqueous lead citrate, were examined by transmission EM. Figure 3.1A illustrates aggregate cultures free floating in serum free DMEM just prior to harvesting while the semi-thin section shown in Figure 3.1B features a cross-section of a homogenous population of 36 day old spherical aggregate cultures and reveals a densely stained nuclear outer border with fewer positively stained cells towards the centre of the aggregate section.

EM examination of standard and macrophage-enriched aggregate cultures revealed the presence of several highly differentiated cell types including astrocytes, oligodendrocytes and neurons. There was evidence of astrocytes displaying typical features including cytoplasmic intermediate filaments and glycogen granules. Astrocytic cell processes appeared to predominate towards the periphery of the aggregate cultures as revealed by the EM view of cells at the outer edge of the aggregates (Figure 3.2A). Neurons tended to be located more towards the centre of the aggregate cultures (Figure 3.2B) as did oligodendrocytes (Figure 3.2C). The neuronal cell body shown in Figure 3.2B has a prominent nucleus containing a nucleolus while other organelles include a rich supply of mitochondria, rough endoplasmic reticulum and Golgi apparatus.
Figure 3.1. **Standard mixed-cell aggregate cultures.**  
A. Standard aggregate cultures free floating in serum free DMEM just prior to harvesting.  
B. A semi-thin section featuring a cross-section of a 36 day old spherical aggregate culture with a diameter of 340-400µM.
Figure 3.2. Electron micrographs of mature aggregate cultures (DIV=48). A. Astrocytic cell processes predominate towards the periphery of the aggregate cultures. B. A neuronal cell body featuring a prominent nucleus containing a nucleolus. C. An oligodendrocyte cell body with a characteristic large dense nucleus containing chromatin and dense cytoplasm and large widely dispersed Golgi apparatus, rough endoplasmic reticulum and groups of free ribosomes. D. A synapse with clearly visible synaptic vesicles contained within the pre-synaptic cells. Scale bar = as indicated.
Oligodendrocytes appeared to be fully differentiated with the fine structure having the characteristic appearance of a large dense nucleus containing chromatin and dense cytoplasm containing microtubules, large and widely dispersed Golgi apparatus, rough endoplasmic reticulum along with groups of free ribosomes and myelin. Synapses with synaptic vesicles were clearly visible within the presynaptic terminals (Figure 3.2D). It was also evident that there were large extracellular spaces between the cells which would allow easy diffusion of molecules to and from the centre of the aggregate.

EM examination revealed the presence of axons in standard and macrophage-enriched aggregate cultures and myelin was associated with these axons. Furthermore, there appeared to be a substantial increase in the number of myelinated axons from DIV=22 to DIV=48. At the earlier time point there were relatively few myelinated axons and the myelin at this stage appeared to be loosely compacted (Figure 3.3A) and there was also evidence of what appeared to be free myelin that was not obviously associated with any axons. By DIV=48 the number of myelinated axons appeared to have increased with the multi-lamellar myelin looking more compact (Figure 3.3B). In most instances the myelin sheaths, with several concentric layers of myelin lamellae, surround axons containing mitochondria and cytoskeletal elements such as microtubules and neurofilaments (Figure 3.3C). Close examination of the myelin revealed that the myelin membranes had compacted with the presence of well formed characteristic major and minor dense lines corresponding to in vivo CNS myelin (Figure 3.3D). There was also evidence of nodes of Ranvier where the myelin sheath segments terminate in paranodal loops and the axolemma is exposed (Figure 3.3E). Occasionally necrotic cells and loose myelin figures were
Figure 3.3. Electron micrographs depicting myelinated axons in foetal rat brain aggregate cultures at differing ages. Myelinated axons at DIV=22 (A) are wrapped in several layers of poorly compacted myelin compared with DIV=48 (B) where compact myelin lamellae were evident. C, D. Cross section through myelinated axon of DIV=48 aggregate culture showing normal compaction of myelin with formation of major dense and intraperiod lines. Note that there are large numbers of neurofilaments and microtubules in the axon and the axolemma is visible. E. A myelin segment terminating at a heminode of Ranvier. Scale bar = as indicated.
observed with the frequency of necrotic cells increasing by DIV=48 particularly towards the core of the aggregate.

### 3.2.2 Immunocytochemical analysis of standard and macrophage-enriched foetal rat brain aggregate cultures

Immunocytochemical staining, in conjunction with EM analysis, was used to assess the spatial distribution and morphological differentiation of standard mixed-glial cell aggregate cultures and those that were enriched with 10% peritoneal macrophages. It was shown that undifferentiated foetal telencephalon cells develop and organise into a uniform population of aggregates that contain astrocytes, oligodendrocytes, neurons and myelinated axons.

#### 3.2.2.1 Peritoneal macrophage incorporation into foetal rat brain aggregate cultures

Immunocytochemistry demonstrated that there are a population of endogenous macrophages or microglia present within standard aggregate cultures as identified by the positive OX-42 staining which is directed against the rat type 3 complement receptor (CR3) present on macrophages and microglia (Figure 3.4A). However, as can be seen from Figure 3.4A the number of stained cells varied between aggregate cultures sections with some containing much fewer positive cells than others while no apparent staining was evident in some standard aggregate sections.
Figure 3.4. OX-42 (A, B) and ED1 (C, D) immunocytochemical staining in standard (A, C) and macrophage-enriched (B, D) aggregate cultures on DIV=21. x20. Inset on (D) shows a range of morphologies exhibited by ED1 positive cells from ramified branched cells to compact amoeboid cells, x40
OX-42 staining confirmed that peritoneal macrophages added to the telencephalon cell suspension at the start of the culture period did successfully incorporate into the aggregates and remained integrated throughout the entire culture period (Figure 3.4B). Thus macrophage-enriched aggregate cultures contained numerous positively stained OX-42 cells. EM analysis shows the ultrastructural appearance of a macrophage added to the telencephalon cell suspension within a few hours of seeding (Figure 3.5).

It was further demonstrated that standard aggregate cultures contained a few ED1 positive cells (Figure 3.4C) which recognises a glycoprotein expressed predominantly on the lysosomal membrane of most circulating rat monocytes, macrophages and microglia and is therefore indicative of cells in an activated state. The number of ED1 positively stained cells was increased in macrophage-enriched aggregate cultures (Figure 3.4D). There was evidence of a range of morphologies from ramified branched cells with variations in the length and complexity of their branching to compact amoeboid cells. A quantitative immunocytochemical examination of the number of OX-42 and ED1 positive cells in standard and macrophage-enriched aggregate cultures is shown below.

3.2.2.2 Diameter of standard and macrophage-enriched aggregates

Mature standard and macrophage-enriched aggregate cultures were harvested on DIV=35 as described in Chapter 2 section 2.3.5 before their diameters were assessed by Dr N.A. Gregson (UMDS, London, UK). Non-enriched aggregate cultures were found to have diameters ranging from 235 to 584μM with a mean of 387.7 ± 29.86μM which
Figure 3.5. Electron micrograph of a macrophage in contact with telencephalic aggregate culture cells just after seeding. This cell has an irregularly shaped nucleus containing chromatin while the cytoplasm contains numerous vacuoles and dense lysosomes. Scale bar = 1000nm.
was not significantly different from the 415.6 ± 44.09 mean diameter measured for macrophage-enriched cultures (Figure 3.6).

3.2.2.3 Total cell number per standard and macrophage-enriched aggregate culture section

Standard and macrophage-enriched aggregate cultures were harvested and snap frozen for immunocytochemical staining on DIV=28 and 42 as described in Chapter 2, section 2.3.3 before sections were cut and stained in Mayer’s haematoxylin as described in section 2.12.2.1. The number of stained nuclei were counted in aggregate sections with diameters of approximately 400 ± 10μM to determine the total cell number per aggregate section.

Macrophage-enriched aggregate culture sections at DIV=28 contained 107 more cells than the 319 ± 25.99 positively stained nuclei counted in standard aggregate culture sections although this difference was not significant (Figure 3.7). By DIV=42, 14 days later, the total number of cells had fallen in both standard and macrophage-enriched aggregate culture sections. The number of cells per standard aggregate culture section had fallen significantly over this time period by 31% (p<0.05) to 220 ± 20.51 while the number of cells in macrophage-enriched aggregate cultures had fallen by 39% to 260 ± 18.15 so that they contained on average only 40 more cells per aggregate section at this late time point.
Figure 3.6. Diameters (μM) of standard and macrophage-enriched aggregate cultures on DIV=35 (n=8-12).
Figure 3.7. Total cell number per aggregate culture section in standard and macrophage-enriched aggregate cultures on DIV=28 and 42. The number of stained nuclei were counted in 4 aggregate sections with diameters of approximately 400 ± 10μM.
3.2.2.4 Determination of OX-42 and ED1 positive cells per standard and macrophage-enriched aggregate culture section

Standard and macrophage-enriched aggregate cultures were harvested and processed for immunocytochemical staining on DIV=28 and 42 as discussed above before sections were cut and stained with the monoclonal antibodies OX-42 and ED1. Positively stained cells were counted per aggregate section with diameters of approximately 400 ± 10µM and expressed as a percentage of the total cell number as described in Chapter 2, section 2.12.4.2.

The number of OX-42 positive cells per section in macrophage-enriched aggregate cultures at DIV=28 was over 5 times higher than the number of positive cells detected in standard cultures (p<0.001; Figure 3.8). This pattern did not change significantly over time such that by DIV=42, 14 days later, the number of OX-42 positive cells in standard and enriched cultures remained relatively constant.

The number of ED1 positive cells per section in both standard and enriched cultures was lower than OX-42 stained positive cells at both time points. In 3 of the 5 standard aggregate sections that were counted on DIV=28 there were no ED1 positive cells present (Figure 3.8) hence the ED1 positive cells per aggregate section expressed as a percentage of total cell count per aggregate section was 0.19 ± 0.13. In contrast the number of ED1 positive cells in macrophage-enriched cultures was significantly 13 times higher than the number in standard flasks (p<0.05). By DIV=42 the number of ED1 positive cells in standard flasks had risen 4 fold while the number of positive cells in macrophage-enriched flasks had halved so that there was no significant difference between the 2 groups.
Figure 3.8. OX-42 and ED1 positive cells in standard and macrophage-enriched aggregate culture sections. The number of OX-42/ED1 positive cells was expressed as a percentage of total cell number in 5 - 7 aggregate culture sections, with diameters of approximately 400 ± 10μM, on DIV=28 and 42.
3.2.2.5 Spatial arrangement of cell types within standard and macrophage-enriched aggregate cultures

Cell types present within the aggregates were characterised using fluorescent and peroxidase immunocytochemistry. The aggregates develop from undifferentiated single cells into extremely organised structures containing a population of highly differentiated oligodendrocytes, neurons and astrocytes that have a characteristic spatial distribution.

The presence and distribution of astrocytes was determined by using an antibody directed against glial fibrillary acidic protein (GFAP) which is expressed predominantly by differentiated astrocytes (Trimmer et al., 1982). Analysis of standard mixed-cell aggregate cultures showed that GFAP immunoreactivity increased with time in culture (Figure 3.9A and D). Furthermore, while there was evidence of astrocytic processes throughout the aggregate by the latter stages of the culture period the periphery of the aggregate was intensely fluorescent (Figure 3.9D) revealing a well defined outer astrocytic border around the circumference of the aggregate. Similar developmental changes were observed in macrophage-enriched aggregate cultures with initial indications that there were no apparent differences compared with standard cultures.
although this needs to be further investigated. Oligodendrocyte cell bodies were found to be almost randomly scattered throughout the aggregate culture sections (Figure 3.9B and E), as assessed using the monoclonal antibody 14E which reacts with a membrane or cytoskeletal component located in the cell body of human and rat white matter section oligodendrocytes (Newcombe & Cuzner, 1988). There were approximately 10 to 15 positively stained cells in both standard and macrophage-enriched aggregate culture sections by DIV=28 and no obvious changes in the distribution or number of cells with ongoing time.

An antibody directed against neuronal RNA binding proteins (anti-Hu) was used to assess the arrangement of neuronal cell bodies within the cultures. It was determined that immunofluorescently stained positive cells increased progressively with time accompanied by a clustering towards the centre of the aggregate section which was evident by DIV=28 (Figure 3.9C and F). From DIV=28 to DIV=42 neuronal nuclei appeared to become increasingly centrally orientated while no discernible difference was observed between standard and macrophage-enriched aggregate cultures at any of the time points assessed although this has yet to be investigated further. The distribution of neuronal processes within the aggregate cultures was assessed by using a pan-axonal antibody directed against phosphorylated neurofilament proteins located in the axons and some dendrites of mature neurons. Immunofluorescent staining revealed that by the fourth week in culture neurofilament positive staining was highly organised predominantly forming a concentric ring towards the periphery of the aggregate but notably inside the outer astrocytic border (Figure 3.10A - D) in both standard and
Figure 3.10. Immunofluorescence staining for neurofilament on DIV=28 (A, B) and DIV=42 (C, D) in standard (A, C) and macrophage-enriched (B, D) aggregate cultures. x20.
macrophage-enriched aggregate cultures. This layer became increasingly fluorescent with time such that by DIV=42 neurofilament positive structures had formed a dense circular ring around the edge of the aggregate inside of the astrocytic border (Figure 3.10C). A preliminary study revealed that there was a marked increase in the intensity and breadth of the neurofilament staining in macrophage-enriched aggregates compared to standard cultures at this late time point (Figure 3.10D) however, this needs to be confirmed.

Immunofluorescent staining for MBP, a major protein component of myelin, demonstrated developmental increases over time such that by DIV=28 there was some clustering of immunoreactive cells evident throughout the aggregate culture (Figure 3.11A). By DIV=28 staining appeared to be more intense in macrophage-enriched aggregate cultures and as was evident with neurofilament staining there was a predominant population of MBP-positive cells beginning to form around the edge of the aggregate, again, just inside of the astrocytic border indicating accelerated organisation (Figure 3.11B) compared to standard mixed-cell cultures. By DIV=42 MBP positive staining had increased in all cultures (Figure 3.11C and D) and immunoreactive cells had become increasingly organised. In standard aggregate cultures an inner MBP band was evident by this stage (Figure 3.11C, E) while in macrophage-enriched cultures the MBP ring was better defined and more confluent than at DIV=28 (Figure 3.11D, F). Furthermore, there was evidence of enhanced MBP immunoreactivity in macrophage-enriched cultures compared to standard cultures. Similarly immunofluorescent staining for the myelin protein proteolipid protein, was found in clusters throughout the aggregate at DIV=28 but became increasingly organised with time such that by DIV=42
Figure 3.11. Immunofluorescence staining for MBP in standard and macrophage-enriched aggregate cultures. Clustering of MBP reactive cells by DIV=28 in standard cultures (A) compared to more intense staining in macrophage-enriched aggregate cultures (B). In standard aggregate cultures at DIV=42 an inner MBP band is evident (C, E) while in macrophage-enriched cultures (D, F) MBP immunoreactivity is enhanced compared to standard cultures. x20 (A, B, E, F). x10 (C, D).
cultures exhibited a PLP-positive ring (data not shown). Hence, supplementing aggregate cultures with peritoneal macrophages accelerated the process of cellular organisation and increased MBP staining without changing the number of oligodendrocyte or neuronal cell bodies.

In accord with the EM studies, double staining of standard and macrophage-enriched cultures at DIV=42 revealed that the myelin protein MBP was associated with neurofilament positive cells (Figure 3.12) as was PLP.

3.2.3 Myelinating properties of standard and macrophage-enriched aggregate cultures

Aggregate samples were harvested for analysis at pre-determined time points throughout the culture period. The samples were homogenised before total protein assays were completed as described in Chapter 2. Subsequently the MBP content of the aggregate homogenates, expressed as μg per mg of total protein ± 1 SEM, was determined using the RIA method described in Chapter 2 to assess the myelinating properties of the aggregate culture system and to ascertain the effect of supplementing peritoneal macrophages at the time of seeding.

3.2.3.1 Myelin basic protein accumulation in standard aggregate cultures

The MBP content (μg/mg total protein) was found to increase with ongoing time in standard aggregates during the first 4 weeks in culture as illustrated in Figure 3.13A and B from 2 representative time course studies. Subsequently a plateau level was reached which
Figure 3.12. Double immunofluorescence staining for MBP and neurofilament in standard aggregate cultures on DIV=42. A. MBP. B, D. Neurofilament. C, E. Double staining of MBP and neurofilament showing co-localisation. x 20 (A, B, C). x100 (D, E).
Figure 3.13. Accumulation of MBP (μg/mg total protein) over time in standard aggregate cultures. Results are expressed as mean ± 1 SEM. A, B. Representative time course experiments where n = 4-15 for each time point and * denotes significance (p ≤ 0.05) from previous time point as determined by paired Student's t-test. C. From multiple aggregate culture preparations where each data point represents mean MBP of at least 3 flasks.
was a trend observed in half of the 22 aggregate culture preparations examined (Figure 3.13C). In the experiment illustrated in Figure 3.13A the MBP content rose significantly \((p<0.0001)\) from \(\text{DIV}=14\) to reach near maximal levels by \(\text{DIV}=28\) with no marked change in the MBP content detected thereafter. However, there were slight variations between preparations in the pattern and extent of myelination such that while in some instances the greatest period of MBP accumulation occurred between \(\text{DIV}=14\) to 21 (as in Figure 3.13A) in other cases the greatest increase in MBP was between \(\text{DIV}=21\) and 28 (Figure 3.13B). Hence in Figure 3.13B the rate of MBP accumulation between \(\text{DIV}=14\) and 21 was 0.16\(\mu\text{g/mg total protein per day} (p<0.005)\) compared with 0.40\(\mu\text{g/mg total protein per day} (p<0.005)\) over the next 7 day period before tailing off in a fashion resembling Figure 3.13A. Furthermore, MBP levels attained in the preparation illustrated in Figure 3.13A were double those reached in the experiment depicted in Figure 3.13B. Such variations highlight the importance of assessing the pattern of MBP accumulation in each individual aggregate culture preparation.

3.2.3.2 Increased myelin basic protein content in macrophage-enriched aggregate cultures

Initially telencephalon cell cultures were supplemented with 5 or 10\% peritoneal macrophages as described in Chapter 2 section 2.1.4 to assess their role in aggregate cell culture myelination. The MBP content of standard cultures and those enriched with 5\% or 10\% macrophages was assessed in two separate aggregate culture preparations with samples removed for analysis on \(\text{DIV}=11, 20\) and 29 in the first case and from the later time points \(\text{DIV}=25, 29\) and 40 in the second instance (Figure 3.14A and B). Standard aggregates and those supplemented with macrophages had similar low levels of MBP on
Figure 3.14. Accumulation of MBP (µg/mg total protein) over time in standard and macrophage-enriched aggregate cultures. Results are expressed as mean ± 1 SEM. Samples harvested on DIV=11, 20 and 29 (A), DIV=25, 29 and 40 (B) and DIV=14, 21 and 48 (C), where n = 4-8 for each time point. * denotes significance (p ≤ 0.05) from standard culture at relevant time point as determined by Student's t-test. D. From multiple aggregate culture preparations where each data point represents mean MBP of at least 3 flasks.
DIV=11 (Figure 3.14A). By DIV=29, 18 days later, the MBP content had risen in all of the aggregate cultures. However, macrophage-enriched flasks accumulated more MBP than standard cultures over this time rising to levels of 1.61 ± 0.37 and 1.46 ± 0.13μg MBP/mg total protein in 5% and 10% macrophage-enriched flasks respectively which were higher than the 0.93 ± 0.2μg MBP/mg total protein reached in standard flasks.

A second time course experiment was conducted to determine the extent of myelination over the later time points DIV=25 to 40 (Figure 3.14B). At DIV=25 and 29, the MBP content in aggregate cultures enriched with 10% macrophages was found to be higher than non-enriched flasks although this was not significant. In contrast, at DIV=20 and 29 in the earlier experiment illustrated in Figure 3.14A the MBP content of macrophage-enriched flasks were already significantly higher than standard cultures. During the next 11 days from DIV=29 to DIV=40 the MBP content in standard flasks almost doubled rising from 0.63 ± 0.12 to 1.24 ± 0.08μg MBP/mg total protein while the MBP content in 10% macrophage-enriched flasks rose significantly (p<0.01) by just under 4 fold to reach levels that were 3 times higher than those reached in standard flasks (p<0.001). In contrast, MBP levels appeared to plateau in aggregate cultures enriched with 5% peritoneal macrophages after DIV=29 with the content of MBP remaining comparable to non-enriched flasks. Hence the pattern of MBP accumulation in 5% macrophage-enriched aggregate cultures was variable between preparations with MBP levels either reaching those obtained in flasks enriched with 10% macrophages (Figure 3.14A) or remaining closer to the levels observed in non-enriched flasks (Figure 3.14B). Thereafter it was decided to supplement all further aggregate cultures with 10% peritoneal macrophages.
Subsequently the pattern of MBP accumulation was determined in a continuous time course study from DIV=14, when MBP protein can just be measured in the cultures, to the end of the culture period, DIV=48 (Figure 3.14C). As depicted in Figure 3.14C all aggregate cultures have similar low levels of MBP on DIV=14 while over the next 7 days MBP accumulated at a higher rate in macrophage-enriched aggregates than in standard cultures. This pattern was continued to the end of the culture period with the MBP content in enriched flasks increasing significantly at a rate of 0.27µg/mg total protein per day (p<0.05) to reach levels that were more than twice those measured in standard cultures (p<0.05). The MBP content was found to continue to accumulate during the later stages of the culture period in three quarters of the macrophage-enriched aggregate culture preparations (Figure 3.14D) while, as described above, non-enriched cultures reached a plateau over these late stages in half of the preparations.

3.2.3.3 Increased myelin basic protein mRNA expression in macrophage-enriched aggregate cultures

The MBP mRNA content of standard and macrophage-enriched aggregate cultures was assessed using Northern blot analysis followed by hybridisation using nonradioactive digoxigenin labelled cDNA probes as described in Chapter 2, section 2.10. In the representative time course experiment illustrated in Figure 3.15 samples were removed for analysis on DIV=5, 8, 14, 28 and 48.

Evidence of MBP mRNA was not apparent in standard or macrophage-enriched aggregate cultures until DIV=8 (Figure 3.15B and C). In standard aggregate cultures MBP
Figure 3.15. Accumulation of MBP mRNA over time in standard and macrophage-enriched aggregate cultures.  

A. A representative Northern gel membrane stained with methylene blue demonstrates equal loading of 10µg total RNA per lane.  

B. The subsequent autoradiograph showing hybridisation signal for MBP (~2.1kb).  

C. MBP mRNA data was normalised to methylene blue staining. Results are expressed as mean ± 1 SEM where n=3-5 for each time point. * denotes significance (p ≤ 0.05) from standard culture at relevant time point as determined by Student’s t-test.
mRNA expression increased significantly (p<0.005) over the 6 days from DIV=8 to DIV=14 before reaching plateau levels. Levels of MBP transcript also increased significantly over this period in macrophage-enriched cultures (p<0.005), but in contrast to standard cultures, levels continued to rise up to DIV=48 and were significantly elevated relative to standard cultures at both DIV=28 and 48 (p<0.05). Hence by DIV=48 levels of MBP mRNA in macrophage-enriched flasks were more than double those in standard cultures (p<0.0005).

3.2.3.4 2',3'-Cyclic nucleotide 3'-phosphodiesterase activity in standard and macrophage-enriched aggregate cultures

2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP) is localised predominantly in oligodendrocytes and was used as a marker for mature oligodendrocytes. CNP activity was assessed by the method of Sogin, as described previously in Chapter 2, section 2.8. Standard and macrophage-enriched aggregate cultures from the same preparation were analysed for both MBP content and CNP activity on DIV=34, 36, 42 and 48 (Figure 3.16A and B). In this representative experiment the MBP content in non-enriched aggregate cultures increased marginally from DIV=34 to 48 which was in contrast to the significant MBP rise in macrophage-enriched aggregate cultures over this time (p<0.01; Figure 3.16A).

On the other hand the CNP activity of standard aggregate cultures was not markedly altered despite a slight increase from DIV=34 to 42 which was not significant (Figure 3.16B). The significant MBP rise in macrophage-enriched cultures from DIV=34 to 48 was not associated with any such changes in CNP activity, which
Figure 3.16. Changes in MBP (A) and CNP activity (B) over time in standard and macrophage-enriched aggregate cultures. Results are expressed as mean ± 1 SEM where n=2-4 for each time point. * denotes significance (p ≤ 0.05) from standard culture at relevant time point as determined by Student's t-test.
remained relatively unchanged over this time and furthermore, no significant difference was found between standard and macrophage-enriched cultures at any time point.

3.2.3.5 MBP degradation peptide content over time in standard and macrophage-enriched aggregate cultures

The content of the synthetic MBP degradation peptide 34T was assessed within the aggregates using a RIA as described in Chapter 2. The synthetic peptide was based on the human amino acid MBP sequence 75-89, but with antibody recognition restricted to the epitopes 80 to 89. The rabbit polyclonal antibody used had operational specificity for human MBP 80-89 detecting peptides with a carboxyl terminus of phenylalanine 89, while reacting poorly with peptides extending beyond this residue and negligibly with intact human MBP. Hence the level of 34T in the cultures indicates the extent of the proteolytic breakdown of MBP following myelin phagocytosis.

The level of 34T (ng/μg MBP) was assessed in standard and macrophage-enriched cultures (Figure 3.17) during the later stages of the culture period from DIV=34 to 48. While only minimal 34T changes were evident in standard aggregate cultures over this 12 day time period the 34T content in macrophage-enriched cultures was significantly reduced by more than half over this time (p<0.05). Hence, by DIV=48 34T levels in macrophage-enriched aggregates were over 3 times lower than those in standard cultures (p<0.05).
Figure 3.17. Changes in 34T content (ng/µg MBP) over time in standard and macrophage-enriched aggregate cultures. Results are expressed as mean ± 1 SEM where n = 3-6 for each time point from a representative experiment. * denotes significance (p ≤ 0.05) from standard culture at relevant time point as determined by Student's t-test.
3.2.3.6 Effect of macrophage-conditioned medium on the MBP content of standard and macrophage-enriched aggregate cultures

Macrophage-conditioned medium (MCM) was prepared and added to standard aggregate cultures as described in Chapter 2, section 2.1.6. In a preliminary experiment MCM was added to standard aggregate cultures from DIV=21 up until DIV=40 and MCM stimulated with lipopolysaccharide (MCM-LPS) was added to these MCM cultures from DIV=32 for 8 days. Preliminary results indicated that standard aggregate cultures that had been exposed to MCM contained augmented levels of MBP comparable to those reached in macrophage-enriched aggregate cultures (Figure 3.18). The increased MBP content in standard aggregate cultures that had been exposed to MCM, relative to non-treated standard aggregates, was apparent both at DIV=32 and 40, 11 and 19 days after treatment respectively although this did not reach significance. MCM cultures that were subsequently exposed to MCM-LPS on DIV=32 showed the greatest increase in MBP content with levels 56% higher than standard aggregate cultures and a third higher than flasks receiving MCM alone (Figure 3.18).

3.2.3.7 Effect of exogenous macrophage addition to mature myelinated standard and macrophage-enriched aggregate cultures

Preliminary studies were performed to determine the effect of adding 10% peritoneal macrophages to mature aggregate cultures. Standard and macrophage-enriched aggregate cultures were prepared and maintained up to DIV=21 before rat peritoneal macrophages were prepared and counted as described in Chapter 2, section 2.1.4 to enable 10% macrophage cells to be added to standard non-enriched aggregate
Figure 3.18. MBP content (µg/mg total protein) in standard and macrophage-enriched aggregate cultures. Macrophage-conditioned medium (MCM) was added to standard aggregate cultures from DIV=21 up until DIV=40 and MCM stimulated with lipopolysaccharide (MCM-LPS) was added to these MCM cultures from DIV=32 for 8 days. Results are expressed as mean ± 1 SEM where n = 4-8 for each time point.
cultures. After 2 days 5ml of medium was exchanged in all aggregate flasks and cultures were left for a further 2 days before a sample was harvested from each flask (DIV=25) and again 17 days later on DIV=42.

On DIV=25 the MBP content of macrophage-enriched aggregate cultures was over 2.5 times that of standard flasks. At this stage the MBP content of aggregates that had received peritoneal macrophages 4 days earlier was not markedly different from non-enriched aggregate cultures (Figure 3.19A). Additionally, at this time point there was no difference in the level of the degradation peptide 34T in standard and macrophage-enriched aggregate cultures while the 34T content in cultures that had received exogenous macrophages on DIV=21 was significantly greater than that of standard and enriched cultures (p<0.05; Figure 3.19B). By DIV=42 the MBP content in aggregate cultures that had received 10% peritoneal macrophages on DIV=21 had risen by over 3.00μg/mg total protein to reach levels that were significantly higher than both standard and macrophage-enriched aggregate cultures (p<0.05). Over this same time period the 34T content in these flasks fell by 58% to reach levels similar to standard and enriched flasks.
Figure 3.19. MBP content (µg/mg total protein) (A) and 34T content (ng/µg MBP) (B) in standard and macrophage-enriched aggregate cultures. 10% peritoneal macrophages were added to standard non-enriched aggregate cultures on DIV=21. Results are expressed as mean ± 1 SEM where n = 4-6 for each time point. * denotes significance (p ≤ 0.05) from standard culture at relevant time point as determined by Student's t-test.
3.3 DISCUSSION

Mechanically dissociated foetal rat brain telencephalon cells maintained under constant rotation re-assemble spontaneously to form three-dimensional aggregate cultures that mature in a manner resembling the developing brain both morphologically and biochemically. Ultrastructural electron microscopy examination run in conjunction with immunocytochemical studies determined that mature telencephalic aggregates contain an extremely organised population of highly differentiated astrocytes, oligodendrocytes and neurons which have a characteristic spatial distribution. Supplementing aggregate cultures with peritoneal macrophages increased myelin deposition over time compared to standard cultures, as determined by morphological, biochemical and mRNA analyses, with no apparent rise in the activity of the mature oligodendrocyte marker CNP implying that oligodendrocytes are producing more myelin in the presence of macrophages. Similarly, preliminary results indicate that both addition of macrophage-conditioned medium to standard aggregates and exogenous macrophage supplementation to mature myelinated cultures resulted in augmented MBP levels.

A decrease in total cells after the fourth week in culture accompanied by reduced numbers of activated macrophages and microglia and a decline in the content of MBP degradation products in macrophage-enriched cultures may be attributable to the enhanced phagocytic activity of macrophages in the process of programmed cell death and the removal of cellular debris as these cultures become increasingly organised.

This was the first comprehensive morphological study to determine the temporal and spatial distribution of cell types within standard and macrophage-enriched aggregate cultures obtained from the telencephalon brain region of embryonic day 16 (E16) rat foetuses. Foetal cells are used in order to minimise cellular damage during
preparation and to obtain a high proportion of undifferentiated cells which have the capacity to grow and differentiate \textit{in vitro} (Honegger, 1985). The developmental age and region from which the starting tissue is taken affects factors such as the mitotic and enzymatic activity of the aggregates. Hence, E15 foetal telencephalon cells exhibit a higher mitotic activity than cultures prepared from hind brain or from E17 telencephalic cells possibly due to their less differentiated state at the time of excision (Lenoir & Honegger, 1983). Similarly, E15 telencephalon aggregate cultures display higher activities of glutamine synthetase (GS), CNP and choline acetyl transferase, found in astrocytes, oligodendrocytes and cholinergic neurons respectively, than aggregates prepared from hind brain while glutamic acid decarboxylase, localised in GABAergic neurons, and acetyl choline esterase activities were lower (Honegger, 1985). Inclusion of different brain regions also influences the population of cells present in aggregate cultures with for example, triiodothyronine and nerve growth factor (NGF) sensitive cholinergic neurons found in a restricted region of the rat telencephalon (Honegger, 1985).

The presence of endogenous macrophages and microglia within standard aggregate cultures prepared from E16 telencephalon cells may be expected as mature monocytes are evident in mice as early as E9 and have been observed entering tissues one day later, as soon as a circulation is established (Perry & Gordon, 1991). Microglia are present throughout the adult brain but their distribution varies from approximately 5% of the total cell population in the mouse cerebral cortex to as much as 12% in the substantia nigra (Perry & Gordon, 1991). In humans microglia comprise 13-15% of total white matter cells which is nearly three times higher than in mice (Hayes \textit{et al}., 1987). It was found that the endogenous macrophages and microglia in standard
aggregate cultures accounted for approximately 1% of the total cell number which is slightly lower than documented for the adult CNS. This may be attributable to the developmental age of the starting foetal tissue (E16) as the greatest number of macrophages infiltrating the developing mouse CNS occurs during the late embryonic and early postnatal period (Perry & Gordon, 1991) suggesting that blood borne macrophages had not yet been fully acquired by E16. Accordingly, macrophages and microglia located in the retina and brain of E16 mice in close association with blood vessels (Hume et al., 1983; Perry et al., 1985) were found to increase in number and become more widespread by the time of birth.

Macrophages added to the telencephalon cell suspension at the start of the culture period successfully incorporated and remained integrated throughout the culture period accounting for approximately 5% of the total cells which was over 5 times that detected in standard cultures. Macrophage enrichment did not alter the mean diameter of mature standard telencephalic aggregate cultures which was approximately 400µM and comparable with whole brain foetal aggregates whose mean diameter increased by 90µM between DIV=4 and DIV=40 to 430µM (Matthieu et al., 1978; Trapp et al., 1979). Similarly, macrophage enrichment did not markedly change the total number of cells present in each aggregate culture section.

The finding that GFAP, which is expressed principally by differentiated astrocytes, increased with time to form a well defined dense outer glial border surrounding the circumference of the aggregate was in agreement with other aggregate culture studies prepared from whole brain and telencephalon cells (Trapp et al., 1979; Monnet Tschudi & Honegger, 1989). Astrocytes constitute the preponderant cell type
in aggregating CNS cultures comprising 57% of total cells in the central half of the aggregate and over 71% around the periphery (Trapp et al., 1979; Honegger et al., 1979). In fact, there are a disproportionately high number of these cells present in 4 week old aggregates compared with adult rat brain (Honegger, 1985; Riederer et al., 1992). Initial indications suggesting that the developmental increase and organisation of GFAP in macrophage-enriched cultures was not markedly different to that in standard cultures needs to be further investigated as GS activity was reduced by as much as 30% in macrophage-enriched aggregate cultures (Loughlin et al., 1994) implying that macrophage enrichment may affect astrocytes.

The outer astrocytic border in the aggregates may be broadly likened to the limiting glial membrane that surrounds the external surface of the CNS in vivo and could have a similar function by acting as a protective barrier forming a boundary between the outside of the aggregate and more central regions while possibly regulating the passage of molecules to and from the aggregate. Additionally they may provide structural neuronal support particularly during maturation where, as occurs in the developing CNS, astrocytes extend radial glial fibres across the full thickness of the cortex providing a guiding pathway for the migration of neurons (Rakic, 1971).

The cell bodies of both oligodendrocytes and neurons tended to be orientated towards the medullary region of the aggregate. The finding that oligodendrocyte cell bodies were not as abundant as neurons, with only 10 to 15 detected per aggregate section, is in line with Trapp et al., (1979) who determined that the proportion of oligodendrocytes in whole brain aggregate cultures was indeed very low. It has been suggested that oligodendrocytes develop from a small pool of progenitor cells which proliferate during the first 2 weeks in vitro and become postmitotic thereafter unlike the
majority of neurons which enter terminal differentiation during the first few days in culture (Honegger & Matthieu, 1990). Neuronal processes were highly organised by the fourth week in culture predominantly forming a concentric ring just inside the outer astrocytic border. Accordingly, the specific activity for enzymes associated with neuronal differentiation was highest between DIV=20 and 30 in whole brain aggregate cultures (Matthieu et al., 1980). In support of our morphological findings detailed studies of the developmental expression of the neurofilament protein subunits with heavy (NF-H) and medium (NF-M) molecular mass revealed that they accumulate sequentially in the aggregate cultures over time with the expression of NF-M preceding that of phosphorylated NF-H which closely resembled the pattern observed in vivo (Riederer et al., 1992). Furthermore, the importance of cellular interactions within the cultures is highlighted by the finding that neuron-enriched aggregates, which contain very few glial cells, have much lower levels of NF-M and phosphorylated NF-H implying that glial cells are critical for the development of the neuronal cytoskeleton. Preliminary studies revealed that while there was no obvious difference in the number of neuronal cell bodies in macrophage-enriched aggregates there was a marked increase in the intensity and breadth of neurofilament staining compared to standard cultures which may indicate the presence of increased neuronal processes although these findings need to be investigated further.

MBP was increasingly orientated into a ring towards the perimeter of the aggregate just inside of the astrocytic layer and in association with neurofilament positive cells. Accordingly, EM revealed that several concentric compact layers of myelin lamellae present in standard and macrophage-enriched aggregate cultures were associated with axons. The well defined layering of the aggregates resembles the brain to some
extent, despite discrepancies in the anatomical location, with the centre of the aggregate
heavily populated with neuronal cell bodies broadly comparable to the surface of the
cerebrum, the grey matter of the cerebral cortex. Similarly, beneath the cerebral cortex
lies the cerebral white matter which consists of many myelinated axons and as such may
resemble the region comprising the majority of axons and myelin in the aggregate
despite its more external location adjacent to the astrocytes bordering the entire
periphery. These findings are in accord with other morphological studies of
differentiating rat brain aggregates (Trapp et al., 1979; Monnet Tschudi & Honegger,
1989) and support the idea that foetal cells are indeed capable of migration and re-
arrangement within the three-dimensional aggregate structure.

Myelin accumulated in the aggregates over time as indicated by increasing levels
of MBP transcript and protein using mRNA, biochemical and morphological analyses.
MBP mRNA paralleled the developmental changes observed in vivo where maximal
MBP gene expression occurs between 16 and 21 days postnatally (Campagnoni et al.,
1978; Sorg et al., 1987; Kruger et al., 1999). Similarly, the appearance of translated
MBP protein and the timing of myelination in the aggregates was comparable to that
which occurs in vivo. Furthermore, the pattern of MBP isoform expression in the
aggregate cultures was also similar to that of the developing brain (Kruger et al., 1999).
Accordingly, in whole brain aggregate cultures myelination commences between
DIV=18 and 19 (Matthieu et al., 1978) followed by a period of intense myelin
formation (Matthieu et al., 1978; Almazan et al., 1985) when the myelin lipid
synthesising enzymes, ceramide galactosyltransferase and galactosylceramide
sulphotransferase are at their peak (Matthieu et al., 1980).
In support of these observations EM revealed that myelin which was not axonally associated at early time points gradually disappeared as the number of myelinated axons substantially increased which may reflect ongoing tissue modelling and suggests that myelin specific mRNA expression and MBP accumulation does represent myelin deposition around axons in the ordered structure of the aggregates. In agreement with our findings, morphometric and biochemical studies have demonstrated that MBP mRNA and protein levels in mice with a range of MBP deficiencies correspond to the degree of CNS myelination (Shine et al., 1992). Thus, MBP levels give an indication of the amount of myelin formed and MBP accumulation parallels myelin synthesis and deposition around axons. Accordingly, from the fourth week onwards in whole brain aggregate cultures compact myelin sheaths with up to 22 spirally wrapped lamellae were observed surrounding axons with periodicity similar to that found in vivo (Trapp et al., 1982). Furthermore, the chemical composition of myelin membranes isolated and purified from these aggregates is similar to that isolated from adult rat brain (Matthieu et al., 1979). In line with our findings immunocytochemical studies have detected MBP in the oligodendrocyte cytoplasm of rat whole brain aggregate cultures at early time points (Matthieu et al., 1978; Trapp et al., 1979). This precedes morphological evidence of myelin sheath formation implying that myelin components may first accumulate in the oligodendrocyte cell body cytoplasm prior to insertion into sheath membranes as the number of MBP positive cells increase and myelination proceeds (Trapp et al., 1982).

Furthermore, aggregate cultures enriched with peritoneal macrophages accumulated significantly more MBP mRNA and protein than non-enriched cultures suggesting increased myelin deposition over time. That there is no concomitant rise in the number of oligodendrocyte cell bodies or in the activity of the mature oligodendrocyte marker
CNP suggests that oligodendrocytes are predominantly differentiating and producing more myelin in the presence of macrophages (Loughlin et al., 1994; Loughlin et al., 1997). This is in line with reports that the majority of the enzyme is associated with single membrane structures such as the oligodendrocyte cell body membrane and paranodal regions of myelin, and is essentially absent from compacted, multi-lamellar myelin (Sprinkle, 1989). Therefore, CNP activity is a reflection of oligodendrocyte number, however, minor amounts may also be present in CNS myelin. Additionally, there may be an increase in the synthesis of CNP or in the amount of enzyme that is solubilised which may obscure the interpretation of results.

In line with the observation that macrophage supplementation enhanced MBP mRNA and protein, the expression of the myelin protein genes proteolipid protein and myelin-associated glycoprotein were also elevated (Loughlin et al., 1997). This suggests that the augmented MBP content in response to macrophage addition is occurring at the level of transcription as well as translation and involves the co-ordinate expression of myelin proteins, consistent with the deposition of structurally intact myelin. Although from EM studies it appears that the numbers of myelinated axons are greater in macrophage-enriched cultures variation in the size and level of internal organisation of the individual aggregates, both within and between samples, limits opportunities for objective quantitation (Loughlin et al., 1997).

The increased myelin deposition in macrophage-enriched cultures was somewhat unexpected as macrophages and microglia are both implicated in demyelination (Prineas & Wright, 1978). However, macrophages and microglia display a wide range of effector properties implicating them not only in CNS damage and cell death but also in its morphological and functional development as well as repair. Hence, it has been
demonstrated *in vitro* that macrophages, with their phagocytic and cytotoxic capacity, can kill developing neurons by numerous mechanisms (Piani *et al.*, 1991; Chao *et al.*, 1992) while *in vivo* reducing macrophage recruitment by early injections of colchicine or chloroquine following ischaemic damage lessened axonal degeneration and improved recovery (Giulian & Robertson, 1990). The paradoxical nature of microglia is highlighted by the findings that microglial-conditioned medium was toxic to mesencephalic dopaminergic neurons *in vitro* while microglia or microglial-conditioned medium cocultured with dopaminergic neurons enhanced their survival (Zietlow *et al.*, 1999) implying that contact with neuronal cells, or diffusible factors secreted by them, alters the repertoire of microglial secretory molecules thus converting them from toxic to neuroprotective. Hence macrophages and microglia are identifiable in the developing CNS where they are thought to have a role in tissue modelling, cellular homeostasis and the facilitation of axonal growth and myelination (Perry & Gordon, 1991). Accordingly, at a time corresponding to the period of considerable natural cell death in the developing CNS there is an invasion of monocytes resulting in a rapid accumulation of macrophages which can be ultrastructurally observed phagocytosing dying cells and debris (Hume *et al.*, 1983; Perry *et al.*, 1985). These cells are also implicated in the selective elimination of misdirected, redundant and non-functional neuronal projections particularly prior to myelination (Cowan *et al.*, 1984; Ferrer *et al.*, 1990) while they may also play a part in tissue modelling in the adult CNS (Pow *et al.*, 1989). In addition to their phagocytic capacity macrophages and microglia produce a variety of secretory products which may regulate the proliferation, differentiation and survival of multiple cell types in the developing CNS including myelin-producing oligodendrocytes (Mallat & Chamak, 1989; Nathan, 1987; Shimojo *et al.*, 1991; Diemel *et al.*, 1998). Their
ability to secrete peptides which are mitogenic for astrocytes such as interleukin-1 (IL-1) implicate them in gliogenesis (Giulian & Lachman, 1985; Giulian & Baker, 1985) while they may also stimulate angiogenesis via transforming growth factor-β1 (TGF-β1) (Finch et al., 1993), tumour necrosis factor-α (TNF-α) (Knighton et al., 1983; Leibovich et al., 1987) or IL-1 production (Giulian et al., 1988b). The close association of macrophages and microglia to pathways of developing axons also implicates them in promoting neuronal growth (Perry et al., 1985; Cuadros et al., 1993). In support of this notion, microglial conditioned medium is associated with the development of neurons in vitro by enhancing the survival and neurite extension of cultured mesencephalic neurons from embryonic rat brain (Nagata et al., 1993) while also promoting cholinergic differentiation in the basal forebrain (Jonakait et al., 1996). Similarly, embryonic brain macrophage-conditioned medium promotes neurite outgrowth of embryonic rat striatal neurons and regeneration of differentiated neurons following injury by secreting the extracellular matrix glycoprotein thrombospondin in vitro (Chamak et al., 1994). Microglial-derived IL-1 and interleukin-6 (IL-6) may also favour the survival and growth of neurons with, for example, IL-6 able to stimulate differentiation of the neuronal cell line PC12 (Satoh et al., 1988) while both cytokines enhance the astrocytic production of nerve growth factor (NGF) and fibroblast growth factor-2 (FGF-2) in vitro (Frei et al., 1989; Araujo & Cotman, 1992). Macrophages and microglia produce FGF-2 (Shimojo et al., 1991; Araujo & Cotman, 1992) and NGF (Mallat et al., 1989; Rappolee & Werb, 1990) as well as a multitude of other cytokines and trophic factors known to influence not only neuronal growth but also the proliferation, migration, differentiation, survival and myelinating capacity of oligodendrocytes. Hence, in
addition to NGF and FGF-2, platelet-derived growth factor (PDGF) is released by macrophages in vitro (Bonner et al., 1991) as is transforming growth factor-β1 (TGF–β1) (Wahl et al., 1990; Morgan et al., 1993; Kiefer et al., 1998; Lehrmann et al., 1998), insulin like growth factor-I (IGF-I) (Nagaoka et al., 1991), EGF (Plata-Salaman, 1991) and the neurotrophins brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Elkabes et al., 1996). Furthermore, oligodendrocytes are known to express receptors and respond to these factors (Bansal et al., 1996; Miyake et al., 1996; Pringle et al., 1992; Otero & Merrill, 1994; Masters et al., 1991; Simpson et al., 1982; Barres et al., 1994c; Wetmore & Olson, 1995; Cohen et al., 1996).

During the period before myelinogenesis numerous microglia are found in developing fibre tracts (Ling et al., 1982; Leong et al., 1983; Innocenti et al., 1983). Furthermore, the increase in invading monocytes and their morphological changes into fully differentiated microglia coincides with the time of peak myelination in the rat (postnatal day 20) supporting a role for macrophages in contributing to and enhancing myelin production as was observed in the aggregate cultures. In line with our findings, in foetal spinal cord aggregate cultures enriched with macrophages, enhanced myelination was observed by EM, while the addition of menadione-induced oxidative stress resulted in complete disruption of the aggregate. This implies that oxidative stress exacerbates the inflammatory response of the macrophage while in the absence of stress macrophage enrichment enhances myelinogenesis (Bartnik et al., 2000). Similarly, microglia or microglia-conditioned medium co-cultured with purified oligodendrocytes at physiological ratios of 1:1 elevated levels of the myelin membrane components.
sulphatide, MBP and PLP while CNP activity was essentially unaffected (Hamilton & Rome, 1994). A contributory role for macrophage-derived growth factors in accelerating maturation and enhancing myelination in enriched aggregate cultures is further supported by the finding that exogenous growth factor addition to telencephalic aggregates accelerated cellular maturation. In particular, immature cultures exposed to EGF exhibited enhanced developmental increases of astroglial, neuronal and oligodendrocyte markers reflecting accelerated neuronal and glial cell maturation (Monnet Tschudi & Honegger, 1989). Hence, the apparent enhanced organisation in macrophage-enriched aggregate cultures could be due to their ability to secrete growth factors which influence cell maturation and provoke changes in cellular organisation and tissue morphology. That macrophages may be enhancing myelinogenesis by releasing soluble factors is further supported by our preliminary findings that addition of macrophage-conditioned medium (MCM) to standard aggregate cultures enhanced MBP levels as effectively as macrophage enrichment.

The total number of cells per aggregate section fell after the fourth week in culture suggesting that natural cell death occurs in the aggregates as they become increasingly organised which is comparable with the situation in vivo. Additional support for this hypothesis is provided by the observation that there was little evidence of necrosis in the aggregates at this time. Previous studies have shown that the total DNA content rises steadily during the first two weeks in telencephalic aggregate cultures when the majority of cells are engaged predominantly in cellular proliferation (Honegger et al., 1979; Lenoir & Honegger, 1983; Guentert Lauber et al., 1985). This is followed by a prolonged period of progressive cellular differentiation. However, in line with our findings, Lenoir et al., (1983) report a reduction in the total DNA of
aggregate cultures after 14 days in vitro indicative of natural cell death. Natural cell death is a widespread phenomenon occurring among a large number of cells in most animal tissues at some stage of their development (Oppenheim, 1991). In almost all regions of the peripheral and central nervous system between 20 and 80% of the original neuron and oligodendrocyte population degenerate during specific time periods in embryonic and postnatal development prior to the formation of a stable mature nervous system (Oppenheim, 1991; Barres et al., 1992a). Regulation of the timing and extent of cell death is controlled, at least in part, by influences derived from or associated with synaptic targets with neurotransmitter release, electrical activity and competition for a limited number of synaptic sites and limited amounts of trophic agents likely to influence survival (Barde, 1988; Barde, 1989; Oppenheim, 1989). Additionally, developing neuronal and glial cells may be competing for limited amounts of survival factors derived from afferents, glia or the extracellular matrix (Oppenheim, 1991; Barres et al., 1992a).

Macrophages and microglia, as mediators of phagocytosis, are implicated in naturally occurring cell death and can be observed phagocytosing dying cells (Hume et al., 1983; Perry et al., 1985). Hence, it has been suggested that monocytes invade the CNS during natural cell death in response to products of cellular degeneration acting as chemoattractants although other signals may also be involved. Accordingly, in macrophage-enriched aggregates the content of the MBP degradation peptide 34T, used to assess the degree of myelin breakdown following phagocytosis, decreased significantly during the later period of the culture while levels in standard cultures remained relatively unchanged further indicating the removal of cellular debris with ongoing organisation particularly in the presence of macrophages.
This is supported by the finding that in macrophage-enriched cultures at a time corresponding to a period of extensive organisation and myelination in the aggregates, the number of phagocytic macrophages and microglia was significantly higher than in standard cultures. However at the time of total cell reduction and decline in MBP degradation products, the number of activated macrophages and microglia had halved to levels comparable with those in standard cultures which remained relatively constant. Therefore, the enhanced myelination evident in macrophage-enriched aggregates may be attributable to the increase in phagocytic macrophages and microglia indicative of a role for these cells in natural cell death while reverting to a resting state once this process is completed. Hence, the presence of exogenous macrophages with enhanced phagocytic activity may lead to more efficient removal of myelin, as indicated by the reduced levels of MBP degradation products, as well as other cellular debris. The release of soluble substances such as trophic factors may then act to shape the gross morphology of the aggregate thereby leading to enhanced myelination.

That the content of MBP degradation peptide rose immediately in mature standard aggregates that had received exogenous macrophages at a time corresponding to the period of peak myelination followed by elevated MBP levels suggests that macrophages are able to influence myelination in the cultures even when it is already underway. Similarly, microglia cocultured with oligodendrocytes at multiple time points up to their peak period of myelin synthesis stimulated myelination. However, microglia added to oligodendrocytes after this time were not stimulatory suggesting that oligodendrocytes become refractory to microglia once they pass their peak period of myelin synthesis (Hamilton & Rome, 1994).
In conclusion, supplementing aggregate cultures with peritoneal macrophages increased myelin deposition over time while CNP remained unchanged, suggesting that oligodendrocytes are producing more myelin in the presence of macrophages. These results have implications for demyelinating diseases such as MS where macrophages form a primary component of the inflammatory process and as cells capable of stimulating oligodendrocyte development, myelination and long term survival may provide a potential strategy for therapeutic intervention in CNS demyelinating diseases such as MS.
CHAPTER 4

DEMYELINATION OF STANDARD AND MACROPHAGE-ENRICHED AGGREGATE CULTURES

4.1 Introduction

Cytokines may have a role in the pathogenesis of multiple sclerosis (MS) and other inflammatory diseases as it is associated with enhanced levels of interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-12 in central nervous system (CNS) lesions and peripheral blood (Olsson, 1995; Navikas & Link, 1996). Cytokines with their diverse range of cell regulatory activities within the CNS have the ability to activate macrophages and microglia, possibly promoting inflammation and phagocytosis of myelin leading to demyelination. The functional properties of macrophages and microglia are subject to regulation by cytokines with, for example, expression of the receptor for the Fc portion of immunoglobulin (FcR) up-regulated by IFN-γ, TNF-α and interleukin-1α (IL-1α) (Loughlin et al., 1992). The cytokines IL-1α, IFN-γ and TNF-α have all shown demyelinating potential when investigated previously in the aggregating rat brain cell cultures and this demyelination was additively increased by a monoclonal antibody directed against myelin oligodendrocyte glycoprotein (MOG) in the presence of complement (Loughlin et al., 1994).

The demyelinating activity in MS has been linked to the presence of antibodies in the cerebrospinal fluid (CSF) and sera (Walsh & Tourtellotte, 1983; Raine, 1984a) although the antigens responsible for inducing antibody-mediated demyelination have yet to be elucidated. MOG is a CNS specific glycoprotein located preferentially at the external surface of myelin sheaths and oligodendrocytes (Brunner et al., 1989). Extensive CNS demyelination can be induced by the anti-MOG antibody 8-18C5 in vivo (Linnington et al.,
1984) while anti-MOG antibodies are present in chronic relapsing experimental allergic encephalomyelitis (EAE) (Linington & Lassmann, 1987) suggesting that it may be a potential target for antibody-mediated demyelination. Addition of anti-MOG plus complement to the culture system has previously been shown to induce demyelination without the loss of oligodendrocytes (Kerlero de Rosbo et al., 1990).

The demyelinating potential of the cytokines IFN-γ, IL-1α and TNF-α was investigated, as was the effect of treating cultures with anti-MOG antibody in the presence of complement in myelinating aggregate rat brain cultures. The capacity for remyelination was also assessed. Furthermore, the influence of macrophages during the course of both demyelination and remyelination was investigated.

4.2 RESULTS

4.2.1 Cytokine induced demyelination in aggregate cultures

4.2.1.1 IFN-γ, IL-1α and TNF-α induced demyelination in standard aggregate cultures

Non-enriched aggregate cultures were treated with the demyelinating cytokines IFN-γ (25U/ml), IL-1α (200U/ml) and TNF-α (5 and 25ng/ml) plus 0.1% BSA from DIV=32 to 36 while untreated control aggregates received 0.1% BSA during the same 4 day period. Aggregate samples were harvested for analysis prior to cytokine addition (DIV=32), at the end of the treatment period (DIV=36) and 4 days after the cytokines had been washed out (DIV=40). The samples were homogenised before total protein and MBP assays were completed as described in Chapter 2 with MBP content expressed as µg per mg of total protein ± 1 SEM.
Four day exposure to IFN-γ, IL-1α and TNF-α resulted in a reduction in the MBP content while MBP synthesis resumed once the demyelinating cytokines had been washed out. On DIV=36, at the end of the 4 day treatment period, a significant 69% reduction in MBP content was observed in aggregate cultures exposed to IFN-γ (25U/ml) while IL-1α (200U/ml) induced a significant 57% decrease in MBP compared to untreated control flasks (p<0.05; Figure 4.1A). Addition of TNF-α to aggregate cultures on DIV=32 and 34 at concentrations of 5 and 25ng/ml resulted in a dose-dependent reduction in MBP content such that 25ng/ml caused a 63% significant loss of MBP (p<0.05) compared to only a 27% reduction at the lower 5ng/ml dose (Figure 4.1B).

Once the cytokines had been washed out the cultures were maintained up to DIV=40 and MBP continued to accumulate during these 4 days in control and previously demyelinated flasks, hence the demyelination was reversible. During this period the MBP content in untreated flasks continued to accumulate each day by an average of 0.05µg MBP/mg total protein while MBP also increased in flasks previously exposed to demyelinating cytokines. The MBP content in flasks that had received IL-1α doubled over this 4 day period (p<0.05) increasing at a rate of 0.11µg/mg total protein a day to reach levels that were only slightly lower than untreated controls (Figure 4.1A).

In those flasks previously exposed to TNF-α (5ng/ml) MBP accumulated at the same rate as non-treated flasks (Figure 4.1B) while the MBP content significantly increased by 2.5 fold (p<0.01) in cultures that had received TNF-α (25ng/ml) with both groups reaching MBP levels similar to untreated controls.
Figure 4.1. MBP content of aggregate cultures treated with IFN-γ (25U/ml), IL-1α (200U/ml) (A) and TNF-α (5 and 25ng/ml) (B) from DIV=32 to 36. All of the flasks received 0.1% BSA. Results are expressed as mean MBP content (μg/mg total protein) ± 1 SEM where n = 3 for each time point from a representative experiment sampled at the end of the treatment period (DIV=36) and 4 days after removal of the cytokines (DIV=40). * denotes significance (p ≤ 0.05) from medium controls as determined by unpaired Student’s t-test.
While the MBP content did increase by 56% during the 4 days following IFN-γ removal levels attained were at least half those reached by IL-1α and TNF-α treated flasks and significantly less than that of controls.

4.2.1.2 IFN-γ and IL-1α induced demyelination in standard and macrophage-enriched aggregate cultures

As discussed in Chapter 3 addition of peritoneal macrophages to aggregate cultures at the time of seeding results in a significant increase in MBP which is consistent with the data from the representative demyelination experiment illustrated in Figure 4.2 where the MBP content of macrophage-enriched aggregate cultures was significantly 3 times that of non-enriched cultures at DIV=40 (p<0.001). Both standard and macrophage-enriched cultures were exposed to the demyelinating cytokines IFN-γ (50U/ml) and IL-1α (200U/ml) from DIV=25 to DIV=29 whereupon the treatment was washed out and the cultures were maintained for a further 11 days up until DIV=40. The MBP content in standard and macrophage-enriched cultures was reduced following exposure to both of these cytokines. IFN-γ addition resulted in an MBP reduction of 0.42µg/mg total protein in both standard and macrophage-enriched aggregate cultures, however this was equivalent to a 67% loss of MBP (comparable to the reduction described in the previous section) in standard flasks compared to only a 43% decrease in macrophage-enriched flasks which without treatment contained 1.6 times more MBP than comparable non-enriched cultures.
Figure 4.2. MBP content of standard (A) and macrophage-enriched (B) aggregate cultures treated with IFN-γ (50U/ml) and IL-1α (200U/ml) from DIV=25 to 29. All of the flasks received 0.1% BSA. Results are expressed as mean MBP content (µg/mg total protein) ± 1 SEM where n = 2 to 6 for each time point from a representative experiment sampled prior to treatment (DIV=25), at the end of the treatment period (DIV=29) and 11 days after cytokine removal (DIV=40). * denotes significance (p ≤ 0.05) from medium controls as determined by Student's t-test.
Addition of IL-1α resulted in a 37% and 43% reduction in MBP content in standard and enriched aggregate cultures respectively equivalent to 0.23 and 0.42 μg/mg total protein. By DIV=40, 11 days after any treatment had been washed out, MBP continued to accumulate in control and previously demyelinated flasks. In standard untreated cultures the MBP content increased 2 fold to 1.24 μg MBP/mg total protein by DIV=40 which is equivalent to an MBP increase of 0.05 μg/mg total protein per day, an observation consistent with the increases described from the representative demyelination experiment illustrated in Figure 4.1. Over the same 11 day period the MBP content in macrophage-enriched flasks increased significantly by nearly 4 fold (p<0.01), with an MBP increase of 0.25 μg/mg total protein per day, to reach levels 3 times higher than that of non-enriched cultures. After IL-1α treatment had been removed the level of MBP in standard aggregate cultures rose at a rate of 0.15 μg/mg total protein per day to reach 2 times the level observed in medium controls while the MBP content in similarly treated macrophage-enriched cultures increased by 7 fold to reach levels comparable to enriched medium controls. As shown in the previous section IFN-γ treated flasks did not reach levels obtained in non-treated controls however, over a 4 fold increase in MBP was observed in both standard and enriched aggregate cultures. Hence, while standard and macrophage-enriched cultures previously treated with IL-1α accumulated more MBP per day than respective controls, cultures treated with IFN-γ re-accumulated MBP at the same rate or at a slightly lower rate than controls. Furthermore, the increased potential for MBP accumulation was retained in macrophage-enriched cultures that had been demyelinated such that in this representative experiment during the 11 days from DIV=29 to 40 enriched aggregates previously treated with IL-1α or IFN-γ accumulated MBP at 2 and 2.5 times the rate of standard cultures.
respectively so that at DIV=40 macrophage-enriched flasks previously treated with IL-1α contained 1.8 times more MBP than comparable non-enriched flasks and despite IFN-γ lagging behind the MBP content at DIV=40 was still 2.6 times higher than its non-enriched equivalent and higher than flasks with no added macrophages. However, when expressed as a proportion of their respective untreated medium control it appeared that macrophage-enriched flasks were not recovering as well as standard aggregate cultures.

4.2.2 Anti-myelin oligodendrocyte glycoprotein antibody induced demyelination in aggregate cultures

To elucidate the potential effect of antibody-induced demyelination aggregate cultures were exposed to two anti-MOG antibody clones in the presence of complement (guinea pig serum) on DIV=34 for a period of 48 hours. The antibodies were an IgG1, kappa (IgG1,κ) mouse monoclonal antibody derived from clone 8-18C5 (Linington et al., 1984) and an IgG2a, kappa (IgG2a,κ) MOG specific monoclonal antibody Z12 (Piddlesden et al., 1993) added to the cultures as described in Chapter 2 to give final concentrations of 31.3μg/ml antibody and 25μl/ml of guinea pig serum per flask. Control flasks received the corresponding purified immunoglobulin mouse IgG1,κ or IgG2a,κ in the presence of complement or complement alone. On DIV=36 following a 48 hour treatment period with anti-MOG antibody or appropriate controls aggregate cultures were sampled before treatments were diluted out of the flasks and the cultures maintained.
4.2.2.1 Anti-myelin oligodendrocyte glycoprotein induced demyelination in standard and macrophage-enriched aggregate cultures

The results illustrated below were selected as representative from 10 separate anti-MOG demyelination experiments performed. Addition of both 8-18C5 and Z12 anti-MOG antibodies in the presence of complement for 48 hours resulted in a significant reduction in the MBP content of standard and macrophage-enriched aggregate cultures as determined by RIA (Figure 4.3). Electron microscopy studies confirmed that previously organised myelin was disrupted on addition of 8-18C5 or Z12 anti-MOG antibodies in the presence of complement (Figure 4.4A and B). Myelin surrounding the axons appeared morphologically abnormal with the splitting of lamellae and the formation of large vacuoles around and within the myelin sheath. The presence of disorganised extracellular myelin debris was also evident as was the occasional macrophage containing ingested myelin (Figure 4.4C).

In standard cultures 8-18C5 plus complement resulted in a significant 73% reduction in MBP compared to cultures receiving medium alone (p<0.01) while in macrophage-enriched cultures, which contained 3.8 times more MBP prior to treatment, the MBP content decreased by an absolute value of 3.39μg/mg total protein corresponding to a 68% loss compared to macrophage-enriched flasks receiving medium alone (p<0.05; Figure 4.3A). The IgG2a,κ MOG specific monoclonal antibody Z12 resulted in a significant reduction in the MBP content of both standard and macrophage-enriched aggregate cultures by 88 and 70% respectively compared to medium controls (p<0.01; Figure 4.3B).
Figure 4.3. MBP content of standard and macrophage-enriched aggregate cultures treated with demyelinating 8-18C5 (A) or Z12 (B) anti-MOG antibodies plus complement (GPS) from DIV=34 to 36. Control flasks received IgG1 or IgG2a plus GPS or GPS alone over the same time period. Results are expressed as mean ± 1 SEM where n = 2 - 7 for each time point from a representative experiment sampled at the end of the treatment period (DIV=36). *, V and O denote significance (p ≤ 0.05) from medium, IgG1 plus GPS or GPS alone flasks respectively as determined by Student's t-test.
Figure 4.4. Demyelinating effect of 48 hour exposure to 8-18C5 (A) or Z12 (B) anti-MOG antibodies plus complement on DIV=34 in macrophage-enriched aggregate cultures. Widespread disruption of the myelin sheath can be observed with swelling and splitting of the lamellae and the presence of large vacuoles. C. The occasional macrophage containing ingested myelin was also present. Scale bar = 2000nm.
While the absolute reduction in MBP tended to be greater in macrophage-enriched aggregate cultures the loss, when expressed as a percentage of appropriate medium controls, was similar which was a trend observed in all of the antibody demyelinated preparations (Figure 4.5). In standard and macrophage-enriched cultures the MBP loss following treatment with Z12 was significantly greater than the reduction observed in cultures exposed to 8-18C5 anti-MOG antibody (p<0.05; Figure 4.5). Hence non-enriched Z12 flasks contained only 18% the MBP content of their appropriate medium control compared to 45% in 8-18C5 flasks and only 24% compared to 40% in macrophage-enriched Z12 and 8-18C5 treated flasks respectively.

Addition of the non-specific IgG1,κ antibody in the presence of complement caused a 33% reduction in the MBP content of cultures that were not enriched with macrophages and only a 16% reduction in those that were, with neither significant from medium controls in this experiment (Figure 4.3A). Exposure to the non-specific IgG2a antibody plus complement reduced the MBP content in standard flasks by 56% while the MBP content in macrophage-enriched flasks remained comparable to medium controls (Figure 4.3B). The demyelinating potential of IgG1 or IgG2a was variable between individual experiments with IgG1 addition to standard aggregate cultures resulting in a reduction in the MBP content in 4 of the 7 demyelination experiments performed while IgG2a reduced the MBP content in two thirds of cases (Figure 4.5). In macrophage-enriched cultures IgG1 or IgG2a reduced the MBP content in 7 out of 9 experiments.
Treatments

Figure 4.5. MBP content of standard and macrophage-enriched aggregate cultures treated with 8-18C5 or Z12 anti-MOG antibodies plus complement (GPS) from DIV=34 to 36. Control flasks received IgG1 or IgG2a plus GPS or GPS alone over the same time period. Mean MBP content of at least 3 flasks from individual aggregate culture preparations was expressed as a % of medium control with data shown in each group from multiple aggregate culture preparations.
Whether addition of non-specific IgG antibodies plus complement resulted in a reduction in MBP compared to medium controls, as it did in both standard cultures and to a lesser extent in macrophage-enriched IgGl plus complement treated flasks (Figure 4.3A and B), the MBP content in anti-MOG treated aggregates was significantly lower than respective IgG treatment a trend apparent in all other demyelination preparations and reaching significance in 74% of cases.

Addition of complement alone, in the form of guinea pig serum, had no discernible effect on the MBP content of standard or macrophage-enriched aggregate cultures in the representative experiment shown in Figure 4.3A while it resulted in a significant 75 and 48% reduction in MBP compared to medium controls (p<0.05) in the demyelination experiment illustrated in Figure 4.3B. Hence, as is the case with IgG, the demyelinating potential of complement alone was variable with a loss of MBP detected in flasks treated with guinea pig serum in two thirds of standard and half of the macrophage-enriched demyelination experiments conducted (Figure 4.5). Therefore, the MBP content in standard and macrophage-enriched flasks treated with 8-18C5 anti-MOG antibody was less than those treated with guinea pig serum while no significant difference was observed when comparing Z12 and guinea pig serum flasks. During all treatment conditions macrophage-enriched cultures maintained higher levels of MBP than comparative non-enriched flasks (Figure 4.3) a trend observed in 67% of cases.

Due to the variable demyelinating potential of both the non-specific IgG antibodies and complement it was important to assess their effect on MBP loss in each preparation to ascertain the degree of demyelination resulting from addition of these 2 treatments. Furthermore, since both were capable of inducing some demyelination in the aggregate cultures it seemed appropriate to use the respective standard or
macrophage-enriched medium controls to determine the extent of demyelination resulting from exposure to the anti-MOG antibodies.

4.2.2.2 Remyelination following anti-myelin oligodendrocyte glycoprotein induced demyelination in standard and macrophage-enriched aggregate cultures

After a 48 hour treatment period demyelinating agents and respective controls were washed out and standard and macrophage-enriched aggregate cultures were maintained for a further 12 days during which time MBP continued to accumulate. As previously mentioned in Chapter 3, section 3.2.3.2 the absolute MBP increase in non-treated macrophage-enriched cultures from DIV=36 to DIV=48 is greater than in standard cultures (Figure 4.6) which was a pattern consistent with the MBP increases observed in other preparations. However, the proportional increase during this time period was similar between the two groups (Figure 4.7). Over the same time period aggregates exposed to various treatments re-accumulated MBP to reach levels that were comparable or surpassed those obtained in medium controls. Moreover, in the representative experiment illustrated in Figure 4.6, MBP accumulated at a higher rate in both standard and macrophage-enriched aggregates that had previously been exposed to various treatments than those cultured in medium implying that MBP synthesis was enhanced following the removal of any given treatment. In standard aggregate cultures, in which a significant 31 and 69% MBP loss was incurred on exposure to 8-18C5 and
Figure 4.6. MBP content of standard (A) and macrophage-enriched (B) aggregate cultures treated with demyelinating 8-18C5 and Z12 anti-MOG antibodies plus complement (GPS) from DIV=34 to 36. Control flasks received IgG1 or IgG2a plus GPS or GPS alone over the same time period. Results are expressed as mean MBP content (µg/mg total protein) ± 1 SEM where n = 2 to 4 for each time point from a representative experiment sampled at the end of the 48 hour treatment period (DIV=36) and 12 days after antibody removal (DIV=48). *, V and O denote significance (p ≤ 0.05) from medium, IgG1 plus GPS or GPS alone flasks respectively at relevant time point as determined by Student's t-test.
Figure 4.7. Proportional MBP increase of standard and macrophage-enriched aggregate cultures from DIV=36 to 48 following removal of 8-18C5 anti-MOG antibody plus GPS or controls. Results are expressed as proportional MBP increase from DIV=36 to 48 of at least 3 flasks from individual aggregate culture preparations.
Z12 anti-MOG antibodies plus complement respectively, MBP synthesis resumed once the demyelinating antibodies had been washed out at similar average rates of 0.26 and 0.28µg/mg total protein per day which were approximately 2.5 times higher than the rate of MBP accumulation in medium controls. This was consistent with observations in other preparations where MBP accumulation during the 12 days following 8-18C5 anti-MOG plus complement removal was greater than for any other treatment group (Figure 4.7) and significantly 2.5 times higher than the increase measured in medium controls over the same time period (p<0.05). Hence, by DIV=48 the MBP content in aggregate cultures previously treated with 8-18C5 or Z12 antibodies had significantly surpassed medium controls by 81 and 71% respectively (p<0.05) which was a finding reflected in data from other 8-18C5 anti-MOG experiments (Figure 4.8). Electron microscopy studies confirmed that the myelin disruption incurred in aggregate cultures on exposure to the demyelinating antibodies 8-18C5 or Z12 anti-MOG antibodies in the presence of complement was reversible, hence the ultrastructural appearance of the myelin sheath correlated to the biochemically detected increase in MBP associated with the re-establishment of myelination (Figure 4.9A and B).

As discussed in the previous section the demyelinating potential of non-specific IgG antibodies and complement were variable and in the experiment shown in Figure 4.6 IgG1 and IgG2 treated cultures were not demyelinated. Despite this the rate of MBP accumulation in standard flasks following IgG1 and IgG2a removal was higher than that of any other treatment group with increases of 0.37 and 0.31µg/mg total protein per day so that by DIV=48 MBP levels had risen to over twice those observed in medium.
Figure 4.8. MBP accumulation of standard and macrophage-enriched aggregate cultures from DIV=36 to 48 following treatment with 8-18C5 anti-MOG antibody plus complement (GPS) or appropriate controls. Mean MBP content of at least 3 flasks from individual aggregate culture preparations was expressed as a % of medium control with data shown in each group from multiple aggregate culture preparations.
Figure 4.9. Longitudinal section of macrophage-enriched aggregate cultures on DIV=40, 6 days after removal of 8-18C5 (A) or Z12 (B) anti-MOG antibodies plus complement. Myelination has been re-established and the amount of extra-cellular myelin debris is greatly reduced. Scale bar = 1000nm.
controls (p<0.05). It can be deduced from Figure 4.7 that the proportional MBP increase following IgG1 treatment removal was twice that of medium controls so that MBP levels surpassed medium controls regardless of whether this treatment had resulted in measurable demyelination or not (Figure 4.8). Addition of complement to standard aggregate cultures resulted in a significant 37% MBP loss in this experiment, subsequently, in the 12 days after diluting out this treatment MBP, increased at twice the rate observed in medium controls (Figure 4.6) which was a pattern conserved among all the demyelinating preparations, as illustrated in Figure 4.7, although final levels attained were not as high as those observed in 8-18C5 or IgG1 treated cultures.

MBP synthesis did resume once treatments had been washed out of macrophage-enriched aggregate cultures, however, macrophage-enriched flasks exposed to anti-MOG or IgG antibodies in the presence of complement did not gain as much MBP as similarly treated standard cultures (Figure 4.6) a trend which was apparent in all of the demyelination experiments performed (Figure 4.7). Exposure to 8-18C5 or Z12 antibodies in the presence of complement did significantly reduce the MBP content in macrophage-enriched cultures (p<0.05) while following removal of the demyelinating antibodies MBP synthesis resumed at a rate of 0.23µg/mg total protein per day which was higher than the rate in macrophage-enriched medium controls (Figure 4.6). Furthermore, this was consistent with the trend observed in other 8-18C5 anti-MOG demyelinating experiments where the proportional MBP increase following 8-18C5 antibody removal in macrophage-enriched aggregates was almost 4 times that in non-treated medium cultures (Figure 4.7). However, despite accumulating more MBP than their medium controls the absolute and proportional increment in MBP by DIV=48 was considerably less than had accumulated in similarly treated standard cultures (Figure
which was consistent with the findings from all the 8-18C5 anti-MOG demyelinating experiments (Figure 4.7). Furthermore, while, in the representative experiment illustrated in Figure 4.6 MBP levels in macrophage-enriched flasks previously treated with anti-MOG antibodies (8-18C5 or Z12) did surpass their medium controls analysis of all 8-18C5 anti-MOG experiments showed that on average macrophage-enriched cultures had re-accumulated enough MBP to reach levels comparable to medium controls, but not to surpass them (Figure 4.8) which was in contrast to standard cultures.

The MBP content in macrophage-enriched cultures previously treated with IgGl and IgG2a antibodies increased at a higher rate than medium controls although they did not reach the levels attained in comparative standard flasks (Figure 4.6). Similarly, it can be seen in Figure 4.7 that the proportional MBP increase in macrophage-enriched flasks previously treated with IgGl antibodies was greater than the increase in medium controls although, as was observed in anti-MOG treated flasks, the proportional MBP increase was much lower than that in standard cultures (Figure 4.7). Hence, by DIV=48 MBP levels had not reached levels attained in medium controls (Figure 4.8). Addition of complement alone resulted in a slight increase in MBP in macrophage-enriched cultures in contrast to the demyelination that resulted on addition to standard cultures. Following complement removal MBP increased significantly at a rate of 0.21μg/mg total protein per day to reach levels that were 1.5 times higher than medium controls (Figure 4.6). Results from all of the demyelination preparations confirmed that in macrophage-enriched flasks the proportional MBP increase was almost 4 times higher than in medium controls (Figure 4.7) so that by DIV=48 MBP levels were comparable between these two groups (Figure 4.8).
4.2.2.3 Anti-myelin oligodendrocyte glycoprotein does not induce demyelination in the absence of complement in standard aggregate cultures

To assess the contribution of complement to antibody-induced demyelination, standard aggregate cultures were exposed to 8-18C5 or Z12 anti-MOG antibody clones both in the presence of and in the absence of complement on DIV=34 for a period of 48 hours. As described in Chapter 2, complement was added to the cultures in the form of guinea pig serum while cultures treated with anti-MOG antibodies in the absence of complement received heat-inactivated guinea pig serum (HIGPS) which destroys complement. Control flasks received the corresponding purified immunoglobulin IgG1,κ or IgG2a,κ in the presence or absence of complement and complement or heat-inactivated complement alone. On DIV=36 aggregate cultures were sampled before treatments were diluted out of the flasks and the cultures maintained until DIV=48.

It was found that neither 8-18C5 or Z12 anti-MOG antibodies in the absence of complement resulted in a significant reduction in the MBP content of standard aggregate cultures (Figure 4.10) despite causing significant demyelination in the presence of complement. Addition of complement alone had resulted in a significant 37% reduction in MBP (p<0.005) compared to only a minimal reduction on addition of heat-inactivated complement. Exposure to the non-specific IgG1 or IgG2a antibodies in the presence of complement had no discernible effect on MBP content in this experiment and neither did addition of these non-specific antibodies in the absence of complement.
Figure 4.10. Effect of 8-18C5 or Z12 anti-MOG antibodies on the MBP content of standard aggregate cultures in the presence of complement (GPS) or in the absence of complement (HIGPS) from DIV=34 to 36. Control flasks received IgG1 or IgG2a with GPS or HIGPS over the same time period. Results are expressed as mean MBP (µg/mg total protein) ± 1 SEM where n = 2-4 for each time point from a representative experiment sampled at the end of the treatment period (DIV=36) and again 12 days later (DIV=48). * denotes significance (p ≤ 0.05) from medium flasks at relevant time point as determined by Student's t-test.
As discussed previously in section 4.2.2.2 MBP continued to increase once the treatments were washed out rising at a rate of 0.11µg/mg total protein per day in standard untreated cultures from DIV=36 to DIV=48 while MBP accumulation was at least double this following removal of antibody plus complement or complement alone. In contrast, the rate of MBP accumulation following antibody treatments in the absence of complement and respective controls were comparable to that in non-treated medium flasks with the exception of IgG1 antibody addition and to a lesser extent IgG2a (Figure 4.10). Following IgG1 and IgG2a plus complement removal MBP increased at rates of 0.37 and 0.31µg/mg total protein per day respectively and unlike the pattern of MBP accumulation following removal of other treatments in the absence of complement after washing out IgG1 or IgG2a plus heat-inactivated complement MBP increased at a enhanced rates of 0.29 and 0.18µg/mg total protein per day respectively (Figure 4.10).

4.2.2.4 MBP degradation peptide content following anti-myelin oligodendrocyte glycoprotein induced demyelination in standard and macrophage-enriched aggregate cultures

The content of MBP peptide was determined within the aggregates by assessing the level of the synthetic MBP degradation peptide 34T using a RIA as described in Chapter 2 to assess the extent of the proteolytic breakdown of MBP. The 34T content (ng/µg MBP) was measured in the aggregates immediately after exposure to anti-MOG demyelinating treatments and at subsequent time points once MBP synthesis had resumed. In the representative experiment illustrated in Figure 4.11A the 48 hour
Figure 4.11. 34T content (ng/μg MBP) of standard and macrophage-enriched aggregate cultures treated with demyelinating 8-18C5 or Z12 anti-MOG antibodies plus complement (GPS) from 2 representative experiments. A. Treated for 48 hours from DIV=32-34 while (B) was treated from DIV=34-36. Control flasks received IgG1 or IgG2a plus GPS or GPS alone over the same time period. Results are expressed as mean ± 1 SEM where n = 2-6 for each time point sampled at the end of the treatment period (DIV=34, 36) and at the indicated times thereafter. *, ∇ and O denote significance (p ≤ 0.05) from medium, IgG1 plus GPS or GPS alone flasks respectively as determined by Student's t-test.
treatment period was from DIV=32 to 34 with both 8-18C5 and Z12 anti-MOG antibodies resulting in an 88% MBP loss while IgG1, IgG2a and complement treatment reduced the MBP content by 73, 68 and 31% respectively. In combination with the MBP reduction, addition of 8-18C5 anti-MOG antibody plus complement resulted in a significant 4 fold increase (p<0.001) in 34T MBP degradation peptide relative to medium controls while a 7.2 fold increase was detected in flasks treated with Z12 plus complement (p<0.05; Figure 4.11A). The content of the degradation peptide 34T was also significantly increased by approximately 3 fold in IgG1 and IgG2a plus complement treated flasks (p<0.001) and by just under 2 fold (p<0.05) in flasks that received complement alone compared to medium controls.

Just 6 days after the demyelinating treatments had been washed out the level of 34T had decreased in all previously treated flasks. The level of 34T in flasks that had been exposed to complement had fallen to reach levels that were comparable to medium controls. The 34T content in flasks previously treated with 8-18C5 and Z12 antibodies plus complement had fallen by 52 and 78% respectively despite remaining significantly higher than medium controls (p<0.05). Similarly, a reduction in the 34T content in flask treated with IgG1 and IgG2a antibodies was observed although levels were still significantly higher than medium controls (p<0.05).

In a separate anti-MOG experiment, where the resulting demyelination corresponds to that shown in Figure 4.3A, the 34T content was assessed in standard and macrophage-enriched aggregate cultures at DIV=36 just prior to treatment removal and again 12 days later at DIV=48. The 34T content in untreated standard and macrophage-enriched aggregate cultures was not significantly different at DIV=36 as discussed in Chapter 3 section 3.2.3.5. It was found that addition of 8-18C5 anti-MOG antibody plus
complement significantly increased the level of 34T by 2 fold (p<0.01) in both standard and macrophage-enriched aggregate cultures compared to respective medium controls (Figure 4.11B). The significant rise in 34T levels associated with standard flasks previously treated with complement exceeded the increase measured in macrophage-enriched flasks. Similarly the increment in the 34T content of standard cultures previously exposed to IgG1 was greater than the slight increase in 34T observed in macrophage-enriched flasks compared to respective medium controls.

Twelve days after any treatment had been washed out of standard cultures the 34T content had fallen to levels comparable to medium controls. While the level of 34T in macrophage-enriched flasks previously treated with 8-18C5 plus complement was reduced it remained higher than medium controls (Figure 4.11B) as opposed to the 34T content in aggregates previously exposed to IgG1 plus complement which fell to levels similar to medium controls.

4.2.2.5 2',3'-Cyclic nucleotide 3'-phosphodiesterase activity following anti-myelin oligodendrocyte glycoprotein induced demyelination in standard and macrophage-enriched aggregate cultures

2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP) which is localised predominantly in oligodendrocytes was used as a marker for mature oligodendrocytes. Standard and macrophage-enriched aggregate cultures were exposed to either 8-18C5 anti-MOG antibody or IgG1 isotype control antibody both in the presence of complement or complement alone from DIV=34 to 36. CNP activity was assessed by the method of Sogin, as described in Chapter 2, on DIV=34 prior to antibody addition, 48 hours later, at the end of the treatment period, and again 6 and 12 days later after the
treatments had been washed out, with CNP activity expressed as \( \mu g/mol/minute/mg \) of total protein ± 1 SEM.

As previously discussed in Chapter 3 section 3.2.3.4 CNP activity in non-treated macrophage-enriched aggregates was not significantly different from standard cultures. While in this representative experiment addition of anti-MOG antibody, IgG1 isotype control antibody or complement alone resulted in a 47, 23 and 42 % reduction in the MBP content of standard aggregate cultures respectively it did not markedly alter CNP activity (Figure 4.12A). Even more strikingly, in the same preparation on exposure to the same treatments, the MBP content of macrophage-enriched flasks was reduced by 62 to 77% and still no change in CNP activity was detected (Figure 4.12B).

During the 12 days following treatment removal the MBP content was found to increase in aggregate cultures as has been discussed in the preceding sections. Furthermore, over this same time period CNP activity was also found to increase in all previously treated standard and macrophage-enriched aggregate cultures to surpass medium controls. In standard aggregate cultures between DIV=36 and 48 there was only minor changes in the CNP activity of medium control flasks (Figure 4.12A). Over this 12 day period, after anti-MOG and IgG1 antibodies in the presence of complement and complement alone treatments had been washed out of standard aggregate cultures, CNP activity was found to have increased significantly to reach levels that were at least twice those of medium controls (Figure 4.12A).
Figure 4.12. CNP activity of standard (A) and macrophage-enriched (B) aggregate cultures treated with demyelinating 8-18C5 anti-MOG antibody plus complement (GPS) from DIV=34 to 36. Control flasks received IgG1 plus GPS or GPS alone over the same time period. Results are expressed as mean CNP activity (μmol/min/mg total protein) ± 1 SEM where n = 2 to 4 for each time point from a representative experiment sampled immediately prior to treatment (DIV=34), at the end of the treatment period (DIV=36) and at the indicated times thereafter. * denotes significance (p ≤ 0.05) from medium flasks at relevant time point as determined by Student's t-test.
In macrophage-enriched cultures between DIV=36 and 48 only minimal changes were observed in the CNP activity of medium only flasks as reported for non-enriched aggregate cultures despite continued MBP accumulation over this time period. As observed in non-enriched cultures, CNP levels in macrophage-enriched aggregates previously exposed to IgG1 plus complement or complement alone increased steadily so that by DIV=48 the activity in these aggregates had reached more than double that of medium controls (p<0.05; Figure 4.12B). In contrast CNP activity did not change markedly once anti-MOG plus complement had been washed out of macrophage-enriched cultures with levels remaining comparable to medium controls.
4.3 DISCUSSION

Treatment of myelinating aggregate cultures with the pro-inflammatory cytokines IFN-\(\gamma\), IL-1\(\alpha\) and TNF-\(\alpha\) or anti-MOG antibodies in the presence of complement resulted in reversible demyelination, indicated by a reduction in MBP content, while CNP activity was largely unaffected indicating a selective destruction of the myelin sheath with preservation of oligodendrocytes. These findings are in agreement with previous work from this laboratory by Loughlin et al. (1994). Demyelination was associated with increased levels of a MBP degradation peptide representative of proteolytic breakdown of MBP. The inability of anti-MOG antibodies to cause demyelination in the absence of complement suggests that this process was, at least in part, mediated by complement. Following removal of demyelinating agents aggregate cultures were found to have the capacity to remyelinate, with resumed MBP synthesis. However, non-enriched aggregate cultures that had been exposed to demyelinating antibodies accumulated more MBP than their comparable macrophage-enriched flasks.

While it has been suggested that it is the direct action of cytokines on oligodendrocytes or the myelin sheath causing tissue damage and demyelination (Brosnan et al., 1988) there is increasing evidence that the predominant mechanism of demyelination is by activating macrophages and microglia thereby influencing the effector mechanisms of these cells (Zajicek et al., 1992) such that they may either actively phagocytose the myelin lamellae or release cytotoxic factors and further cytokines with potentially damaging effects on the myelin sheath. The T-cell derived cytokine IFN-\(\gamma\) is one of the most potent macrophage and microglial cell activators and also enhances their cytotoxic and phagocytic activities, as confirmed by the observation that in standard aggregate
cultures it resulted in the greatest reduction in MBP. This cytokine may act by inducing the release of other cytokines such as IL-1 (Giulian et al., 1986), IL-6 (Frei et al., 1989) and TNF-α (Sawada et al., 1989). TNF-α, IL-1α and IFN-γ all induce FcR expression on microglia and macrophages while immunocytochemical studies have shown that TNF-α also induces complement receptor 3 (CR3) expression on macrophages and microglia in aggregate cultures (Loughlin et al., 1994) suggesting activation of these cells. A role for macrophages is supported by observations that in demyelinating lesions in EAE there is marked absence of organelles in mononuclear cell processes adjacent to the myelin suggesting considerable secretory activity on the part of these cells (Lampert, 1983).

TNF-α induced delayed onset necrosis of oligodendrocytes in mouse myelinated spinal cord cultures and gradual myelin breakdown (Selmaj & Raine, 1988) while it also caused human oligodendrocytes to undergo programmed cell death in vitro (Wilt et al., 1995). The cytokines IFN-γ, TNF-α and IL-1 as well as IL-2 and IL-3 have been detected in the CNS of mice with active EAE while they could not be identified in control mice or during the remission phase in mice with chronic relapsing EAE (Baker et al., 1991). Furthermore TNF-α and IL-1 administration exacerbated EAE (Kuroda & Shimamoto, 1991; Jacobs et al., 1991) while the appearance of TNF-α and IFN-γ mRNA coincided with the onset of clinical signs (Issazadeh et al., 1995a; Issazadeh et al., 1995b). Transgenic mice with TNF-α over-expressed in astrocytes or neurons developed neurological disorders including CNS inflammation and demyelination (Probert & Selmaj, 1997). Over-expression of this cytokine in oligodendrocytes, using the MBP promoter, had no effect under normal conditions, however, CNS impairment was intensified following induction of EAE compared to control mice (Taupin et al., 1997). IFN-γ and TNF-α are the principal
cytokines elevated in acute MS lesions (Traugott & Lebon, 1988; Hofman et al., 1989; Selmaj et al., 1991a; Cannella & Raine, 1995) and treatment of MS with IFN-γ exacerbated the disease (Panitch et al., 1987). Increased TNF-α, which has been identified in MS brain lesions (Selmaj et al., 1991a; Hofman et al., 1989) and in the CSF and serum of MS patients, appears to correlate to clinical disease activity (Sharief & Hentges, 1991; Navikas et al., 1996; Rieckmann et al., 1995). Immunocytochemical studies have revealed that TNF-α in MS lesions is expressed mainly by microglia but can also be localised to astrocytes (Selmaj et al., 1991a; Cannella & Raine, 1995).

The presence of macrophages in the aggregates did not markedly alter the reduction in MBP elicited by demyelinating cytokines. Although it may have been expected that exogenous macrophage supplementation would have exacerbated the MBP reduction in response to inflammatory cytokines given their documented role in demyelination the enhanced myelinogenesis observed in macrophage-enriched aggregate cultures (Loughlin et al., 1994; Loughlin et al., 1997) may disguise, to some extent, the influence of macrophages in demyelination. Alternatively, the endogenous macrophages and microglia present in the aggregates may be sufficient to produce a maximal MBP loss or macrophages may not be the principal agents of myelin phagocytosis in cytokine-induced demyelination. While peritoneal macrophages exposed to the pro-inflammatory cytokine IL-1α induced a moderate stimulation of myelin phagocytosis in vitro there was no response to TNF-α and phagocytic activity was reduced by IFN-γ although proteolytic enzyme release was enhanced (Smith et al., 1998). In contrast, the phagocytosis of myelin was greatly increased in microglia exposed to these three inflammatory cytokines while having minimal effect on proteolytic enzyme release leading Smith et al., (1998) to
speculate that microglia may be the principal agents of myelin destruction in cytokine-driven demyelinating diseases such as MS. Macrophages may produce most of the proteolytic enzyme activity thought to contribute to myelin destruction while also having a supplemental phagocytic role. Accordingly, microglia were found to be the main population of phagocytes present in the early stages of MS plaques (Li et al., 1996).

Following removal of demyelinating cytokines MBP continued to accumulate in the aggregate cultures with electron microscopy studies confirming ultrastructurally the re-establishment of myelination. The recovery process, however, is variable following cytokine removal depending on the nature of the demyelinating action. In both standard and macrophage-enriched aggregate cultures recovery following IL-1α induced demyelination was more successful than that following IFN-γ induced demyelination. The delayed response following IFN-γ treatment may reflect the potent macrophage activating properties of this cytokine, which include enhancing the release of reactive oxygen intermediates and proteases as well as up-regulating FcR expression (Woodroofe et al., 1989). IL-1α, however, has less profound effects on the functional properties of macrophages and microglia (Loughlin et al., 1993) which may explain why MBP levels are able to recover more swiftly following demyelination by this cytokine as opposed to IFN-γ. In these experiments, while the absolute amount of MBP accumulated in macrophage-enriched cultures that had been demyelinated was greater than in standard cultures the proportional gain was similar. Moreover, when expressed as a proportion of untreated medium controls it appeared that macrophage-enriched aggregates did not recover as well as standard cultures, a finding consistent with the recovery following anti-MOG mediated demyelination.
Despite a reduction in the MBP content of standard and macrophage-enriched aggregate cultures exposed to anti-MOG or IgG1 antibodies in the presence of complement or complement alone CNP activity, a marker for mature oligodendrocytes, remained largely unaffected. These findings are consistent with those of Loughlin et al., (1994) and indicate a selective destruction of the myelin sheath with preservation of oligodendrocytes. Kerlero de Rosbo et al., (1990) found that CNP activity in aggregating cultures was reduced to some extent following exposure to anti-MOG antibody plus complement, although less so than MBP, implying that myelin is more susceptible to attack than oligodendrocytes. It is possible that the trophic effects of guinea pig serum could disguise any oligodendrocyte loss in antibody-mediated demyelination given that addition of normal serum to culture medium increased oligodendrocyte differentiation and myelin production (Honegger & Matthieu, 1980). Hence, the differential loss of MBP compared with CNP on exposure to anti-MOG antibody plus complement could arise from a selective loss of myelin while oligodendrocytes are spared or alternatively the oligodendrocyte loss may be disguised by the trophic effects of guinea pig serum and this needs to be further investigated.

The ability of anti-MOG antibodies to mediate demyelination may be a direct consequence of the topographical organisation of MOG at the external surface of the myelin membrane (Brunner et al., 1989). Previous studies investigating the effect of anti-MOG plus complement in the aggregate culture system, found that increasing concentrations of the antibody produced a dose-related loss of MBP (Kerlero de Rosbo et al., 1990). The monoclonal antibody 8-18C5 directed against a glycoprotein exposed on the external surface of the myelin sheath and oligodendrocytes (MOG) has a strong demyelinating effect in vivo (Linnington et al., 1984; Lassmann & Linnington, 1987) and
in aggregate cultures (Honegger et al., 1989; Kerlero de Rosbo et al., 1990; Loughlin et al., 1997). The presence of anti-MOG antibodies in chronic relapsing EAE supports a role for it as a target antigen in demyelinating disease (Lington & Lassmann, 1987). Furthermore, anti-MBP antibodies in the presence of complement showed no demyelinating activity in aggregate cultures while those antibodies directed against the myelin lipid galactocerebroside plus complement did induce demyelination (Honegger et al., 1989).

In line with our results that Z12 has a greater demyelinating capacity than 8-18C5 in the aggregate cultures, Piddlesden et al., (1994) showed that administration of Z12 anti-MOG antibody caused more extensive demyelination in animals with EAE than anti-MOG antibodies of the IgG1 isotype such as 8-18C5. Although both antibodies exhibit complement-dependent demyelinating activity in vitro (Piddlesden et al., 1993) antibodies of the IgG2a isotype have been shown to fix complement more effectively than IgG1 antibodies (Burton, 1985). A clear correlation was observed between the extent of demyelination and C9 complement deposition within the CNS in vivo implying that Z12 and other IgG2a monoclonal antibodies were the most pathogenic due to their enhanced ability to activate complement compared to IgG1 antibodies highlighting the central role of complement in the demyelinating process. Hence, enhanced complement fixation by Z12 in the aggregates may lead to increased demyelination via elevated levels of inflammatory mediators or from direct effects of membrane attack complex (MAC) damage to the myelin sheath as well as C3b receptor-mediated phagocytosis of myelin by macrophages (Goldenberg et al., 1989; Mosley & Cuzner, 1996).
Limited demyelination was also detected in aggregate cultures exposed to the IgG1 or IgG2a isotype control antibodies although the demyelinating potential was variable between individual experiments. This is in line with previous observations that a myelin protein-antigen specific antibody is not an absolute requirement for demyelination in vitro (Mosley & Cuzner, 1996). Certainly macrophages possess a wide variety of receptors which are implicated in myelin phagocytosis in vitro including the Fc, lectin, scavenger and complement receptors (Mosley & Cuzner, 1996).

It was found that complement alone, as well as in the presence of anti-MOG antibodies, induced detectable MBP loss in the aggregate cultures when added in the form of guinea pig serum which contains complement with a high haemolytic activity. Serum complement components are implicated in myelin phagocytosis by macrophages (Bruck & Friede, 1991) enhancing both the ability of macrophages to invade degenerating nerve and their ability to ingest myelin particles. Complement and its cleavage products are also thought to induce the locomotion of neutrophils and monocytes (Rollins & Springer, 1985) and to opsonise degenerating myelin (Goldenberg et al., 1989). C3b coating of myelin membranes has been shown to enhance antibody-induced phagocytosis and antibody-mediated cell cytotoxicity (Epstein et al., 1983). MBP itself is a potential complement activating protein and along with myelin can activate both the classical antibody-dependent and alternative complement pathways (Cyong et al., 1982; Vanguri et al., 1982; Koski et al., 1985; Silberberg et al., 1984). Evidence supporting a contributory role for serum complement components in demyelination in humans includes the localisation of complement activation product C3 in demyelinating MS plaques (Lumsden, 1971) and the detection of MAC around inflamed capillary endothelial cells and in areas of active demyelination in MS.
Further studies in experimental models have shown that demyelination and the clinical expression of EAE (Linington et al., 1989) and EAN (Feasby et al., 1987) were reduced on administration of cobra venom factor which depletes serum complement components. Similarly, soluble recombinant human complement receptor 1 (sCR1), which has potent complement inhibiting activity \textit{in vivo} and \textit{in vitro}, significantly reduced the demyelination, inflammation and clinical deficits associated with demyelinating antibody-mediated EAE (ADEAE), while also markedly inhibiting deposition of the complement components C1, C3 and C9 further implicating complement in the pathogenesis of the disease (Piddlesden et al., 1994).

The contribution of complement to antibody-mediated demyelination was further suggested by the finding that no demyelination was detected in standard aggregate cultures exposed to anti-MOG antibodies or their appropriate controls in the absence of complement. Accordingly, Kerlero de Rosbo et al., (1990) found that addition of anti-myelin antibodies alone did not induce detectable demyelination in aggregate cultures. Since demyelination is induced with anti-MOG in the presence of complement it is possible that the antibody-initiated classical complement pathway is activated in this system whereby a membrane attack complex is generated (Liu et al., 1983) which creates pores in the membranes of myelin lamellae disrupting the intracellular ionic environment and eventually destroying the sheath.

The absolute MBP reduction in macrophage-enriched aggregate cultures exposed to 8-18C5 or IgG1 antibodies in the presence of complement was greater than that in standard cultures indicating a putative role for macrophages in antibody-mediated demyelination, however, the percentage decrease relative to medium controls was similar between the two groups. It might be assumed that the incorporated macrophages
would have enhanced antibody-mediated demyelination although, as already suggested for cytokine-induced demyelination, the endogenous macrophages and microglia present in the aggregates may be sufficient to produce a maximal MBP loss so that increasing their number would have no effect. However, macrophages are able to produce complement components which in turn are able to activate microglia and macrophages causing them to produce an array of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 (Stahel et al., 1998). Anti-MOG coating the myelin sheath may activate the phagocytic response of macrophages via the FcR receptor or it is possible that C3b opsonises the myelin enabling receptor mediated phagocytosis via the type 3 complement receptor (CR3).

Previous reports have shown that the demyelinating effects of anti-MOG plus complement were greater than that of the cytokines IFN-γ, TNF-α or IL-1α while simultaneous addition of anti-MOG plus complement together with one of the cytokines resulted in an approximately additive MBP reduction, with no obvious CNP change (Loughlin et al., 1994) suggesting that they may be acting by different mechanisms. Cytokines may act directly on the myelin sheath or activate macrophages to produce various factors which contribute to myelin disruption such as further cytokines, lipases, leukotrienes, reactive oxygen intermediates and proteases (Werb, 1986; Nathan, 1987) while antibody-mediated demyelination appears to occur via the complement classical pathway or by FcR and CR3 receptor mediated endocytosis.

From these studies, the possibility that the MBP reduction on exposure to cytokines or antibody is not the result of demyelination cannot be excluded. However, our EM studies have demonstrated that addition of demyelinating antibodies resulted in disruption of the organised myelin lamellae, vacuolation and an increase in extracellular
‘myelin’ debris suggesting myelin membrane degradation (Loughlin et al., 1997). Furthermore, the content of a degradation peptide fragment of MBP with a carboxy terminal at phenylalanine 89 was significantly increased at a time when the MBP content in aggregate cultures was reduced. This novel finding supports the notion that myelin was not only disrupted but that MBP was phagocytosed and underwent proteolytic digestion by lysosomal hydrolases such as cathepsin D and/or secreted neutral proteinases including matrix metalloproteases (Copelman et al., 2000)

Support for this comes from studies in MS and EAE which have shown that myelin debris, phagocytosed by receptor mediated mechanisms into cells such as macrophages, are susceptible to a range of proteinases which have myelinolytic activity. MBP is particularly susceptible to proteolytic digestion (Cuzner & Norton, 1996) and this would be expected to play a major role in its catabolism in actively demyelinating MS plaques since MBP peptides are detectable in the blood (Paterson et al., 1981), urine (Whitaker, 1987) and CSF during the active phase of this disease (Whitaker & Herman, 1988). Among other enzymes the lysosomal enzyme Cathepsin D, which cleaves MBP at various sites including between the phenylalanine-phenylalanine 89-90 peptide bond, (Whitaker & Seyer, 1979) is increased in EAE and MS tissue lesions (Einstein et al., 1972; Rauch et al., 1973) implicating this enzyme in the pathological degradation of CNS proteins. Immunocytochemical studies have shown that MBP residues 80 to 88 contain an antigenic determinant which is inaccessible or absent from the intact molecule (Whitaker et al., 1977; Whitaker & Seyer, 1979) but becomes exposed during the degradation of MBP by cathepsin D. Our antibody did not detect intact MBP supporting the view that MBP did indeed undergo proteolytic digestion on exposure to
demyelinating antibody to expose a previously unrecognised or inaccessible portion of the protein.

As MBP synthesis resumes after demyelination of the cultures and EM studies confirm ultrastructurally the re-establishment of myelination it seems that neither cytokine or antibody treatments are causing irreversible damage and the laying down of new myelin can occur. Attempts at CNS remyelination do occur in both acute nascent (Prineas et al., 1993a; Raine & Wu, 1993) and chronic MS lesions (Prineas & Connell, 1979). However, early MS plaques containing a high density of oligodendrocytes (Raine et al., 1981; Prineas et al., 1993a; Ozawa et al., 1994; Bruck et al., 1994) exhibit greater signs of spontaneous remyelination (Bruck et al., 1994) than areas that have undergone extensive demyelination where it would seem that there is a progressive loss of oligodendrocytes associated with disease chronicity and continual repeated demyelination in MS (Raine et al., 1981; Prineas et al., 1989). It seems increasingly likely that adult oligodendrocyte progenitor cells, which have been identified in both the human and rat adult CNS (Armstrong et al., 1992; Scolding et al., 1995; ffrench Constant & Raff, 1986; Wolswijk et al., 1990), are responsible for remyelinating demyelinated areas rather than undamaged pre-existing mature oligodendrocytes. Accordingly, the density of cells positive for NG2, a chondroitin sulphate proteoglycan found on oligodendrocyte progenitor cells, increased locally in normal white matter following demyelination (Keirstead et al., 1998). Furthermore, Gensert et al., (1997) has shown that endogenous proliferating oligodendrocyte progenitor cells located in adult rat white matter, which probably correspond to these NG2 positive precursors, differentiated into myelinating oligodendrocytes following lysolecithin-induced demyelination (Gensert & Goldman, 1997).
It is possible that the induction of demyelination or the addition of non-specific antibodies has a positive effect on remyelination as, at least in standard cultures, MBP accumulated at an accelerated rate once these treatments had been washed out. Accordingly, administration of immunoglobulins has been found to promote remyelination in the Theiler’s virus model of MS (Rodriguez & Lennon, 1990). It is possible that addition of these antibodies to standard cultures may result in the recruitment and activation of endogenous macrophages and microglia which subsequently phagocytose and proteolytically degrade any myelin debris. This is in line with our findings that the content of the MBP degradation peptide 34T declined to levels comparable to controls in standard cultures as MBP continued to accumulate once the demyelinating treatments had been washed out. Macrophages and microglia are agents of myelin phagocytosis and degradation (Prineas & Wright, 1978) while paradoxically they are also associated with the microscopic hallmarks of remyelination in demyelinating MS lesions (Prineas et al., 1993a). In the PNS macrophage invasion following nerve lesion is an important part of the repair process with regenerating axons evident within a macrophage rich environment (Perry & Gordon, 1991). Hence, the phagocytic clearance of myelin debris may be a necessary prerequisite for successful remyelination (Perry & Gordon, 1991) and their recruitment to demyelinated areas may positively affect the repair process, promoting remyelination beyond levels observed in medium controls, since they are also a documented source of growth factors which regulate the proliferation, migration, differentiation, survival and myelin synthesising capacity of oligodendrocytes (Diemel et al., 1998). Furthermore, the presence of several hormones and trophic factors contained within guinea pig serum may also be
having a proliferative effect on oligodendrocytes in the aggregate cultures thus explaining the increases observed in CNP activity during the remyelination phase.

Inflammation in itself is a necessary response to injury and can promote regeneration. For example, neuronal death is increased following ischaemic injury in knockout mice where microglial recruitment or activation was reduced (Bruce et al., 1996; Fedoroff et al., 1997) suggesting a neuroprotective role. Similarly, eliminating macrophages from the wound site by injection of anti-leukocyte serum slows the process of healing (Rappolee & Werb, 1992) while microglia implanted into spinal cord lesions have been shown to stimulate CNS axon regeneration (Rabchevsky & Streit, 1997). Studies of toxin-induced demyelination have indicated that there is a relationship between the efficient removal of myelin debris by macrophages and enhanced remyelination (Ludwin, 1980; Triarhou & Herndon, 1985; Graca & Blakemore, 1986). Accordingly, following ethidium bromide induced demyelination the capacity for remyelination is reduced in the absence of extensive inflammatory episodes (Graca & Blakemore, 1986) with morphological studies confirming that remyelination is not very efficient when demyelinated axons are in the continued presence of myelin debris (Graca & Blakemore, 1986; Gilson & Blakemore, 1993; Shields et al., 1999). Substances derived from embryonic brain macrophages stimulate neurite outgrowth and regeneration of cultured CNS neurons (Chamak et al., 1994) and macrophage-derived mitogens appear to be responsible for Schwann cell proliferation following myelin degeneration in vitro and in vivo (Baichwal et al., 1988; Clemence et al., 1989). Transplantation of blood-derived monocytes, pre-activated by incubation with regenerating peripheral but not CNS sciatic nerve segments, into transected rat CNS nerve leads to efficient clearance of myelin, axonal regrowth and partial functional
recovery (Lazarov Spiegler et al., 1996; Lazarov Spiegler et al., 1998; Rapalino et al., 1998).

While exogenous macrophage enrichment did not enhance demyelination in aggregate cultures their presence in additional numbers appeared to reduce MBP accumulation and prolong the period of myelin phagocytosis and degradation following a demyelinating episode so that, as occurs in MS lesions, attempts at remyelination are counter-balanced by continuing demyelination. Hence, the phagocytic nature of the additional macrophages and the continued presence of degraded myelin does not render an environment that is fully conducive to remyelination enabling the enriched aggregates to achieve the myelin status of medium controls. Therefore, the macrophage, with its multiple effector properties, may influence the intricate balance between the breakdown and repair of myelin.
CHAPTER 5

TEMPORAL ANALYSIS OF GROWTH FACTOR mRNA EXPRESSION DURING MYELINATION AND REMYELINATION IN STANDARD AND MACROPHAGE-ENRICHED AGGREGATE CULTURES

5.1 Introduction

Myelinogenesis, which consists of oligodendrocyte proliferation and differentiation followed by a period of myelin synthesis and deposition, is a tightly controlled process regulated in part by multifunctional growth factors. Growth factors mitogenic for oligodendrocyte progenitor cells in vitro include platelet-derived growth factor (PDGF) (Noble et al., 1988; Raff et al., 1988), fibroblast growth factor-2 (FGF-2) (McKinnon et al., 1990; Eccleston & Silberberg, 1985), insulin-like growth factor-I (IGF-I) (McMorris & Dubois Dalcq, 1988; McMorris et al., 1990), neuregulin (Canoll et al., 1996) and neurotrophin-3 (NT-3) (Barres et al., 1994c). Transforming growth factor-β1 (TGF-β1), synthesised by oligodendrocyte precursor cells, promotes differentiation (McKinnon et al., 1993) as does IGF-I (McMorris et al., 1990; McMorris et al., 1993) and FGF-2 when in the presence of astrocytes (Mayer et al., 1993). IGF-I, PDGF and epidermal growth factor (EGF) stimulate myelin basic protein (MBP) synthesis in single cell and aggregate cultures (Saneto et al., 1988; Mozell & McMorris, 1991; Grinspan et al., 1993a; Honegger & Tenot Sparti, 1992; Almazan et al., 1985) while FGF-2 encourages oligodendrocyte process outgrowth (Oh & Yong, 1996) and myelin membrane construction following demyelination (Fressinaud & Vallat, 1994). Ciliary neurotrophic factor (CNTF), IGF-I and PDGF are potent oligodendrocyte survival factors in vitro (Barres et al., 1992a; Barres et al., 1993) while the latter two are
also implicated in myelin repair (Yao et al., 1995; Liu et al., 1995; Fressinaud et al., 1996; McKay et al., 1997).

Macrophages and microglia are associated with CNS modelling during development and remyelination in MS lesions where paradoxically they participate in myelin phagocytosis and degradation. As a source of growth factors implicated in oligodendrocyte maturation and myelination, macrophages have the potential to promote myelin formation and repair.

The effects of these growth factors have been identified primarily from in vitro cell culture studies and highlight a need to consider more carefully the time frame and interactions between different growth factors during both myelinogenesis, demyelination and remyelination. Therefore, in this study a three-dimensional aggregating culture system was used which matures in a manner that is similar to the developing brain including cell to cell interactions and morphological maturation including myelination (Honegger, 1985; Loughlin et al., 1997; Kruger et al., 1999). Pro-inflammatory cytokines or anti-myelin oligodendrocyte glycoprotein (MOG) antibodies, in the presence of complement, can induce demyelination in myelinated aggregate cultures while they have the capacity to remyelinate, with resumed MBP synthesis, once the demyelinating agents have been removed (Loughlin et al., 1994; Loughlin et al., 1997).

Therefore, in the present study growth factors, which have been documented most extensively to promote oligodendrocyte proliferation and differentiation, were investigated to determine their pattern of expression over time in standard myelinating CNS aggregate cultures and in those that had been enriched with peritoneal macrophages at the start of the culture period. Furthermore, changes in growth factor
expression during remyelination following short term exposure to the demyelinating anti-MOG 8-18C5 antibody plus complement were assessed in standard and macrophage-enriched aggregate cultures and comparisons were made to ascertain whether any of the growth factors associated with myelination were implicated in remyelination.

5.2 RESULTS

5.2.1 Growth factor mRNA expression over time in myelinating aggregate cultures

Growth factor mRNA expression was determined in samples from standard and macrophage-enriched three-dimensional myelinating rat brain aggregate cultures at predetermined time points throughout the culture period from DIV=1 to DIV=48 using semi-quantitative RT-PCR. Aggregate samples were harvested and processed for mRNA analysis as described in Chapter 2 prior to total RNA extraction and RT-PCR. Verification of the product sizes for individual growth factors within the aggregate cultures amplified using RT-PCR and analysed by size separation using ethidium bromide stained agarose gel electrophoresis with subsequent visualisation under ultraviolet light was shown previously in Chapter 2, section 2.11.2.4, Figure 2.16. The semi-quantitative PCR product yields were standardised to 25 amplification cycles and 100ng starting cDNA template before they were expressed over GAPDH to enable the relative amounts of each growth factor to be directly comparable to one another.
5.2.1.1 Growth factor mRNA expression in standard myelinating aggregate cultures

The pattern of growth factor mRNA expression in myelinating aggregate cultures over time shown in Figure 5.1 is representative time course data from one of the 14 experiments performed. Growth factor mRNA expression over time from all of the aggregate culture preparations are shown in Figure 5.1i. As discussed in Chapter 3 it was important to assess the pattern of MBP increases in each preparation to assess when the peak rate of MBP accumulation occurred. In the same preparation from which the growth factor mRNA data is shown it was determined that the MBP content doubled from 1.32 to 2.80µg/mg total protein between DIV=14 and 21. However, the peak rate of MBP accumulation occurred over the next 7 days from DIV=21 to 35 when the MBP content rose significantly by 0.26µg/mg total protein per day (p<0.05). Over the next 13 days MBP increased by 0.16µg/mg total protein per day to reach levels of 8.53µg/mg total protein by DIV=48 (p<0.005).

The corresponding growth factor results show that PDGF-A mRNA expression (Figure 5.1A) was higher than that of any other growth factor tested throughout the culture period with the level of FGF-2 mRNA rising to become the second most abundant growth factor from DIV=14 to the end of the culture period. These observations were consistent with the data from all but one of the other time course experiments where after the first 14 days of culture the expression of PDGF-A or FGF-2 mRNA was greater than that of any other growth factor. The levels of EGF, NGF and NT-3 mRNA were expressed at much lower levels than any of the other growth factors examined such that in the representative experiment illustrated in Figure 5.1C EGF expression was up to fifty times lower than that of PDGF-A at comparable time points.
Figure 5.1. Time course of growth factor mRNA expression in aggregate cultures. All data are represented as mean ± 1 SEM where n = 3-5 for each time point. All densitometry values were expressed over GAPDH and standardised for 25 PCR cycles so that the relative amounts of each growth factor could be compared on a semi-quantitative basis. Initial detection of MBP mRNA and MBP protein in the aggregate cultures are indicated by i and ii respectively, as determined by Northern blotting and radioimmunoassay.
Figure 5.1i. Time course of growth factor mRNA expression from multiple aggregate culture preparations. Results are expressed as mean ± 1 SEM. All densitometry values were expressed over GAPDH and standardised for 25 PCR cycles so that the relative amounts of each growth factor could be compared on a semi-quantitative basis. Each data point represents mean growth factor value of at least 3 flasks. Initial detection of MBP mRNA and MBP protein in the aggregate cultures are indicated by i and ii respectively, as determined by Northern blotting and radioimmunoassay.
PDGF-A mRNA expression rose significantly more than 2.5 fold (p<0.01) from DIV=1 to DIV=8 (Figure 5.1A) with mRNA levels increasing at a rate of 57.84 arbitrary densitometry units per day during the 3 days from DIV=5 up to DIV=8 which, as discussed in Chapter 3, is the earliest time point that MBP mRNA was detectable in the cultures using Northern blot analysis. PDGF-A mRNA levels remained high thereafter with no marked change in the level of expression for the remaining culture period. Assessment of the temporal pattern of PDGF-A mRNA expression during other time course experiments determined that, in all but one instance, after the second week in culture its level of expression was maintained or continued to increase slightly.

During DIV=1 to DIV=8, as observed with PDGF-A, there was a marked increase in TGF-β1 (Figure 5.1B) and NT-3 mRNA expression (Figure 5.1C; p<0.05), by 2.6 and 1.7 fold respectively, to reach maximal levels by DIV=8 before falling at each consecutive time point. Despite falling between DIV=1 and 5 the level of IGF-I mRNA (Figure 5.1B) increased significantly between DIV=5 and DIV=8 at a rate of 15.82 arbitrary densitometry units per day (p<0.05) and continued to rise to reach maximal levels by DIV=14 at a time point when MBP protein, which is indicative of the extent of myelination, can be detected by radioimmunoassay in the aggregate cultures. Over the next 7 days IGF-I mRNA expression fell by 45% and continued to fall significantly during the latter stages of the culture period.

FGF-2 mRNA expression (Figure 5.1A) was low at the beginning of the culture period, however it continued to rise as the culture matured to reach 6.5 times its starting expression (p<0.05). The peak rate of FGF-2 mRNA accumulation was observed during the 6 days from DIV=8 to DIV=14, a time corresponding to the greatest rise in
MBP mRNA in standard aggregate cultures, with levels of FGF-2 rising significantly over 2.54 times at a rate of 21.34 arbitrary densitometry units per day (p<0.001). Correlation studies on the changes in FGF-2 mRNA expression over time from all the preparations performed (Figure 5.2A) demonstrated a significant increase in the level of FGF-2 over time (p<0.005 as determined by an F-test). CNTF mRNA expression (Figure 5.1A) was found to increase significantly (p<0.05) from DIV=1 to 5 before falling again in the 3 days up to DIV=8. From DIV=8 to the end of the culture period CNTF mRNA expression was found to increase significantly 6.5 fold (p<0.05) with the greatest increase per day of 11.08 arbitrary densitometry units detected during the 3rd week in culture. Analysis of all the time course preparations determined that there was a positive correlation between increased CNTF mRNA expression and ongoing time (Figure 5.2B; p<0.0001 as determined by an F-test). Minimal changes were observed in the mRNA expression of EGF up to DIV=14 (Figure 5.1C), however after this time point and up to the end of the culture period its expression fell significantly by a third (p<0.05).

In a separate time course experiment the level of expression of NGF mRNA was measured throughout the culture period on DIV=1, 5, 8, 14, 28 and 48 (Figure 5.3). NGF mRNA was found to be expressed at levels that were comparable to NT-3 and from 50 to almost 150 times less than that of PDGF-A. NGF mRNA expression was found to increase from DIV=1 to DIV=5 before falling significantly by over a third (p<0.005) during the next 3 days in culture. NGF mRNA levels were maintained at this lower level until DIV=14 whereupon a significant 1.7 fold increase was measured from DIV=14 to DIV=28 corresponding to the time when the rate of MBP synthesis in the
Figure 5.2. FGF-2 (A) and CNTF (B) mRNA expression over time from multiple aggregate culture preparations. Demonstrating a significant increase in the mRNA level of both growth factors over time. All data are represented as mean ± 1 SEM where n = 3-7 for each preparation at all time points. All densitometry values were expressed over GAPDH and standardised for 25 PCR cycles so that the relative amounts of each growth factor could be compared on a semi-quantitative basis.
Figure 5.3. Time course of NGF and NT-3 mRNA expression in standard aggregate cultures. All data are represented as mean ± 1 SEM where n = 6-7 for each time point. Densitometry values were expressed over GAPDH and standardised for 25 PCR cycles so that the relative amounts of each growth factor could be compared on a semi-quantitative basis. Initial detection of MBP mRNA and MBP protein in the aggregate cultures are indicated by i and ii respectively, as determined by Northern blotting and radioimmunoassay.
aggregate cultures was at its greatest. Over the next 20 days in culture levels of NGF mRNA decreased significantly by over a half (P<0.05).

5.2.1.2 Growth factor mRNA expression in isolated peritoneal macrophages

Peritoneal macrophages were prepared as described in Chapter 2 section 2.1.4. however, once the cells had been centrifuged for a final time the pellet was frozen at -20°C. Subsequently total RNA was extracted and RT-PCR was performed, as described in Chapter 2, to determine the level of growth factor mRNA expression in peritoneal macrophages.

It was found that these macrophages expressed significant levels of TGF-β1 mRNA while EGF mRNA was also evident but it was expressed at much lower levels (Figure 5.4).

5.2.1.3 Growth factor mRNA expression in standard and macrophage-enriched myelinating aggregate cultures

Growth factor mRNA expression was assessed by semi-quantitative PCR in both standard and macrophage-enriched aggregate cultures at multiple pre-determined time points throughout the culture period in five separate preparations. In the representative experiment illustrated in Figure 5.5A it was found that TGF-β1 mRNA levels were significantly elevated at all time points compared to standard aggregate cultures with the exception of DIV=5 and 14. In this preparation TGF-β1 mRNA expression was high in both standard and macrophage-enriched aggregate cultures on DIV=1 with macrophage-enriched cultures expressing nearly 30% more mRNA than standard cultures (p<0.005). During the next 4 days TGF-β1 mRNA expression was reduced in both standard and
Figure 5.4. Representative ethidium bromide stained gel of growth factor RT-PCR products derived from isolated peritoneal macrophages showing mRNA expression of TGF-β1 and EGF. Equivalent amounts of each PCR product were size fractionated on a 1% agarose gel where n = 2 for all growth factors except TGF-β1 where n = 4. M denotes a 100bp DNA ladder marker with the brightest band indicating a 500bp product.
Figure 5.5. Increased TGF-β1 (A) and FGF-2 (B) mRNA expression in macrophage-enriched aggregates compared to standard cultures over time. All data are represented as mean ± 1 SEM where n = 4-7 for each time point. Densitometry values were expressed over GAPDH and standardised for 25 PCR cycles so that the relative amounts of each growth factor could be compared on a semi-quantitative basis. * denotes significance (p ≤ 0.05) from standard culture at relevant time point as determined by Student's t-test. Initial detection of MBP mRNA and MBP protein in the aggregate cultures are indicated by i and ii respectively, as determined by Northern blotting and radioimmunoassay.
macrophage-enriched aggregate cultures. However, by DIV=8 TGF-β1 mRNA expression had increased again with levels in enriched aggregate cultures accumulating at their highest rate over this time period to reach values that were significantly 59% more than those reached in standard cultures (p<0.001). In this preparation TGF-β1 mRNA expression in standard aggregate cultures did not peak until DIV=14 while levels in macrophage enriched flasks had already begun to fall by this time. While the levels of TGF-β1 mRNA appeared to plateau after DIV=14 they remained significantly higher than those detected in standard flasks at all subsequent time points (p<0.005). These results reflect the finding from other TGF-β1 mRNA time course studies in standard and macrophage-enriched aggregate cultures where when taking into consideration multiple time points throughout the course of the culture period TGF-β1 mRNA expression was augmented in macrophage-enriched flask in two thirds of cases (Figure 5.6A) with the most consistent increases occurring during the 4th week in culture from DIV=21 to DIV=28.

Similarly, it was found that the levels of FGF-2 mRNA (Figure 5.5B) were increased in macrophage-enriched aggregates compared to standard cultures from DIV=5 for the rest of the culture period with enhancement reaching significance at DIV=5, 28 and 48 (p<0.05). As discussed previously in section 5.2.1.1 FGF-2 mRNA expression was low at the beginning of the culture period and continued to rise as the aggregates matured which is consistent with the pattern of FGF-2 expression in both standard and macrophage-enriched aggregate cultures in this separate preparation. While the level of FGF-2 mRNA expression was similar in both standard and macrophage-enriched aggregate cultures at DIV=1 by DIV=5, 4 days later, FGF-2
Figure 5.6. TGF-β1 (A), FGF-2 (B) and CNTF (C) mRNA expression in macrophage-enriched aggregates expressed as a proportion of standard cultures. Densitometry values were expressed over GAPDH and standardised for 25 PCR cycles so that the relative amounts of each growth factor could be compared on a semi-quantitative basis. For each time point in a given preparation the mean macrophage value was then expressed as a proportion of the mean standard value where a value greater than 1 indicates enhanced growth factor mRNA expression. Initial detection of MBP mRNA and MBP protein in the aggregate cultures are indicated by i and ii respectively, as determined by Northern blotting and radioimmunoassay.
mRNA had increased over 3 fold in standard aggregate cultures while expression in macrophage-enriched flasks had increased by over 5 fold at a rate of 4.17 arbitrary densitometry units per day to reach levels that were significantly 58% higher than those in standard aggregate cultures (p<0.05). In this preparation the peak rate of FGF-2 mRNA accumulation occurred during the next 3 days up to DIV=8 with increase per day of 13.08 and 12.60 arbitrary densitometry units in standard and macrophage-enriched aggregate cultures respectively. FGF-2 mRNA continued to rise so that by DIV=48 FGF-2 levels had reached over 20 and 30 times those measured at DIV=1 in standard and macrophage-enriched flasks respectively. Furthermore, by this late time point FGF-2 mRNA expression in macrophage enriched flasks significantly exceeded that of standard cultures (p<0.01). These increases were consistently observed in four other time course experiments and when taking into consideration multiple time points throughout the course of the culture period FGF-2 mRNA expression was found to be increased in macrophage-enriched flask in 63% of cases (Figure 5.6B).

In four out of five time course experiments the expression of CNTF mRNA was reduced in macrophage-enriched aggregate cultures compared to those that did not receive exogenous macrophages. In two representative time course studies aggregate cultures were harvested during the early and later stages of the culture period (Figure 5.7A and B). It can be seen that there was no difference in CNTF mRNA expression at DIV=1 (Figure 5.7A), however at DIV=5 and 8 the levels of CNTF mRNA in macrophage-enriched flasks had fallen by nearly 30% compared to non-enriched cultures although this did not reach significance. In accordance with discussions in the
Figure 5.7. Reduced CNTF mRNA expression in macrophage-enriched aggregates compared to standard cultures from two representative time course experiments (A, B). All data are represented as mean ± 1 SEM where n = 3-8 for each time point. Densitometry values were expressed over GAPDH and standardised for 25 PCR cycles so that the relative amounts of each growth factor could be compared on a semi-quantitative basis. * denotes significance (p ≤ 0.05) from standard culture at relevant time point as determined by Student's t-test. Initial detection of MBP mRNA and MBP protein in the aggregate cultures are indicated by i and ii respectively, as determined by Northern blotting and radioimmunoassay.
preceding section CNTF mRNA was found to increase with ongoing time from DIV=8 in both standard and enriched aggregate cultures. In the experiment depicted in Figure 5.7A it can be seen that while there was no difference in the level of CNTF mRNA expression at DIV=14, by DIV=28 there was significantly less in macrophage-enriched flasks (p<0.05). Studies over the later time points in culture shown in Figure 5.7B show that in this experiment by DIV=14 CNTF mRNA expression had increased in standard flasks and remained significantly lower in macrophage-enriched flask (p<0.05). One culture week later CNTF mRNA expression had increased over 7.6 times in macrophage-enriched flasks to reach levels that were comparable to standard flasks. However, while CNTF mRNA continued to increase by over a third in standard flasks from DIV=21 to DIV=28 there was no change in enriched flasks leaving their CNTF mRNA expression at significantly lower levels than that in standard flasks (p<0.05). A similar pattern of expression was observed at DIV=48 although the reduction in CNTF mRNA in macrophage-enriched cultures did not reach significance at this time point. When multiple time points throughout the course of the culture period were analysed it was determined that CNTF mRNA expression was reduced in macrophage-enriched flask in over 60% of cases (Figure 5.6C).

Macrophage-enrichment did not consistently alter the level of expression of any of the other growth factors tested i.e. PDGF-A, IGF-I, NT-3 or EGF (data not shown).
5.2.2 Growth factor mRNA expression in aggregate cultures following anti-myelin oligodendrocyte glycoprotein antibody induced demyelination

To elucidate the temporal changes in growth factor mRNA expression during demyelination and remyelination both standard and macrophage-enriched aggregate cultures were exposed to 8-18C5 anti-MOG demyelinative antibody in the presence of complement (GPS) on DIV=34 for a period of 48 hours as described in Chapter 2. Control flasks received either the corresponding purified mouse IgG1,κ isotype antibody in the presence of complement or complement alone. On DIV=36 following 48 hours exposure to anti-MOG antibody or appropriate controls plus complement treatments were diluted out of the flasks and the cultures maintained during which time they were able to re-accumulate MBP. Growth factor mRNA expression was assessed by semi-quantitative PCR in two separate aggregate culture preparations on DIV=34, prior to the addition of any demyelinative antibody, on DIV=36 at the end of the forty eight hour treatment period and at various time points following treatment removal during the period of MBP re-accumulation.

5.2.2.1 Growth factor mRNA expression in standard aggregate cultures following anti-myelin oligodendrocyte glycoprotein antibody induced demyelination

Growth factor mRNA expression was assessed by semi-quantitative PCR in two separate aggregate culture preparations on DIV=34, prior to the addition of any antibody or appropriate control, on DIV=36 at the end of the 48 hour treatment period and on DIV=39 and 48 three and twelve days respectively after the treatments had been washed out.
In the same preparation from which the growth factor mRNA data was derived it was determined that in standard aggregate cultures exposure to anti-MOG and IgG1 antibodies plus complement resulted in a 73 and 33% MBP content reduction while MBP levels did not change in flasks that received complement alone. By DIV=48, 12 days after any treatments had been washed out, the MBP content had increased in all treatment groups to reach levels that surpassed medium controls.

The corresponding growth factor results show that on DIV=36, just prior to the removal of any given treatment, the expression of CNTF mRNA in anti-MOG treated cultures was significantly 60% higher than that of medium controls (p<0.005; Figure 5.8A) and over 30% higher than levels in aggregates that had received IgG1 antibody plus complement (p<0.05). The levels of FGF-2 and IGF-I mRNA (Figure 5.8B and C) were also significantly increased in aggregate cultures that had received anti-MOG plus complement when compared to IgG1 plus complement controls (p<0.05).

By DIV=39, 3 days after any treatment had been washed out of the culture medium, it was found that CNTF mRNA expression in anti-MOG treated cultures had risen by 26.94 arbitrary densitometry units per day to reach levels that were still significantly greater than medium controls (p<0.05). FGF-2 and IGF-I mRNA expression in anti-MOG treated cultures had significantly risen to 2 times the levels observed in untreated medium controls (p<0.01) while mRNA levels of these two growth factors also increased significantly in IgG1 treated flasks compared to those that received medium alone (p<0.005; Figure 5.8B and C).
Figure 5.8. CNTF (A), FGF-2 (B) and IGF-I (C) mRNA expression in standard aggregate cultures treated with 8-18C5 anti-MOG antibody in the presence of complement (GPS) from DIV=34 to DIV=36. Control flasks received IgG1 plus GPS or GPS alone. Results are represented as mean ± 1 SEM where n = 2-4 for each time point from a representative experiment sampled immediately prior to treatment (DIV=34), at the end of the treatment period (DIV=36) and 3 and 12 days after removal of the treatment (DIV=39, 48). *, ▽ and O denote significance (p ≤ 0.05) from medium, IgG1 plus GPS or GPS alone flasks respectively at relevant time point as determined by Student's t-test.
On DIV=48, twelve days after the treatments had been washed out FGF-2 and IGF-I mRNA in previously treated anti-MOG flasks continued to be expressed at significantly 1.4 to 1.6 times higher levels than medium controls (p<0.05). FGF-2 and IGF-I mRNA expression was also significantly higher than flasks previously treated with complement (p<0.05) with IGF-I levels also significantly elevated when compared to IgG1 previously treated flasks (p<0.05). From DIV=39 to DIV=48 PDGF-A mRNA in flasks previously treated with anti-MOG antibody accumulated at more than double the rate in medium flasks to reach levels that were significantly greater than both medium and IgG1 plus complement control groups (p<0.05; Figure 5.9A). There was no longer any significant difference in the levels of CNTF mRNA between any of the various groups by this late time point.

In one of the two demyelination experiments performed, where all treatments had resulted in some degree of MBP reduction, it was observed that by DIV=48 there was a significant increase in the expression of NGF mRNA of up to 54% in 8-18C5 anti-MOG treated aggregates relative to IgG1, complement and medium treated cultures (p<0.005; Figure 5.9B). No changes in TGF-β1, EGF or NT-3 mRNA expression was observed during the course of either of these demyelination experiments (data not shown).
Figure 5.9. PDGF-A (A) and NGF (B) mRNA expression in standard aggregate cultures treated with 8-18C5 anti-MOG antibody in the presence of complement (GPS). Control flasks received IgG1 plus GPS or GPS alone. Results are represented as mean ± 1 SEM where n = 2-4 for each time point from a representative experiment sampled prior to treatment (DIV=32, 34), at the end of the treatment period (DIV=36) and as indicated thereafter. *, V and O denote significance (p ≤ 0.05) from medium, IgG1 plus GPS or GPS alone flasks respectively at relevant time point as determined by Student's t-test.
5.2.2.2 Effect of macrophage enrichment on growth factor mRNA expression following anti-myelin oligodendrocyte glycoprotein antibody induced demyelination

Standard aggregate cultures and those that had been enriched with peritoneal macrophages at the time of seeding were exposed to anti-MOG demyelinating antibody and appropriate controls from DIV=34 to DIV=36 before growth factor mRNA expression was assessed by RT-PCR. As has been discussed in the preceding chapters aggregate cultures enriched with peritoneal macrophages accumulate more MBP than cultures that do not receive macrophages. Accordingly, in the preparation from which the growth factor mRNA data is shown it was determined that macrophage-enriched aggregate cultures had accumulated approximately 4 times more MBP than non-enriched cultures at both DIV=36 and DIV=48 (p<0.05). Exposure to anti-MOG antibody plus complement resulted in a significant 70% MBP loss in both standard and macrophage-enriched aggregate cultures (p<0.05) while MBP content was reduced by 33 and 16% respectively after IgG1 plus complement exposure with the addition of complement alone having no discernible effect. By DIV=48, 12 days after any treatments had been washed out the MBP content had increased in all groups however, macrophage-enriched flasks contained almost twice as much MBP as comparably treated standard aggregate cultures.

The corresponding growth factor results show that by DIV=39, 3 days after any treatment had been washed out, IGF-I mRNA expression in anti-MOG treated macrophage-enriched cultures had risen to levels that were significantly twice those of medium and complement controls (p<0.05) and over two thirds that of IgG1 plus complement controls (p<0.05; Figure 5.10).
Figure 5.10. IGF-I mRNA expression in standard and macrophage-enriched aggregate cultures treated with 8-18C5 anti-MOG antibody in the presence of complement (GPS) from DIV=34 to DIV=36. Control flasks received IgG1 plus GPS or GPS alone. Results are expressed as mean ± 1 SEM where n = 2-4 for each time point from a representative experiment sampled at the end of the treatment period (DIV=36) and 3 and 12 days after removal of the treatment (DIV=39, 48). * denotes significance (p ≤ 0.05) from comparably treated standard culture at equivalent time point as determined by Student's t-test.
Furthermore, these anti-MOG treated macrophage-enriched cultures were found to express significantly double the amount of IGF-I mRNA than comparably treated standard aggregate cultures at this time point (p<0.01). By DIV=48 IGF-I mRNA expression was similar in all macrophage-enriched aggregate culture groups while, as previously shown, expression in standard cultures that had been exposed to anti-MOG antibody was significantly increased relative to all other groups at this time point to reach levels that were significantly higher, by over 80%, than those attained in macrophage-enriched cultures (p<0.001). These results imply that the peak rise in IGF-I mRNA in aggregates exposed to 8-18C5 anti-MOG antibody plus complement in macrophage-enriched flasks precedes the subsequent peak in standard flasks.
5.3 DISCUSSION

Using semi-quantitative RT-PCR it has been shown that myelinating three-dimensional brain aggregate cultures express a wide range of growth factors that regulate oligodendrocyte proliferation and differentiation. Furthermore, developmental maturation including myelination in the aggregates was associated with a specific pattern of growth factor mRNA expression comparable with that of the developing brain, indicating that maturing oligodendrocytes respond to multiple growth factors in a co-ordinated and sequential manner (Copelman et al., 2000).

The increase in PDGF-A mRNA expression was greatest just prior to the detection of MBP mRNA when oligodendrocyte progenitors would be expected to be a prominent population and remained high thereafter while FGF-2 and CNTF mRNA rose steadily as the culture matured and MBP accumulated. The peak of TGF-β1 mRNA expression coincided with the appearance of MBP mRNA, while that of IGF-I was more closely associated with the detection of MBP protein. NT-3, NGF and EGF mRNA were expressed at much lower levels than any of the other growth factors examined. After the first week in culture, prior to MBP mRNA detection, NT-3 mRNA expression peaked as observed in the developing CNS once oligodendrocyte proliferation and neuronal maturation is underway. Early increased EGF mRNA expression may reflect its mitogenic and glial maturation properties. The enhanced myelination in macrophage-enriched cultures was associated with reduced expression of CNTF and increased levels of TGF-β1 and FGF-2 mRNA, compared to standard non-enriched cultures, both of which promote oligodendrocyte development in vitro.

Demyelination induced a distinct pattern of expression of many myelination-associated growth factors. A rapid rise in CNTF mRNA in standard cultures, compared
to controls, closely followed by increases in FGF-2 and IGF-I was in contrast to the delayed induction of PDGF-A and NGF mRNA supporting a role for these factors in oligodendrocyte survival and myelin repair. The rise in IGF-I mRNA expression in anti-MOG treated macrophage-enriched aggregates preceded that in standard cultures suggesting that macrophage-enrichment instigates a faster IGF-I response during remyelination.

The results are in agreement with previous studies demonstrating that mitotic activity within aggregate cultures occurs during the first 2 weeks in vitro (Lenoir & Honegger, 1983) and is then followed by a period of cellular differentiation. This is further supported by findings in our laboratory of a relative abundance at DIV=14 of exon-2 MBP mRNA in aggregate cultures which peaks during the initial stages of myelination (Kruger et al., 1999).

PDGF, which exhibited the greatest increase in mRNA expression during the initial stages of the culture prior to the first detection of MBP mRNA, is a potent mitogen for oligodendrocyte progenitor cells in vitro (Noble et al., 1988; Raff et al., 1988) and in vivo (Richardson et al., 1988; Hall et al., 1996) triggering a limited number of cell divisions before they become postmitotic and differentiate. Furthermore, PDGF-α receptors (PDGF-Rα) are associated with oligodendrocyte progenitors implying that they are responsive to available PDGF produced in the CNS by astrocytes and neurons (Hart et al., 1989; Ellison & de, 1994). In addition to its mitogenic capacity, PDGF stimulates oligodendrocyte migration in vitro by both enhancing motility and acting as a chemotactic factor (Noble et al., 1988; Armstrong et al., 1990; McMorris & McKinnon, 1996) while it also promotes the survival of immature, but not mature cells both in vitro and in vivo (Barres et al., 1992b). Furthermore, exogenous
administration of PDGF significantly augments the expression of MBP and PLP mRNA in rat cerebral white matter oligodendrocyte cell cultures (Grinspan et al., 1993a) and stimulates myelogenesis in CNS aggregating cultures (Honegger & Tenot Sparti, 1992) while PDGF-A null mice display reduced numbers of proliferating oligodendrocyte precursor cells (Calver et al., 1995), defective oligodendrocyte development and hypomyelination (Fruttiger et al., 1999). PDGF-A mRNA expression was higher than that of any other growth factor examined and remained high throughout the culture period which is consistent with in vivo observations (Vignais et al., 1995) and implies that PDGF-AA in the aggregates may initiate not only oligodendrocyte progenitor proliferation but also the onset of myelin gene expression.

FGF-2 mRNA expression rose throughout the culture period which reflects in vivo postnatal rises with continued high expression throughout adulthood (Webster, 1997). Like PDGF-A, FGF-2 is mitogenic for immature oligodendrocytes in vitro and up-regulates oligodendrocyte PDGF-α receptor expression thereby increasing their sensitivity to PDGF driven proliferation (Eccleston & Silberberg, 1985; McKinnon et al., 1990). While simultaneous exposure to both PDGF and FGF-2 results in continuous progenitor cell proliferation in vitro with suspension of differentiation (Bogler et al., 1990) in the presence of astrocytes FGF-2 has been found to promote differentiation (Mayer et al., 1993). In aggregate culture, where astrocytes are a major cell population, a single application of FGF-2 can promote premature oligodendrocyte maturation as determined by CNP activity (Honegger & Tenot Sparti, 1992) while this factor has also been shown to synergise with the astrocytic extracellular matrix to promote adult human oligodendrocyte process outgrowth in vitro (Oh & Yong, 1996). Furthermore, FGF receptors have been localised to the myelinated tract in the rat CNS further implicating
it in myelination (Asai et al., 1993). Thus, FGF-2 may first sustain precursor proliferation in the aggregate cultures and then stimulate differentiation.

CNTF mRNA, like FGF-2, also increased significantly during the culture period reflecting the progressive rise observed during the first postnatal weeks in vivo (Stockli et al., 1991; Dobrea et al., 1992) and in accordance with its ability to enhance PDGF-AA stimulated oligodendrocyte precursor cell proliferation both in vitro and in vivo (Barres et al., 1996). The rate of oligodendrocyte precursor cell proliferation was reduced in CNTF-deficient mice although the number of oligodendrocytes and extent of myelination in these adult mice was indistinguishable from wild-type mice suggesting that while CNTF may help to control the rate of oligodendrocyte generation it is not required for oligodendrocyte differentiation (Barres et al., 1996).

TGF-β1, the mRNA expression of which peaked early in the aggregates coinciding with the first appearance of MBP mRNA, has been shown to inhibit PDGF driven O-2A progenitor proliferation in vitro, thereby causing these cells to stop dividing and differentiate (McKinnon et al., 1993). Similarly, TGF-β1 may up-regulate oligodendrocyte FGF receptors thereby increasing their sensitivity to FGF-2 (Thannickal et al., 1998). IGF-I mRNA expression increased significantly during the early stages of the culture reaching a peak slightly later than PDGF-A, TGF-β1 and NT-3 at a time more closely associated with the detection of MBP protein in the aggregate cultures and consistent with the pattern of mRNA expression in rodents during the first postnatal weeks (Adamo et al., 1989). IGF-I is a potent mitogen for neuronal and oligodendroglial progenitor cells in vitro (McMorris et al., 1986; Lenoir & Honegger, 1983) increasing oligodendrocyte number by 60 fold in developing rat cerebral glial cell
cultures (McMorris et al., 1986). IGF-I also promotes oligodendrocyte differentiation (McMorris et al., 1990; McMorris et al., 1993) and enhances myelin protein gene expression (McMorris & Dubois Dalcq, 1988; Saneto et al., 1988) and myelin production in vitro (Mozell & McMorris, 1991). Similarly, over-expression of IGF-I in transgenic mice causes increased CNS myelination compared to normal non-transgenic littermates (Carson et al., 1993) while severe CNS hypomyelination with fewer oligodendrocytes was apparent in mice with an inactive IGF-I gene (Beck et al., 1995) suggesting a role for IGF-I not only in oligodendrocyte precursor cell proliferation but also in promoting myelin synthesis. However, a more recent study using IGF-I null mice demonstrated that IGF-I is not essential to myelination in vivo (Cheng et al., 1998).

Nerve growth factor and neurotrophin-3, members of the neurotrophin family, were originally identified by their ability to promote the proliferation, differentiation, growth and survival of peripheral and CNS neurons (Davies, 1994) however, they are also thought to regulate glial development and function. The peak of NT-3 mRNA expression in the aggregates coincided with the appearance of MBP mRNA before declining over time, as observed in the developing CNS once oligodendrocyte proliferation and neuronal maturation is underway (Maisonpierre et al., 1990a; Hohn et al., 1990; Murase et al., 1994). Exogenous NT-3 enhances the survival of oligodendrocyte progenitor cells in vitro (Barres et al., 1993) while in conjunction with PDGF it promotes precursor cell proliferation in vitro and in vivo (Barres et al., 1994c). Neutralising anti-NT-3 monoclonal antibodies reduced the number of progenitor and mature oligodendrocytes in the developing rat optic nerve (Barres et al., 1994c) implying that it is necessary for normal oligodendrocyte development in vivo.
Furthermore, recent studies of NT-3 and TrkC knockout mice have shown that the number of oligodendrocytes, astrocytes and NT-3 dependent neurons were reduced (Kahn et al., 1999) which is consistent with the localisation of TrkC on oligodendrocyte precursors and the documented role of NT-3 as a mitogen for these cells (Barres et al., 1994c). NT-3 may therefore be supporting oligodendrocyte as well as neuronal elaboration in the aggregate cultures.

A role for NGF in stimulating the differentiation of forebrain cholinergic neurons in vitro and in vivo has been suggested (Honegger & Lenoir, 1982; Gnahn et al., 1983). Hence, the first rise in NGF mRNA in the aggregates may correspond to peak neurogenesis while during the later stages of the culture it may also be acting as an oligodendrocyte survival factor as has been demonstrated in vitro (Barres et al., 1993; Cohen et al., 1996). The levels of EGF mRNA, along with NT-3 and NGF, were expressed at much lower levels than any of the other growth factors examined reflecting observations in the mammalian brain (Lazar & Blum, 1992). In the developing nervous system EGF is mitogenic for glial cells while also acting as a neurotrophic and survival factor (Fallon et al., 1984; Almazan et al., 1985). Addition of EGF to aggregate cultures during the earliest stages in vitro increased activity of the two glial enzymes glutamine synthetase (GS), located exclusively in astrocytes (Norenberg, 1979a; Norenberg & Martinez, 1979b), and CNP in parallel to enhanced MBP accumulation with only minimal changes in proliferation indicating that EGF accelerates the differentiation of astrocytes and oligodendrocytes (Honegger & Guentert Lauber, 1983; Almazan et al., 1985). It has been suggested that the stimulation of differentiation elicited by EGF in three-dimensional aggregate cultures, where there is a restricted period of proliferation followed by extensive differentiation (Honegger, 1985), is more
likely to correspond to the situation in vivo compared to monolayer cultures which proliferate to confluence with only limited differentiation.

Triiodothyronine, retinoic acid, cAMP and neuregulin are some of the other factors not studied in this project that have been shown to induce oligodendrocyte generation (Barres et al., 1994a; Raible & McMorris, 1993; Canoll et al., 1996). Neuregulin, for example, is a potent mitogen for oligodendrocyte precursors and is also implicated in their survival (Canoll et al., 1996) with oligodendrocytes failing to develop in mice that lacked the neuregulin gene (Vartanian et al., 1999).

The expression of TGF-β1 mRNA by rat peritoneal macrophages is in line with the finding of Rappolee et al., (1988) who found that TGF-β mRNA transcripts were constitutively expressed by mouse peritoneal macrophages. Similarly, both amoeboid and ramified microglia as well as astrocytes were found to express mRNA encoding TGF-β receptors type I and II as well as the mRNA for TGF-β1, 2 and 3 (Jones et al., 1998). Furthermore, this endogenous TGF-β1 and TGF-β3 expression was found to inhibit ramified microglial proliferation (Jones et al., 1998). Macrophages isolated from a wound site expressed TGF-α, TGF-β, PDGF-A and IGF-I mRNA transcripts (Rappolee et al., 1988).

The enhanced myelination observed in macrophage-enriched cultures was associated with increased levels of TGF-β1 and FGF-2 as well as reduced expression of CNTF mRNA compared to non-enriched cultures without consistently altering the mRNA levels of any of the other growth factors tested. Macrophage augmented expression of TGF-β1 may have a role in increasing the myelin content and the rate of organisation in the aggregate cultures since it inhibits PDGF driven proliferation and
promotes oligodendrocyte differentiation (McKinnon et al., 1993). Furthermore while microglia were cytotoxic to oligodendrocytes, pre-incubating them with TGF-β inhibited the cytotoxicity in a dose-dependent fashion implicating TGF-β as an oligodendrocyte protective factor (Merrill & Zimmerman, 1991). TGF-β1 has been shown to afford neuroprotection after CNS injury (Flanders et al., 1998) and to support neuronal survival and neurite outgrowth (Ishihara et al., 1994; Abe et al., 1996). While TGF-β1 is synthesised by astrocytes, macrophages and neurons (Constam et al., 1992; Kriegstein et al., 1995), activated macrophages and microglia were found to be the major source of TGF-β1 produced in the brain following an ischaemic episode (Lehrmann et al., 1998) as well other forms of brain pathology (Lindholm et al., 1992; Kiefer et al., 1993; Morgan et al., 1993; Logan et al., 1994). Interestingly there may be some synergy between the elevation of TGF-β1 and FGF-2 mRNA in macrophage-enriched aggregate cultures since exogenous TGF-β1 markedly and selectively increased the protein expression of FGF receptors 1 and 2 in human lung fibroblast cultures resulting in a potentiation of the mitogenic response of multiple FGF ligands particularly FGF-2 (Thannickal et al., 1998). Furthermore, TGF-β1 induces the astrocytic expression of FGF-2 (Lindholm et al., 1990; Mattson & Cheng, 1993) thus, it may be postulated that in macrophage-enriched aggregates enhanced levels of TGF-β1 may up-regulate not only FGF-2 but also FGFR-1 and FGFR-2 priming cells such as oligodendrocytes to proliferate in response to augmented FGF-2 which ultimately promotes oligodendrocyte maturation and myelinogenesis.

Both rat and human microglial cells produce FGF-2 (Shimojo et al., 1991; Araujo & Cotman, 1992; Presta et al., 1995) in addition to its production in vitro by neurons
(Pettmann et al., 1986; Torelli et al., 1990) and astrocytes (Ferrara et al., 1988; Hatten et al., 1988). FGF-2 is mitogenic for immature oligodendrocytes in vitro (McKinnon et al., 1990) while in the presence of astrocytes also promotes their differentiation (Mayer et al., 1993). Thus, this factor has the potential to increase the number of myelinating oligodendrocytes in macrophage-enriched aggregate cultures. However, this is unlikely as the enhanced MBP levels observed in enriched aggregate cultures were not accompanied by augmented CNP activity (Loughlin et al., 1997) indicating that it is the myelinative capacity of individual oligodendrocytes that is enhanced in these enriched cultures. Hence, FGF-2 has been shown to synergise with the astrocytic extracellular matrix to promote adult human oligodendrocyte process outgrowth in vitro (Oh & Yong, 1996) and to influence the differentiation of immature human oligodendrocytes (Armstrong et al., 1992; Gogate et al., 1994). Similarly, FGF receptors have been localised to the myelinated tract in the rat CNS further implicating it in myelination (Asai et al., 1993). A recent article demonstrates that FGF-2 is a principal regulatory factor governing neuronal as well as glial output during cortical development (Vaccarino et al., 1999).

Macrophage-derived FGF-2 down-regulates astrocytic CNTF expression in vitro (Carroll et al., 1993) indicating that the significant increase in FGF-2 mRNA associated with enrichment may in turn be responsible for the decrease in CNTF expression. Alternatively, given that CNTF protects rodent and human oligodendrocytes from cytotoxic injury in vitro (Louis et al., 1993; D'Souza et al., 1996), and promotes their survival both in vivo and in vitro (Barres et al., 1993) it may be down-regulated in macrophage-enriched cultures where enhanced myelination and increased organisation is evident. Thus, macrophage addition to aggregate cultures on the day of seeding may
result in their activation with consequent phagocytosis of cellular debris and secretion of TGF-β1 and FGF-2 along with down-regulation of CNTF engendering neuroprotection and enhanced oligodendrocyte differentiation.

While remyelination can be widespread and extensive in experimental models of demyelination in diseases such as MS demyelinated lesions that have not remyelinated eventually predominate. The temporal expression of myelin proteins is similar in both developmental myelination and remyelination (Jordan et al., 1990; Woodruff & Franklin, 1999) while both of these processes are likely to be controlled by the same transcription factors. Assuming that adult oligodendrocyte precursors are involved in CNS remyelination then these cells, which have not progressed beyond the progenitor stage, are myelinating an axon for the first time as would be the scenario in the developing CNS, albeit after some time delay. Therefore, it seems probable that those growth factors that are integral to developmental myelination may be central to remyelination, although the environment in which these two processes proceed are clearly different.

Demyelination of aggregate cultures demyelinated by short term exposure to 8-18C5 anti-MOG antibody plus complement induced a distinct pattern of expression of many myelination-associated growth factors (Copelman et al., 2000). A rapid rise in CNTF mRNA in standard cultures closely followed by increases in FGF-2 and IGF-I was in contrast to the delayed induction of PDGF-A and NGF mRNA. Similarly, Hinks and Franklin, (1999) observed large increases in IGF-I and TGF-β1 mRNA expression following lysolecithin-induced demyelination in the rat spinal cord which peaked at a time when new myelin sheaths were beginning to appear while a more modest rise in
the expression of FGF-2 and PDGF-A mRNA was observed with both rapidly rising shortly after the appearance of MBP and PLP mRNA transcripts. In macrophage-enriched aggregates the rise in IGF-I mRNA preceded that in standard cultures suggesting that macrophage-enrichment instigates a faster IGF-I response during remyelination. With minor exceptions the IgG1 isotype did not influence growth factor mRNA expression despite some evidence of demyelination. An early transient increase in the mRNA expression of some growth factors was observed following exposure to guinea pig serum which may be attributable to demyelination although this seems unlikely. A recent report showed that NGF transcription and translation was induced in microglia exposed to complement factor C3a (Heese et al., 1998).

The immediate rise in CNTF mRNA expression observed in anti-MOG treated cultures relative to the IgG1 and medium controls with continued high expression for at least 3 days into the recovery phase supports a role for it as an oligodendrocyte survival factor both during development (Barres et al., 1993) and after demyelinating treatment since it can prevent TNF-α induced oligodendrocyte death in vitro (Louis et al., 1993). In addition to its role as a survival factor CNTF may contribute to the repair process by enhancing the rate of oligodendrocyte generation as has been observed in vitro and in vivo (Barres et al., 1996). The presence of protective factors such as CNTF may be a prerequisite for remyelination in the otherwise hostile environment of a demyelinating CNS plaque.

In a recent study, CNTF injected into adult rat white matter induced transient expression of IGF type I receptor and FGF receptor-1 mRNA in interfascicular oligodendrocytes (Jiang et al., 1999) as well as enhanced FGF-2 and IGF binding protein-2 mRNA expression (Wood et al., 1995). In vitro studies have shown that FGF-
2 reduces MBP mRNA in fully differentiated oligodendrocytes *in vitro* thereby inducing de-differentiation of mature oligodendrocytes which revert to a progenitor cell phenotype thus providing a possible source of cells capable of remyelination (Grinspan *et al.*, 1993b; Fressinaud *et al.*, 1995). Accordingly, addition of exogenous FGF-2 to mature aggregate cultures resulted in a reduction in CNP activity and MBP content combined with a significant increase in total DNA and protein content (Pouly *et al.*, 1997) which was proposed as induction of oligodendrocyte dedifferentiation. However, mature oligodendrocytes exposed to FGF-2 *in vitro* did not divide, as their perinatal counterparts did, but instead underwent cell death by apoptosis (Muir & Compston, 1996) while at the same time stimulating the residual population of progenitor cells to divide. In line with our results following demyelination, FGF-2 increased in response to CNS injury when cell proliferation may be required once again and improved remyelination after chemically induced demyelination (Frautschy *et al.*, 1991; Logan, 1990). Similarly, FGF-2 induced myelin membrane construction following demyelination *in vitro* (Fressinaud & Vallat, 1994).

IGF-I is also a potent survival factor *in vitro* for both progenitor and mature oligodendrocytes (McMorris *et al.*, 1993; Barres *et al.*, 1993) and in organotypic cultures was found to enhance myelin production and inhibit anti-white matter antibody plus complement induced demyelination (Roth *et al.*, 1995). Following demyelination induced by cuprizone intoxication, cryogenic injury and EAE IGF-I mRNA and protein production are up-regulated in reactive astrocytes as determined by *in situ* hybridisation and immunocytochemistry (Komoly *et al.*, 1992; Liu *et al.*, 1994; Yao *et al.*, 1995) with levels returning to normal following recovery. IGF administration to Lewis rats with EAE is effective in increasing oligodendrocyte proliferation and myelin protein mRNA
while reducing the size and number of demyelinating lesions with corresponding clinical improvement (Yao et al., 1995; Liu et al., 1995). Furthermore, the IGF-I receptor, localised in oligodendrocytes, was up-regulated during recovery and remyelination when new myelin sheaths were detected (Komoly et al., 1992; Yao et al., 1995). These studies suggest that IGF-I could act on oligodendrocytes to aid myelin sheath regeneration and survival in the aggregates.

It is somewhat intriguing that PDGF-A mRNA transcription was not increased in demyelinated aggregates until 12 days after the antibody had been washed out suggesting that it is not acting as a primary proliferative or survival factor even though it has been implicated in both processes in vitro and in vivo (Barres et al., 1992a). However, the increase in FGF-2 mRNA prior to that of PDGF-A may increase the sensitivity of progenitors in the aggregates to PDGF driven proliferation. Addition of PDGF to mature aggregate cultures demyelinated by anti-MOG antibody underwent increased cell proliferation followed by a period of enhanced myelination suggesting that PDGF stimulates oligodendrocytes to proliferate and then this larger population of cells is able to produce increased myelin on removal of the mitogenic stimulus (Honegger & Tenot Sparti, 1992). Furthermore, when added to cultured oligodendrocytes PDGF has been shown to improve myelin repair following lysophosphatidyl choline exposure (Fressinaud et al., 1996) while the PDGF antagonist Trapidil reduces the remyelinating capacity of rat spinal cord after lysolecithin demyelination (McKay et al., 1997). In addition increased levels of PDGF-A expression following coronavirus-induced demyelination in mice has been observed (Redwine & Armstrong, 1998) further suggesting a role for this factor in remyelination.
To date, the major effect of macrophage-enrichment on remyelination was an early increase in IGF-I mRNA expression in anti-MOG treated cultures to levels that surpassed all control groups and particularly reached twice those of comparably treated standard aggregate cultures before falling with time. This decline was accompanied by increased IGF-I mRNA levels in standard cultures such that the rise in IGF-I mRNA expression in macrophage-enriched flasks following demyelination precedes the subsequent peak in standard flasks suggesting that macrophage-enrichment instigates a faster IGF-I response during remyelination.

In conclusion, these studies have shown that growth factors implicated in oligodendrocyte development *in vitro* are expressed in multi-cellular CNS aggregates in a pattern which is similar to that found *in vivo*. Multiple extracellular factors appear to interact to regulate myelinogenesis in the aggregates with macrophage-enhanced myelination associated with increased expression of both TGF-β1 and FGF-2. Growth factors are also a prerequisite for remyelination, however the extent and timing of their expression varies with some, but not all, of myelination-associated growth factors increased during remyelination, a factor to be taken into account when optimising conditions to promote remyelination. These results have implications in demyelinating disease where macrophages form a primary component of the inflammatory process and secretion of protective growth factors may be central to regeneration. Favourably altering macrophage activation to promote oligodendrocyte survival as well as stimulating progenitor cell proliferation, differentiation and myelination may provide potential therapeutic strategies for demyelinating diseases such as MS.
CHAPTER 6
CONCLUSIONS AND FUTURE STUDIES

6.1 Conclusions

Supplementing three-dimensional aggregate cultures with peritoneal macrophages increased myelin deposition over time as determined by morphological, biochemical and mRNA analyses, with no apparent rise in the activity of the mature oligodendrocyte marker CNP suggesting that oligodendrocytes are producing more myelin in the presence of macrophages.

Treating myelinated aggregate cultures with pro-inflammatory cytokines or anti-MOG antibodies in the presence of complement resulted in demyelination, indicated by a reduction in the MBP content, while MBP synthesis resumed following removal of demyelinating agents. Exogenous macrophage enrichment did not enhance demyelination in aggregate cultures but their presence in additional numbers appeared to reduce MBP accumulation and prolong the period of myelin phagocytosis and degradation following a demyelinating episode so that, as occurs in MS lesions, attempts at remyelination are counter-balanced by continuing demyelination.

Developmental maturation and myelination in the aggregate cultures was associated with a specific pattern of growth factor mRNA expression comparable with that of the developing brain. Demyelination induced a distinct pattern of expression of many myelination-associated growth factors. A rapid rise in CNTF mRNA in standard cultures closely followed by increases in FGF-2 and IGF-I was in contrast to the delayed induction of PDGF-A mRNA. However, in macrophage-enriched aggregates the rise in IGF-I mRNA preceded that in standard cultures suggesting that macrophage-enrichment instigates a faster IGF-I response during remyelination.
The precise physiological role of most of these growth factors remains to be elucidated and this task is complicated by the fact that in addition to growth factors extracellular matrix molecules and cell membrane constituents may participate in the control of normal oligodendrocyte development and function. Furthermore, some of these factors may only influence the development of these cells for a limited period of development while others may be required more continuously. Presumably exogenous addition of growth factors would affect other cell types including neurons and astrocytes such that if IGF-I promoted the survival and growth of neurons then this could indirectly increase the number of oligodendrocytes. Astrocytes would also be responsive to factors like FGF-2 and IGF-I which in turn could influence oligodendrocyte maturation. It is likely that initiation of successful remyelination after a demyelinating episode would similarly require the interaction of several growth factors.

In summary, these results have implications for demyelinating diseases such as MS since macrophage-rich demyelinating multiple sclerosis lesions also display signs of remyelination. Therefore, the macrophage, with its multiple effector properties, has the potential to influence the intricate balance between the degradation and repair of myelin. Favourably altering macrophage activation to promote oligodendrocyte development, myelination and repair may provide a potential strategy for therapeutic intervention in CNS demyelinating diseases such as MS.

6.2 Future studies

Future studies could involve the optimisation of *in situ* hybridisation in combination with immunocytochemistry to determine the spatio-temporal pattern of
growth factor expression within particular cell types with particular reference to FGF-2 and TGF-β1 transcripts and proteins. Oligodendrocyte growth factor receptor expression in the aggregates could also be investigated using this technique along with the determination of the maturational stages of the oligodendrocytes. It is hoped that by correlating growth factor expression with particular stages of oligodendrocyte development key macrophage-derived growth factors influencing myelination and remyelination will be identified. The application of specific neutralising antibodies to the culture system would elucidate the role of particular growth factors in myelination. Depleting pre-existing macrophages and microglia from the cultures and determining the effects of microglia enrichment at the start of the culture period and at later time points should provide further insight into the role of these cells in myelination.

The long term aim of this work is to provide more information about the mechanisms promoting myelination and remyelination in the developing and adult CNS. It is hoped that by correlating growth factor expression with oligodendrocyte proliferation and differentiation during myelination and remyelination and identifying the cellular source of these factors targets for therapeutic intervention will become evident. Any treatment that enhances myelination and/or remyelination would be likely to promote functional recovery alleviating and preventing further disability.

6.3 Evaluation of the aggregate culture system

Three-dimensional foetal rat brain aggregate cultures undergo morphological and biochemical differentiation in a manner that resembles the developing brain in vivo (Honegger, 1985). Thus, this culture system accurately resembles myelinogenesis in the developing CNS in vivo (Honegger, 1985, Loughlin et al., 1997; Kruger et al., 1999)
thereby providing a model to study oligodendrocyte development and myelination from the early stages of progenitor cell proliferation through to the formation of compact myelin sheaths. Furthermore, the presence of myelinated axons provides an ideal system to study the potential demyelinating effects of cytokines and antibodies whilst avoiding complex systemic interactions. An in vitro approach was employed to study myelination, demyelination and remyelination in an attempt to reduce cellular complexity, to manipulate the cellular environment and to characterise extrinsic influences.

In comparison to other in vitro systems the three-dimensional aggregate culture system has distinct advantages. In contrast to monolayer cell cultures, aggregate cultures form regular three-dimensional cell arrangements retaining histotypic structure, signalling capacity, cell to cell contacts and interactions as well as histological characteristics. Aggregate cultures provide a system where cells of the appropriate lineage, at the appropriate developmental age and in the appropriate regulatory environment can migrate and interact as well as exchange nutritional and signalling factors. As the multiple cell types present mature they can secrete growth factors in addition to extracellular matrix components and cell membrane constituents which are appropriate for particular developmental stages and may participate in normal oligodendrocyte development in vivo. Hence, the response of integrated tissue may be different from single cell cultures because of the interactions between associated cells. It has been suggested that the three-dimensional structure and cellular interactions are necessary for optimal differentiation. For example, the response of a given cell type to an exogenous stimuli may be influenced by cell to cell interactions and may be more likely to correspond to the situation in vivo compared with that in monolayer cultures.
which proliferate to confluence with only limited differentiation (Honegger, 1985). Furthermore, the importance of cellular interactions within the cultures is highlighted by the finding that neuron-enriched aggregates, which contain few glial cells, have lower levels of NF-M and phosphorylated NF-H implying that glial cells are critical for the development of the neuronal cytoskeleton (Riederer et al., 1992). Therefore, while monolayer cell cultures may be easier to handle and to interpret they are fairly restricted in cell to cell contacts and cell to matrix interactions.

In organ, slice and explant cultures, potential alternative in vitro models, the original structural integrity of different cells and their interactive functions are also largely preserved. Therefore, these in vitro models retain a high degree of organisation which may preserve the cellular interactions present in vivo as well as maintain the chemical configurations of the extracellular matrix. These cultures can also be initiated from both developing and adult animals and undergo cell differentiation. However, tissue yield and sample reproducibility are limiting factors and they are therefore unsuitable for quantitative temporal and multi-disciplinary studies. Aggregate cultures have the advantage of high yield and reproducibility producing a large number of culture flasks each containing a homogenous population of aggregates. Additionally, while in many culture models cells adhere to the surface the three-dimensional aggregates remain in suspension allowing easy and sequential manipulation for prolonged periods. Furthermore, with each culture flask containing thousands of spherical aggregates that can be maintained for several months, samples can be harvested sequentially during the course of the culture period enabling temporal analyses. Similarly, this in vitro system provides relatively large amounts of material to enable parallel biochemical, molecular biological and morphological studies at multiple time points providing a unique system
to investigate developmental events and changes in cellular composition over time. The capacity to maintain the aggregates in a highly differentiated state for prolonged periods also enables long term studies with chronic or repeated acute exposures while maintaining the advantages of an *in vitro* system in terms of ease of manipulation and accessibility.

The use of chemically defined medium allows a rigorous definition of the chemical requirements of the cells and enables manipulation of the culture environment to favour optimal cell maturation and survival. Additionally, there are advantages of using a serum free culture system as serum contains multiple proteins, lipids and other factors which have the potential to considerably affect cell growth. The wide range of minor components present in serum may include growth factors, adhesion factors, hormones, minerals, vitamins, fatty acids, and lipids. The concentrations of these constituents can vary considerably between batches introducing inter-experimental errors. Consequently, the cellular responses are less likely to be varied or obscured if chemically defined serum free medium is used enabling greater reproducibility and ease of interpretation. Additionally, as with other *in vitro* models, the physiochemical environment including pH, environment, oxygen and carbon dioxide can be controlled.

The main disadvantage of the aggregate system is the structural complexity. As is the case for organ, slice and explant cultures, the multiple cell types present in the aggregates renders direct observation and manipulation of individual cells types difficult. Furthermore, the complexity of the system means that it may be difficult to accurately interpret results. However, in the aggregates, this can be partly overcome by correlating the results of multiple parallel analyses whereas for organ, slice and explant cultures only more qualitative histochemical or immunocytochemical analyses are suitable.
Furthermore, it is possible to eliminate a class of endogenous cells or add exogenous cells, such as macrophages. Alternatively, the cellular composition of standard mixed-glial cell aggregate cultures can be modified and compared with cultures enriched with either neurons or glial cells. Technically, to set up aggregate cultures a relatively large amount of starting foetal tissue is required of a defined gestational age and the preparation and maintenance can be laborious.

In summary, this well characterised model provides a system to study the factors influencing developmental myelination, demyelination and remyelination while eliminating some of the complexities associated with *in vivo* studies. Simplification of the experimental system enables easier manipulation, however, as with other in vitro systems the culture environment lacks several components involved in homeostatic regulation *in vivo* principally the nervous, endocrine and vascular systems. This model, therefore, serves as a link between single cell *in vitro* systems and the *in vivo* situation in which modulation such as macrophage-enrichment and addition of potentially toxic cytokines and antibodies cannot be easily undertaken. As long as the limits of the model are appreciated it is a valuable tool ideal to study the processes of myelinogenesis, demyelination and remyelination.
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