

Inflammatory Cascades in the Pathogenesis of Multiple Sclerosis Lesions.

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by

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Abstract

Multiple sclerosis (MS) is a disease of the human central nervous system (CNS) characterised by inflammation and demyelination. Initially the MS lesion has a distinct histopathological picture with myelin-positive microglia in the midst of apparently intact myelin but minimal perivascular inflammation. Inflammatory mediators produced by these activated microglia may precipitate the infiltration of mononuclear cells and the overt myelin loss seen in actively demyelinating MS lesions.

Nuclear factor- κ B (NF- κ B) is a transcriptional regulator of proteolytic enzymes, adhesion molecules and inflammatory cytokines which rapidly translates extracellular signals into protein synthesis. The immunocytochemical detection of the transcriptionally active form of NF- κ B, but not the inhibitory protein I κ B α , in the nuclei of microglia in normal human CNS white matter indicates the capability of microglia to respond rapidly to pathological stimuli in the CNS. Activation of NF- κ B in MS plaques, evident from the nuclear localisation of the NF- κ B subunits RelA, c-Rel and p50 in macrophages, may propagate inflammatory demyelination through upregulation of NF- κ B-controlled macrophage genes for inflammatory mediators.

In demyelinating disease the plasmin-matrix metalloprotease (MMP) enzymatic cascade promotes blood-brain barrier (BBB) damage, generation of encephalitogenic myelin peptides and activation of pro-inflammatory cytokines. Constitutive expression of MMPs 1, 2, 3 and 9 in glial cells in normal control white matter was demonstrated by immunocytochemistry. However, the lack of tissue (t-PA) and urokinase (u-PA) plasminogen activators in glial cells and the absence of caseinolytic activity as shown by *in situ* zymography emphasises the latent nature of the plasmin-MMP cascade in normal CNS tissue. In contrast, the co-localisation of t-PA and u-PA, rate-limiting serine-proteases, and MMPs in macrophages and astrocytes in active MS lesions forms the basis of a functional enzymatic cascade. Furthermore, increased amounts and activity of u-PA and MMP-9 in

homogenates of active MS plaques coupled with the presence of caseinolytic activity in foamy macrophages implicates these cells as the major source of MMPs, which cause proteolytic damage in MS.

Insulin-like growth factors (IGFs) play an important role in development and myelination in the CNS but can also stimulate phagocytosis and production of inflammatory mediators by macrophages. In active MS lesions binding of IGF-II to the IGF receptor on foamy macrophages may induce mitogenic responses and invasiveness of macrophages which can be further enhanced by MMP-mediated proteolytic removal of inhibitory IGF-binding proteins. Similarly, the potent mitogens IGF-I and insulin may stimulate astrocytosis and gliosis. In contrast, oligodendrocytes in normal-appearing white matter do not express IGFs or IGF-I receptor which implies that the oligodendrocyte response to these remyelinating growth factors is impaired. Therefore, the prevailing role of IGFs in MS lesions may be in line with pro-inflammatory mediators promoting macrophage and astrocyte responses to tissue damage.

In conclusion, NF- κ B activation in microglia and macrophages upregulates the production of PAs and inflammatory cytokines which trigger the plasmin-MMP cascade, leading to BBB damage and enhanced inflammatory cell migration and demyelination in white matter. Influx of IGFs through the damaged BBB and their increased local production may promote myelin phagocytosis and reactive astrocytosis. In turn IGF-mediated upregulation of PAs in glial cells could provide a feedback amplification of the MMP cascade. Therefore, the findings from these studies bring together three systems of mediators, NF- κ B, MMPs and IGFs, into a hypothetical model for the propagation of demyelination in MS lesions.

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Abbreviations

AL	acute lesion
AMPA	9-aminophenylmercuric acetate
APC	antigen-presenting cell
AT	ataxia telangiectasia
ATM	ataxia telangiectasia mutated gene
BBB	blood-brain barrier
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
CL	chronic lesion
CNP	2,3-cyclic nucleotide phosphodiesterase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
cpm	counts per minute
CSF	cerebrospinal fluid
DAB	3,3'-diaminobenzidine tetrahydrochloride
DFT	death to snap-freezing time
EAE	experimental allergic encephalomyelitis
EAN	experimental allergic neuritis
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
FITC	fluoresceine isothiocyanate
<i>g</i>	centrifugal force symbol
GFAP	glial fibrillary acidic protein
GFAP	glial fibrillary acidic protein
GM	grey matter
G-PBS	2% glucose phosphate buffered saline
h	hour
HSP	heat shock protein
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IGF	insulin-like growth factor
IGF-BP	insulin-like growth factor binding protein
IGF-IIR	insulin-like growth factor II/mannose-6-phosphate receptor
IGF-IR	insulin-like growth factor I receptor
IL	interleukin
KW	Kruskal-Wallis test
LDL	low density lipoprotein
LFA-1	leukocyte function-associated antigen 1
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MCP	macrophage chemotactic protein
MHC	major histocompatibility complex
min	minute
MIP	macrophage inflammatory protein
MMP	metalloprotease

MOG	myelin-oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
MW	molecular weight
NAWM	normal-appearing white matter
NBT	nitroblue tetrazolium
NF- κ B	nuclear factor kappaB
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PI3	phosphoinositol 3 kinase
PLP	proteolipid protein
PMSF	phenylmethylsulphonyl fluoride
RA	rheumatoid arthritis
ROI	reactive oxygen intermediates
rpm	rotations per minute
RT	room temperature
SAL	subacute lesion
sec	seconds
TCR	T-cell receptor
TNF- α	tumour necrosis factor alpha
t-PA	tissue plasminogen activator
TRITC	tetramethylrhodamine isothiocyanate
u-PA	urokinase plasminogen activator
v/v	volume per volume
VCAM	vascular cell adhesion molecule
VLA	very late antigen
w/v	weight per volume
WM	white matter

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Chapter I

1. Introduction.

1.1. Multiple sclerosis: an overview.

Multiple sclerosis (MS) is a chronic inflammatory disease of the human central nervous system (CNS) characterised by selective damage to myelin, inflammatory infiltration and impairment of axonal conduction. MS was first described as a separate disease by Carswell and Cruveilhier whilst Charcot (Medaer, 1979) recognised a demyelinating process as the major pathological feature of MS. Advancements in the field of immunology and development of animal models of MS deepened insight into the autoimmune nature of demyelination and the underlying mechanisms of CNS inflammation. From the immunological point of view MS represents a molecular conundrum in which components of cellular and humoral immunity interact with endogenous CNS cells leading to initiation and propagation of the demyelinating process in the CNS.

1.1.1. Epidemiology and genetics of MS.

The environmental influence on the disease has been documented and geographic position, migration, diet and infections represent important epidemiological factors. MS is the third most common neurological disease after brain tumours and cerebrovascular insults and represents the major demyelinating disease. It predominantly affects Caucasians in the Northern hemisphere with a prevalence rate of 60 per 100,000 (Ebers & Sadovnick, 1994). MS is a disease of temperate climates with the highest prevalence in the geographic region delimited by 45° and 65° latitude on both hemispheres (Kurtzke, 1980). Migrants from areas of high prevalence before the age of 15 adopt the prevalence rate of their destination area whereas migrants after this age retain the high risk of the country of

origin (Dean & Kurtzke, 1970, Alter *et al*, 1978). There is a less certain connection between dietary factors and MS although a role for lipid abnormalities and deficiency in polyunsaturated fatty acids has been proposed by several researchers (Swank, 1970, Fisher *et al*, 1987). The occurrence of demyelination with axonal sparing in a number of viral infections of the CNS and the increased incidence of upper respiratory tract infections found to be associated with MS have fuelled the hypothesis on involvement of an infectious agent in MS pathogenesis (ter Meulen *et al*, 1984). Furthermore, several viral proteins have been detected in brain and other tissue of MS patients as well as increased anti-viral antibody titres in sera and cerebrospinal fluid (CSF) (Allen & Brankin, 1993).

MS is approximately twice more frequent in women with the first onset likely to be during adolescence (Duquette *et al*, 1987). The sex-related bias reflects the influence of the neuroendocrine system on immune responsiveness and is described in other autoimmune diseases (Compston, 1992). Family and twin studies have provided further insight into the role of genetic factors. The risk of developing MS within a family increases with the degree of relatedness, being highest for first-degree relatives, and is markedly higher compared with the general population (Sadovnick & Ebers, 1995). Such findings are supported by a low disease occurrence in adopted family members, therefore excluding the role of microenvironmental factors within families (Ebers *et al*, 1995). The results from twin studies indicate that concordance rates for monozygotic twins are on average 27% compared to 4.7% of dyzygotic pairs, which is similar to the rate for non-twin siblings (Sadovnick & Ebers, 1993). Association and linkage studies of candidate genes showed a significant genetic effect with nineteen regions harbouring MS susceptibility genes, each contributing to the overall risk.

1.1.2. Clinical aspects and diagnosis of MS.

The symptoms of MS can be broadly divided into three categories (Hallpike, 1983). Initial symptoms predominantly arise from affected visual pathways and the spinal cord and include weakness in one or more limbs (in 40% of cases), optic neuritis (22%), paraesthesiae (21%), diplopia (12%) vertigo (5%) and disturbance of micturition (5%). Secondary symptoms involve complications that arise as a consequence of impaired organ function such as bladder infections and skin disorders whereas tertiary symptoms include psychological, social and vocational difficulties. Clinical manifestations of MS are not unique and there is a substantial overlap with other primary demyelinating, neurological and systemic autoimmune diseases. The criteria formulated by Poser *et al.* (1983), which take into account both clinical and laboratory evidence, have greatly facilitated the diagnosis of MS. Diagnosis of clinically definite MS is based on the finding of objective abnormalities of CNS function that predominantly reflect white matter involvement from two or more areas of the CNS. Further, the disease manifests itself either in two or more episodes each lasting more than 24h and at least one month apart or as a stepwise progression over a minimum of six months. Immunoglobulin oligoclonal bands in cerebrospinal fluid (CSF) and anatomical and functional abnormalities detected by magnetic resonance imaging (MRI) and evoked potentials supplement the diagnosis of definite MS.

The temporal course of the disease varies individually and can be categorised into four main types. A relapsing-remitting course is initially found in 90% of patients and is characterised by periods of remission interspersed by relapses which on average occur at the rate of 1.5 per year. Approximately 40% of patients continue to follow this course whilst the other 40% develop secondary progressive MS and experience a gradual onset without further remissions. In 10% of cases MS has a primary progressive course with

rapid onset of symptoms and no remissions. The remaining 10% experience a benign form of MS with few or no symptoms after the initial diagnosis. Disability usually increases with the duration of MS but mortality remains low and 75% of patients survive 25 years (Hallpike, 1983). Concurrent infections, commonly pneumonia or urinary sepsis, are the leading causes of death in the affected population.

1.2. Pathological features of MS.

Disorders that affect human CNS myelin are classified as dysmyelinating or demyelinating depending on their association with genetic, metabolic or infectious elements. Dysmyelinating disorders, best represented by leucodystrophies, are caused by a defect in myelin formation due to genetically controlled metabolic or glial dysfunction in which demyelination is secondary to myelin degeneration. In contrast, in demyelinating diseases loss of myelin is the main pathological feature and can occur without an obvious cause, as in primary demyelinating diseases, or following vaccination or viral infection in secondary demyelination. The inflammatory component plays an important role in most demyelinating diseases although non-inflammatory demyelination such as central pontine myelinolysis and Marchiafava-Bignami disease have been described. Chronic MS with the characteristic relapsing-remitting course and disseminated white matter lesions is the classic example of primary inflammatory demyelinating disease. Several subtypes of MS have also been described and are characterised by a fulminant disease course (Marburg disease), a specific demyelination pattern (Balo's concentric sclerosis), the extent of demyelination (Schilder's diffuse sclerosis) or predominant involvement of spinal cord and visual pathways (Devic's disease) (Adams, 1989a).

1.2.1. Anatomical pathology and distribution of MS lesions.

The major gross pathological characteristic of MS is the presence of disseminated

lesions in the CNS white matter which vary in size, shape and activity (Adams *et al*, 1989b). Smaller lesions are usually rounded and frequently associated with veins along which they may spread in the form of a Dawson's finger. As the disease progresses, lesions tend to coalesce forming large irregular demyelinated areas. Macroscopically, active lesions are red-brown in colour and soft in texture while chronic lesions are grey and glassy usually with a firm texture due to astrogliosis. Lesions are predominantly found in white matter but those affecting basal ganglia and cortical grey matter have been described (Lumsden, 1970). The number of lesions and distribution pattern varies considerably from case to case and there is no particular predilection site for lesion development. On post-mortem examination most cases show asymmetrical lesions in the cerebral periventricular white matter and optic pathways. Some areas such as the lateral angle of the lateral ventricles and optic chiasma are more frequently affected than the rest of the white matter. Similarly, in the brain stem, lesions are found around the IV ventricle with often extensive demyelination in the pons parenchyma. Spinal cord involvement is common, particularly in the cervical segment, and areas of demyelination range from small subpial lesions to complete demyelination. It is important to note that the extent of demyelination and lesion distribution are not necessarily reflected in the clinical manifestation of MS and a number of lesions remain clinically silent.

1.2.2. Histopathology and classification of MS lesions.

The main histopathological features taken into account when grading MS lesions are the extent of myelin loss, the presence of lipid breakdown products within macrophages, perivascular inflammatory infiltration, astrogliosis and the degree of axonal preservation (Li *et al*, 1993). Depending on these features MS lesions can be broadly categorised as initial, active or chronic. There is a substantial disagreement about the character of the initial MS lesion, particularly as significant pathological and biochemical

changes may be present in macroscopically normal-appearing white matter (NAWM). According to some authors the earliest stage in MS lesion development is represented by perivascular mononuclear cell infiltration with minimal glial reaction and no myelin involvement (Adams *et al*, 1989b). Although this is logical especially if MS is an autoimmune disease, perivascular cuffing is frequently found in NAWM which does not necessarily lead to demyelination. Disruption and uptake of myelin by activated microglia is considered by other groups to be an early event in lesion development (Li *et al*, 1996, Gay *et al*, 1997). The presence of immuno- or histochemically detectable myelin degradation products within activated microglia coupled with a lack of blood-brain barrier (BBB) damage and minimal inflammatory infiltration are the main characteristics of this type of early lesion (Li *et al*, 1996, Gay *et al*, 1997). Oligodendrocyte and axon numbers are usually unaffected at this stage.

Formation of an established active MS lesion is a gradual process characterised by increased inflammatory response and different degrees of myelin loss. Active lesions are generally hypercellular due to the astrocyte and macrophage reaction and increased perivascular inflammatory infiltration. Lymphocytes are the most numerous cell type in the perivascular space although substantial numbers of macrophages and plasma cells are also present (Cuzner *et al*, 1988). Endothelial cell vesiculation and increased leakage of plasma proteins indicate damage to the BBB (Gay & Esiri, 1991). Small islands of apparently intact myelin can be seen at earlier stages of lesion development surrounded by lipid-laden macrophages (Li *et al*, 1993). Proteolytic degradation of myelin results in accumulation of sudanophilic lipid breakdown products in macrophages and formation of foam cells. The astrocyte reaction is pronounced, forming a meshwork of processes throughout the lesion parenchyma. Oligodendrocyte numbers are decreased in active lesions but the loss of axons is less evident. Active lesions are usually not well defined and their borders exhibit a

shelving effect with intermittent bands of myelinated and demyelinated tissue and in some areas lipid-laden macrophages can be seen infiltrating the surrounding NAWM. Increased cellularity in NAWM is mostly due to higher numbers of activated microglia and hypertrophic astrocytes although a rim of proliferating oligodendroglia can be frequently found close to the active plaque border (Raine *et al*, 1988, Prineas *et al*, 1989a). With further lesion development the inflammatory reaction subsides to the hypercellular plaque border and perivascular space leaving the hypocellular plaque parenchyma occupied by densely packed astrocyte processes. Oligodendrocytes within lesions are usually depleted at this stage whilst axons are decreased in numbers and display changed morphology.

The final stage of lesion development in MS is the formation of chronic lesions which are hypocellular, sharply demarcated from adjacent NAWM and entirely occupied with gliotic scar. Histopathological changes are also present throughout NAWM suggesting that MS is a widespread rather than focal CNS disease (Allen & McKeown, 1979). These changes include activated phenotypes of glial cells and diffuse gliosis in white matter, mononuclear cell infiltration, thickening and fibrosis of veins and small foci of demyelination or a generalised myelin pallor.

1.3. Immunopathology of CNS inflammation in MS.

MS has a multifactorial background in which genetic, environmental and infectious components may act simultaneously to provoke a disease in a susceptible individual. Regardless of the aetiology, inflammation plays the important part in the development and progression of MS lesions. However, the nature of inflammation in MS as either the initial process that precipitates demyelination or as a tissue response to myelin damage is still a matter of debate. Experimental data suggest that the inflammatory process which arises from the interactions between primed immune cells and endogenous glia frequently precedes demyelination. The development of animal models, especially experimental

allergic encephalomyelitis (EAE), has provided valuable tools for studying such interactions.

EAE is an autoimmune disorder commonly induced in susceptible rat, mice and guinea pig strains by active immunisation with CNS tissue homogenate, myelin or myelin proteins emulsified in Freund's complete adjuvant. Injection of spleen or lymph node cells from EAE animals or T lymphocytes stimulated *in vitro* with myelin proteins gives rise to the transfer form of EAE (Ben & Cohen, 1981, Pettinelli & McFarlin, 1981). This disease shares several pathological and clinical features of MS including perivascular inflammatory infiltration, a macrophage and astrocyte reaction, and clinical signs of impaired axonal conduction. However, there is a significant polymorphonuclear component in inflammatory infiltrates not found in MS and minimal demyelination. Clinically both active and transfer EAE have a monophasic course and animals fully recover due to complete remyelination (Pender, 1987). The relapsing-remitting nature and histopathological characteristics of the inflammatory process in MS have been better reproduced in chronic relapsing EAE in mice and strain 13 guinea pigs by adoptive transfer technology and active immunisation, respectively (Lassmann *et al*, 1983b). A condition similar to EAE has also been described in humans treated with lyophilised brain tissue or as a complication of rabies vaccination (Lassmann, 1983a). Similarly to EAE in animals, these were monophasic diseases with histologically more pronounced perivenular inflammatory reaction, limited demyelination and mild gliosis. Although these findings suggest that EAE is not an accurate representation of the spontaneous human demyelinating disease, it has proven to be a good model of immune and inflammatory processes that may occur during lesion development in MS.

1.3.1. Immunological abnormalities and the role of immune system in the pathogenesis of multiple sclerosis.

The evidence linking immune system abnormalities with the pathogenesis of MS is based on genetic evidence and the histological demonstration of immune system cells and molecules in MS lesions and NAWM. T-cells recognise antigen in the context of the major histocompatibility (MHC) class I and II molecules on antigen-presenting cells (APC). Immunogenetic susceptibility to MS is frequently associated with a specific HLA-DR2 haplotype, found in about 60% of MS patients, and the rearrangement of restricted numbers of T-cell receptor (TCR) V α and V β genes in myelin basic protein (MBP)-specific T-cells (Wucherpfennig *et al*, 1990, Kotzin *et al*, 1991). Striking preferential usage of the TCR gene V β 8.2 is reported for MBP-reactive T-cell in Lewis rats but a similar degree of restriction was not observed among patients with MS (Chluba *et al*, 1989, Hafler *et al*, 1996). Systemic abnormalities of T-cell subsets and reactivity are found in peripheral blood and CSF of MS patients. The T-suppressor to T-helper cell ratio is reduced during relapse and in progressive MS and there is a higher incidence of myelin protein-reactive T-cells (Bach *et al*, 1980, Antel *et al*, 1986). Similarly, the increase in immune complexes in peripheral blood and elevated IgG levels and oligoclonal bands in CSF suggest involvement of the humoral immune system (Newcombe *et al*, 1985, Tourtellotte *et al*, 1988). However, autoantigen-specific T-cells and antibodies are frequently reported in normal control subjects, suggesting that inappropriate regulation of immune cells can lead to autoimmune disease.

The majority of cells in perivascular inflammatory infiltrates in MS and EAE are mononuclear cells and all lymphocyte subsets are represented in the perivascular space with a preponderance of CD4⁺ and CD8⁺ cells (Traugott *et al*, 1983, Woodroffe *et al*, 1986). Studies in mice with EAE showed that a subset of MHC class II-restricted CD4⁺ T-helper cells (Th1) are pivotal to disease manifestation through production of cytokines

interleukin (IL)-2, tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) which activate macrophages and promote inflammation (Renno *et al*, 1995). Although the functional T-cell dichotomy has not been demonstrated in humans, increased numbers of IL-2 receptor-positive T-cells and enrichment of the CD4 subset for memory CD45-RO⁺ cells were found in perivascular infiltrates (Woodroffe *et al*, 1986). In active lesions higher numbers of CD4 T-cells are present and with the decrease in inflammatory activity they are replaced by the cytotoxic/suppressor CD8 cells (Raine, 1991). Similarly $\gamma\delta$ T-cells accumulate in subacute and chronic lesions and appear to use a restricted number of V γ and V δ genes, suggesting a clonal expansion within lesions (Selmaj *et al*, 1991, Wucherpfennig *et al*, 1992).

Expression of the class I and II MHC antigens within normal rodent and human CNS is minimal and professional APCs are not found (Raine, 1994, Hart & Fabry, 1995). MHC class I molecules, which present antigen to cytotoxic CD8⁺ T-cells, are seen only on endothelium in normal control CNS tissue (Lampson & Hickey, 1986, Woodroffe *et al*, 1986) whereas low constitutive expression of class II MHC molecules is found on microglial cells in brain parenchyma (Traugott *et al*, 1983, Cuzner *et al*, 1988). In MS lesions immunoregulatory molecules are upregulated on both perivascular and parenchymal cells and numbers of MHC class II-positive macrophages and lymphocytes are present in the hypercellular border of active lesions and perivascular inflammatory infiltrates (Hauser *et al*, 1986, Woodroffe *et al*, 1986). Expression of MHC class II extends beyond the lesions into NAWM where positive activated microglia and hypertrophic astrocytes are found. MHC class I antigens are highly elevated on endothelial cells and infiltrating mononuclear cells (Cuzner *et al*, 1988, Hayashi *et al*, 1988). Recently expression of an alternative antigen presenting molecule CD1 has been reported in MS lesions (Battistini *et al*, 1996). The interesting feature of CD1 is presentation of lipid

antigens to a subset of T-cells which possess the natural killer cell marker NK1 (Porcelli *et al*, 1998). Prominent expression of the CD1b subtype was seen on perivascular cells and hypertrophic astrocytes in active MS lesions but not in normal-appearing or control white matter. Inflammatory cytokines are the major stimulators of MHC molecule expression in MS lesions as shown in astrocytes, microglia and endothelial cells stimulated *in vitro* with TNF- α and IFN- γ (Fontana *et al*, 1984, Male & Pryce, 1988a and 1988b).

The low affinity of TCR-MHC interaction is increased by accessory molecules such as LFA-1, CD2 and CD28 which provide a co-stimulatory signal for lymphocyte activation. The expression of B7-1 (CD80), a CD28 ligand, in β -islets of transgenic mice provoked autoimmune tissue destruction and diabetes (Harlan *et al*, 1994) whilst in chronic relapsing EAE inhibition of CD28/B7-1 binding prevented relapse and limited the scope of the autoimmune response (Miller *et al*, 1995). Upregulation of B7-1 and B7-2 (CD86) gene expression was found in active MS lesions and the proteins were immunolocalised on perivascular cells and macrophages (Windhagen *et al*, 1995). Hence, the presence of molecules involved in antigen presentation and T-cell activation in the perivascular space and MS lesion parenchyma suggests that stimulation of myelin-specific T-cells can be achieved locally in the CNS.

An increase in B-cell numbers and deposition of immunoglobulin (Ig) and complement were also described in MS lesions. B-cells are often found in perivascular infiltrates where they can form up to 40% of cells (Woodroffe *et al*, 1986). However, Ig-producing plasma cells are more frequent and have a wider distribution than B-cells especially in chronic lesions, being found in both perivascular space and lesion parenchyma (Esiri, 1977, Lassmann *et al*, 1994). Oligoclonal Ig bands and an increase in MBP-specific antibodies are found in CSF and can also be demonstrated in protein extracts from MS lesions (Martino *et al*, 1991).

Antibody-mediated tissue injury can be enhanced by activation of the complement cascade. The influx of complement components is facilitated by BBB damage but an increase in local synthesis in microglia and astrocytes also takes place concomitantly to the increase in expression of complement receptors on these cells (Barnum, 1995, Gasque *et al*, 1995). Deposits of C3 and the membrane attack complex are found in affected areas of the CNS co-localised with Ig (Gay & Esiri, 1991, Zajicek *et al*, 1992). These data emphasise that both cellular and humoral immune mechanisms are involved in generation of tissue damage in MS lesions.

1.3.2. Blood-brain barrier: an interface between the immune system and the CNS.

Anatomical properties and events at the BBB influence the immune privilege of the CNS and the characteristics of the inflammatory process within nervous tissue. The luminal side of the brain microvasculature is lined by specialised endothelial cells characterised by tight intercellular junctions and a lack of fenestration and vesicular transcytosis. The collagenous basement membrane and interspersed pericytes, contractile cells functionally similar to smooth muscle cells, represent the next layer whilst the abluminal side of the BBB contains specialised perivascular microglia and is ensheathed by astrocyte foot processes. Developmental studies have indicated that BBB features are acquired gradually during development and through contacts with nervous tissue cells (Janzer & Raff, 1987).

The BBB is impermeable for serum proteins, as demonstrated by use of the vascular tracer horse radish peroxidase (Reese & Karnovsky, 1967, Brightman, 1991) although, specialised transport systems have developed in order to meet brain metabolic requirements for glucose and other metabolites. Under basal conditions the high electrical resistance of $>2000 \Omega/\text{cm}^2$ and low adhesion surface of endothelium ensures a smooth passage of circulating blood cells. Such barrier properties and the lack of conventional

lymphatics in the brain were thought to form the basis of the CNS immune privilege. These views were challenged by demonstration of MHC molecules in normal CNS tissue and experiments which showed that activated T-cells can cross the intact BBB irrespective of their specificity (Wekerle *et al*, 1987, Hickey *et al*, 1991). It was subsequently observed that following the intracerebral injection of radio-labelled protein, a high percentage appeared in the cervical lymph nodes of injected animals thus indicating the existence of lymphatic drainage of interstitial fluid from the CNS (Knopf *et al*, 1995). The use of dye tracers showed that drainage pathways are comprised of perivascular (Virchow-Robin) spaces around brain arteries which interconnect with lymphatics in the nasal submucosa at the cribriform plate (Zhang *et al*, 1990, Kida *et al*, 1993). These studies have established the way in which CNS-specific antigens can activate autoimmune T-cells in regional lymph nodes enabling them to cross the BBB and initiate an autoimmune response in the CNS.

Inflammatory changes at the BBB are characterised by two related events, increased permeability for serum proteins and mononuclear cell infiltration of the perivascular space. In serial MRI studies, new clinical exacerbations in MS were shown to occur simultaneously with focal BBB damage (Kermode *et al*, 1990) and subsequent studies on biopsy and post-mortem material indicated that BBB damage was associated with brain inflammation (Estes *et al*, 1990, Nesbit *et al*, 1991). Increase in BBB permeability is detectable histochemically in MS lesions as heavy accretions of fibrinogen, complement proteins and low-density lipoproteins (LDL) on the abluminal side of the BBB and in perivascular parenchyma (Gay & Esiri, 1991, Newcombe *et al*, 1994). Cotran *et al*. (1965) have demonstrated that the vasoactive mediators histamine and serotonin induce leakage specifically from post-capillary venules which are frequently associated with early MS lesions. Inflammatory cytokines TNF- α , IL-1 and IL-6 may also have a direct effect

on the barrier permeability evident *in vitro* through a decline in transendothelial electrical resistance (DeVries *et al*, 1996). The underlying mechanisms of increased permeability involve enhanced transendothelial vesicular transport of proteins or an increase in molecular movement associated with mononuclear cell transmigration. In addition inflammatory cytokines expressed by endothelial and mononuclear cells induce upregulation of adhesion molecules and chemokines leading to establishment of an inflammatory BBB phenotype (Male *et al*, 1990).

Under physiological conditions the small numbers of lymphocytes crossing the BBB ensure a permanent immune surveillance of the CNS. In contrast during the neuroinflammatory reaction the number of cells in perivascular spaces increases sharply. Mononuclear cell infiltrates in MS and EAE consist predominantly of T-cells and monocytes suggesting that transmigration across the BBB is a selective process (Cuzner *et al*, 1988). The lymphocytes interact with endothelium in a stepwise fashion which takes place in postcapillary venules and involves several types of adhesion molecules (Albelda *et al*, 1994). The initial phase, rolling, is mediated by E and P selectins which interact with carbohydrate ligands on lymphocytes. The initial contacts are strengthened through binding of integrins on lymphocytes (LFA-1, $\alpha_L\beta_2$ integrin) and monocytes (Mac-1, $\alpha_M\beta_2$ integrin) to intercellular adhesion molecules (ICAM)-1 and -2 on endothelial cells. Further interactions ensue between very late antigen-4 (VLA-4, $\alpha_4\beta_1$ integrin) and vascular cell adhesion molecule-1 (VCAM-1) on lymphocyte and endothelium, respectively. The expression of adhesion molecules in normal brain microvasculature is low but temporally and spatially-regulated increase in expression occurs in active MS and EAE lesions (Washington *et al*, 1994, Brosnan *et al*, 1995) under the influence of the inflammatory cytokines TNF- α , IL-1 β and IFN- γ (Male *et al*, 1990, Wong & Dorovini, 1992). Upregulation of ICAM-1 and LFA-1 expression is an early event and was found in both

MS lesions and NAWM whilst VLA-4 and VCAM-1 expression occurs later in subacute lesions. Furthermore, VCAM-1 is expressed over the whole surface of the endothelial cell whereas ICAM-1 is concentrated at parajunctional areas. The effects of increased adhesion molecule expression on lymphocyte adherence were elegantly demonstrated in a frozen section assay in which isolated peripheral blood lymphocytes incubated on unfixed frozen sections attached specifically to blood vessels in active lesions but not normal white matter (Vora *et al*, 1995). Treatment of EAE animals with antibodies against adhesion molecules such as α 4-integrin and LFA-1 prevented development of EAE (Yednock *et al*, 1992, Kent *et al*, 1995), emphasising the functional significance of lymphocyte-endothelial interactions in the induction of CNS inflammation.

An additional degree of specificity for inflammatory cell transmigration is conveyed through the action of chemokines which attract and activate specific subsets of mononuclear cells (Ransohoff & Tani, 1998). Members of the β family of chemokines, monocyte chemoattractant peptide-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1) and RANTES are relatively specific for monocytes and T-cells which predominate in MS inflammatory infiltrates. As with adhesion molecules, chemokine expression is regulated by inflammatory cytokines and increases with the onset of CNS inflammation (Simpson *et al*, 1998, McManus *et al*, 1998). Furthermore, treatment of animals with anti-chemokine antibodies was shown to prevent development of EAE (Eng *et al*, 1996, Karpus *et al*, 1995). Hence, cells of the BBB play an active role in inflammatory responses in brain parenchyma through regulation of cell and protein entry from the peripheral blood into the CNS parenchyma.

1.3.3. Mechanisms of myelin and oligodendrocyte damage in MS.

Autoimmune responses in MS against yet unknown antigens specifically damage the myelin layer and myelin-producing cells, oligodendrocytes, resulting in impaired

axonal conduction. Myelin membranes are special extensions of oligodendrocyte plasma membranes which form an insulating layer around axons allowing rapid saltatory conduction of nerve impulses. Unusual characteristics of myelin are a tight lamellar structure and a high lipid content with approximately 70 - 75% lipid and 25 - 30% protein (Cuzner & Norton, 1996). Myelin basic (MBP) and proteolipid protein (PLP) are two major components responsible for myelin compaction and are frequently hypothesised to be autoantigens in MS. Other less abundant proteins which may be a target of autoimmune responses include the myelin-associated glycoprotein (MAG) and myelin-oligodendrocyte glycoprotein (MOG). Biochemical studies have revealed extensive loss of myelin proteins in MS lesions but also more widespread changes in NAWM (Newcombe *et al*, 1982). Selective loss of MAG is found in NAWM suggesting that there is an ongoing subtle demyelination throughout white matter which is focally potentiated by an inflammatory reaction (Itoyama *et al*, 1980, Moller *et al*, 1987).

Destruction of oligodendrocyte cell bodies is closely associated with inflammatory demyelination although it is not clear which process is primary. Ozawa *et al*. (1994) demonstrated that clinically different forms of MS show histologically distinct patterns of oligodendrocyte and myelin damage. Lesions in primary progressive MS were characterised by marked destruction of cellular elements and severe axonal pathology whereas those from a relapsing-remitting type exhibited predominantly demyelination with the preservation of tissue structures. Such findings illustrate the heterogeneity of mechanisms that cause oligodendrocyte and myelin damage. Earlier studies pointed to the role of humoral factors showing that MS serum can selectively damage the oligodendrocyte-myelin unit in CNS explant cultures (Diaz *et al*, 1978). A co-operative role for myelin protein-specific antibodies and complement have been deduced from transfer EAE in which animals were co-injected with MBP-specific T-cells and anti-MOG

antibody (Piddlesden *et al*, 1993). The ability of antibody to fix complement correlated with the demyelinating potential of such treatment. Similarly, injection of anti-MAG antibodies and membrane attack complex into rabbit sciatic nerve opens the pores in the myelin membrane that allow access to other inflammatory mediators and proteases causing demyelination (Willison *et al*, 1988, Monaco *et al*, 1995). MHC class I restricted cytotoxicity is controversial since oligodendrocytes do not express class I antigens *in vivo* although a non-MHC restricted mechanism incorporating direct binding of CD8 cytotoxic cells to glycoproteins on the oligodendrocyte surface, has been proposed (Jewtougoff *et al*, 1992). Cytotoxic activity towards oligodendrocytes is also a characteristic of T-cells expressing $\gamma\delta$ -TCR (Freedman *et al*, 1997), known to respond to heat shock proteins (HSP) by activation and production of the macrophage-activating cytokine IFN- γ (Follows *et al*, 1992). HSPs are molecular chaperones with highly conserved sequences across species (Brosnan *et al*, 1996). In the CNS of EAE rats, HSP-reactive T-cells are enriched and some may cross-react with the MBP-derived peptides implicating in part a molecular mimicry in initiation of immune response in EAE (Mor & Cohen, 1992). HSPs are expressed on several types of glial cell in MS lesions and co-localisation of $\gamma\delta$ T-cells and oligodendrocytes expressing HSP-65 was found in the borders of active lesions (Selmaj *et al*, 1991, Bajramovic *et al*, 1997). Demyelination mediated by CD4 T-cells, especially Th1 subset, is more indirect and includes release of inflammatory cytokines which are toxic to oligodendrocytes. Furthermore, Th1 cytokines orchestrate the progression of inflammatory process through recruitment and activation of macrophages.

1.3.4. Effector cells of inflammatory demyelination.

Microglia and macrophages are major effector cells during an inflammatory process in the CNS. They are represented by a heterogeneous cell population that include microglia, perivascular phagocytes, macrophages in the leptomeninges and blood-borne

macrophages (Cuzner, 1997, Bauer *et al*, 1996). The term microglia is reserved for the resident CNS macrophages present in both white and grey matter parenchyma although in higher numbers in the latter compartment (Lawson *et al*, 1990). The mesodermal origins of microglia are in line with other members of the mononuclear phagocyte system which all derive from bone marrow precursors, have a characteristic phenotype and high phagocytic activity (Hickey *et al*, 1992, Ling & Wong, 1993). Under basal conditions white matter microglia have a small elongated cell body and highly branched processes arranged along myelinated nerve fibres. The level of expression of macrophage cell markers prompted the view that resting microglia represent functionally downregulated macrophages (Perry & Gordon, 1988). Constitutive expression of MHC class II, CD4, Fc receptor and complement receptor 3 (CR3) integrin in resting microglia was described in normal brain white matter (Hayes *et al*, 1987, Perry & Gordon, 1987, Peress *et al*, 1993). Such morphological and phenotypic characteristics of microglia are influenced by the brain microenvironment as elegantly shown in studies of Sievers and colleagues (Sievers *et al*, 1994, Kloss *et al*, 1997). Cultured macrophages usually take on an ameboid shape and display a number of macrophage properties but when grown on astrocyte monolayers, the cells revert to the ramified phenotype of resting microglia.

The precise function of microglia in normal CNS remains to be elucidated although roles in neurotransmitter metabolism and extracellular fluid cleansing have been proposed (Thomas, 1992). Microglia respond rapidly to even subtle pathological changes in the CNS such as spreading depression (Bastuardo *et al*, 1993) implying a further role in tissue surveillance. Activation of microglia is a process that involves adoption of macrophage-like properties which renders cells fully immuno-competent. This process involves proliferation of microglia and gradual transformation from activated to activated/phagocytic cells (Streit *et al*, 1988). *In vitro* studies have identified a growing

number of microglial mitogens. Treatment of rodent microglia in culture with macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage-CSF (GM-CSF) or IL-3 strongly stimulates cell proliferation (Giulian & Ingeman, 1988, Frei *et al*, 1986). The crucial role for M-CSF has been further illustrated in an experimental model of facial nerve axotomy in the osteopetrosis mouse strain in which M-CSF expression is prevented due to a genetic mutation (Raivich *et al*, 1994). Microglial cells in the facial nucleus of osteopetrosis mice are fewer, display changed morphology and do not proliferate if challenged by facial nerve axotomy. The inflammatory cytokine IFN- γ and bacterial cell wall product lipopolysaccharide (LPS) prime microglial cells for activation and upregulate a number of immunologically relevant molecules. Increase in expression of MHC class II antigens and Fc receptors was achieved upon treatment with IFN- γ whereas co-stimulation with other cytokines antagonised or had no effects on IFN- γ -induced expression of these molecules (Woodroffe *et al*, 1989, Loughlin *et al*, 1993). The kinetics of the microglial response to IFN- γ were similar to peripheral blood monocytes and peritoneal macrophages. However, most of these studies have been performed on amoeboid microglia which display considerable differences to the resting ramified cell population. The experiments on microglial cells co-cultured on astrocyte monolayers confirmed the proliferation effects of CSFs. Surprisingly, of a number of inflammatory cytokines used, only IL-1 could induce proliferation of ramified microglia and its effects were indirect as shown by blocking with antibodies against the CSFs (Kloss *et al*, 1997). These findings suggest that other mechanisms besides increased local concentrations of cytokines are responsible for the high sensitivity and rapid activation of resting microglia *in situ*.

Macrophages represent a major cell type in parenchyma and perivascular inflammatory infiltrates in actively demyelinating MS lesions (Esiri & Reading, 1987, Cuzner *et al*, 1988, Li *et al*, 1993). Myelin-laden macrophages are also found in early MS

lesions which are characterised by no apparent demyelination and minimal perivascular inflammatory infiltration, implying a role for these cells in the initial phase of the demyelinating process (Li *et al*, 1996, Gay *et al*, 1997). Data showing that severity of EAE correlates with the number of macrophages infiltrating the CNS parenchyma but not with the number of T-cells strengthen such a view (Berger *et al*, 1997). Furthermore, depletion of macrophages in a rat EAE model suppresses clinical disease and histopathological changes in the CNS (Huitinga *et al*, 1990). During inflammatory CNS disease microglia and macrophages play primarily a role in myelin phagocytosis and debris clearance but also complement the array of inflammatory mediators produced by T-cells. Phagocytic myelin-laden macrophages are histochemically detectable at several stages in MS lesion development and are a good indicator of MS lesion activity (Li *et al*, 1993). Previous studies have shown that macrophages actively phagocytose myelin *in vitro* and that this is mediated to a large extent by Fc receptors (Goldenberg *et al*, 1989). However, the capacity of microglia for myelin uptake is significantly greater than that of other tissue macrophages and does not solely depend on myelin opsonisation by antibodies (Mosley & Cuzner, 1996). Activation of microglia and macrophages as the consequence of myelin phagocytosis is evident through increased respiratory burst activity and production of cytokines IL-1, IL-6 and TNF- α (Williams *et al*, 1994, Mosley & Cuzner, 1996).

Processing of CNS antigens in microglia and inducible expression of antigen presenting and co-stimulatory molecules indicate that these cells can function as APCs. IFN- γ induces expression of MHC class II on microglia both *in vitro* and when injected into rodent brain (Steiniger & van der Steen, 1988). Similarly, activated microglia increase the expression of molecules critical for effective antigen presentation, including LFA-1, ICAM-1 and B7-1 (Windhagen *et al*, 1995). A number of studies have demonstrated that microglial cells are effective APCs *in vitro* and can present myelin antigens in a MHC-

restricted manner (Woodroffe *et al*, 1989, Frei *et al*, 1994, Walker *et al*, 1995). However, there are some discrepancies on the antigen presenting role of microglia *in vivo* and whether this role is better fulfilled by perivascular and infiltrating macrophages (Hart & Fabry, 1995).

Activated macrophages and microglia release a number of cytotoxic mediators that may damage oligodendrocytes and myelin. Production and release of ROI and reactive nitrogen species is of importance as these mediators may oxidatively modulate a number of macromolecular systems (Merrill & Murphy, 1997). In active MS lesions macrophages release increased amounts of IL-1 which may further potentiate inflammatory reaction through activation of endogenous glial cells (Cannella & Raine, 1995). Similarly there is an increased production of TNF- α which is cytotoxic for oligodendrocytes (Selmaj & Raine, 1988) and IL-6 which modulates the humoral branch of the immune system enhancing the production of antibodies (Woodroffe & Cuzner, 1993). Recently a role for macrophage-derived IL-12 has been proposed in EAE as this cytokine was capable of inducing a relapse in Lewis rats. The recurrence of clinical and pathological signs of disease is considered to be a consequence of re-activation of residual inflammatory T-cells and macrophages in the CNS (Leonard *et al*, 1997), suggesting that IL-12 may similarly contribute to disease exacerbation in patients with MS.

Astrocytes are the most numerous glial cells and are implicated in the preservation of homeostasis in the CNS through their roles in glycogen metabolism, potassium buffering and maintenance of the BBB (Aschner, 1998). The major result of astrocyte activation in neuropathological conditions is formation of scar tissue. The astrocyte reaction in variety of neuropathological conditions is stereotypical and incorporates astrogliosis and subsequent astrogliosis which can be followed histochemically by upregulation of the astrocyte-specific cytoskeletal proteins, glial fibrillary acidic protein

(GFAP) and vimentin (Bignami & Dahl, 1974). The reactivity of astrocytes resembles that of microglia and substantial astrocytosis is detected in early stages of demyelination (Kraig *et al*, 1991). Hypertrophic astrocytes produce a number of inflammatory mediators and growth factors and they are the CNS source of CSFs, potent microglial mitogens (Aschner, 1998). Astrocytes are also capable of phagocytosis, although on a much lower scale in comparison with macrophages, and can present CNS antigens to immune cells (Fontana *et al*, 1984).

These data indicate that both macrophages and astrocytes can directly contribute to the progression of the inflammatory process in the CNS and consequently to myelin damage. However, at the same time both cell types are capable of producing anti-inflammatory mediators and growth factors which may dampen down the inflammation and support repair in the CNS (Diemel *et al*, 1998). Glial and inflammatory cells specifically respond to the type of damage in the CNS. This has been elegantly demonstrated in the model of facial nerve axotomy in which activated microglia produce growth factors that stimulate neuronal survival whereas occurrence of neuronal apoptosis in the facial nucleus prompts microglia to become phagocytic and release inflammatory mediators (Graeber *et al*, 1998). The exact nature of the insult which leads to recruitment of inflammatory cells and demyelination is not clear although autoimmunity is probably a major element underlying the pathogenesis of MS. Thus, further investigations are needed to elucidate the components of the autoimmune and inflammatory response in the CNS in order to prevent loss of oligodendrocytes and myelin.

1.4. Nuclear factor kappaB: transcriptional regulator of inflammatory mediators in macrophages.

Transcription of eukariotic genes is regulated via the interaction of specific DNA sequences known as cis-factors with DNA-binding proteins described as trans-factors (Hill & Treisman, 1995). In addition to core promoter elements that engage basic transcriptional machinery, binding of transcription factors to specific parts of the DNA sequence (cis) in the promoter, enhancer or silencer regions confers a degree of specificity to gene regulation. As transcription factors provide a means of modulation of gene expression by up- or downregulating transcription of a target gene they are divided into those with activating or repressing potential. Primary transcription factors, which are stored in cells in an inactive form, respond to extracellular signals directly and can be considered as an end-point of signal transduction pathways, spanning the distance between cell surface and the nucleus. In contrast, following the initial stimulus secondary transcription factors require protein synthesis for their activation, making them slow-reacting.

Nuclear factor κ B (NF- κ B) was initially described as the transcriptional regulator of the kappa light chain in B lymphocytes and was subsequently found to be involved in the regulation of many other genes, particularly those involved in inflammatory processes (Baeuerle & Henkel, 1994). NF- κ B is a ubiquitously expressed primary transcription factor which exists in the cytoplasm in a complex with inhibitory I κ B proteins. NF- κ B is activated by many diverse exogenous and endogenous pathogenic stimuli and on activation leads to a rapid induction of genes encoding defence and signalling proteins. Due to such characteristics it is particularly utilised by the cells of the innate and acquired immune system such as macrophages and lymphocytes which have to respond promptly to a variety of pathogenic challenges in their environment.

1.4.1. *NF- κ B family: structure-function relationship of NF- κ B subunits.*

The NF- κ B/Rel transcription factor family consist of several protein subunits which share structural and functional characteristics. There are five mammalian proteins described to date: RelA (p65), c-Rel (p75), RelB, p50 and p52 of which the latter two are synthesised as the larger molecular weight precursors p105 and p100 (May & Ghosh, 1997). All subunits share a Rel homology domain located in their C-termini and are characterised by sequence homology and capability to bind κ B motifs on DNA molecules (Urban & Baeuerle, 1991, Schmitz & Baeuerle, 1991). Additional features are the nuclear location signal (NLS), a lysine-rich charged region of the molecule immediately C-terminal to the Rel domain which is responsible for interaction with nuclear receptors and mediates nuclear translocation of the activated NF- κ B complex (Beg *et al*, 1992). The C-terminal contains transcription activation domains which are variably expressed in NF- κ B subunits and are not present in p50 and p52. NF- κ B subunits form homo- or heterodimers and all combinations have been described with the exception of RelB homodimers. The most abundant form of NF- κ B is a RelA-p50 heterodimer which was first described and therefore designated as the NF- κ B prototype (Urban & Baeuerle, 1991). Due to the lack of transcription activation domains, the p50 and p52 subunits are considered to be transcriptionally inactive and consequently homodimers of p50 are inhibitory and can terminate NF- κ B activity by displacing transcriptionally active dimers from κ B sites (Schmitz & Baeuerle, 1991, Franzoso *et al*, 1992). It is thought that a role for p50 in heterodimers would be to provide a stable link to a DNA κ B sequence with the NF- κ B.

The specific cis-element on DNA that binds NF- κ B is designated as the κ B site and is the decameric consensus sequence 5'-GGGPuNNPyPyCC-3'. This element was first described in the intronic κ -light chain enhancer (Sen & Baltimore, 1986). An interesting characteristic of κ B sequence is the slight asymmetry and only a small proportion of

palindromic sites exist. Numbers of κ B sites vary from gene to gene and the fine differences in sequence show preferences for particular combinations of NF- κ B subunits. A crystal structure of the p50 homodimer bound to a κ B site revealed that subunits in the dimer contact the DNA through residues in their N-termini whilst C-termini form a dimerisation surface (Ghosh *et al*, 1995). Using the recombinant p50 homodimer and a random pool of κ B oligonucleotides Kunsch *et al*. (1992) showed preferential binding of p50 to a highly symmetrical consensus sequence whereas RelA homodimers bound markedly different and more asymmetric oligonucleotides.

1.4.2. I κ B proteins: endogenous regulators of NF- κ B activity.

NF- κ B activity is regulated through interactions with inhibitory I κ B proteins by two basic mechanisms: retention of NF- κ B in the cytoplasm and inhibition of DNA binding (Whiteside & Israel, 1997b). A common characteristic of I κ Bs is the presence of five to seven ankyrin repeats in their C-termini which mask the NLS of NF- κ B subunits retaining the NF- κ B complexes in the cytoplasm (Beg *et al*, 1992, Ganchi *et al*, 1992). Additional functionally important features are the PEST (single letter amino acid code) domain, found in proteins with a rapid turnover, and a nuclear export signal (NES) which plays a role in the active transport of NF- κ B out of the nucleus (Nigg, 1997). The I κ B α (37kD) protein is the major species which preferentially interacts with transcriptionally active subunits RelA and c-Rel (Baeuerle & Baltimore, 1989). Additional proteins include I κ B β and I κ B ϵ which also preferentially inhibit NF- κ B complexes containing RelA and c-Rel (Link *et al*, 1992, Whiteside *et al*, 1997a). Due to the presence of ankyrin repeats in their C-termini, the p50 and p52 precursors are functionally I κ B proteins (Rice & Ernst, 1993, Naumann *et al*, 1993). The proto-oncogene Bcl-3 is a nuclear I κ B protein which forms transcriptionally active complexes with p50 and p52 homodimers (Franzoso *et al*, 1992).

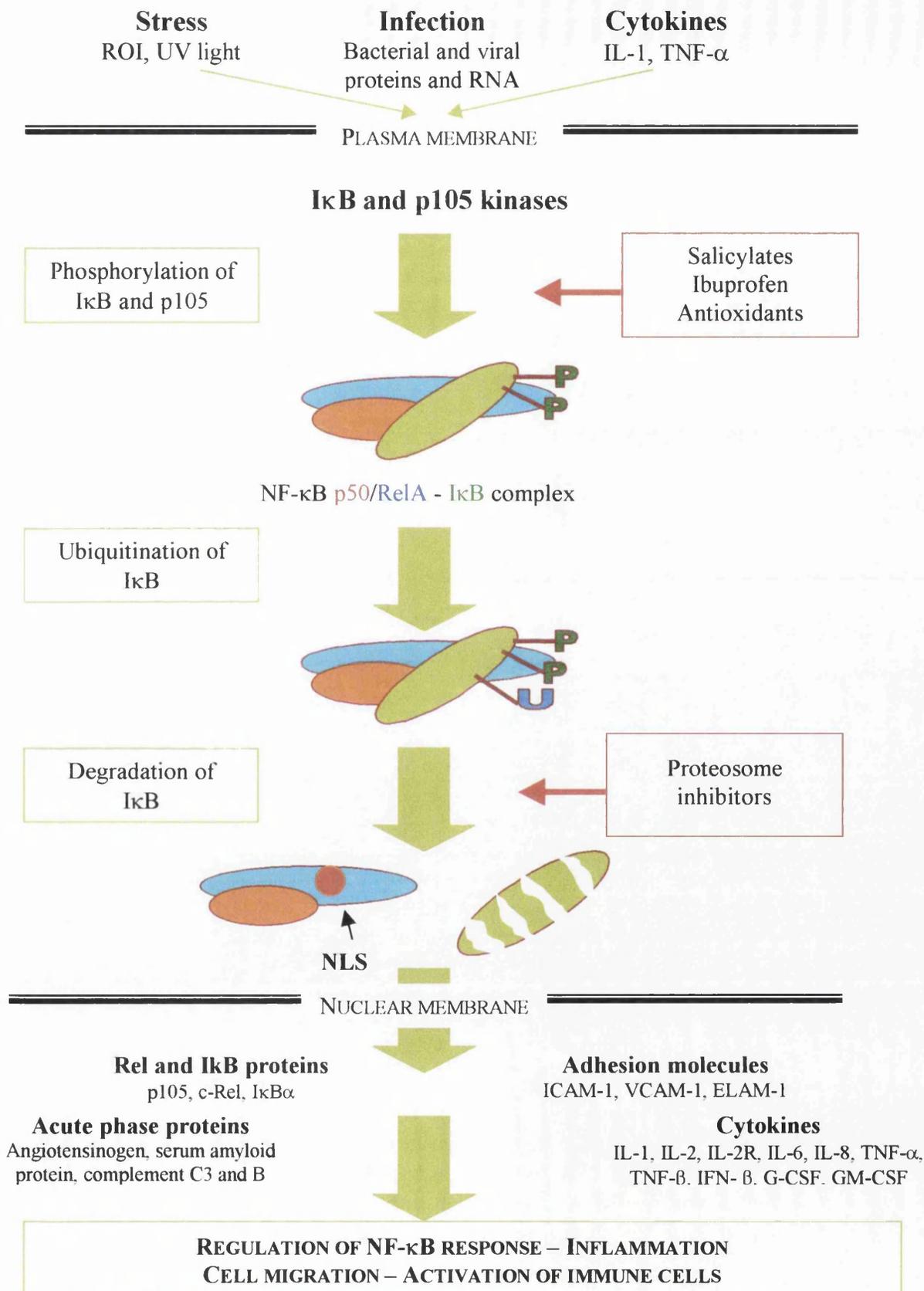


Figure 1.4.1: A schematic representation of proposed mechanisms for induction of NF- κ B transcription factors in eukaryotic cells. Inhibitors are highlighted in red. Adapted from Ghosh *et al*, 1998.

1.4.3. Mechanisms of NF- κ B activation.

The activation of NF- κ B is a rapid process comprising the phosphorylation of key residues in both I κ B and NF- κ B subunits. Phosphorylation of the I κ B on serine residues triggers poly-ubiquitination of the I κ B molecule and targets it for rapid degradation by the 26S proteasome which reveals the NLS of NF- κ B (May & Ghosh, 1998). The specific kinase responsible for I κ B phosphorylation has been purified and is a macromolecular complex composed of several serine kinase subunits (Didonato *et al*, 1997, Rothwarf *et al*, 1998). The kinase activity is largely increased on treatment of HeLa cells with IL-1 or TNF- α , well-characterised activators of NF- κ B (Regnier *et al*, 1997, Didonato *et al*, 1997). Proteolytic removal of I κ B allows the activated NF- κ B complex to translocate into the nucleus and bind specific κ B sequence in target genes. A variety of naturally occurring and synthetic activators of NF- κ B have been described, the most potent being pro-inflammatory cytokines and ROI which stimulate NF- κ B with similar kinetics (Schreck *et al*, 1991, McKean *et al*, 1995). In cultured cells nuclear localisation of NF- κ B occurs within 10 minutes of application of stimulus and is preceded by rapid degradation of I κ B (**Fig. 1.4.1**). Activation of NF- κ B is closely followed by upregulation of I κ B α , a NF- κ B dependent gene, and its redistribution into the nucleus (Zabel *et al*, 1993). In this way an autoregulatory loop is established in which nuclear I κ B α binds active NF- κ B complexes, rapidly terminating the transcriptional activity, following which the whole complex is exported into the cytoplasm. However, some stimuli such as LPS result in prolonged activation of the NF- κ B which escapes the feedback inhibition by the I κ B α (Thompson *et al*, 1995). This phenomenon can occur due to a sustained I κ B α degradation or protection of NF- κ B by I κ B β (Miyamoto *et al*, 1994, Suyang *et al*, 1996). The newly synthesised and hypophosphorylated I κ B β forms stable complexes with NF- κ B but it does not mask NLS

or interfere with the DNA-binding capacity of the NF- κ B. Therefore, the kinetics of NF- κ B activation allows signals generated in the extracellular space to be rapidly translated into the cell response through upregulation of stress and defence genes.

1.4.4. NF- κ B is a transcriptional regulator of macrophage and T-cell responses to environmental stimuli.

The cells of the innate and acquired immune system, i.e. macrophages and lymphocytes, rapidly respond to pathological stimuli by expression of immunomodulatory and defence genes. In each of these different genes, NF- κ B is a common transcriptional activator which facilitates their co-ordinated expression during inflammation and stress.

Macrophages are versatile cells which form a first line of defence to noxious stimuli from the environment. All aspects of macrophage activation: proliferation, phagocytosis and antigen presentation, production of pro-inflammatory mediators and growth factors are regulated by the NF- κ B-dependent genes. Macrophage growth factors GM-CSF and M-CSF, which play an important role in proliferation of microglia and macrophages, are NF- κ B dependent genes (Grigoriadis *et al*, 1996). NF- κ B is also involved in transcriptional regulation of the pro-inflammatory cytokines TNF- α , IL-1 and IL-6 and chemotactic factors MCP-1 and IL-8 (Collart *et al*, 1990, Antal *et al*, 1996, Nicholson *et al*, 1996). In addition genes for the proteolytic enzymes u-PA and MMP-9 which are involved in migration and extracellular proteolysis are NF- κ B controlled (Reuning *et al*, 1995, Bond *et al*, 1998). In addition to the inducible form of NF- κ B, macrophages also have constitutively active nuclear NF- κ B which can be responsible for basal expression of some genes or may facilitate macrophage activation in response to pathological conditions (Collart *et al*, 1990).

The common features of all Rel protein knock-out animals are degenerative changes in lymphoid organs and an inability of these animals to mount full scale immune

responses, thus indicating a strong association between NF- κ B and immune cell function (Attar *et al*, 1997). In T-cells antigenic stimuli and protocols which utilise specific antibodies against the co-stimulatory molecules CD2, CD28 and CD3 induce activation of NF- κ B (Baeuerle & Henkel, 1994). Furthermore, cytokine receptor-induced signals synergise with TCR signals to activate NF- κ B-dependent gene transcription (McKean *et al*, 1995). The full activation of NF- κ B in T-cells requires both signals which induce calcium mobilisation and those which activate protein kinase C (Tong *et al*, 1989). In T-cells NF- κ B participates in transcriptional activation of the T-cell growth factor IL-2 and the α -chain of the high affinity IL-2 receptor. There is some indication that NF- κ B can be differentially regulated in Th1 and Th2 cells (Lederer *et al*, 1996). Antigen stimulation of Th1 cells induces rapid translocation of RelA/p50 heterodimers into the nucleus which coincides with the upregulation of IL-2 production. In contrast, TCR signalling in Th2 cells exhibits a defect in NF- κ B activation which correlates with the failure to induce IL-2 gene expression in this cell type. It can be speculated that in the context of an inflammatory cell infiltrate in the brain perivascular space antigen stimulation may selectively activate NF- κ B in the Th1 subtype leading to increased production of pro-inflammatory mediators and stimulation of cellular immunity.

1.4.5. Apoptosis and the NF- κ B.

Apoptosis is the type of cell death in which cells are removed in an orderly and selective fashion without leaking the cell contents into the surrounding environment (Fraser & Evan, 1996). This process is particularly important during development and also occurs during inflammatory processes. A number of mediators which cause cells to undergo apoptosis also activate NF- κ B. It has been found that depending on the cell type the activation of NF- κ B can counteract apoptotic changes or be a part of that process. A

potent NF- κ B activator TNF- α induces apoptosis in a number of cells types including oligodendrocytes (Selmaj & Raine, 1988). Using the RelA knockout mice Beg and Baltimore (Beg & Baltimore, 1996) demonstrated that macrophages and fibroblasts from these mice, treated with TNF- α , undergo apoptosis shortly after exposure to this cytokine. Similarly transfection of cells with the mutated form of I κ B α which is resistant to proteolysis resulted in permanent inhibition of NF- κ B and higher susceptibility to apoptosis, whereas transfection of RelA into these cells reconstituted cell resistance to TNF- α -induced apoptosis (van Antwerp *et al*, 1996, Wang *et al*, 1996). The role of NF- κ B activation in response to pro-apoptotic stimuli would be to avoid unnecessary cell death and to ensure that only damaged cells, for example those infected by a virus, are removed from the organism. Interestingly, some viruses produce I κ B-like molecules which inactivate NF- κ B rendering infected cells more susceptible to cytotoxic stimuli.

The anti-apoptotic role of NF- κ B in the CNS has been challenged. In contrast to other tissues, NF- κ B activation in neuronal cells occurs simultaneously with the onset of apoptotic changes (Grilli *et al*, 1996). Several neurotoxins including glutamate and β -amyloid peptide are associated with NF- κ B activation and neuronal cell death (Kaltschmidt *et al*, 1995a and 1997). Apoptosis is one of the mechanisms by which the depletion of oligodendrocytes is brought about in MS lesions (Ozawa *et al*, 1994). The oligodendrocytes in MS lesions express Fas (CD95), a receptor for an apoptosis promoting member of the TNF family, on their surface and when challenged by TNF- α *in vitro* undergo apoptosis (Selmaj & Raine, 1988, Raine *et al*, 1998). Although NF- κ B is the ubiquitously expressed transcription factor its role in oligodendrocytes is presently unknown. Interestingly in Schwann cells, the myelinating cells of the PNS, treatment with nerve growth factor induced activation of NF- κ B (Carter *et al*, 1996) which resulted in

increased production of ECM and enhanced mobility. Therefore, in oligodendrocytes association of NF- κ B with both anti-apoptotic effects and cell activation may be envisaged.

1.4.6. The activation of NF- κ B in autoimmune and inflammatory conditions.

NF- κ B may play a role in the pathogenesis of chronic inflammatory diseases as well as in acute inflammatory reactions. The activation of NF- κ B amplifies the inflammatory response through rapid increase in expression of genes for pro-inflammatory cytokines, chemokines, adhesion molecules and proteolytic enzymes in effector cells. The connection is further strengthened by the fact that a number of immunosuppressive and anti-inflammatory drugs including salicylates, ibuprofen and glucocorticoids mediate their effects through inhibition of NF- κ B (Kopp & Ghosh, 1994, Scheinman *et al*, 1995, Scheuren *et al*, 1998). Activation of NF- κ B has been observed in macrophages in rheumatoid arthritis, chronic intestinal disease and atherosclerosis (Handel *et al*, 1995, Brand *et al*, 1997, Neurath & Pettersson, 1997). Increased phagocytosis of lipids and transformation of macrophages into foam cells are common characteristics of both atherosclerotic and MS lesions. The accumulation of oxidised LDL, a major component of early atheromas, leads to formation of foamy macrophages and the activation of NF- κ B in this cell type. This can be extrapolated to the pathogenesis of MS as early lesions are characterised by myelin- and oxidised LDL-containing macrophages surrounded by apparently intact myelin (Li *et al*, 1993, Newcombe *et al*, 1994). Phagocytosis of myelin in these lesions could activate NF- κ B in macrophages resulting in upregulation of NF- κ B-controlled adhesion molecules, cytokines and chemokines which could attract inflammatory cells to the site leading to further amplification of the inflammatory process and the onset of overt demyelination.

In the CNS constitutively active NF- κ B is found in some neuronal cells whereas the inducible form is found in synapses where it may be part of a novel synapse to nucleus signalling system (Kaltschmidt *et al*, 1993 and 1994b). The NF- κ B activity in neurones can also be induced *in vivo* by glutamate and may play a role in neuroprotection. On the other hand, microglia and astrocytes predominantly contain the inducible form of NF- κ B (Diehl *et al*, 1995, Bonaiuto *et al*, 1997). *In vitro* studies have demonstrated that NF- κ B in brain cells is activated by similar mediators and with similar kinetics to those in the cells in other tissues. Enhancement of immunoreactivity for NF- κ B in the cortex has been observed in animal models of ischemic disease, experimental traumatic brain injury and kainate-induced neurotoxicity (Salminen *et al*, 1995, Yang *et al*, 1995). The effect of injury was enhanced in adrenalectomised animals suggesting that glucocorticoids play a role in the negative regulation of NF- κ B activity in the CNS (Scheinman *et al*, 1995). In EAE, the activation of NF- κ B in the CNS was found to correlate with disease progression and the activated form was detected in macrophage and astrocyte nuclei at the peak of clinical disease (Kaltschmidt *et al*, 1994a). However, there was a lack of NF- κ B activation in perivascular inflammatory cells, in particular lymphocytes, which was attributed to intrinsic CNS mechanisms (Irani *et al*, 1997).

In human neuropathological conditions NF- κ B activation occurs in the vicinity of lesions. In Alzheimer's disease NF- κ B was immunolocalised in nuclei of neuronal cells and astrocytes in early senile plaques (Terai *et al*, 1996b, Kaltschmidt *et al*, 1997). Similar findings were reported for brain infarcts in which NF- κ B was predominantly found in reactive astroglia in the penumbra whilst NF- κ B-positive macrophages were rare (Terai *et al*, 1996a). In contrast, in brains from children with HIV-1 encephalitis there was an increase in the number of microglia and macrophages with nuclear immunoreactivity for

NF- κ B (Dollard *et al*, 1995). These data suggest that the nature of neuropathological conditions influences the activation of NF- κ B in a particular cell type, i.e. astrocytes and neurones in neurodegenerative diseases and microglia and macrophages in diseases with autoimmune and inflammatory background. However, these data do not provide the clues as to whether NF- κ B activation is a cause or only a consequence of a pathological process. Recently a naturally occurring defect in NF- κ B activation has been described in the human autosomal recessive disease ataxia telangiectasia (AT), caused by genetic mutation of the AT gene encoding kinase homologous to PI3 kinase, which is characterised by neurological, immunological and radiobiological deficiencies. Fibroblasts from AT patients are extremely sensitive to ionising radiation and exhibit constitutive NF- κ B activation (Jung *et al*, 1995). Interestingly AT patients and I κ B α -knockout mice share some similarities in phenotype, notably a small thymus and spleen indicating that a defect in I κ B regulation may be a contributing factor in disease pathogenesis which acts downstream of the AT kinase representing the primary defect.

NF- κ B is therefore a candidate for regulating the expression of cytokine and adhesion molecule genes in immune and glial cells during neurodegenerative and inflammatory conditions in the CNS. In that context, the upstream elements of the NF- κ B activation cascade, I κ B and I κ B kinase, may be potential targets for therapeutic manipulation of CNS disease.

1.5. Matrix metalloproteases: mediators of demyelination in MS.

1.5.1. The proteolytic profile of MS lesions.

Proteolytic degradation of extracellular proteins is an essential component of physiological processes in the CNS, including cell migration, axonal sprouting and tissue remodelling and repair. Uncontrolled excessive proteolytic activity, on the other hand, may play a role in a number of inflammatory and neurodegenerative conditions. Proteases implicated in these conditions are derived from both glia and mononuclear cells and are classified according to their active site, preferred substrate and optimum pH into four enzyme families – serine, aspartic, cysteine and metalloproteases (**Table 1.5.1**). In both human and animal demyelinating diseases proteases are part of a non-specific mechanisms responsible for myelin and BBB damage. Several proteases were found to be associated with myelin membranes forming an intrinsic enzyme system which is primarily involved in myelin turnover (Cuzner & Norton, 1996). However, a myelin-associated proteinase, calpain, can be rapidly activated catalysing proteolysis of MBP and MAG (Shields *et al*, 1998) suggesting that a dormant autodigestive potential of myelin may be initiated in demyelinating disease.

Proteolytic damage and degradation of myelin during inflammatory demyelination is predominantly mediated by neutral and acid proteases produced by mononuclear cells and microglia (Cuzner *et al*, 1975). Ultrastructural studies of EAE lesions showed macrophages closely apposed to myelin membranes with their processes intercalating with myelin layers, and displacement of lamellae towards coated pits of macrophages (Epstein *et al*, 1983). The early changes of myelin in MS and EAE lesions such as vesiculation and swelling that may be a consequence of proteolytic attack were identified in contact areas with phagocytes (Prineas, 1975). The intrinsic stability of myelin membranes resists proteolytic attack but the opsonisation by antibody or complement facilitates macrophage

engagement and phagocytosis and may also initiate a signalling cascade leading to secretion of proteases (Mosley & Cuzner, 1996). Furthermore, the terminal complement complex was found to form pores in myelin which may facilitate the influx of extracellular proteases (Monaco *et al*, 1995). The initiation of proteolysis is primarily due to the neutral proteinase activity which is complemented by lysosomal acid endopeptidases of macrophages and astrocytes (Cuzner & Opdenakker, 1999). In the case of frustrated phagocytosis, macrophages discharge lysosomal enzymes into the extracellular space followed by secretion of lactic acid and acidification which forms optimal conditions for acid protease activity. These local pockets of acidification with limited access for protease inhibitors may lead to disruption of myelin membranes and demyelination.

Protease family	Active site	pH optimum	Members*
Aspartic proteases	Asp-Asp	pH 2-5 (acidic) lysosomal	Cathepsin D
Cysteine proteases	Cys-His	pH 3-6 (acidic) ?	Cathepsins S, L, B and H
Serine proteases	Asp-His-Ser	pH 7-9 (neutral) secreted	Cathepsin G u-PA and t-PA
Metallo-proteases	Zn ²⁺ atom in active site	pH 7-9 (neutral) secreted	MMP-1 – 19

Table 1.5.1: Proteolytic enzymes of mononuclear cells classified according to the biochemical characteristics of their active site and pH optimum. *Only members involved in MS and EAE pathogenesis are shown. Compiled from Owen & Campbell, 1999, and Cuzner & Opdenakker, 1999.

Earlier studies on actively demyelinating MS plaques have found an increase in a wide spectrum of proteases, including cathepsin A and D, neutral proteinase, acid phosphatase, β -glucuronidase and carboxypeptidase A and B (Cuzner *et al*, 1975, Hirsch, 1981). In chronic plaques the predominant activity of acid phosphatase and acid proteinase

has been described. Activities of proteolytic enzymes studied in rats and primates with acute EAE have indicated elevation of cathepsin D, cathepsin A and neutral proteinase and corresponded with lymphocytic infiltration of lesions. Higher acid protease activity predominantly reflects cell activation in demyelinating lesions and excluding frustrated phagocytosis, these proteases remain confined to the lysosomal compartment. In contrast, the neutral pH of the extracellular fluid represents the optimal conditions for secreted neutral proteases, plasminogen activators (PAs) and metalloproteases (MMPs), which extensively interact in a cascade-like fashion.

1.5.2. Structure and function of MMPs.

MMPs are a distinct family of zinc-dependent enzymes with a primary role in remodelling connective tissue during development and in inflammatory and reparative processes (Cuzner & Opdenakker, 1999). All MMPs share a common basic structure with propeptide and N-terminal catalytic domains which both contain zinc-binding amino acid residues (Massova *et al*, 1998). The conserved amino acid sequence (PRCGXPD) in the propeptide which contains the Zn²⁺-binding cysteine is responsible for blocking of the catalytic zinc atom in the active site of the zymogen form. The catalytic domain contains two Zn²⁺ atoms, structural and catalytic, of which the latter is necessary for enzyme activity and is co-ordinated with three histidine residues found in all MMPs (Lovejoy *et al*, 1994). This simple two domain structure is found only in matrilysin (MMP-7) (**Fig. 1.5.1**) (Browner *et al*, 1995). The rest of the MMP family evolved into different subtypes by incorporating additional structural domains. The hemopexin-like domain is the most conserved among MMPs and plays a role in substrate binding and interaction with tissue inhibitors of MMPs (TIMP) (Willenbrock *et al*, 1993, Gomis-Ruth *et al*, 1997). Membrane-type MMPs (MT-MMPs, MMP-14 to 17) have in addition a C-terminal transmembrane domain and furin recognition motif at the end of a propeptide (Cao *et al*,

1995, Sato *et al*, 1996). Gelatinases A (MMP-2) and B (MMP-9) have the most complex structure of all MMPs with two specialised substrate-binding domains, fibronectin-like repeats located within the catalytic domain which bind to denatured collagen and the proline-rich V-collagen-like domain found in MMP-9 which may function as a spacer between catalytic and hemopexin domains (Murphy *et al*, 1994).

Multiple control mechanisms of MMP activity have evolved in the organism due to the potentially deleterious consequences of excessive proteolysis. Cytokines, growth factors, cell surface and ECM molecules regulate MMP synthesis at the level of transcription (Woessner, 1991). MMP genes contain binding sites for AP1 and NF- κ B transcription factors which transmit signals initiated by the proinflammatory cytokines IL-1 β and TNF- α , potent stimulators of MMP synthesis (Bond *et al*, 1998). Production of MMPs is also stimulated by several growth factors whereas in macrophages cell-cell or cell-ECM binding represents an additional pathway for MMP upregulation (Shapiro *et al*, 1993, Lacraz *et al*, 1994b, Hosono *et al*, 1996). Anti-inflammatory mediators glucocorticoids, TGF- β and IFN- β generally downregulate MMP production whilst IL-10 and IL-4 effects appear to be cell-type specific (Wahl & Corcoran, 1993, Mertz *et al*, 1994). Post-translational modifications such as glycosylation may modify tissue localisation and substrate specificity of MMPs and interfere with inhibitor activity (van den Steen *et al*, 1998). Differences exist in the rate of MMP production, for example MMP-2 is constitutively produced by fibroblasts at low levels whereas MMP-9 is inducible by inflammatory mediators. Neutrophils store MMP-9 in granules which can be rapidly mobilised in response to IL-8 and other chemokines (Kjeldsen *et al*, 1992). The majority of MMPs, with the exception of MT-MMPs and stromelysin-3 (MMP-11), are secreted as zymogens which are proteolytically activated in the pericellular space. The cysteine switch mechanism is a stepwise process of MMP activation in which an intermediate active form

is generated by disrupting the connection of a propeptide cysteine with the catalytic Zn^{2+} followed by proteolytic removal of the entire propeptide (van Wart & Birkedal-Hansen, 1990). The first step is mediated by plasmin and other trypsin-like proteases and *in vitro* can be imitated by organomercurials, SDS and chaotropic agents. After exposure of the active site, autocatalytic cleavage results in elimination of the propeptide whereas further processing by exogenous MMPs may generate an enzyme with higher catalytic activity (Suzuki *et al*, 1990). MT-MMPs are activated intracellularly by the serine-protease furin and exported to the cell surface where they take part in activation of other members of the MMP family (Strongin *et al*, 1995, Sato *et al*, 1996). Sequential activation of MMPs which involves multiple types of enzymes suggests the existence of an enzymatic cascade with several rate-limiting steps.

The MMP activity in the extracellular space is principally regulated by endogenous TIMPs which form high affinity 1:1 non-covalent complexes with active enzymes (Murphy & Willenbrock, 1995). There are four known species of TIMP (TIMP-1 to 4) described to date which share a common structure represented by 12 conserved cysteine residues and characteristic tertiary folding essential for inhibitory activity (Willenbrock & Murphy, 1994). TIMPs have two functional domains, N- and C-terminal, which both take part in TIMP interaction with the catalytic and hemopexin-like domains of MMPs. They are not fully interchangeable in their interactions with pro-MMPs or as inhibitors of the active MMPs. TIMP-1 specifically binds to pro-MMP-9, regulating the rate of proenzyme conversion, whereas TIMP-2, 3 and 4 form complexes with the pro-MMP-2 (Olson *et al*, 1997). TIMP-1 is also a poor inhibitor of the MT-MMPs compared to the other family members. The potential of TIMPs for dual function is well illustrated by effects of TIMP-2 which at low concentrations promotes formation of pro-MMP-2/MMP-14 complexes on the cell surface and activation of MMP-2, whilst higher concentration are inhibitory

(Shofuda *et al*, 1998). TIMP-3, on the other hand, displays several characteristics that set it apart from the other TIMPs, notably its association with ECM and apoptosis-promoting effects on many cell types (Vranka *et al*, 1997, Baker *et al*, 1998). The expression of inducible TIMP-1 and 3 is a part of the autoregulatory loop and their synthesis is enhanced by anti-inflammatory mediators such as glucocorticoids and IL-10 (Lacraz *et al*, 1995, Rosenberg *et al*, 1996). Plasmin and related enzymes decrease TIMP concentrations in the extracellular space by proteolytic degradation, thus influencing the formation of a molecular excess of MMPs over TIMPs. Therefore, extracellular activity of MMPs is modulated by multiple factors including concentration of specific TIMPs and upstream proteolytic enzymes as well as by associations with ECM or cell surface molecules.

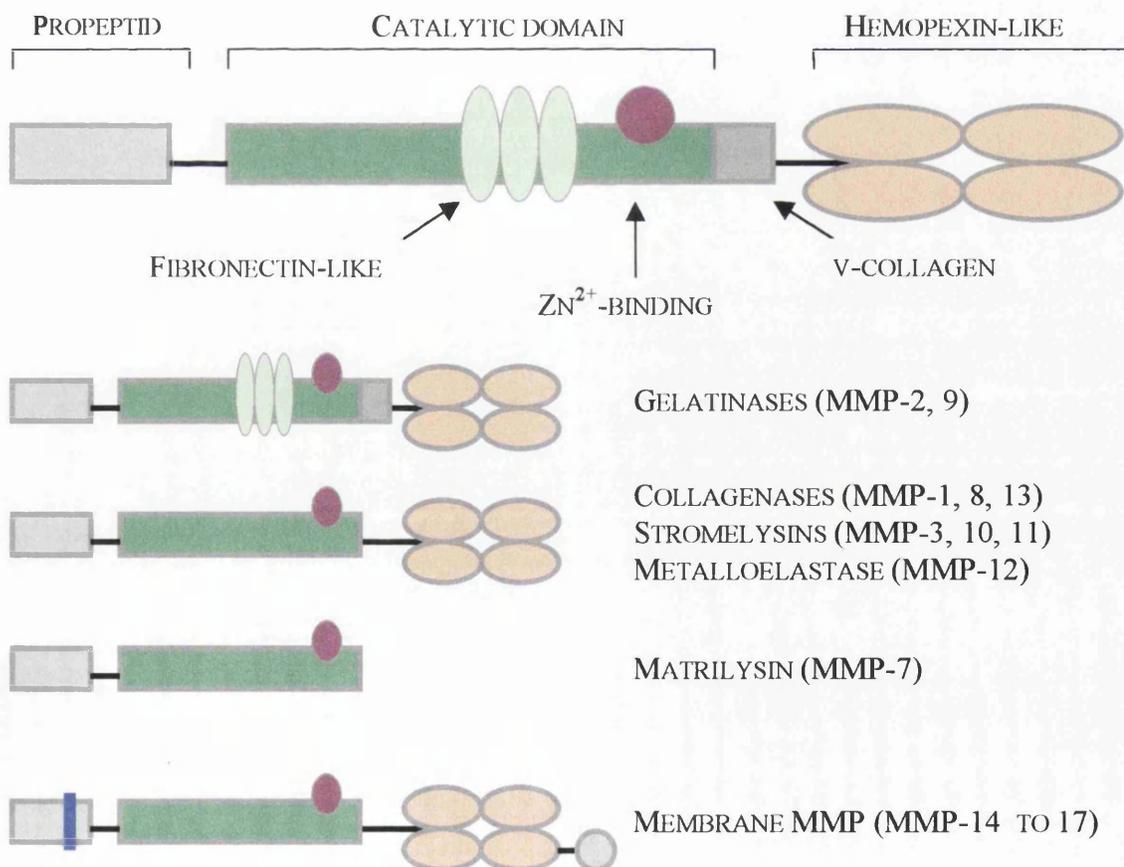


Figure 1.5.1: Schematic representation of MMP structure. Transmembrane domain (grey circle) and furin cleavage site (blue line) are indicated for MT-MMPs. The original names of MMPs frequently indicate the substrate specificity.

1.5.3. Structure and function of plasminogen activators: the upstream elements of the plasmin/MMP cascade.

Plasminogen activators (PAs) and plasmin form a serine protease loop of the ECM-degrading plasmin/MMP cascade. Two types of PAs have been identified in mammals, tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activators, which differ in their non-catalytic domain structure and activation mechanisms. The t-PA molecule consists of an N-terminal fibronectin-binding domain, followed by EGF-like and two kringle regions and a C-terminal serine protease domain (Pennica *et al*, 1983). Vascular endothelium is the primary site of t-PA expression and storage and it is rapidly released into the blood stream following activation of endothelial cells with vasoactive agents (Lijnen *et al*, 1994). Similarly to other serine proteases, t-PA is secreted as a zymogen which is subsequently proteolytically cleaved by plasmin into a two-chain active form (Stubbs *et al*, 1998). Interestingly, enzyme activities of the zymogen and active form of t-PA towards their natural substrate plasminogen are comparable and are significantly increased in the presence of fibrin (Hoylaerts *et al*, 1982).

Similarities between u-PA and t-PA include the overall domain organisation and plasmin-dependent activation of a single-chain enzyme proform (Woessner, 1998). The specificity of the u-PA catalytic activity arises from its interactions, through the EGF-like domain, with the specific glycolipid-anchored u-PA receptor (u-PAR) which leads to the formation of a unique proteolytic and chemotactic complex (Blasi, 1997). u-PA binding to u-PAR with the concomitant plasminogen localisation of the cell surface causes a significant increase in plasmin generation as well as an increased efficiency of the reciprocal activation of pro-u-PA (Ellis *et al*, 1991). Furthermore, cell-associated plasmin generated by this system is protected from inhibition by α 2-antiplasmin indicating the existence of an intrinsic amplification loop which promotes u-PAR-mediated plasminogen activation (Plow & Miles, 1990). The role of u-PA/u-PAR in pericellular proteolysis is

closely linked to cell adhesion and migration through the binding of distinct u-PAR domains to integrins and vitronectin (Waltz & Chapman, 1994, Deng *et al*, 1996) and the u-PA-mediated exposure of a chemotactic epitope on the u-PAR molecule (Resnati *et al*, 1996).

Activity of PAs and plasmin is regulated by specific endogenous plasminogen activator inhibitors (PAIs) and nexin-1 which inhibit PA and plasmin and specific plasmin inhibitor α 2-antiplasmin (Vassalli *et al*, 1991). PAI-1 is the main inhibitor species in plasma and extracellular fluid which equally well inhibits pro- and active forms of both t-PA and u-PA and is found bound to plasma and ECM vitronectin which stabilises it in the active conformation (Seiffert *et al*, 1990). PAI-1, similarly to TIMPs, forms high affinity 1:1 stoichiometric complexes with tPA. In the case of the u-PA, PAI-1 binds to the u-PA/u-PAR complex interfering with the proteolytic activity and vitronectin binding (Kanse *et al*, 1996, Deng *et al*, 1996), and promotes internalisation of the complex (Cubellis *et al*, 1990). Two additional inhibitory proteins PAI-2 and 3, exist as soluble molecules and preferentially inhibit u-PA. PAs share similar levels of regulation with MMPs including cytokine-mediated transcription and post-transcriptional modifications which are in many cases cell-specific. The available evidence suggests that PAs may have distinct functional roles in the organism. t-PA is an important component of the fibrinolytic system with the major role in fibrin clearance from the circulation and maintenance of fluidity in the extracellular space (Carmeliet *et al*, 1994). On the other hand u-PA, through its interactions with u-PAR, is predominantly involved in pericellular proteolysis which is necessary for cell migration (Blasi, 1997).

1.5.4. MMPs and PAs in non-diseased CNS and neuropathological conditions.

MMPs are implicated in number of physiological processes especially during development when their controlled activity contributes to the proper formation of tissue

structures. Processes such as neuronal migration and axonal outgrowth during rat brain development are known to involve the plasmin/MMP enzymatic cascade (Dent *et al*, 1993, Muir *et al*, 1998). Similarly u-PA and MMP-9 may be involved in extension of oligodendrocyte processes and consequently myelination of axons (Dent *et al*, 1993, Uhm *et al*, 1998). A widespread distribution of u-PA and t-PA as well as of several MMPs has been described in adult rodent brain and included both neuronal and glial cells (Soreq & Miskin, 1981, Ware *et al*, 1995). In the normal human CNS the expression of PAs and PAI-1 is low and predominantly found in endothelial cells of brain microvessels (Arai *et al*, 1998). In contrast, MMP-1, 2, 3, 9 and MT-MMP-1 are constitutively expressed in white matter microglia (Yamada *et al*, 1995, Maeda & Sobel, 1996) and weak gelatinolytic activity has been detected in extracts from normal brain tissue (Nakagawa *et al*, 1994). TIMP-1 immunoreactivity is generally absent in the CNS parenchyma and only low level expression in endothelial cells was observed (Peress *et al*, 1995, Nakagawa *et al*, 1994).

The expression and activity of PAs and MMPs sharply increase in a variety of neuropathological conditions such as brain tumors, ischemic and neurodegenerative diseases (Romanic & Madri, 1994). In invasive CNS processes PAs and MMPs function on two fronts, decreasing cell to cell contacts and remodelling ECM which facilitates cell migration and formation of metastases. In malignant glioma, the most frequent tumor of the CNS, increased expression of MMP-9 and gelatinolytic activity were found to correlate with higher invasiveness and spreading of tumor cells (Nakagawa *et al*, 1994). Similarly, upstream activator of the plasmin/MMP cascade, u-PA was also found in increased amounts in aggressive spreading of human brain tumors (Arai *et al*, 1998). Such expression patterns of PAs and MMPs are accompanied by an imbalance in PAI and TIMP production suggesting that reduction in local concentrations of inhibitors contributes to their dysregulation in tumor cells. MMPs and particularly gelatinases (MMP-2 and 9) also

contribute to haemorrhagic injury and brain oedema in ischemic disease (Clark *et al*, 1997, MunBryce & Rosenberg, 1998b). In an experimental model of ischemic brain injury in rats an hydroxamate-based inhibitor of MMP was shown to prevent the formation of oedema and neuronal damage (Rosenberg & Navratil, 1997, Rosenberg *et al*, 1998). Deposition of amyloid proteins in neuronal cells is the cause of neuronal damage and dementia in Alzheimer's disease. Metalloproteases can cleave amyloid precursor protein at the α -secretase site releasing the β -amyloid peptide and thus favouring amyloidogenesis (Qiu *et al*, 1997). Furthermore, the exposure of mixed glial and neuronal cultures to amyloid beta results in upregulation of several MMPs (Gottschall, 1996a). Increased concentrations of TIMPs in senile plaques and the activity of MMP-9 are additional evidence implicating MMPs in the pathogenesis of Alzheimer's disease (Backstrom *et al*, 1992, Peress *et al*, 1995).

1.5.5. MMPs and PAs in inflammatory demyelination.

In MS lesions extracellular proteases could be involved in infiltration of immune cells, BBB and myelin damage and generation of pro-inflammatory mediators (Cuzner & Opdenakker, 1999) (**Fig. 1.5.2**). MBP, a major myelin protein, is cleaved *in vitro* by MMPs into encephalitogenic fragments whereas the supernatant of cultured macrophages was shown to induce proteolytic cleavage of MBP via a plasminogen dependent mechanism (Gijbels *et al*, 1993, Chandler *et al*, 1995). The REGA model which stands for "remnant epitopes that generate autoimmunity" has indicated that proteolytic activity in MS lesions may increase the concentration of encephalitogenic myelin peptides which in turn may increase their presentation in the context of MHC molecules thus further amplifying the immune response in the brain parenchyma (Opdenakker & Van Damme, 1994). Transmigration of inflammatory cells, in particular lymphocytes, requires degradation of the collagenous basement membrane of the BBB. T-cells were shown to

upregulate MMP-9 when stimulated with mitogens and when in direct contact with macrophages and endothelial cells (Zhou *et al*, 1993, Lacraz *et al*, 1994b, Stuve *et al*, 1996). The expression of MMP-9 in lymphocytes is stimulated by chemokines but inhibited by IFN β , a cytokine used in therapy of MS (Stuve *et al*, 1997). All cellular components of active MS lesions, *in vitro* stimulated with inflammatory mediators, are capable of expressing PAs and a wide range of MMPs (Gottschall & Deb, 1996b). The distribution and kinetics of MMP involvement have been characterised in rodent models of neuroinflammation such as EAE and delayed-type hypersensitivity response to Bacillus Calamette-Guérin (Hewson *et al*, 1995, Clements *et al*, 1997, Anthony *et al*, 1998). All these studies have identified MMP-7 and 9 as the major MMPs produced during immune-mediated neuroinflammation and showed that specific inhibitors of these enzymes attenuate the disease process.

There is an accumulation of data relating to the increased production and activity of MMPs in the brains from MS patients. Serum and CSF levels of MMP-9 are increased in MS patients whereas TIMP-1 levels were significantly lower compared to those for MMP-9 (Gijbels *et al*, 1992, Leppert *et al*, 1998). Similarly, concentrations of t-PA and u-PA in serum and CSF were higher in MS patients compared to non-diseased subjects and were present in their active form as shown by casein zymography (Akenami *et al*, 1997) stressing the importance of the PAs in the initiation of the plasmin/MMP cascade. Such findings indicate that therapeutic intervention in MS may include the inhibition of metalloproteases. However, the proteolytic activity of PAs and MMPs may also have a beneficial side including activation of growth factors such as IGFs and TGF β or may be utilised by oligodendrocytes for process extension and remyelination. The knowledge that microglia and macrophages are subject to a similar paradox being involved in both damage and repair (Diemel *et al*, 1998) suggest that a balanced and specific therapeutic approach will have to be devised to target the detrimental side of the plasmin/MMP cascade in MS.

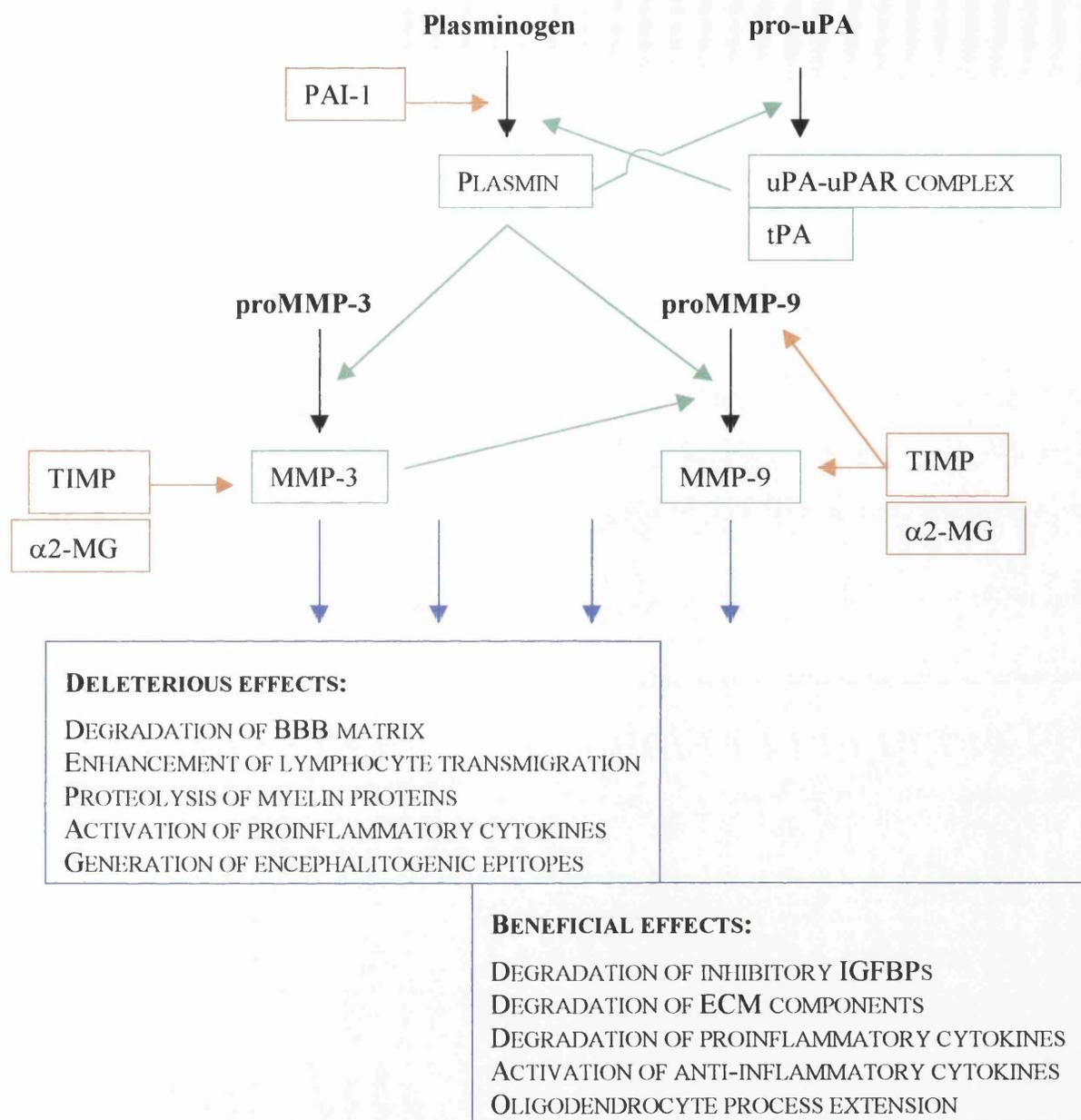


Figure 1.5.2: Some aspects of the plasmin/MMP cascade and potential deleterious and beneficial consequences of its activation in MS lesions. Active enzymes and their interaction are indicated in green, inhibitors are indicated in red and enzyme precursors are given in bold.

1.6. Remyelination and reparative processes in MS.

The major difference between MS and the majority of experimental demyelinating models is the degree of remyelination within lesions and the extent of functional recovery. The most frequent findings on post-mortem in MS cases are various degrees of demyelination which often correlate with the disease duration. Imaging techniques provided evidence that in parallel with appearance of new lesions, previously formed lesions may decrease in size or disappear altogether, indicating that some form of repair occurs in MS brains. Histopathological studies confirmed the existence of remyelination in both active and chronic MS lesions (Raine & Wu, 1993, Prineas *et al*, 1993a and 1993b). In active lesions remyelination was more extensive and coupled to a repopulation of lesions by oligodendrocytes which frequently resulted in formation of so called shadow plaques. In contrast, chronic lesions were characterised by limited remyelination confined to the lesion edge. Electron microscopy of remyelinated fibres revealed shortened internodes of myelin segments and decreased myelin thickness to axon diameter ratio.

The relationship between recurrent inflammatory episodes and the extent and timing of myelin repair in MS lesions is not known. Studies in chronic relapsing EAE indicated that the first signs of remyelination can be observed approximately 4 days after the induction of demyelination when inflammatory cells and myelin debris are still found within lesions (Raine *et al*, 1984, Stanley & Pender, 1991). Chronic demyelinated lesions in this model are the final outcome of repeated episodes of myelin destruction combined with a diminishing capacity for myelin repair. In their studies on shadow plaques in post-mortem MS brains, Prineas *et al*. (1993a and 1993b) showed that these lesions result from a single previous episode of focal demyelination. Similarly to chronic relapsing EAE, recurrent inflammatory activity within shadow plaques, which interrupts the remyelinating process, appears to be responsible for persistence of demyelination.

The failure of myelin repair in MS may also be maintained by the lack of oligodendrocytes or trophic factors that promote oligodendrocyte survival and synthesis of myelin components. Significantly, the degree to which myelinating cells are destroyed in MS lesions does not parallel demyelination (Bruck *et al*, 1994). An increased number of oligodendrocytes survive in and around developing or established MS lesions (Prineas *et al*, 1989b, Morris *et al*, 1994). Furthermore, in early lesions oligodendrocyte-like cells that expressed early differentiation markers were found in areas of newly formed myelin indicating that myelinating cells not only survive but also proliferate, even during periods of active demyelination. Recently, the existence of oligodendrocyte precursors was demonstrated in chronic lesions (Wolswijk, 1997). It became evident from these studies that remyelinating cells are recruited from a pool of undifferentiated progenitors and that depletion of these cells by the recurrent inflammatory activity results over time in a decrease of the remyelinating potential within lesions (Dubois-Dalcq & Armstrong, 1990, Prineas *et al*, 1993b).

A number of growth factors have been implicated in oligodendrocyte development and maturation. A functional classification divides them into competence factors which prepare cells for the cell cycle and progression factors which drive cells through to the DNA-synthesising S phase. Platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF), neurotrophin-3 (NT-3) and IL-2 are mitogenic for rodent oligodendrocyte precursors *in vitro* (Raff *et al*, 1988, Besnard *et al*, 1989, Barres *et al*, 1993). PDGF also promotes migration and provides chemotactic stimuli for oligodendrocyte precursors (Noble *et al*, 1988, Armstrong *et al*, 1990). On the other hand, transforming growth factor- β (TGF β) inhibits mitotic effects of PDGF and promotes maturation of developing oligodendrocytes (McKinnon *et al*, 1993). During CNS development expression of growth factors and receptors correlate spatially and temporally with differentiation of

oligodendrocyte precursors and the onset of myelination, resulting in the formation of mature myelin-producing oligodendrocytes. Many growth factors also act as survival factors for oligodendrocyte precursors, for example ciliary neurotrophic factor (CNTF) protects precursor cells from TNF- α -induced injury (Louis *et al*, 1993). However, treatment of mature cells with growth factors fails to induce dedifferentiation and results in the activation of the apoptotic cascade (Muir & Compston, 1996). The benefits of growth factor use in remyelination of the human CNS have been extrapolated from animal models in which treatment with growth factors promoted remyelination whilst use of specific growth factor inhibitors enhanced disease severity (Tourbah *et al*, 1992, McKay *et al*, 1997). Data from such studies provide a rationale for therapeutic use of trophic factors which promote proliferation and survival of oligodendrocytes and their precursors in human demyelinating disease.

1.6.1. Insulin-like growth factors.

Insulin-like growth factors (IGFs) I and II are mediators of cell growth and differentiation which are structurally and functionally related to the anabolic hormone insulin (Jones & Clemmons, 1995). Mature IGF-I is a basic 7.5kD peptide whilst IGF-II is a slightly acidic 7.4kD peptide with approximately 60% sequence similarity to IGF-I. The human genes for IGF-I and -II are located on chromosomes 6 and 11, respectively and they share a similar structure to other mammalian IGF genes in terms of size and complexity which gives rise to a significant cross-species similarity (Rotwein *et al*, 1986). Transcription of both IGF-I and II genes yields multiple mRNA species due to differential exon usage and several IGF-I and IGF-II variants are described in human serum presumably arising from the differentially spliced mRNA (Daughaday & Rotwein, 1989). As with many other hormones and growth factors, IGFs are produced in pre-pro-form and initial translation products undergo extensive endoproteolysis and glycosylation in order to

produce mature peptide. There is evidence that IGF-I and -II undergo a similar maturation sequence involving cleavage at a specific basic site by subtilisin-related proprotein convertases (Duguay *et al*, 1998). In the adult organism, liver is the primary source of serum IGF-I but it can be also produced locally in many other tissues (Lund *et al*, 1986). Tissue levels of IGF-I in postnatal life are regulated by growth hormone (Clemmons, 1991). In serum, IGF-I is stored in a form of circulating ternary complex which also includes IGF-binding protein 3 (IGF-BP3) and acid-labile subunit (ALS). Production of IGF-II is growth hormone-independent and less centralised, although higher levels are produced in some adult tissues such as the choroid plexus in the CNS. Stimulating actions of IGFs on cells *in vitro* include effects on cell cycle progression, cell proliferation and differentiation, migration and cell survival, some of which are dependent on other growth factors (Jones & Clemmons, 1995).

The majority of IGF effects on cell growth and metabolism are mediated through IGF-I receptor (IGF-IR) which has the highest affinity for IGF-I but also binds IGF-II and insulin at higher ligand concentrations (Leroith *et al*, 1994). IGF-IR is expressed on the cell surface as a heterotetramer of two α and β subunits. The extracellular ligand-binding α subunit is linked through a disulphide bridge to a transmembrane β subunit which forms contacts with the intracellular signalling machinery. IGF-IR is structurally similar to the insulin receptor and hybrid forms have been described. IGF-I binding initiates a common signalling sequence which involves autophosphorylation of the β -subunit tyrosine kinase domain and engagement of the insulin receptor substrate-1 (Chuang *et al*, 1993). IGF-II binds with high affinity to IGF-II/cation-independent mannose-6-phosphate receptor (IGF-IIR), a single chain protein with multiple functions (Kornfeld, 1992). IGF-IIR mediates clearance of IGF-II from the extracellular space and intracellular sorting of mannose-6-phosphate-expressing lysosomal enzymes. There is evidence that IGF-IIR is coupled to a

signalling machinery and that high affinity binding of IGF-II or specific antibodies induces cellular and metabolic responses (Kornfeld, 1992).

1.6.2. IGF-binding proteins: endogenous regulators of IGF functions.

Only a small percentage of IGFs exist free in serum and extracellular fluid, the rest is bound by the high affinity IGF-binding proteins (IGF-BP). The six well characterised IGF-BPs, designated by their cloning order, share sequence homology including two conserved cysteine clusters at their N- and C-termini (Kelley *et al*, 1996, Collett-Solberg & Cohen, 1996) (**Fig. 1.6.1**). They all bind IGF-I and -II with high specificity and similar affinity, with the exception of IGF-BP6 which has up to 70-fold higher affinity for IGF-II (Rechler, 1993). Affinities of IGF-BPs for IGF-I are also 10-40 times higher than that of the IGF-IR. Recently another two putative members of the IGF-BP family, have been characterised indicating existence of an extended IGF-BP superfamily. IGF-BP7 and IGF-BP8 are structurally similar to the rest of the IGF-BPs but have lower affinity for IGFs and are capable of binding insulin (Kim *et al*, 1997).

The best documented role of IGF-BPs is that of a carrier proteins for IGFs in peripheral blood and extracellular fluid. Circulating ternary complexes containing IGF-BP3 and ALS carry the bulk of IGFs which prolongs their half-life several fold. Bound IGFs represent the pool of growth factors that can be mobilised under stress conditions through the proteolytic cleavage of IGF-BP3 (Zapf *et al*, 1990) or the action of proteoglycans on the surface of endothelium which disrupt ternary complexes releasing the free IGF (Baxter, 1990). In contrast to the 150kD ternary complex, which is confined to the intravascular compartment, smaller molecular weight IGF-BP1, 2 and 4 may cross the blood-endothelial barriers carrying IGFs into the tissue (Bar *et al*, 1990). IGF-BP1 and 2 are unsaturated in peripheral blood and avidly bind free IGFs. Release of IGFs from low molecular weight IGF-BPs in the circulation and tissue is also regulated by the specific

proteases (Conover *et al*, 1993, Fowlkes *et al*, 1997). This suggests a sequential release of IGFs under stress conditions from relatively stable circulating ternary complex over to low molecular weight IGF-BPs which transport IGFs to the tissue compartment where specific proteases release IGFs in the vicinity of the target cells.

In addition to their function as carrier proteins IGF-BPs were found, when in molar excess, to inhibit binding of IGFs to IGF-IR and consequently mitogenic responses of cells (Jones & Clemmons, 1995). Inhibition of IGF function is due to high affinity IGF-BP binding and can be alleviated by proteolytic cleavage of IGF-BPs into low affinity protein fragments (Collett-Solberg & Cohen, 1996). The initially described proteolytic activity in pregnancy serum has since been expanded to other tissue compartments and includes serine, aspartic and metalloproteases (Angelloz-Nicoud & Binoux, 1995, Conover & Deleon, 1994, Fowlkes *et al*, 1997). Other mechanisms such as dephosphorylation of IGF-BP1 on serine residues also result in decreased affinity for IGF-I and higher local growth factor bioavailability (Jones *et al*, 1991). Further, binding of IGF-BPs to cell surface receptors or ECM molecules generally decreases their affinity for IGFs but can also generate IGF-independent effects. IGF-BP1 has the Arg-Gly-Asp (RGD) sequence which allows it to bind to the $\alpha 5\beta 1$ integrins on the cell surface resulting in IGF-independent stimulation of cell migration (Jones *et al*, 1993b) or alternatively the enhancement of IGF-I effects through slow release of IGF-I from the cell surface pool (Clemmons, 1991, Koistinen *et al*, 1990). Furthermore, Conover (1992) showed that IGF-I-induced downregulation of IGF-IR can be prevented by surface-bound IGF-BP3. In the case of IGF-BP5, stimulation of IGF-I effects was achieved upon interaction with the ECM, again through decreased IGF affinity of the bound IGF-BP5 (Jones *et al*, 1993a). Therefore a number of local factors influence the interaction of IGFs with their binding proteins with the net effect being either inhibition or stimulation of IGF actions (**Table 1.6.1**).

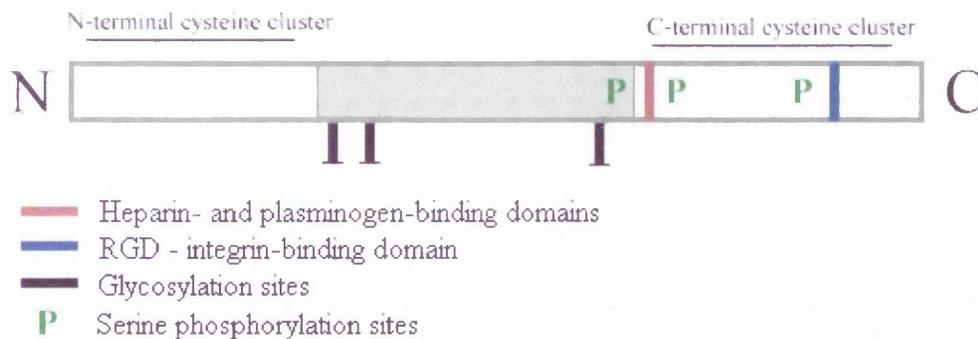


Figure 1.6.1: Schematic representation of the IGF-BP structure. Ligand-binding domains are located within cysteine clusters at N- and C-termini. The heparin-binding domain in IGF-BP3 and 5 mediates binding to ECM whereas the RGD sequence in IGF-BP1 and 2 mediates binding to integrins on the cell surface. Modulation of serine phosphorylation in IGF-BP1 changes its affinity for IGFs whilst the glycosylation influences tissue compartmentalisation of proteins.

Function	Binding protein
Prolongation of IGF half-life in the circulation.	Ternary complex of IGF-BP3 and ALS
Transport of IGFs to extravascular space.	IGF-BP1, 2 and 4
Limitation of IGF bioavailability (inhibition). a. molar excess b. phosphorylation	a. IGF-BP1 to 6 b. IGF-BP1
Enhancement of IGF effects. a. cell receptor binding b. phosphorylation c. ECM binding d. proteolysis	a. IGF-BP1 and 2 b. IGF-BP1 c. IGF-BP3 and 5 d. IGF-BP2 to 6
IGF-independent effects.	IGF-BP1 and 3

Table 1.6.1: Summary of mechanisms by which IGF-BPs modulate IGF effects.

1.6.3. The effects of IGFs on CNS and immune cells.

A specific interest in IGFs in MS stems from their role during oligodendrocyte development and myelination. *In vitro* IGF-I acts as a progression factor for oligodendrocyte progenitors stimulating DNA synthesis, proliferation and commitment of O-2A precursors to the oligodendrocyte cell lineage (McMorris *et al*, 1990). In cultured oligodendrocytes and rat aggregate brain cultures IGF-I increases oligodendrocyte numbers and myelin content (Saneto *et al*, 1988, Mozell & McMorris, 1991). IGF-I also functions as a survival factor for oligodendrocytes and their precursors (Barres *et al*, 1992 and 1993) partially through attenuation of cytotoxic effects of TNF- α (Ye & Dercole, 1997). IGF-II also stimulates oligodendrocyte development and growth but with lower potency than IGF-I (McMorris *et al*, 1990).

Spatial and temporal co-ordination of IGF, its receptor and binding protein expression occurs during CNS development and peaks in the first few weeks after birth concomitantly with the onset of myelination (Adamo *et al*, 1989, Bondy, 1991). IGF transgenic and knock out animals have provided evidence for an *in vivo* role of IGF-I in myelination. In IGF-I transgenic mice myelination of axons was initiated upon transgene activation (Ye *et al*, 1995a) resulting in a largely increased brain size and myelin content but only modest increase in oligodendrocyte numbers (Carson *et al*, 1993, Ye *et al*, 1995b). In contrast extensive hypomyelination and decreased brain size were evident in IGF-I and IGF-IR gene knock-out animals (Beck *et al*, 1995, Cheng *et al*, 1998) whereas IGF-BP1 transgenics also exhibited a significant decrease in MBP and PLP mRNA levels and oligodendrocyte numbers (Dercole *et al*, 1994, Ye *et al*, 1995a) suggesting an inhibitory role for IGF-BP1 on brain myelination. On the other hand, absence of IGF-II results in normally proportioned dwarf mice with proportionally sized brains without histological abnormalities (Dercole *et al*, 1996). Similarly, brain abnormalities are lacking in IGF-II

transgenic mice although thymus and spleen size and numbers of lymphocytes in these organs are increased (Vanbuuloffers *et al*, 1995).

IGFs are also developmental and survival factors for a variety of neuronal cell types (Feldman *et al*, 1997). Treatment of neuronal cell lines and primary neurons results in cytoskeletal changes and vigorous neurite outgrowth. Neuroprotective effects of IGF-I and inhibition of programmed cell death are mediated in part through regulation of intracellular Bcl protein levels and interference with the apoptotic caspase cascade (Feldman *et al*, 1997). Astrocyte proliferation is also stimulated by IGFs although combination with other growth factors such as EGF generally yields more potent effects (Ballotti *et al*, 1987, Tranque *et al*, 1992, Chernauek, 1993, Matsuda *et al*, 1996). IGF-I promotes astrocyte migration directly through effects on actin polymerisation and indirectly through stimulation of PA synthesis (Faberelman *et al*, 1996, Tranque *et al*, 1994). In addition, some metabolic functions such as glucose utilisation and glycogen synthesis in astrocytes are also under the control of IGF-I (Dringen & Hamprecht, 1992).

The production of IGFs in the adult CNS is low except in the choroid plexus in which IGF-II is constitutively synthesised and released into the CSF (Logan *et al*, 1994). The circulating pool represents an additional source of tissue IGFs as they can cross in small amounts the blood-tissue barriers including the BBB (Reinhardt & Bondy, 1994). A postnatal decrease of IGF-IR numbers follows completion of myelination and a fall in IGF levels, however, they remain expressed at low levels in both grey and white matter in the adult CNS (Dekeyser *et al*, 1994a). Two forms of IGF-IR were found in the adult CNS, a peripheral type receptor in glial cells and a smaller molecular weight CNS type receptor in neurons, but the significance of this finding is not clear.

IGFs have a significant input on development and function of immune cells. IGF-I enhances mitogen-induced lymphocyte proliferative responses and promotes maturation

and antibody production of B-cells (Robbins *et al*, 1994). IGFs and IGF-IR were found to be differentially expressed on peripheral blood mononuclear cells with the highest levels reported on monocytes and low levels on T-cells (Stuart *et al*, 1991, Kooijman *et al*, 1992b) although resting human lymphocytes express high levels of IGF-BP2 and 3 (Nyman & Pekonen, 1993). The expression of IGFs and the number of receptors increases upon mitogen stimulation of lymphocytes or engagement of T-cell antigen receptors (Xu *et al*, 1995, Hartmann *et al*, 1992). In rodents and Rhesus monkeys IGF-I treatment produces an increase in the size of lymphoid organs and the number of lymphocytes (Clark *et al*, 1993, Leroith, 1996). Tissue macrophages produce high levels of IGFs particularly when differentiated into a mature phenotype (Arkins *et al*, 1993). The inflammatory mediators TNF- α and prostaglandin E₂ upregulate, but the interferons inhibit macrophage production of IGF-I (Noble *et al*, 1993, Fournier *et al*, 1995, Arkins *et al*, 1995, Lake *et al*, 1994). In turn IGF-I enhances macrophage inflammatory properties, production of TNF- α and phagocytosis of lipoproteins (Hochberg *et al*, 1992, Renier *et al*, 1996) but decrease macrophage adhesion to endothelium (Motani *et al*, 1996). There is no direct evidence of IGF effects on microglial cells although murine microglia *in vitro* produce IGF-I (Arkins *et al*, 1993). Data generated from studies on other tissue macrophages can be extrapolated to microglia and the enhancement of proliferation and cell migration may be envisaged. Overall, it is evident that IGFs can stimulate development and proliferation of both oligodendrocytes and immune cells which in the context of an inflammatory demyelinating disease may result in either remyelination or enhancement of inflammatory processes.

1.6.4. Alterations of the IGF/IGF-BP system in neuropathological conditions.

The upregulation of different components of the IGF/IGF-BP system have been described in brain tumors and a number of human neurodegenerative and inflammatory diseases and their animal models (Dercole *et al*, 1996). A summary of available

histopathological data is given in **Table 1.6.2**.

Support for a therapeutic application of IGFs in inflammatory demyelination stems from findings in experimental models of MS. In mouse spinal cord explants, an *in vitro* model of demyelination, treatment with IGF-I abrogated serum-induced myelin loss (Roth *et al*, 1995). A variety of neuropathological conditions are characterised by the upregulation of IGF-I mRNA and protein synthesis in hypertrophic astrocytes (Liu *et al*, 1994). In all three demyelinating animal models astrocyte IGF-I production was accompanied by upregulation of IGF-BP2 and astrocytosis (Komoly *et al*, 1992, Liu *et al*, 1994, Yao *et al*, 1995b). Furthermore, with the onset of remyelination high IGF-IR levels on oligodendrocytes were observed simultaneously with an increase in myelin protein production. The authors hypothesised that astrocyte IGF-I, delivered to the target cells via IGF-BP2, is directly responsible for remyelinating effects. Further proof came from animals with acute and chronic relapsing forms of EAE in which treatment with recombinant human IGF-I significantly reduced clinical scores and the number and size of lesions (Yao *et al*, 1995a, Liu *et al*, 1997, Li *et al*, 1998). Importantly, numbers of macrophages and lymphocytes were significantly decreased in lesions of treated animals with markedly reduced parenchymal invasion of macrophages. The underlying mechanism of IGF-I actions in both models appeared to be restoration of the BBB and consequently limitation of mononuclear cell infiltration. However, in the mouse chronic relapsing EAE model treatment with an IGF-I/IGF-BP3 complex induced a relapse by expanding the population of encephalitogenic T-cells (Lovett-Racke *et al*, 1998). This model highlighted the importance of IGF-BPs as naturally occurring regulators of IGF function and indicated that the IGF/IGF-BP system can also accentuate inflammatory demyelinating disease.

In MS patients there are no detectable changes in any of the IGF/IGF-BP components in serum or CSF (Wilczak *et al*, 1998, Torres-Aleman *et al*, 1998).

Furthermore, in post-mortem MS brain samples IGF-IRs are found in chronic plaques and their numbers were comparable to those in NAWM (Wilczak & Dekeyser, 1997). In contrast, in amyotrophic lateral sclerosis (ALS), a spinal motoneuron disease with a demyelinating component, significant increases were found in circulating IGF-BP1 to 3, whereas serum IGF-I and insulin levels were significantly reduced (Torres-Aleman *et al*, 1998). Similarly, numbers of IGF-IRs were significantly higher in grey matter of the ALS spinal cord (Adem *et al*, 1994) indicating that neuronal injury may be a stimulus for IGF upregulation and redistribution into the tissue. The relative inactivity of the myelinogenic IGF system during MS may be one of the reasons for incomplete remyelination. On the other hand upregulation of IGFs in the CNS during an exacerbation of MS may be detrimental through stimulation of inflammatory cells and may lead to further tissue damage.

Neuropathology	Findings	References
Normal CNS tissue		
Human	High IGF-IR density in choroid plexus and pituitary gland, moderate in GM and low in WM.	Dekeyser <i>et al</i> , 1994a Dekeyser <i>et al</i> , 1994b
Rodent	IGFs and IGF-IR found in neuronal cells in cortex but not in white matter glia.	Garcia-Segura <i>et al</i> , 1991
Physical and hypoxic-ischemic brain injury		
Rodent brain trauma	Increased expression of IGF-I in astrocytes.	Li <i>et al</i> , 1998
Cerebral infarcts (stroke)	Decreased plasma levels of IGF-I and IGF-BP3.	Schwab <i>et al</i> , 1997
Ischemic-hypoxic injury	Initial decrease in expression of IGFs and IGF-BPs followed by early increase in IGF-I and IGF-BP5 in astrocytes and late increase in IGF-II in macrophages. IGF-IR and IGF-BP2 remain low.	Beilharz <i>et al</i> , 1995 Lee <i>et al</i> , 1996 Hammarberg <i>et al</i> , 1998
Inflammatory demyelinating diseases		
Multiple sclerosis	Normal IGF-IR density in NAWM and chronic plaques. No alteration in IGF-I or IGF-BP1 to 3 concentration in serum. IGF-BP1 not found in CSF.	Wilczak & Dekeyser, 1997 Wilczak <i>et al</i> , 1998
EAE, Cuprizone and cryogenic injury	Increased IGF-I and IGF-BP2 expression in astrocytes and IGF-IR in oligodendrocytes and neurones.	Liu <i>et al</i> , 1994 Webster, 1997 Komoly <i>et al</i> , 1992 Yao <i>et al</i> , 1995b
Neurodegenerative diseases		
Alzheimer's disease	No alterations in IGF-IR expression. Increased IGF-I and II in serum and increased IGF-II and IGF-BP2 and 6 in CSF.	Dekeyser <i>et al</i> , 1994a Tham <i>et al</i> , 1993
Amyotrophic lateral sclerosis	Increased IGF-IR expression in spinal cord GM. Serum IGF-I and insulin low but IGF-BP1-3 higher compared to controls.	Adem <i>et al</i> , 1998 Torres-Aleman <i>et al</i> , 1998
Tumours		
Gliomas and meningiomas	Markedly increased expression of IGF-I, IGF-II, IGF-IR and IGF-BP1 in tumour cells.	Merrill & Edwards, 1990 Unterman <i>et al</i> , 1991 Glick <i>et al</i> , 1993

Table 1.6.2: Alterations in the IGF/IGF-BP system in different neuropathological conditions. Where available histopathological data are given first, followed by alterations of IGF/IGF-BP concentrations in serum and CSF. GM – grey matter, WM – white matter.

1.7. Aims of the thesis.

The initiation and progression of MS lesions is influenced by inflammatory mediators from immune and glial cells which interact in a cascade-like fashion. NF- κ B is one of these mediators which upon activation rapidly induces a number of effector molecules, including cytokines, adhesion molecules and proteolytic enzymes, leading to several-fold amplification of the initial stimulus. Furthermore, the enzymes of the plasmin/MMP cascade, may paradoxically recruit IGFs to fuel the inflammatory reaction, therefore diminishing the remyelinating capacity in the lesions.

In order to test such a hypothesis the cellular distribution and activity of the NF- κ B transcription factor, the plasmin/MMP cascade and myelinogenic IGFs at different stages of MS lesion evolution was investigated using immunocytochemistry and biochemical assays. Data on MS lesions were compared with those in normal control white matter, other neurological diseases and isolated adult human microglia in order to examine the specificity of findings to MS. The main aim of this approach was to identify rate-limiting steps in each cascade which can be used as potential therapeutic targets.

Chapter II

2. Materials and methods.

2.1. The NeuroResource tissue bank: sampling and assessment of tissue.

Human tissue is an irreplaceable resource in medical research and the understanding of the exact nature of pathogenic mechanisms relies on access to CNS tissue which is representative of diverse lesion patterns distinguishing individual MS cases. The classification and selection of CNS tissue dissected accurately according to neuropathologically accepted criteria is pivotal to data interpretation in research studies.

All post-mortem tissue samples were obtained from the NeuroResource tissue bank at the Institute of Neurology, London. CNS tissue was transported on wet ice from mortuaries and dissection was carried out on arrival to minimise the interval between post-mortem and the cryoprotection of tissue samples. A flow chart showing the routine procedures for dissection and classification of tissue obtained from normal control and neurological disease cases is given in **Figure 2.1**. Whole brain was placed in a specially designed Perspex box to facilitate accurate slicing of the fresh tissue into 1cm thick coronal slices starting from the occipital pole. From each slice of MS brain, brain stem and spinal cord 0.5-1cm³ blocks containing plaque and apparently normal white and grey matter were dissected and a map of lesion activity was prepared for each case. As pathology is widely disseminated in MS and not all lesions are macroscopically visible, normal-appearing white and grey matter were extensively sampled to cover all predilection sites for plaque development. CNS tissue samples taken for snap-freezing also included samples of meninges, choroid plexus and spinal roots of peripheral nerves. Samples of CNS infarcts and brain tumours found as primary or an accompanying pathology in MS cases were used as the non-inflammatory neurological disease controls. Liver, pancreas, spleen and lymph nodes from MS cases and normal controls were also dissected and used as control tissue

when relevant to a particular study. Matching areas to those routinely sampled from MS cases were dissected from normal control human brain and spinal cord with similar clinico-pathological characteristics (**Tables 2.4a and 2.4b**).

For each immunocytochemical study, tissue blocks from normal control and MS cases were matched as closely as possible for age, sex and death to snap-freezing time (DFT). Careful comparison of staining patterns obtained with glial cell markers and research antibodies in tissue section from cases with short and long DFTs revealed that this factor had a negligible influence on immunostaining. This is in accord with the findings of de Groot and colleagues (1995) who concluded that only minor enhancement of immunostaining occurs in rat brains with prolonged post-mortem delay. Furthermore, the brain retains a balanced environment even during prolonged post-mortem delay and the brain lysosomal fraction is considerably more stable than in other organs (Cuzner & Davison, 1973).

Following dissection, tissue blocks were mounted on cork discs using OCT compound (Merck, Lutterworth, UK) and snap-frozen by immersion for 9 sec in isopentane (Merck) cooled in liquid nitrogen. Blocks were wrapped in aluminium foil and stored at -70°C in air-tight containers until further use. A total of 63 blocks were sampled from 32 MS cases and cases with other neurological diseases and 18 blocks from 16 normal control cases (**Table 2.4a and 2.4b**).

The clinical diagnosis of MS was established from the patients' medical records and cases were classified into following five clinically defined groups:

- (i) **Early relapsing remitting MS:** duration less than 5 years with minimal disability.
- (ii) **Malignant MS:** duration less than 5 years with severe disability.
- (iii) **Benign MS:** duration greater than 10 years with minimal or no disability.
- (iv) **Secondary progressive MS:** disease initially manifests as relapsing-remitting and subsequently enters progressive course resulting in increased disability.
- (v) **Primary progressive MS:** disease is progressive on onset and is accompanied by increased disability.

The majority of MS cases from which samples were used in the studies described in this thesis were those with secondary progressive disease, while only a small but significant number had primary progressive disease.

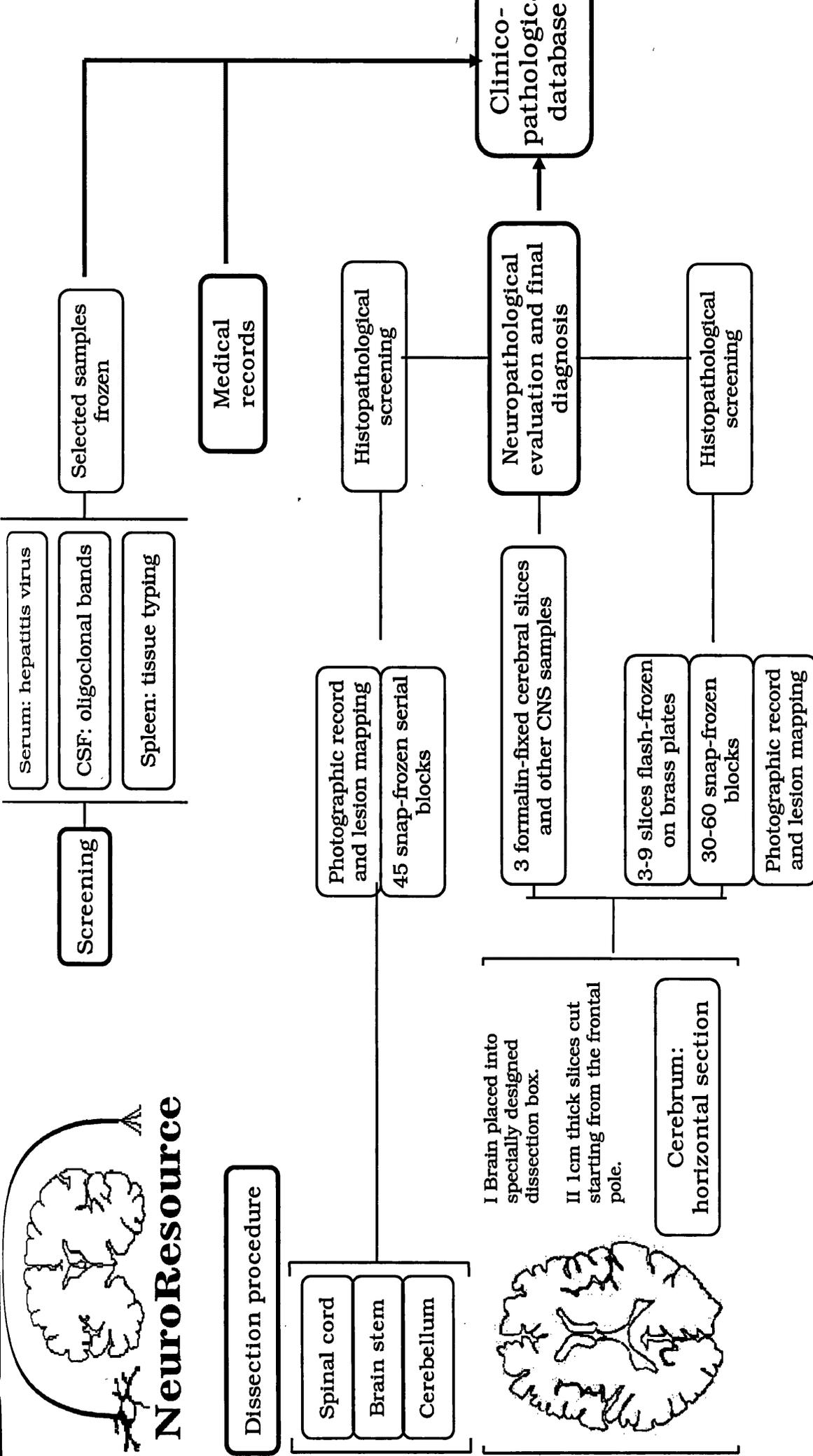
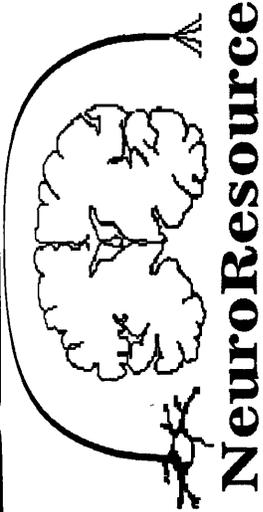


Figure 2.1: Dissection and classification procedure for MS and normal control tissue samples. The procedure incorporates (a) dissection of samples and lesion mapping, (b) written and photographic evidence, (c) neuropathological confirmation of existing pathology and screening for other neurological diseases and (d) database storage and update.

2.2. Histochemistry and immunocytochemistry.

2.2.1. Slide preparation.

Glass slides for immunocytochemical staining were coated with Vectabond (Vector Laboratories, Peterborough, UK) according to the manufacturers instruction. Briefly, 2% Vectabond solution was prepared by mixing the 7ml of Vectabond with 350ml of acetone. Slides were thoroughly washed in distilled water, air-dried and immersed in acetone for 5 min. A coating solution of 2% (v/v) Vectabond in acetone was applied for another 5 min. Slides were briefly washed in distilled water and air-dried overnight at 37°C.

2.2.2. Haematoxylin and eosin staining.

Haematoxylin and eosin (H&E) staining was used to assess the extent of inflammatory infiltration and cellularity in MS sections. In addition H&E was used in the study on transcription factor NF- κ B (Chapter III) to visualise the morphology and distribution of glial and inflammatory cell nuclei.

Unfixed cryostat sections (10 μ m), thaw-mounted on glass slides, were placed in Harris haematoxylin (Merck) for 3 min and then washed briefly with running tap water. Slides were differentiated in acid alcohol (1% v/v HCl in 100% ethanol) and then washed again for 2 min in cold running tap water. Finally, slides were counterstained in eosin for 30 sec, dehydrated through graded ethanol solutions (95% followed by 2 x 100% solution) and xylene for 45 sec each and mounted in DPX (Merck).

2.2.3. Oil red O staining.

Oil red O (ORO) is Sudan-type lipid-soluble dye routinely used to demonstrate neutral lipids in histological preparations. ORO staining was performed in order to assess the number and distribution of myelin-laden macrophages in MS and other types of CNS lesions.

A saturated ORO solution was prepared by dissolving 5g of ORO powder (R. Lamb, London, UK) in 500 ml of 60% (v/v) triethyl-phosphate (Sigma-Aldrich, Poole, UK) in de-ionised water. This solution was incubated for 3h on 60°C in water bath with constant stirring. After filtering through a Whatman No.1 filter (Merck) the ORO solution was stored in the dark. Sections mounted on glass slides were fixed in 4% (v/v) formaldehyde (Merck) for 10 min and washed in running tap water for 2 min with constant agitation. Following fixation sections were immersed 3x in 60% (v/v) triethyl phosphate, incubated in the ORO solution for 15 min and than again briefly immersed 3x in 60% triethyl phosphate. After further 2 min washing in tap water slides were counterstained in Mayers haematoxylin (Sigma) for 3 min, washed in tap water for 5 min and mounted in glycerol jelly (Dako, Cambridge, UK). The edges of the coverslips were sealed with nail varnish.

2.2.4 Preparative techniques: choice of tissue fixative and antibody titration.

Adequate and complete fixation forms the foundation of all good histological preparations and its aim is to preserve the tissue antigens as far as possible in their native structure. Four protocols (**Table 2.1**) were routinely tested for each immunocytochemical study to assess the effects of different fixatives on antigen-antibody reactions. Each reagent was initially tested under three different temperature conditions with antibody markers for macrophages, oligodendrocytes and astrocytes (**Table 2.2**) as internal controls. Based on the clarity of cellular staining, tissue structure preservation, low background signal and suitability for both single and double immunocytochemistry, methanol fixation at -20°C for 10 min was adopted as the protocol of choice.

Fixative (10 min)	RT	4°C	-20°C
Ethanol	-	+	++
Methanol	+	++	+++
Acetone	+	++	++
Paraformaldehyde	+	N/A	N/A

Table 2.1: Assessment of different fixation protocols. The symbols stand for - poor + good, ++ satisfactory and +++ excellent. All the antibodies listed in Table 2.2 were tested under these conditions.

Optimal binding conditions for primary antibodies were established by applying a range of four dilutions to sections of normal control white matter, active and chronic MS lesions. The initial doubling dilutions for mouse supernatant reagents were usually from 1:2 to 1:20, concentrated mouse ascites from 1:50 to 1:1000 and purified monoclonal antibodies from 1:100 to 1:1000. Polyclonal rabbit reagents were tested from 1:200 for immunoaffinity-purified antisera and from 1:1000 for whole antisera. The optimum dilution was considered to be the antibody concentration which gives a clear staining pattern and the low background signal. Marker antibodies were used to distinguish different cell populations in the CNS parenchyma and blood vessels, and their specifications are given in **Table 2.2**.

Antibody	Cell specificity	Isotype	Dilution	Source and reference
14E	Oligodendrocytes and reactive astrocytes	Mouse IgG1	1:10	Newcombe <i>et al</i> , 1991
α -CNPase	Oligodendrocytes and myelin	Rabbit antiserum	1:1000	Prineas <i>et al</i> , 1989a
α -GC	Galactocerebroside in myelin	IgG culture supernatant	1:10	Wolswijk, 1998
5.2E4	Astrocytes (GFAP)	Mouse IgG1	1:1000	Newcombe <i>et al</i> , 1986
α -GFAP	Astrocytes	Rabbit antiserum	1:500	Newcombe <i>et al</i> , 1986
EBM-11	Microglia and macrophages	Mouse IgG1	1:25	Dako Esiri & Wilcock, 1986
α -CD2	T lymphocytes	"	1:100	Dako
α -collagen IV	Type IV collagen in basement membrane	"	1:100	Dako Esiri <i>et al</i> , 1991
α -SMC actin	Smooth muscle cells	"	1:50	Dako Esiri <i>et al</i> , 1991

Table 2.2: The characteristics and source of marker antibodies.

2.2.5. *Single immunocytochemical staining.*

All chemicals used for the preparation of antibody solutions were of AnalaR grade (Merck) unless stated otherwise and the whole staining procedure was performed at room temperature (RT). A three-step peroxidase method was used for single immunocytochemical staining. Cryostat sections (10 μ m), cut onto Vectabond-coated slides, were fixed in methanol (-20°C, 10 min) and blocked with 2.5% normal serum diluted in phosphate-buffered saline (PBS, 0.15M NaCl, 100mM K₂HPO₄ x 3H₂O and 20mM KH₂PO₄, pH 7.4) for 30 min. Blocking serum was tipped off and primary antibodies in PBS incubated on the sections for 1h. After washing 3 x 5 min in PBS on a magnetic stirrer sections were incubated with biotin-labelled secondary antibody diluted 1:200 in PBS for 45 min. Peroxidase-labelled avidin and biotin reagents (Vector) were mixed at 1:100 in PBS and preincubated for 30 min according to the manufacturer's instructions to ensure formation of complexes. After a further three washes in PBS, the avidin-biotin solution was incubated on sections for 45 min. The peroxidase substrate was prepared by mixing 0.05% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma), 0.04% (w/v) NiCl₂ and 0.02% (v/v) hydrogen peroxide in 400ml of PBS immediately before use. Sections were incubated in the nickel-enhanced peroxidase substrate for 5 min giving a black coloration where antigen was immunolocalised. Sections were not routinely counterstained in haematoxylin, but when nuclear localisation of antigens was studied (Chapter III), sections were developed in substrate without nickel and subsequently counterstained with Mayers haematoxylin for 20 sec. In both instances following a 5 min wash in tap water sections were dehydrated and cleared in the same way as described for the H&E-stained sections.

2.2.6. Double immunocytochemical and histochemical staining.

Double immunocytochemical staining was performed either using immunofluorescence or a combination of two differently coloured peroxidase substrates. For double staining using immunofluorescence, sections were fixed in methanol (10 min, -20°C) and blocked with 10% normal goat serum. After incubation with a mixture of two primary antibodies raised in different species, usually rabbit and mouse, at appropriate dilutions, sections were incubated with fluorescence-labelled goat anti-mouse (1:50, FITC or TRITC, Sigma) and biotin-labelled goat anti-rabbit (1:200, Vector) secondary antibodies. Finally, FITC- or TRITC-labelled avidin (1:200, Vector) in sodium bicarbonate buffer (100mM NaH₂CO₃, pH 8.5) was used to reveal bound rabbit antibodies, and sections were mounted in Citifluor anti-fade reagent (University of Kent, UK). Sections were examined under a Leitz Laborlux microscope equipped with fluorescence optics.

Double staining using peroxidase substrates was performed by repeating twice the single immuno-labelling as described in Section 2.2.5. The first antibody was developed for 5 min in DAB substrate without nickel enhancement to give brown coloration. The second antibody was developed using Vector VIP colour substrate (Vector), which gives a purple colour, prepared according to the manufacturer's instructions and incubated for 5 min, then sections were dehydrated and mounted as for H&E staining. Omission of one of the primary antibodies was used as a negative control but peroxidase and biotin blocking steps were not routinely performed.

Combined ORO and immunofluorescent staining was used to demonstrate the co-localisation of antigens in lipid-laden macrophages (Li *et al*, 1993). Unfixed, air-dried sections were stained for 2 min in 5% ORO solution, washed 3x in PBS and then immunofluorescence stained with an appropriate FITC-labelled secondary antibody. After a final wash in PBS, sections were post-fixed in 4% paraformaldehyde, washed in distilled

water and mounted in Citiflour. Staining was visualised under a FITC excitation filter as a green fluorescence of antibody-labelled antigen and red fluorescing ORO-stained neutral lipids.

2.2.7. Immunocytochemical controls.

Both methodology and antibody specificity controls were routinely employed. Omission of each step in the immunocytochemical staining procedure was used as a negative control. Additional controls included non-immune mouse ascites (NS-1, Sigma) and non-immune isotype controls IgG1, IgG2a and IgG3 (Sigma) for monoclonal antibodies, and non-immune rabbit serum (Dako, Cambridge, UK) for polyclonal reagents applied at the same immunoglobulin concentration as the primary antibodies. The antigen specificity of antibodies and antisera was assessed by blocking with their complementary antigens whenever these were available. All reagents were pre-incubated with a tenfold excess of antigen by weight for 2h at RT or overnight at 4°C with constant rotation. Following incubation antibody-antigen solution was centrifuged for 3 min at 10,000 rpm to precipitate immune complexes and supernatant was applied on sections.

2.3. Classification of MS lesions.

The criteria for classification of MS lesions were adopted from Li *et al.* (1993) and a schematic representation of different stages in lesion development based on ORO staining is given in **Figure 2.2**. This classification is based purely on histopathological criteria and can accommodate all clinical types of MS. The following histological and immunocytochemical reagents were used as a basis for the classification of MS lesions:

ORO	distribution of myelin-laden macrophages
H&E	extent of inflammatory infiltration
anti-GC	extent of demyelination and lesion definition
EBM-11	number and morphology of microglia and macrophages
CD2	number and distribution of T-cells
14E	number and distribution of oligodendrocytes
GFAP	number and morphology of astrocytes

On the basis of these criteria lesions were classified in groups from 1 to 5 (**Table 3.2**). Group 1 represented an initial or early MS lesions. Group 2 and 3 represented two subsequent stages in active lesion development i.e. early and late active. Group 4 depicted subacute or chronic-active lesions whereas Group 5 represented chronic inactive lesions. The same criteria were used to assess the age of brain infarcts which were all recent lesions as ORO-positive macrophages, mononuclear cell infiltration and reactive astrocytes were present.

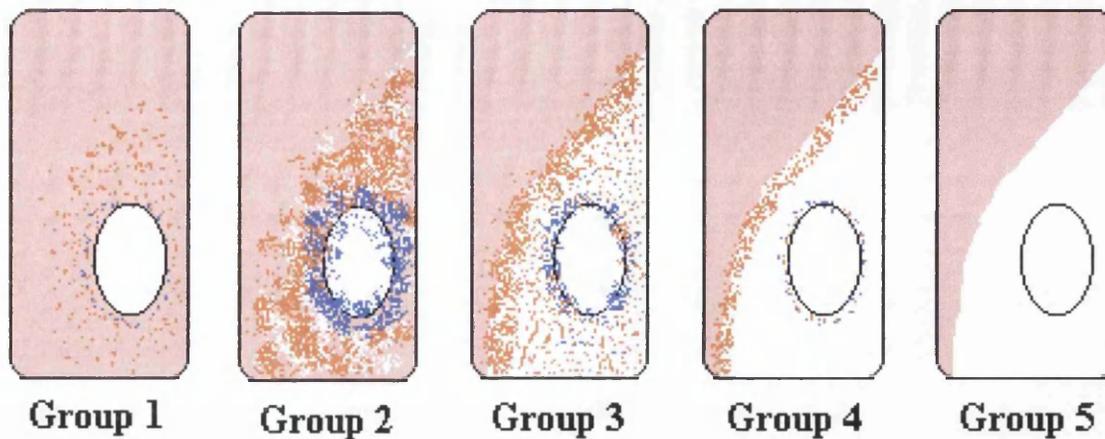


Figure 2.2: Schematic representation of sequential stages in MS lesions development. Colour coding: pink – normal-appearing white matter, white – demyelinated areas, red – ORO⁺ macrophages and blue – perivascular inflammatory cell infiltrates. The circle in the middle represents a blood vessel.

GROUP 1 LESION (INITIAL, EARLY)	Scattered ORO ⁺ macrophages surrounded by apparently intact myelin and limited perivascular infiltration. No established lesions.
GROUP 2 LESION (ACUTE, EARLY ACTIVE)	Partially demyelinated lesion with numerous ORO ⁺ macrophages throughout lesion parenchyma and pronounced perivascular inflammatory infiltration. Lesion edge is not well defined and exhibits shelving effect.
GROUP 3 LESION (ACUTE, LATE ACTIVE)	Demyelinated lesion with ORO ⁺ macrophages evenly distributed within parenchyma and less pronounced perivascular infiltration. Lesion well defined with ORO ⁺ edge.
GROUP 4 LESION (SUBACUTE, CHRONIC ACTIVE)	Demyelinated plaque with a limited number of ORO ⁺ cells confined to active border and perivascular space.
GROUP 5 LESION (CHRONIC, SILENT)	Completely demyelinated lesion with no evidence of inflammatory activity.

Table 2.3: Description of individual group characteristics. The terms in brackets indicate the equivalent terminology used by the other authors (Ozawa *et al*, 1994, Raine *et al*, 1998).

Case No.	Age (sex)	DFT	MS duration	MS type	Study	Cause of death	No. of blocks
540	43 f	25	22	RR	I	Status epilep.	1
489	77 f	8	30	RR	M,I	BP/CVA	1
488	47 f	9	20	RR	N,M,I	BP	4
486	69 m	70	36	RR	M,I	Renal failure	1
485	51 m	8	20	RR	I	BP	1
484	59 f	12	28	RR	N,M,I	Glioma	1
483	66 f	18	30	RR	M	Long MS	1
468	82 m	60	27	RR	M,I	CVA	3
465	66 f	15	26	RR	N,I	BP	1
463	37 m	67	15	RR	M	BP	2
462	40 f	19	15	RR	N,M,I	BP	3
444	29 f	11	8	PP	N,M,I	BP	5
441	46 f	98	10	RR	M,I	BP	1
440	43 m	48	18	RR	N	BP	2
413	59 f	13	20	RR	N,M,I	BP	3
412	58 f	26	27	RR	N,I	BP	2
410	64 f	51	14	RR	N,M	Haemorrhage	3
405	47 f	24	7	PP	I	Carcinoma	1
400	29 f	14	7	RR	I	BP	2
399	42 f	34	11	RR	N,I	BP	1
396	37 f	24	10	RR	N,M	BP	2
395	58 f	52	14	RR	N,M	Lung Ca.	2
342	50 m	29	15	RR	N,M,I	BP	2
335	66 m	75	21	RR	N,M	CA	3
332	53	56	22	RR	M	Septicaemia	2
330	42	48	16	RR	M	BP	2
318	76 m	28	41	RR	N,I	CVA	1
303	22 f	11	2	PP	N,M,I	BP	2
302	71 f	42	50	RR	N,M,I	CVA	2
300	74 f	35	16	RR	N,M	BP	1
285	35 m	28	1	PP	N,M	Asphyxia	2
194	26	18	2	PP	N,M	BP	3

Table 2.4a: Clinico-pathological data for MS cases. Abbreviations: BP – bronchopneumonia, CA – coronary arrest, CVA – cerebro-vascular accident, RTA – road traffic accident. PP – primary progressive MS and RR – relapsing-remitting MS. Codes for studies: N – NF-kB, M – MMP and I – IGF study.

Case No.	Age (sex)	DFT	Study	Cause of death	No. of blocks
563	41 f	41	I	CA	1
546	59 m	15	I	Renal failure	1
532	71 m	40	I	CA	1
524	80 m	24	I	CA	1
523	67 m	38	I	Haemorrhage	1
520	78 f	32	M	CA	1
504	49 m	11	M	CA	1
502	34 m	21	N,I	CA	1
501	65 m	15	M	CA	1
449	60	19	M	CA	1
408	39 f	9	N	Peritonitis	1
407	54	61	M	CA	1
401	38	75	N,M,I	RTA	2
393	37 m	40	N,M,I	RTA	2
392	40	30	N	CA	1
385	60	45	N	BP	1

Table 2.4b: Clinico-pathological data for normal control cases. For abbreviations see Table 2.4a.

Chapter III

3. Nuclear factor NF- κ B: transcriptional regulator of pro-inflammatory cytokines and adhesion molecules.

3.1. Introduction.

3.1.1. *Molecular basis of microglial activation.*

Microglial cells are the resident CNS macrophages which form a sensitive cell system for detection of pathological changes in the CNS (Cuzner, 1997). Under physiological conditions resting microglia have a ramified morphology and display a downregulated macrophage phenotype. Early and rapid activation of microglia occurs in a number of neuropathological conditions ranging from subtle changes in spreading depression to severe tissue damage during an acute inflammatory episode (Bastuardo & Kreutzberg, 1994). Activation is a graded response which results in expression of many macrophage-like properties including a whole array of secretory products and cell surface molecules and increased phagocytic capacity. A distinct pattern of inward rectifying potassium channels and electrophysiological properties which increases microglial sensitivity to ionic changes in the microenvironment may facilitate rapid activation (Kettenmann *et al*, 1990 and 1993). Membrane depolarisation and receptor-generated signals are then translated into changes in gene expression. Transcription factors are the part of the signalling cascade which bridge the gap between the cytosol and nucleus directly modulating the expression of target genes. Primary transcription factors such as NF- κ B are stored in the cytoplasm in an inactive form and can be rapidly activated by a number of mediators (Baeuerle & Henkel, 1994). They are particularly utilised by the cells of the innate and specific immune systems which have to respond to sudden changes in their environment.

3.1.2. *NF- κ B: transcriptional co-ordinator of macrophage gene expression in inflammation and stress.*

NF- κ B is a transcription factor family of five subunits p50, p52, RelA (p65), c-Rel (p75) and RelB which form NF- κ B dimers (May & Ghosh, 1997). RelA, c-Rel and RelB all have transcription activation domains and dimers containing these subunits are transcriptionally active. In contrast, p50 and p52 are truncated and can not activate transcription but are important for the stabilisation of NF- κ B/DNA complexes. In the majority of cell types NF- κ B is present in the cytoplasm in an inactive form coupled to inhibitory I κ B proteins. Downstream signalling cascades of inflammatory and stress factors involve activation of specific I κ B kinases which phosphorylate I κ B rendering it susceptible to ubiquitination and proteolysis in proteosomes (Didonato *et al*, 1997). Removal of I κ B reveals the NLS sequence on NF- κ B subunits and enables NF- κ B to translocate to the nucleus and bind the specific κ B sequence on the DNA.

NF- κ B plays a central role in co-ordinately controlling gene expression during macrophage activation. Many biological mediators important for macrophage activation and function during the inflammatory process are transcriptionally regulated by NF- κ B. The list is growing rapidly and includes the macrophage growth factors M-CSF and GM-CSF, the inflammatory cytokines TNF α , IL-1 and IFN γ and chemotactic molecules such as MCP-1 and IL-8 (Baeuerle & Henkel, 1994). Beside the inducible form, macrophages also possess the constitutively active, nuclear form of NF- κ B (Collart *et al*, 1990). Because differentiation of cells is defined by the production of cell-type specific proteins, the constitutive activation of NF- κ B may allow macrophages to maintain a differentiated phenotype in the tissue in which they reside and may facilitate rapid cell-specific responses to various types of tissue injury.

In the CNS constitutively active NF- κ B is found in some neurones (Kaltschmidt *et al*, 1994b) while the form found in microglia and macroglia requires induction by inflammatory mediators (Moynagh *et al*, 1994, Diehl *et al*, 1995). In rodent microglia NF- κ B activation is an essential part of the cell activation cascade (Bonaiuto *et al*, 1997) regulating the expression of inflammatory mediators such as cyclooxygenase-2 and IL-8 (Bauer *et al*, 1997, Ehrlich *et al*, 1998). There is evidence that NF- κ B plays a role in the pathogenesis of several CNS diseases (O'Neill & Kaltschmidt, 1997). In EAE, the activation of NF- κ B was found to correlate with disease progression and the activated form was detected in macrophages at the peak of clinical disease (Kaltschmidt *et al*, 1994a). Activation of NF- κ B also occurs in macrophages in HIV encephalitis (Dollard *et al*, 1995) and affected brain regions in multiple system atrophy (Schwarz *et al*, 1998). In brain infarcts NF- κ B was found to be localised predominantly in reactive astroglia, NF- κ B positive macrophages being rare (Terai *et al*, 1996a). Recent data also indicate that NF- κ B may be a key modulator of TNF α -induced apoptosis in human oligodendrocytes.

3.1.3. Aims of the study.

NF- κ B is a candidate for regulating the expression of cytokines and adhesion molecules in immune and glial cells during inflammatory demyelination in the CNS. The aim of this study was to characterise the localisation and DNA-binding activity of NF- κ B in MS lesions by immunocytochemistry and gel-shift assay. In the light of the specific role of NF- κ B in macrophage gene regulation, the localisation of NF- κ B and I κ B are also studied in adult human microglia isolated from post-mortem CNS white matter from normal control and MS cases and surgical resections.

3.2. Materials and methods.

3.2.1. Tissue for immunocytochemistry and microglial isolation.

Clinical data for individual MS and normal control case are given in Chapter II, Tables 2.4a and 2.4b. In total 32 snap-frozen blocks containing lesions and/or macroscopically normal-appearing white matter from 17 cases of MS were examined. Blocks from MS cases included 10 with active, 7 with subacute and 3 with chronic MS lesions and 8 of NAWM distant from any known lesion. Cryostat sections were also cut from 9 brain infarcts and 2 tumours from the total of 10 cases which were used as other neurological disease controls. Six normal control samples were taken from the brain or spinal cord of six non-neurological disease cases. The clinico-pathological data for all MS and normal control cases used in the NF- κ B study are given in the **Table 3.1**.

TISSUE TYPE	AGE (RANGE)	SEX RATIO (M:F)	DFT	DURATION OF MS
NC CASES (n=6)	49.4 y (22-69)	4:2	36.0 h (8-98)	N/A
MS CASES (n=17)	54.1 y (34-80)	7:10	24.6 h (9-41)	17.6 y (2-36)

Table 3.1: Average age, sex and death to snap-freezing time (DFT) data of MS and normal control cases.

Microglial cells were isolated from brain tissue removed during surgery to ameliorate intractable epilepsy. This tissue included both white and grey matter in approximately 50:50 ratio, weighing between 0.5 and 1.5g and obtained within 2h of surgery. Microglia were also isolated from 20g samples of periventricular white matter from occipital lobe of post-mortem cases with no history of neurological disease and with post-mortem delay of up to 24h and from the same brain area of one MS case. All tissue was dissected free of meninges and large blood vessels to reduce the contamination with monocytes and other blood cells.

3.2.2. *Antibodies and immunocytochemistry.*

The characteristics of the antibody panel used in this study and the dilutions for each antibody are given in **Table 3.2**. Single and double immunocytochemical staining was performed as described in Chapter II (Section 2.2.5. and 2.2.6.). All sections were fixed in methanol (10 min, -20°C). Fixation in acetone (4°C, 10 min) or 4% paraformaldehyde (RT, 5 min) gave the same staining pattern for all the antibodies used in the study. Double immunofluorescence staining of oligodendrocytes in normal control white matter was performed using 14E antibody detected with TRITC-labelled goat anti-mouse IgG and anti-I κ B α antibody detected with biotin-labelled goat anti-rabbit coupled with FITC-conjugated avidin. The same combination of secondary fluorescence-labelled reagents was used for double staining of microglia and macrophages with polyclonal anti-RelA antibody and macrophage marker EBM-11. Routine immunocytochemical controls included omission of primary antibodies and mouse ascites (NS-1, Sigma) and non-immune rabbit serum (Dako) applied at the same immunoglobulin concentration as the primary antibodies. The specificity of antisera against RelA, c-Rel and I κ B α was assessed by blocking with their complementary peptides (Santa Cruz). Antibodies were pre-incubated with a tenfold excess of peptide by weight for 2h at room temperature or overnight at 4°C. Peptides for the anti-p50 and monoclonal RelA antibodies were unavailable. In order to assess NF- κ B expression in lipid-laden macrophages, combined ORO and immunofluorescent staining was also carried out (Li *et al.*, 1994) as described in Chapter II, Section 2.2.6.

3.2.3. *Isolation of adult human microglia.*

Microglial cells were isolated using enzymatic digestion and discontinuous Percoll gradient, using the modified method of Sedgwick *et al.* (1991). Tissue was finely chopped into approximately 1mm³, resuspended in digestion buffer (20mM MgCl₂, 15mM CaCl₂,

50mM KCl, 0.15M NaCl, pH6-7) with added trypsin (0.125%, from bovine pancreas, Sigma), DNase (20 μ g/g wet weight, type II from bovine pancreas, Sigma) and collagenase (100U/g wet weight, type XI from *C. histolyticum*, Sigma) and incubated for 30 min at 37°C with constant agitation. Following digestion, tissue was washed with glucose-PBS/BSA (G-PBS, 0.15M NaCl, 5mM KCl, 10mM Na₂HPO₄, 5mM NaH₂PO₄, 0.2% D(+) glucose and 0.02% BSA) by centrifugation at 500xg for 10 min at 4°C and passed through 80nm nylon mesh (J. Stanier, Birmingham, UK).

ANTIGEN	ISOTYPE	Ig CONCENT.	DILUTION	SOURCE
RelA-NLS	IgG3	1 mg/ml	1:10	Boehringer
RelA	Polyclonal	0.1 mg/ml	1:200	Santa Cruz
c-Rel	“	“	1:200	“
p50	“	0.2 mg/ml	1:100	“
p50	“	2 mg/ml	1:200	UBI
I κ B α	“	“	1:200	Santa Cruz
I κ B β	“	“	1:200	“

Table 3.2: The characteristics of the NF- κ B antibody panel.

After a further two washes, the tissue pellet was resuspended in a mixture of 21.4 ml of G-PBS and 8.6 ml of isotonic Percoll (30% Percoll, $\delta = 0.037$) and centrifuged at 500xg for 30 min at 4°C. Supernatant and myelin were removed and the cell pellet containing mixed glial cells was washed as before and resuspended in 2ml of G-PBS. A discontinuous Percoll gradient was assembled by adding 2ml steps of 1.072, 1.065, 1.055 and 1.030 density Percoll solutions and overlaying the 2ml containing resuspended cells in G-PBS on the top of the gradient. This gradient was centrifuged at 1500xg for 30 min at 20°C in a

swing-out rotor centrifuge (Sorvall RT6000B, Du Pont). Cells were collected from the 1.055 and 1.065 interfaces, washed once with G-PBS, resuspended and counted. Viable cells were enumerated by Trypan Blue exclusion. The cell suspension was prepared with 0.4% Trypan Blue (Gibco) and allowed to settle on ice for 3 min after which cells were counted in a haemocytometer. The level of monocyte contamination of microglial samples was assessed on the basis of erythrocyte numbers and the ratio between erythrocytes and monocytes in peripheral blood (1×10^4 erythrocytes for 1 monocyte).

3.2.4. Immunocytochemistry of cytopins of adult human microglia.

Cytopins represent a rapid method for assessment of the expression of cell-specific antigens and the level of contamination of purified cells with other cell types. For cytopins, cell numbers were adjusted to 5×10^4 per 300 μ l with G-PBS and centrifuged at 1,100 rpm for 5 min onto Polysine slides (Merck) in a cytocentrifuge (Shandon, Basingstoke, UK). After air-drying cytopins were fixed and incubated with the primary antibody overnight at 4°C (~16h). The following day cytopins were stained using a three step avidin-biotin method with DAB as the chromogen, dehydrated in ethanol-xylene and mounted in DPX (Chapter II, Section 2.2.5). In order to ensure adequate cell permeabilisation three fixation protocols were tested with the panel of NF- κ B and marker antibodies: (i) absolute methanol, -20°C, 5 min, (ii) 4% paraformaldehyde, RT, 10 min, followed by 5% acetic acid/ethanol, -20°C, 5 min, and (iii) formalin, RT, 10 min followed by absolute ethanol, RT, 2 min. With all three protocols similar staining patterns and the intensity of staining were obtained for all antibodies used in this study. An additional permeabilisation step with 0.05% Triton X-100 (Sigma) in PBS for 10 min at RT did not improve immunocytochemical staining, indicating that methanol is the fixative of choice which gives a sufficient permeabilisation and antigen preservation in all three cell compartments, plasma membrane, cytoplasm and nucleus. In some experiments cytopins

were lightly counterstained with haematoxylin or nuclear fast red for 20 sec and 2 min, respectively. All antibodies were used at the same concentration as those for single immunocytochemical staining of tissue sections. Data for marker antibodies are given in Chapter II, Table 2.2 whereas NF- κ B antibodies are listed in **Table 3.2**.

3.2.5. The preparation of nuclear protein extracts.

The preparation of nuclear protein extracts from 2 samples each of normal control white matter, NAWM and active MS plaques was performed using a modified method of Sonnenberg *et al.* (1989). 1cm³ snap-frozen tissue blocks were thawed and gently syringe homogenised in buffer A (15mM Tris-HCl, pH7.9, 5mM EDTA, 1mM DTT, 0.5mM PMSF, 1mM MgCl₂ and 0.6M sucrose). The nuclear pellet was formed by brief centrifugation at 2000g in a fixed-angle centrifuge (J2-21, Beckman, High Wycombe, UK). The molarity of sucrose in the buffer A prevented sedimentation of cell debris and myelin. The pellet was washed in buffer B (10mM Hepes, pH7.9, 1.5mM MgCl₂, 10mM KCl, 1mM DTT and 0.5mM PMSF) and collected by centrifugation at 4000g for 10 min. The pellet was resuspended in buffer C (0.5M Hepes, pH7.9, 0.75mM MgCl₂, 0.5mM EDTA, 0.5M KCl, 1mM DTT, 0.5mM PMSF and 12.5% glycerol) and incubated on a rotator for 30 min. After 30 min of salt extraction in buffer C, nuclei were collected by centrifugation at 14,000g for 30 min, and the supernatant was dialysed overnight against buffer D (10mM Tris-HCl, pH7.9, 1mM EDTA, 5mM MgCl₂, 10mM KCl, 1mM DTT, 0.5mM PMSF and 10% glycerol). The entire procedure was carried out at 4°C. The protein concentration in the samples was determined by the method of Lowry (Chapter IV, Section 4.2.6)

3.2.6. κ B-oligonucleotide probe labelling.

A double stranded DNA oligonucleotide containing a consensus κ B site (Promega,

Southampton, UK) was 5'-end labelled with [γ - 32 P] ATP (Amersham, Chalfont, UK) according to the manufacturer's protocol (Promega technical bulletin No. 110). The phosphorylation reaction was assembled in a sterile microfuge tube by adding 2 μ l of kB-oligonucleotide, 1 μ l T4 polynucleotide kinase 10X buffer, 1 μ l of [γ - 32 P] ATP (3000 Ci/mM at 10mCi/ml), 5 μ l of nuclease-free water and 1 μ l of T4 polynucleotide kinase (5-10U/ μ l, Promega) in this order and incubated at 37°C for 10 min. The reaction was stopped by adding 1 μ l of 0.5M EDTA and made up to 100 μ l with 89 μ l of TE buffer (10mM Tris-HCl, pH8.0 and 1mM EDTA).

The percentage of label incorporation was determined by blotting 1 μ l of the labelled primer onto each of four Whatman DE81 filters. Filters were air-dried following which two filters were washed in 50ml of 0.5M Na₂HPO₄ to remove unincorporated label and air-dried again. After all filters were placed in individual vials and appropriate scintillation fluid was added, all samples were counted in a scintillation counter. The average cpm for the total and incorporated filters was calculated from the formula: % incorporation = cpm incorporated/cpm total x 100. On average 60% or more of the radioactivity was incorporated in the 5' end-labelling reaction.

The unincorporated radionucleotides were removed on Microspin G-25 columns (Pharmacia, Chalfont, UK). Columns were placed in sterile microfuge tubes and pre-spun at 735g in a microfuge (Eppendorf 5415C) for 1 min. After transferring the columns into new sterile microfuge tube, 50 μ l samples were carefully applied to the centre of the angled surface of the compacted resin bed and centrifuged at 735g for 2min. The purified sample was collected in the bottom of the support tube and stored at 4°C until further use.

3.2.7. DNA-binding shift assay.

The DNA-shift assay utilises a radio-labelled synthetic oligonucleotide probe

bearing a specific transcription factor-binding site and is used to examine the presence of transcription factor in nuclear protein extracts. The binding reactions were assembled in sterile microfuge tubes in 5x gel shift binding buffer (10mM Hepes, pH7.9, 50mM KCl, 0.2mM EDTA, 2.5mM DTT, 10% glycerol, 0.05% NP-40 and 0.05mg/ml poly(dI-dC) poly(dI-dC)) with 10 μ g or 20 μ g of protein extract per reaction. HeLa cells nuclear extracts (Promega) were used as a positive control at 5 μ g and 10 μ g protein per reaction. The binding reactions were incubated for 10 min at RT and 1 μ l (3.5fM) of ³²P-labelled NF- κ B consensus oligonucleotide was added to each tube and incubated for a further 20 min at RT. Gel loading 10X buffer (1 μ l per reaction, Novex, Frankfurt, Germany) was added to negative controls only and complexes were resolved on a pre-cast 6% (w/v) native polyacrylamide gel (Novex) at 100mV until the dye front reached the last quarter of the gel (~45 min). Gels were exposed to autoradiography films (Kodak SO-163, Sigma) overnight at -70°C and developed using standard methods.

3.3. Results.

3.3.1. Activated RelA immunolocalises in microglial nuclei in normal control white matter.

In normal control white matter anti-RelA-NLS immunoreactivity was predominantly detected in the small irregular nuclei characteristic of resting microglia (**Fig. 3.1b**) which was confirmed by double staining with the macrophage marker EBM-11 (**Table 3.3**). The inhibitory protein I κ B α immunolocalised in oligodendrocyte and astrocyte nuclei as shown by the double staining with 14E (**Fig. 3.1d, g and h**) and anti-GFAP antibodies whilst microglia primarily exhibited cytoplasmic staining (**Fig. 3.1d**). The polyclonal c-Rel (**Fig. 3.1c**), RelA and p50 antibodies immunostained microglial cytoplasm. In addition, fine punctate c-Rel staining was observed in the nuclei of some glial cell in the brain parenchyma and in smooth muscle cell nuclei in blood vessel walls.

Normal rabbit serum applied at the same immunoglobulin concentration as the polyclonal reagents gave only weak staining of small numbers of microglia in white matter and macrophages in plaques, and control mouse ascites fluid applied at the same immunoglobulin concentration as the monoclonal RelA-NLS gave no staining. The pre-incubation of I κ B α (**Fig. 3.1f**), RelA and c-Rel antibodies with specific peptides abolished staining in both normal control and MS sections.

3.3.2. NF- κ B is activated in reactive glia in NAWM surrounding active plaques.

In white matter remote from lesions, or surrounding chronic plaques, numbers and distribution of cell nuclei immunopositive for RelA-NLS and I κ B α were comparable to that in normal controls. However, in white matter adjacent to demyelinating plaque borders, strong I κ B α staining was visualised in microglial cytoplasm (**Fig. 3.2f**) whilst RelA-NLS showed strong staining of microglial and astrocyte nuclei (**Fig. 3.2e**). As in normal control white matter, p50 and c-Rel were confined to microglial cytoplasm.

Perivascular inflammatory infiltrates were characterised by punctate staining of lymphocyte nuclei with c-Rel (**Fig. 3.2b**) whereas p50, RelA (**Fig. 3.2a**) and I κ B α (**Fig. 3.2c**) were found predominantly in the cytoplasm.

3.3.3. NF- κ B subunits and I κ B α co-localise in macrophage nuclei in active MS lesions.

Immunoreactivity of all three NF- κ B subunits and I κ B α was increased in the nuclei of macrophages in actively demyelinating plaques (**Table 3.3**), and in most myelin-laden macrophages a narrow rim of cytoplasm immediately adjacent to the plasma membrane was also stained (**Fig. 3.3c** and **3.4a** and **b**). In addition to foamy macrophages nuclear RelA was found in hypertrophic astrocytes in the parenchyma of some active lesions (**Fig. 3.4b**), whereas I κ B α was predominantly localised in astrocyte cytoplasm (**Fig. 3.3d** and **3.4d**). Strong NF- κ B immunoreactivity was visualised within mononuclear cells in perivascular inflammatory infiltrates. From observations on serial sections, macrophages showed nuclear NF- κ B and I κ B α whilst immunolocalisation of NF- κ B subunits in lymphocytes was similar to that in MS white matter with only c-Rel being found in nuclei. In addition, groups of anti-RelA-NLS-positive mononuclear cells were visualised in the parenchyma of active plaques (**Fig. 3.5d**). As these cells were also CD2-positive (**Fig. 3.5e**) they probably represent lymphocytes migrating into lesions. Immunolocalisation of c-Rel but not other NF- κ B subunits was seen in the nuclei of blood vessel wall cells as punctate staining of numerous large granules in cell nuclei in lesions at all stages of development. In subacute lesions, a shift from macrophage to astrocyte NF- κ B activation (**Fig. 3.5a**) and I κ B α expression (**Fig. 3.5b**) appeared to parallel the reduction of macrophage numbers in the lesion parenchyma. Nevertheless, the remaining macrophages in hypercellular borders of subacute plaques were strongly positive for all of the NF- κ B subunits.

3.3.4. Brain infarcts and tumours.

Brain infarcts and tumours were examined to determine the specificity of the NF- κ B expression patterns in MS lesions. The most numerous NF- κ B-immunoreactive cells within infarcts were macrophages although numerous astrocytes in infarct penumbra were also positive with anti-RelA-NLS (**Fig. 3.6c**, **Table 3.3**). Macrophages were also the major cell type stained with the p50 (**Fig. 3.6f**), c-Rel (**Fig. 3.6e**) and I κ B α antibodies (**Fig. 3.6d**). Tumour cells in both a meningioma and a glioma multiforme were weakly positive for NF- κ B and I κ B α . In contrast, the majority of macrophages infiltrating the tumours were strongly positive with the distribution of individual NF- κ B subunits and I κ B α similar to that in inflammatory infiltrates in MS lesions.

3.3.5. Gel-shift assay reveals no NF- κ B DNA-binding activity in NAWM or plaque.

In order to assess the capacity of nuclear NF- κ B complexes to bind κ B consensus sequence on the DNA, gel shift assays were performed on nuclear protein extracts from normal control white matter, NAWM and plaque (**Fig. 3.7**). No shift was observed with protein extracts from either plaque or white matter even at a protein concentration of 20 μ g per binding reaction. In contrast, 5 μ g and 10 μ g of nuclear extracts from the HeLa cell line showed a characteristic double shift which was abolished by preincubation with 100X molar excess of unlabelled NF- κ B consensus oligonucleotide. However, staining of nuclear pellet cytospins with NF- κ B and I κ B α antibodies revealed the presence of these proteins in isolated glial nuclei. It can be estimated that protein levels of NF- κ B and I κ B α in the CNS tissue nuclear extracts are lower than those found in 5 μ g of HeLa extract and therefore are below the detection limits of the gel-shift assay.

	NC white matter n=6	MS white matter n=10	Active MS lesions n=10	CNS infarcts n=9
Histopathology	Resting glia and intact myelin.	Reactive and non-reactive glia and apparently intact myelin; some mononuclear cells.	Numerous ORO ⁺ macrophages, perivascular cuffs and demyelination.	Numerous ORO ⁺ macrophages variable mononuclear cell infiltration and no apparent demyelination.
NF-κB RelA-NLS	microglia	microglia, *astrocytes	macrophages, *astrocytes	macrophages, astrocytes
NF-κB p50	---	---	macrophages	macrophages, *astrocytes
NF-κB c-Rel	*microglia, smooth muscle cells	*microglia, lymphocytes, smooth muscle cells	macrophages, lymphocytes, smooth muscle cells	macrophages, lymphocytes, smooth muscle cells
IκBα	astrocytes, oligodendrocytes	astrocytes, oligodendrocytes	macrophages	macrophages

Table 3.3: The expression pattern of NF-κB subunits and IκBα in cell nuclei in normal and MS white matter, active MS plaques and CNS infarcts. Both polyclonal anti-RelA and IκBα antibodies stained the cytoplasm of resting microglia in normal control and MS white matter. In MS lesions and CNS infarcts, the polyclonal RelA exhibited a similar staining pattern to that of RelA-NLS. * denotes a subpopulation of cells. The MS white matter category refers to the white matter surrounding actively demyelination lesions.

Figure 3.1: Immunocytochemical staining of neighbouring sections of normal control periventricular white matter. (a) Haematoxylin staining of glial cell nuclei; (b) anti-RelA-NLS staining of microglia nuclei; (c) anti-c-Rel staining of microglial cytoplasm; (d) oligodendrocyte and astrocyte nuclei and microglial cytoplasm stained with anti-I κ B α and (e) cytoplasm of resting microglia stained with the macrophage marker EBM-11. (f) Pre-incubation of anti-I κ B α with antigen completely abolished immunostaining. Double immunofluorescent staining of (g) oligodendrocyte nuclei with anti-I κ B α (FITC) and (h) the nuclear membrane of oligodendrocytes with 14E antibody (TRITC). Magnifications, x512, except (g) and (h) , x1076. No counterstaining.

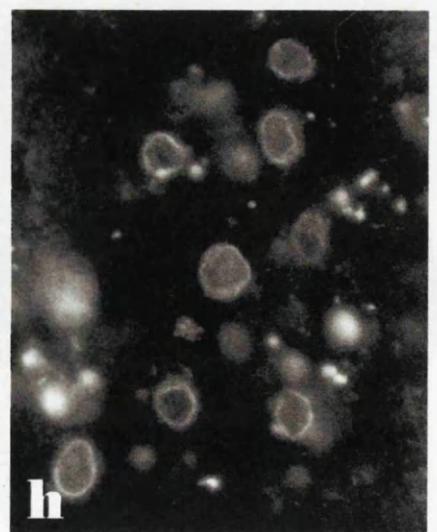
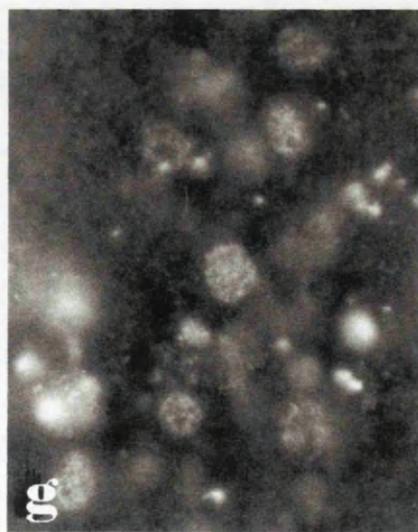
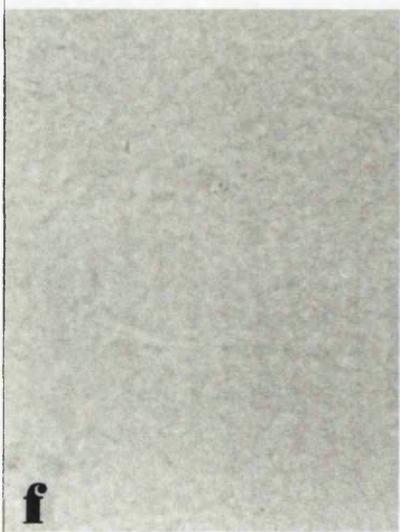
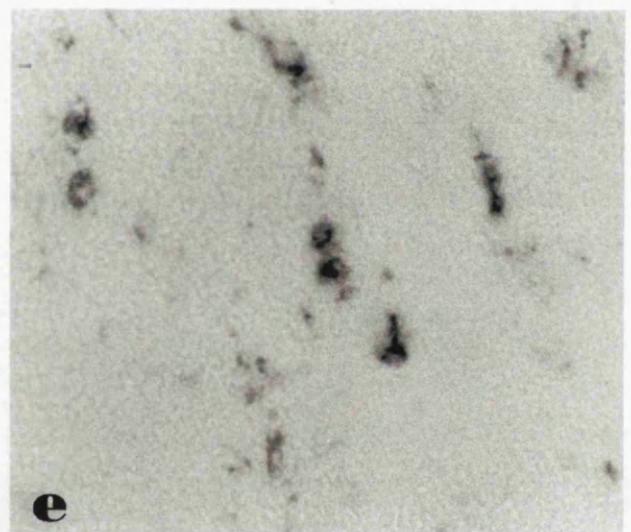
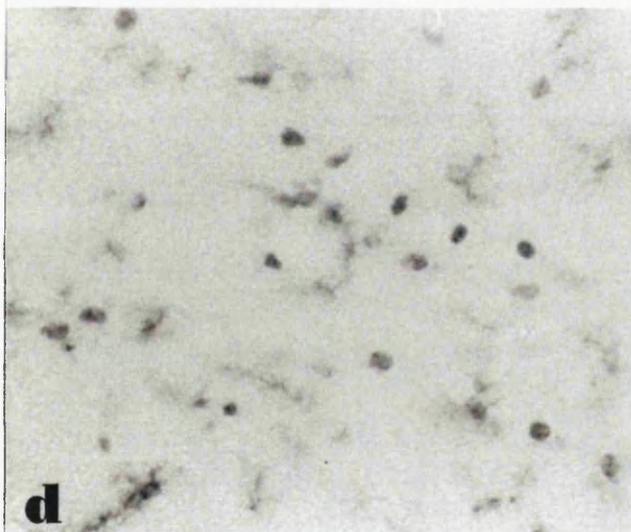
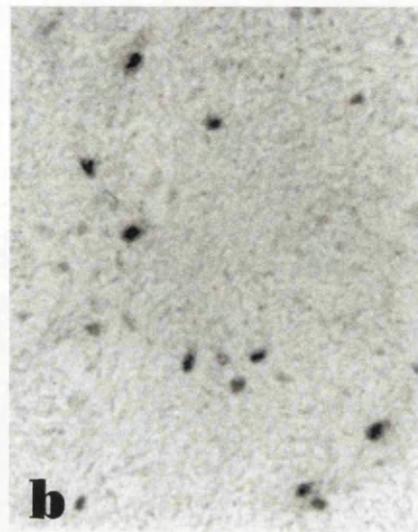
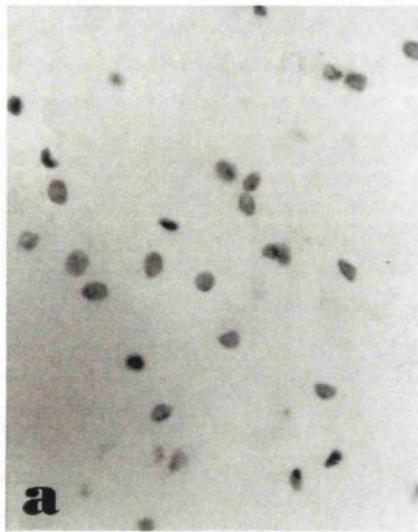


Figure 3.2: Perivascular inflammatory infiltrate in white matter remote from any known MS lesion. Lymphocytes cytoplasm stained with (a) anti-RelA-NLS and (b) punctate staining of lymphocyte nuclei with anti-c-Rel. (c) Lymphocyte cytoplasm stained with anti-I κ B α and (d) lymphocyte plasma membranes stained with anti-CD2 antibody. **Normal-appearing white matter adjacent to an active lesion border.** (e) Microglial nuclei (arrowheads) stained with anti-RelA-NLS and (f) cytoplasm of activated microglia (arrowheads) stained with anti-I κ B α . I κ B α -positive glial nuclei (arrows) are weakly stained and are less numerous in comparison with normal control white matter. All at magnification X512.

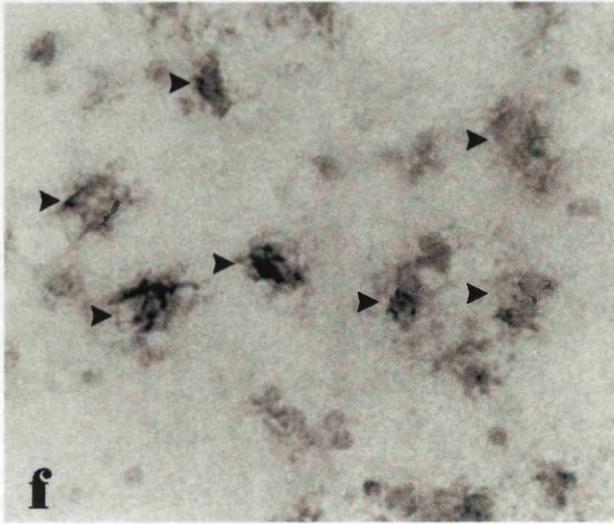
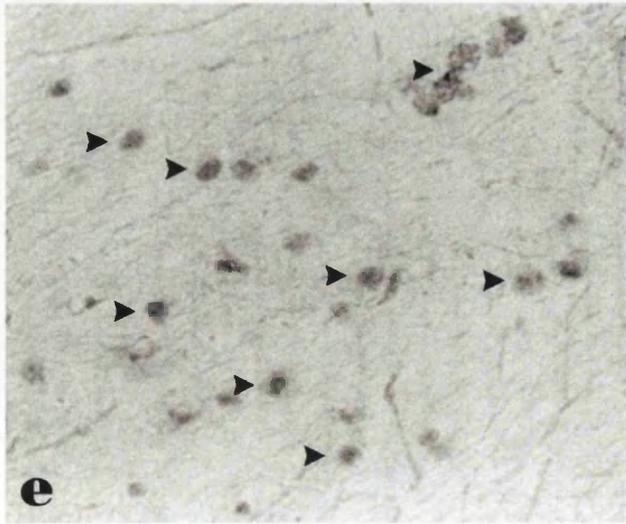
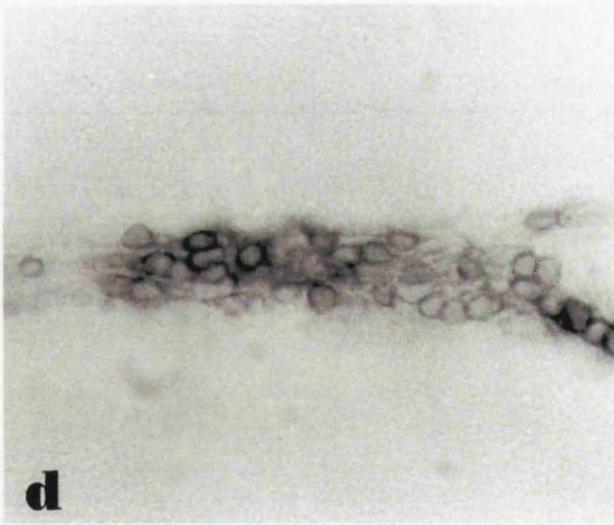
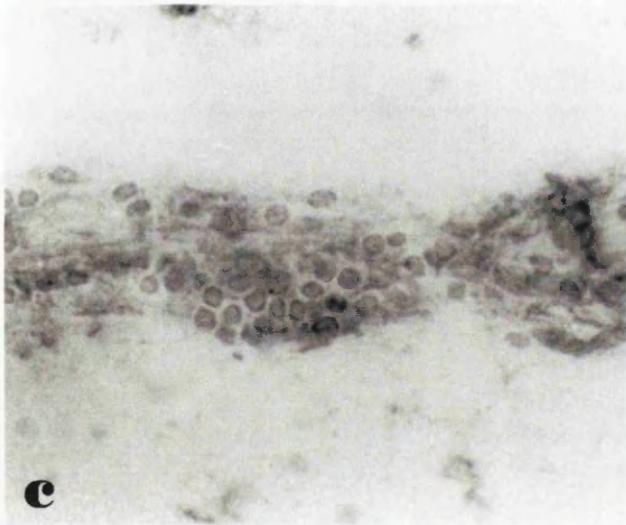
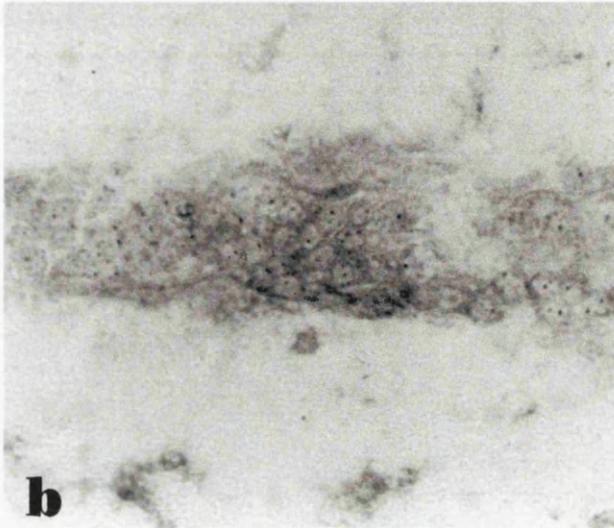
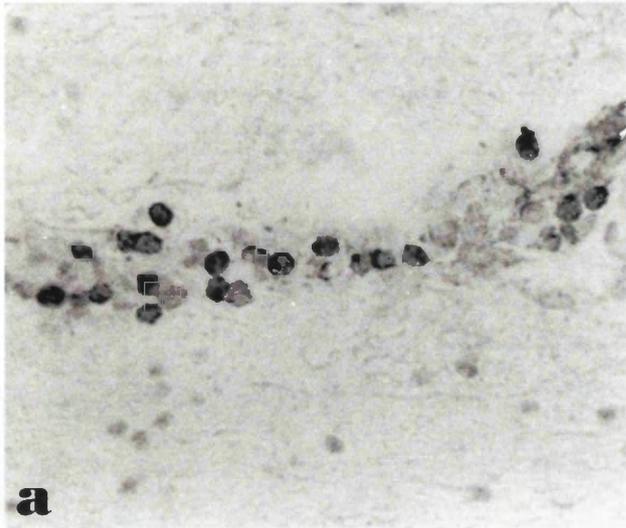


Figure 3.3: Immunolocalisation of NF- κ B and I κ B in actively demyelinating MS lesions. Low power photomicrographs showing a deep ORO-positive border of an active lesion. (a) Macrophages stained with EBM-11 and (b) astrocytes and oligodendrocytes stained with 14E. (c) Macrophage and astrocyte nuclei stained with anti-RelA-NLS. (d) Macrophage cytoplasm and nuclei stained with anti-I κ B α . High power photomicrographs of these areas are shown in Fig. 3.4. Double immunofluorescence staining of macrophages in lesion parenchyma with (e) anti-RelA (polyclonal, FITC) and (f) EBM-11 (TRITC). All x32, except (e) and (f) x512.

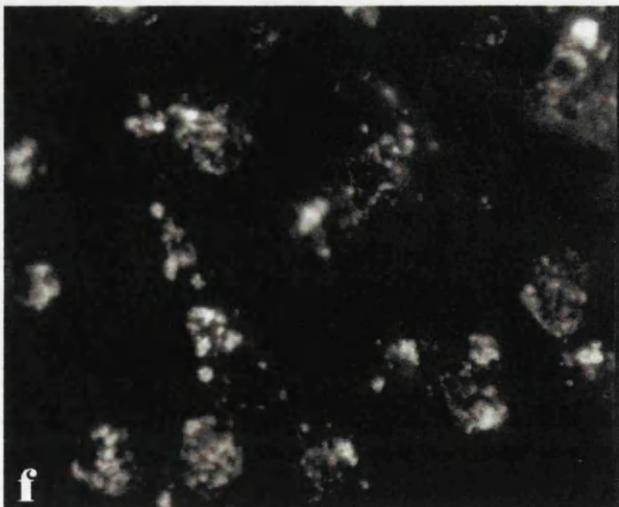
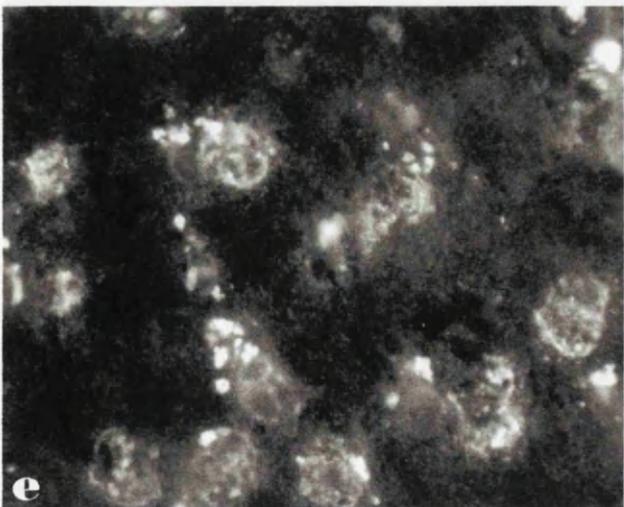
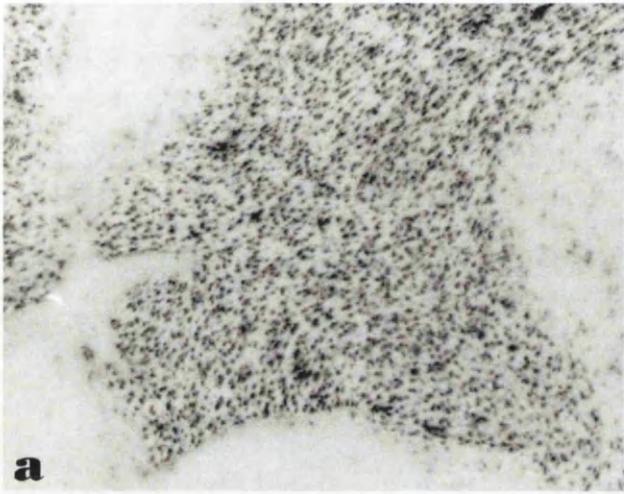


Figure 3.4: Serial sections from an actively demyelinating MS lesion. (a) Macrophage nuclei and cytoplasmic rim adjacent to the plasma membrane stained with RelA-NLS; **(b)** high magnification detail showing a positively stained macrophage nucleus (arrowhead) and cytoplasmic rim and membrane, and a strong astrocyte nucleus (arrow) with weak cytoplasm and processes. The nuclei and cytoplasmic rim of foamy macrophages in a MS lesion stained with **(c)** anti-p50 and **(d)** anti-I κ B α antibodies. **(e)** ORO-positive macrophages and **(f)** macrophage cytoplasm stained with EBM-11. All at magnification X512 except (b) at X1280.

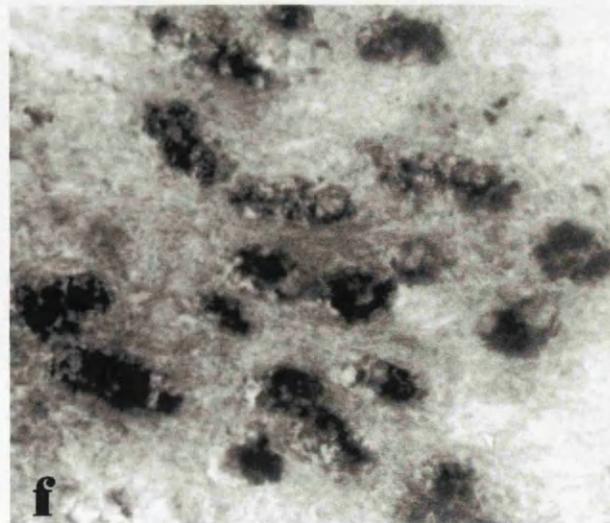
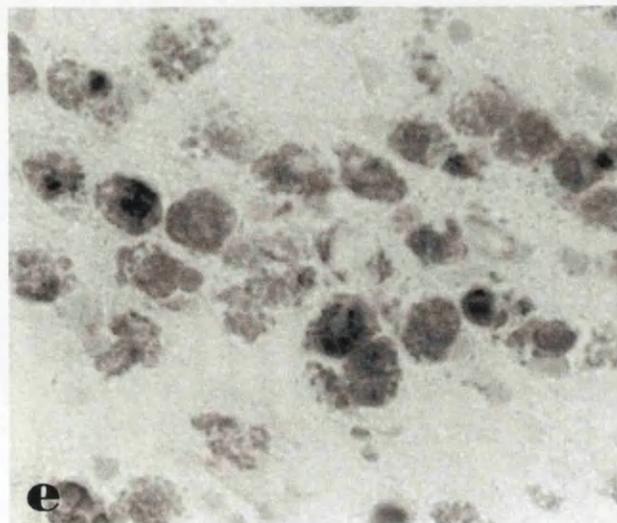
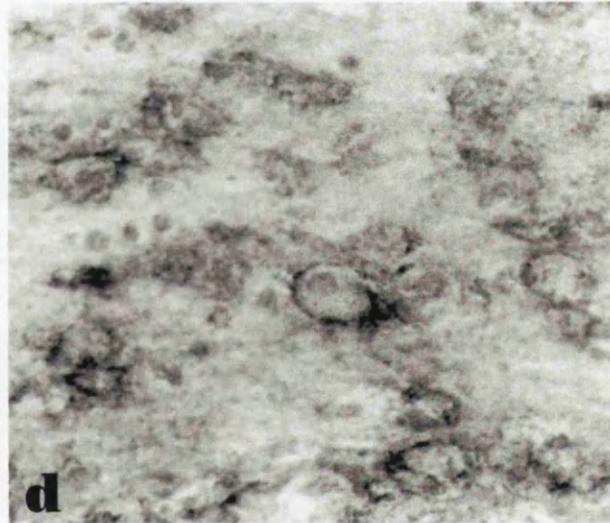
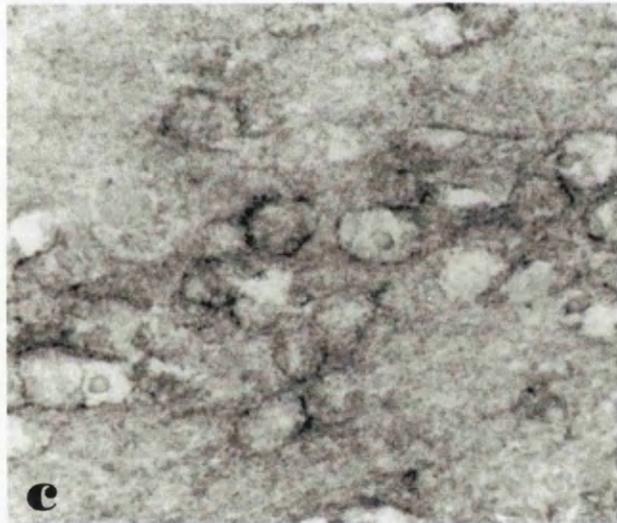
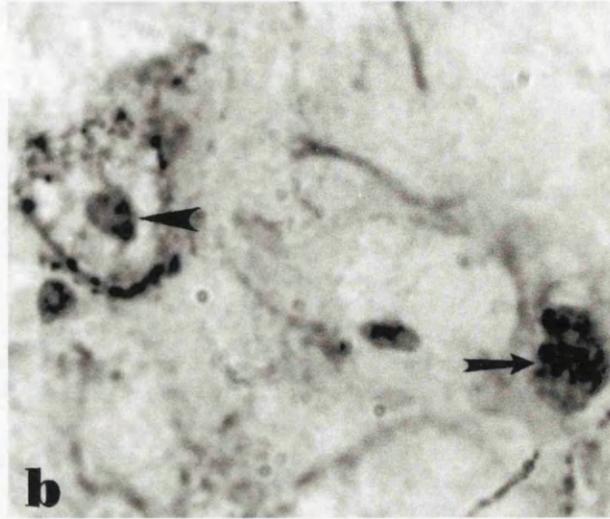
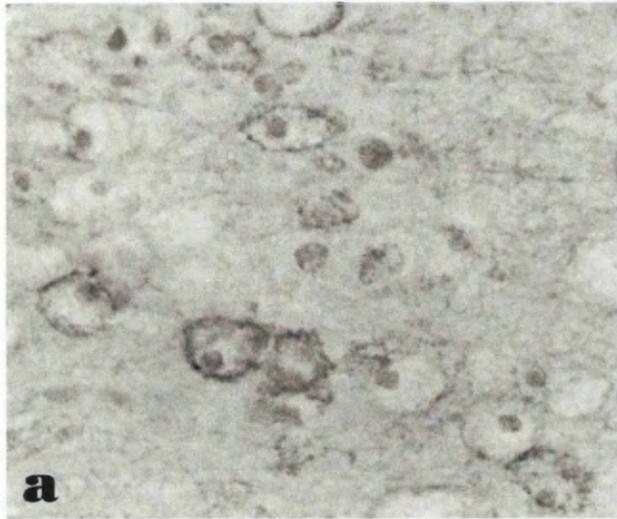


Figure 3.5: Demyelinated parenchyma of a subacute lesion. Astrocytes stained with (a) anti-RelA-NLS; (b) anti-I κ B α and (c) anti-GFAP antibodies. Mononuclear cells in the parenchyma stained with (d) anti-RelA-NLS and (e) anti-CD2. All at magnification X512.

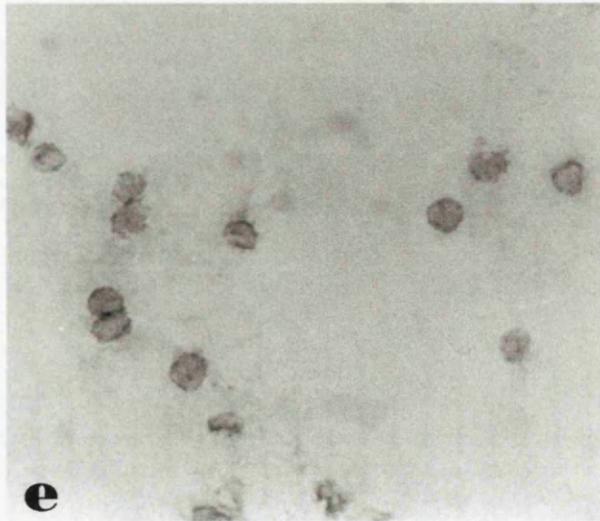
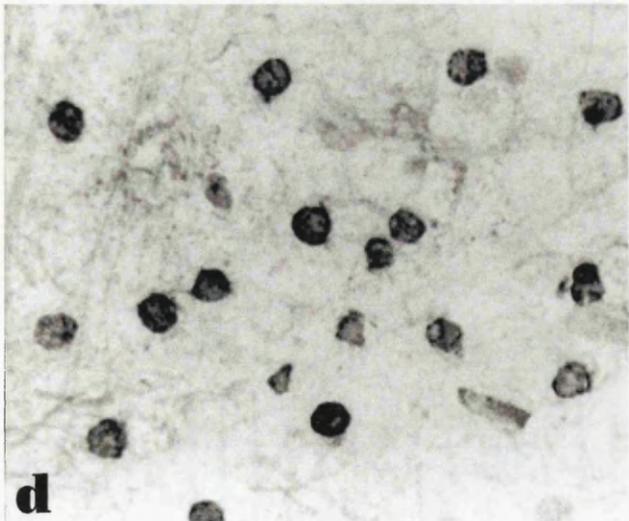
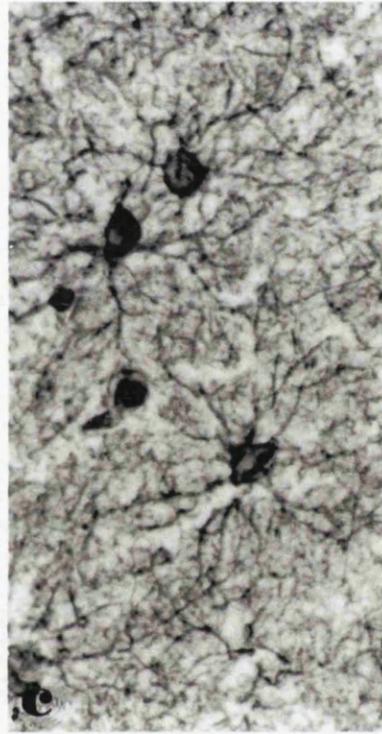
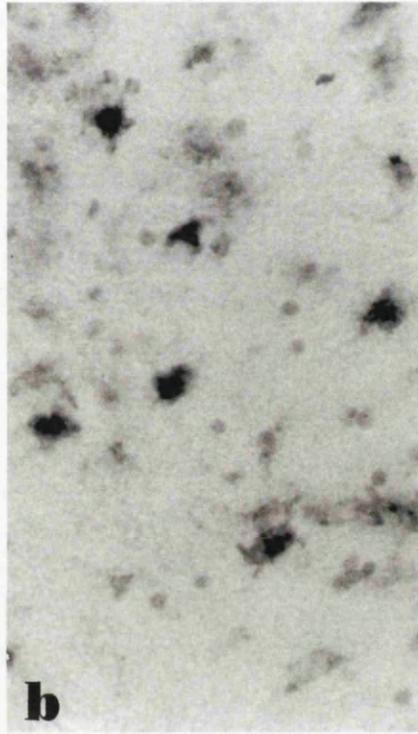
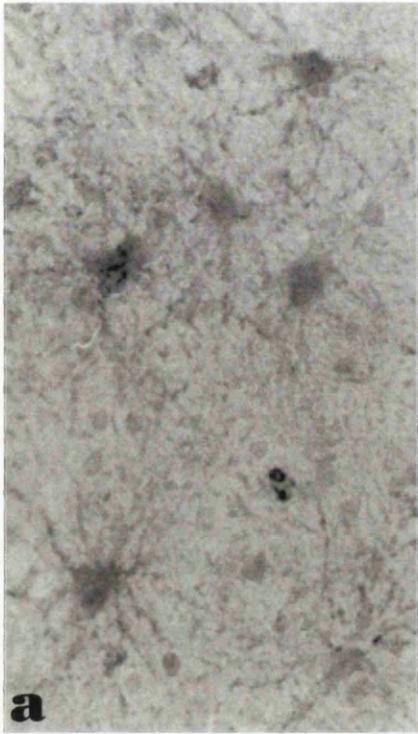
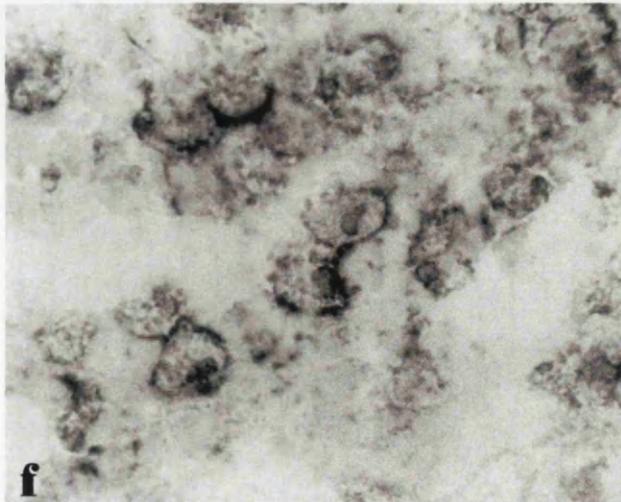
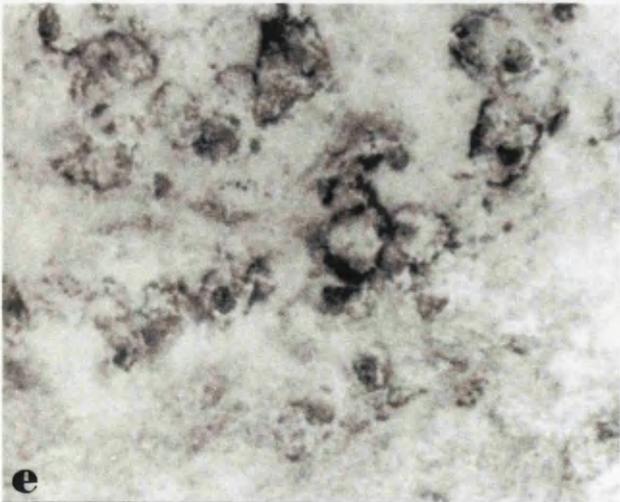
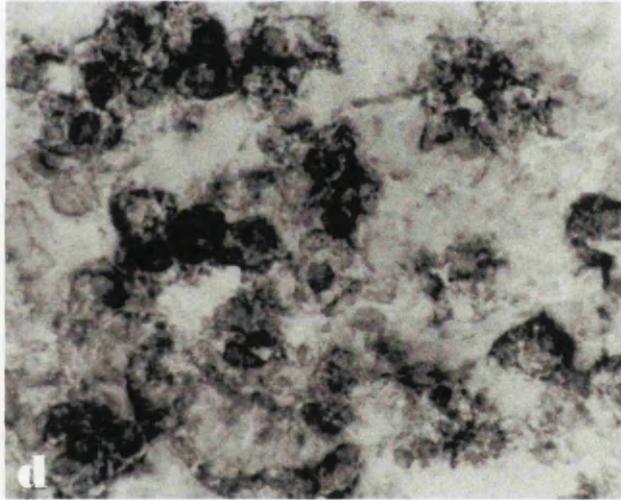
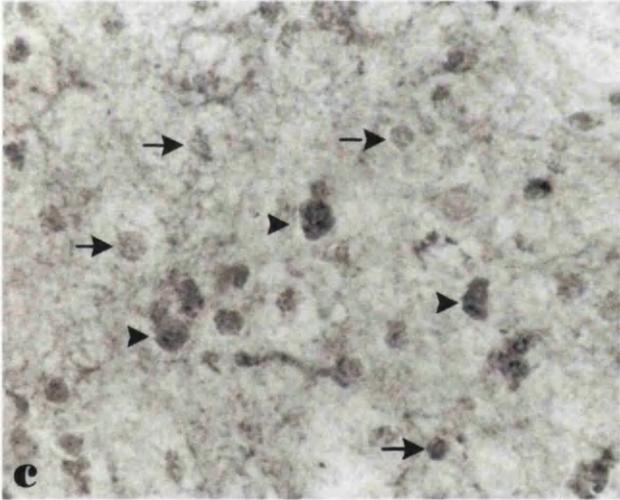
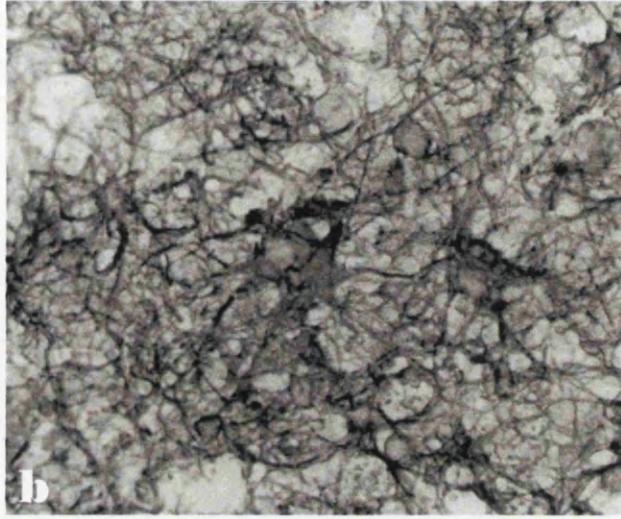
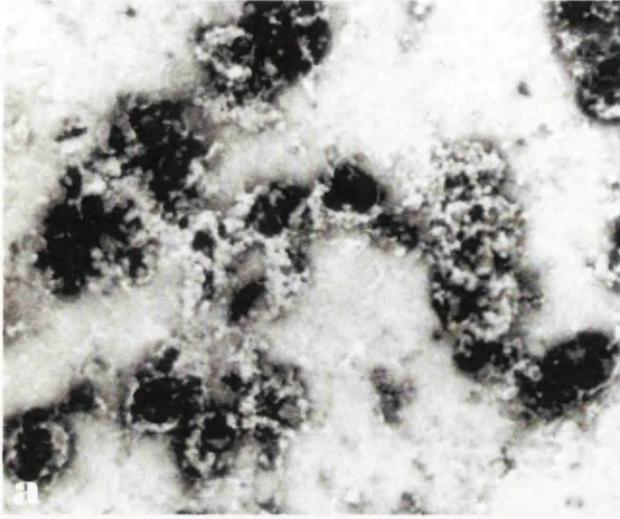


Figure 3.6: A recent brain infarct from a MS case. (a) Macrophage cytoplasm stained with EBM-11 and **(b)** astrocyte cytoplasm and processes stained with anti-GFAP. **(c)** Macrophage (arrows) and astrocyte (arrowheads) nuclei stained with anti-RelA-NLS. Macrophage nuclei and cytoplasm stained with **(d)** anti-I κ B α , **(e)** anti-c-Rel and **(f)** anti-p50. All at magnification X512.



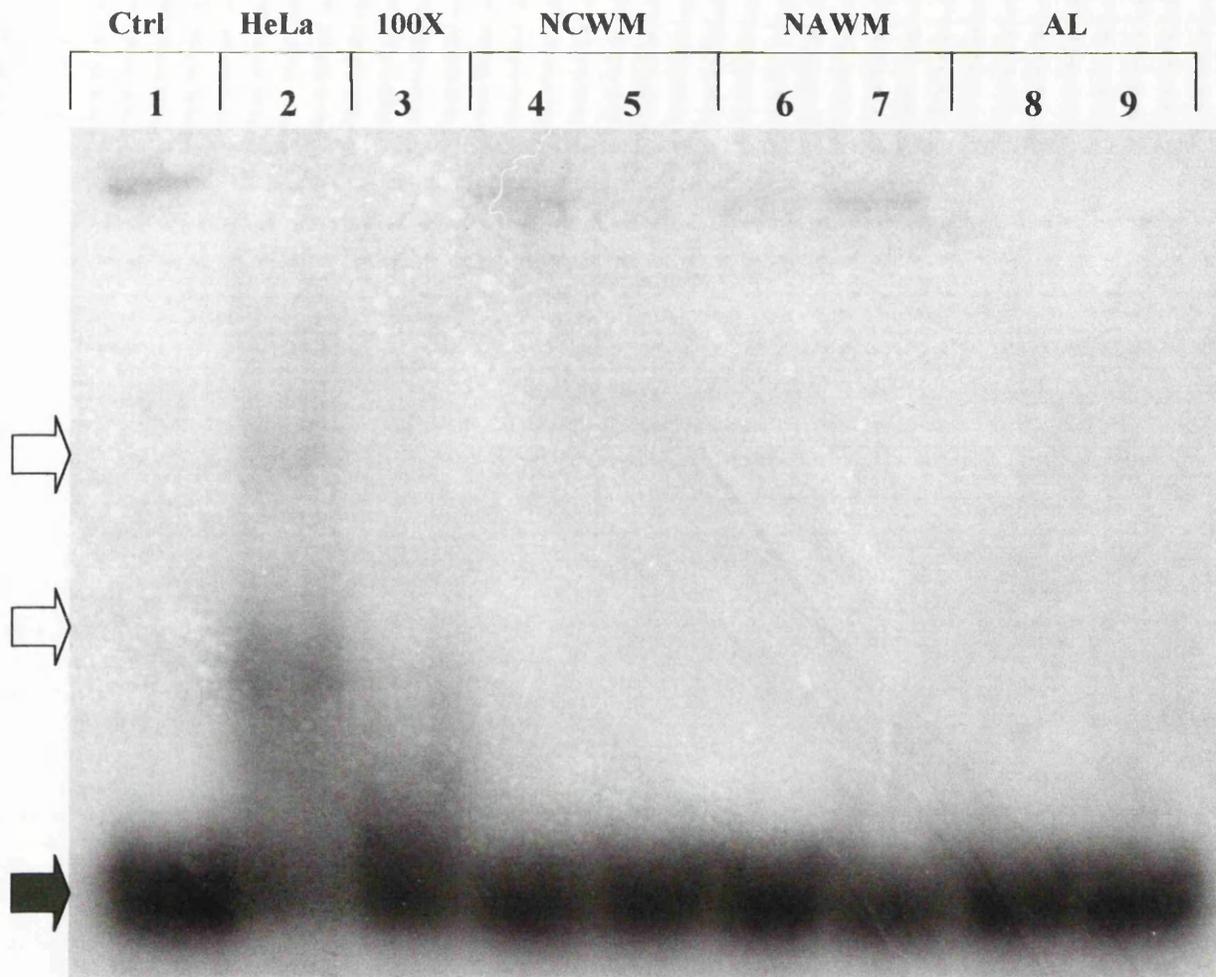


Figure 3.7: Characterisation of the DNA-binding activity in the nuclear protein extracts from NC white matter, NAWM and active MS lesion investigated using the DNA-shift assay. Oligonucleotide probe incubated in the absence of protein extract migrated near the bottom of the gel (lane 1). Lane 2 shows a characteristic double shift obtained with the 10 μ g of HeLa nuclear protein extract which was abolished by pre-incubation with 100X molar excess of unlabelled probe (shown in lane 3). No shift was observed with nuclear protein extracts from normal control (lanes 4 and 5), NAWM (lanes 6 and 7) or active MS lesions (lanes 8 and 9). Open arrows indicate the position of the κ B-specific DNA-binding activity and the filled arrow shows the position of unbound DNA.

3.3.6. Recovery and characterisation of isolated adult human microglia.

A discontinuous Percoll gradient method was used in order to circumvent the problems associated with other isolation techniques which use selective cell adherence or opsonised erythrocytes, resulting in activation of microglia (Woodroffe & Cuzner, 1995). Microglial cells were obtained from three sources: surgical resections within a two hour post-operative period and post-mortem normal control and MS brains within 24h of death. The viable cell recovery was comparable for both surgical and post-mortem material although the total cell yields were 2-fold higher in surgical resection samples than in post-mortem material (Table 3.4). In autopsy material post-mortem delay was the major factor influencing cell recovery with delays of up to 24h giving on average 1×10^6 cell per gram of tissue. However, cell yield was not affected by the cause of death.

Tissue source	Cell yield ($\times 10^6$) per gram of tissue	% cell viability	% monocyte contamination
Surgical resections (n=10)	2.37 ± 1.15	$86.14\% \pm 11.09$	0.04%
Post-mortem normal control (n=5)	1.14 ± 0.87	$75.40\% \pm 22.90$	<0.01%
Post-mortem multiple sclerosis (n=1)	0.82	91.60%	<0.01%

Table 3.4: Average cell yield and viability obtained from surgical resection and post-mortem brain tissue. Data refer only to cells collected from the 1.055 interface.

A reproducible cell separation pattern was obtained on Percoll gradient (Fig. 3.8). The majority of cells were found at the 1.055 interface whereas less than 5% of the total recovered cells were found at the 1.065 interface. Remaining erythrocytes and corpora amylacea remained in the 1.072 layer and the pellet. Staining with the human macrophage marker EBM-11 indicated that 99% of cells from post-mortem and more than 90% of cells

from surgical samples found at 1.055 and 1.065 interface are of macrophage lineage (**Fig. 3.9b** and **e**). Cells from the 1.065 interface also appeared to have stronger stained cell membranes compared to those in the 1.055 interface whilst cells in both layers were weakly HLA-DR and HLA-DQ immunopositive. Staining with 5.2E4, an anti-GFAP antibody, gave only weak background staining similar to that observed with IgG1 isotype control (**Fig. 3.9g**). Small numbers of rounded cells with evenly distributed cytoplasm isolated from surgical samples were stained with 14E, an antibody against oligodendrocyte nuclear membrane protein (Newcombe *et al*, 1991) which immunostains both oligodendrocytes and astrocytes in frozen human brain sections (**Fig. 3.9h**). EBM-11, HLA-DR and CD45 staining of cells collected from the 1.055 interface is characteristic of parenchymal microglia. Stronger anti-CD45 staining, higher cell density and lower numbers of cells from the 1.065 interface indicate that these cells could represent a population of brain macrophages or blood monocytes. 14E-positive cells in the 1.055 layers may be oligodendrocytes although astrocytes, regardless of the absence of GFAP staining, can not be excluded at this stage. When the intensity of staining was compared, cells isolated from post-mortem material were markedly stronger stained with all macrophage markers. Post-mortem microglia were larger and had more abundant cytoplasm with numerous lysosomes as shown with EBM-11 staining (**Fig. 3.9e**). Such morphological differences may be a consequence of microglial activation due to the protracted exposure to hypoxic conditions during the agonal stage and post-mortem delay (**Fig. 3.9a** and **c**). Microglial cells isolated from NAWM of an MS brain morphologically resembled those isolated from normal control post-mortem material.

3.3.7. Immunolocalisation of *NF- κ B* subunits and *I κ B α* in isolated adult human microglia.

In order to extend the immunocytochemical findings of constitutive *NF- κ B* activity in resting white matter microglia, cytopins of freshly isolated adult human microglia were

stained with the panel of NF- κ B and I κ B antibodies. All three NF- κ B subunits RelA (**Fig. 3.10a** and **c**), c-Rel (**Fig. 3.10b**) and p50 were immunolocalised in nuclei of EBM-11-positive microglia. Activation of NF- κ B, probably as a result of the isolation procedure, was confirmed by monoclonal anti-RelA-NLS antibody which strongly stained microglial nuclei but not cytoplasm (**Fig. 3.10a**). The inhibitory proteins I κ B α (**Fig. 3.10d**) and I κ B β predominantly co-localised with the NF- κ B subunits in the nuclei of microglia although in a small percentage of cells they were evenly distributed between cytoplasm and nucleus. There were no significant differences in NF- κ B localisation between microglia from different sources.

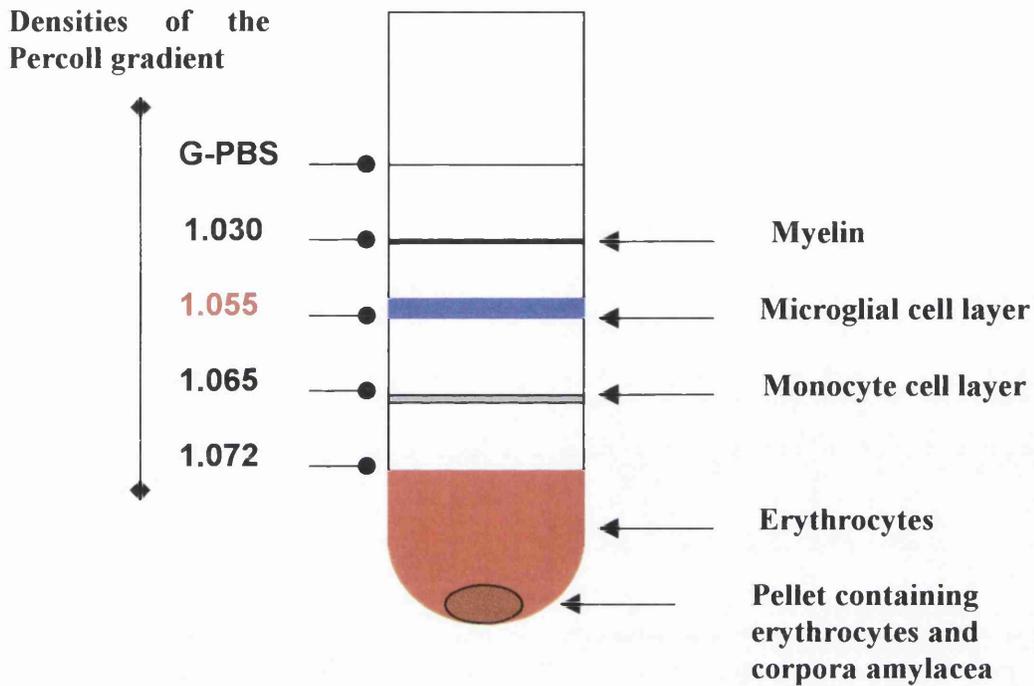


Figure 3.8: Cell separation on a discontinuous Percoll gradient. The major cell layer comprised predominantly of microglia is retained at the 1.055 density interface. Smaller number of cells that could represent brain macrophages and peripheral blood monocytes are found at the 1.065 interface whereas erythrocytes and corpora amylacea rest in the 1.072 density layer and the pellet.

Figure 3.9: Characterisation of adult human microglia isolated from surgical and post-mortem material. (a) Microglial cells in white matter from an epilepsy case stained with EBM-11 antibody and cytopins of adult human microglia isolated from an epilepsy case stained with (b) EBM-11 and (c) anti-CD45 antibodies. (d) Microglial cells in white matter from a post-mortem normal control case stained with EBM-11 antibody and cytopins of adult human microglia isolated from the same post-mortem case stained with (e) EBM-11 and (f) anti-CD45 antibodies. Cytopins of cells isolated from an epilepsy case stained with (g) anti-GFAP, (h) 14E and (i) anti-GC antibodies. Only haematoxylin-stained nuclei are visible in cells stained with anti-GFAP and anti-GC antibodies. All x1280 except (a) and (d) at x512. Cells shown in (g), (h) and (i) are counterstained in haematoxylin.

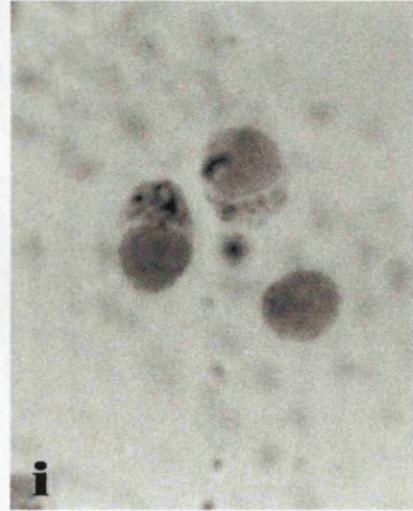
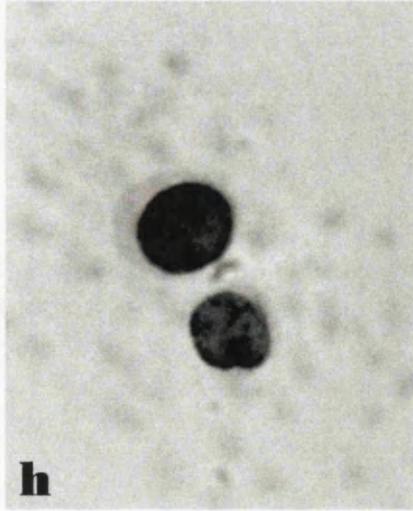
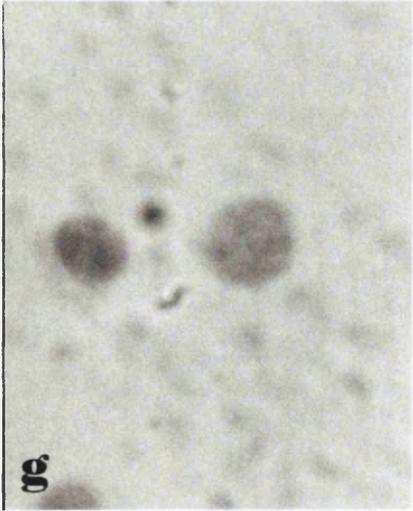
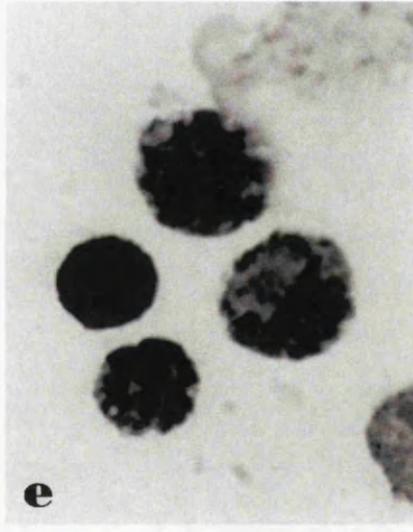
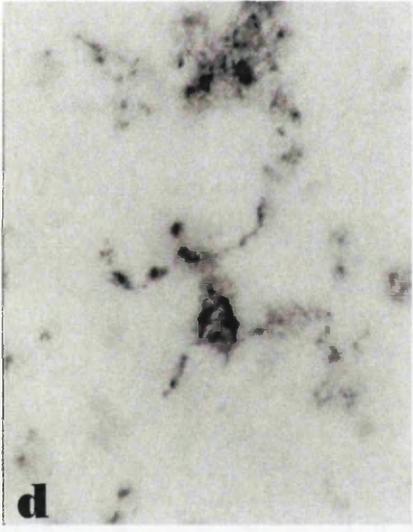
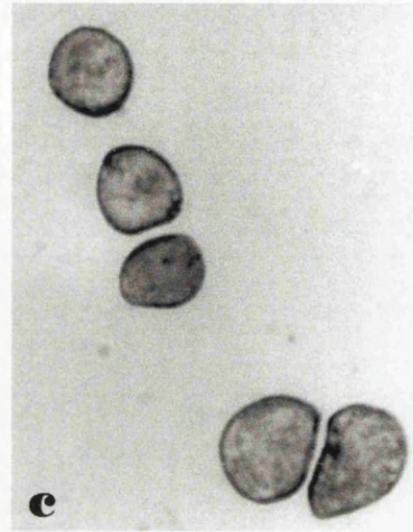
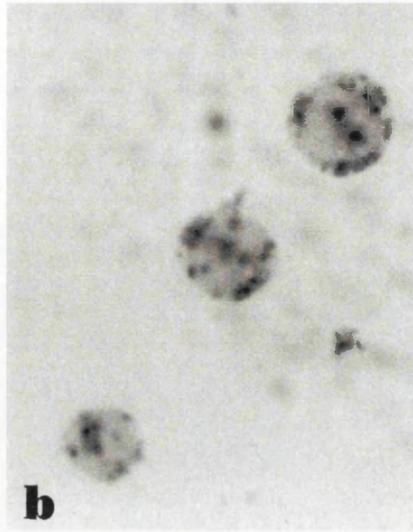
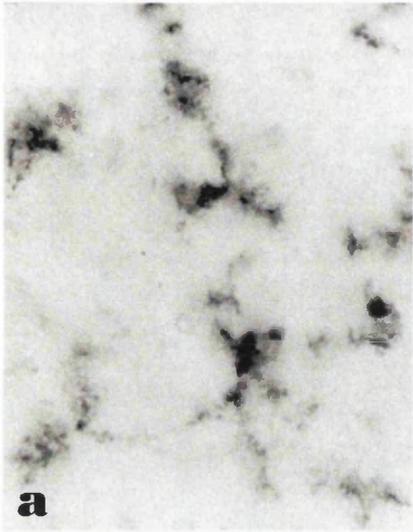
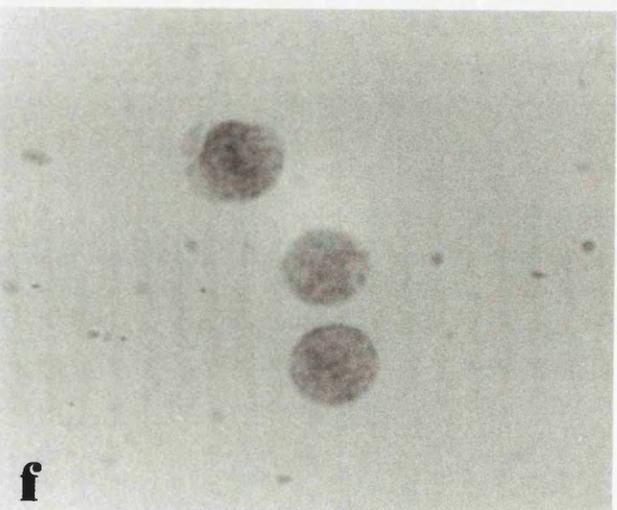
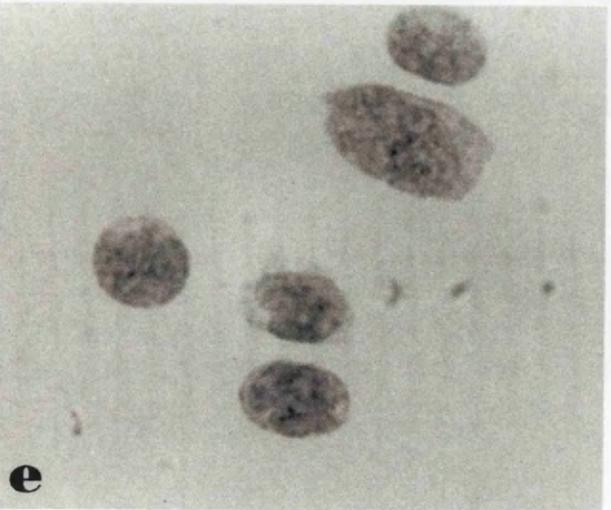
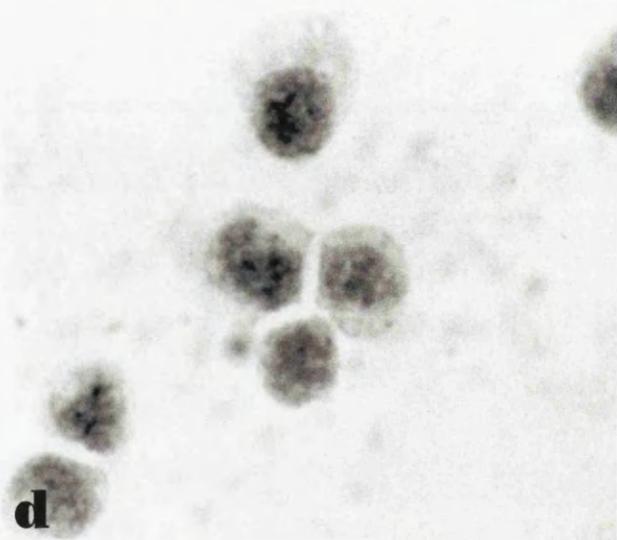
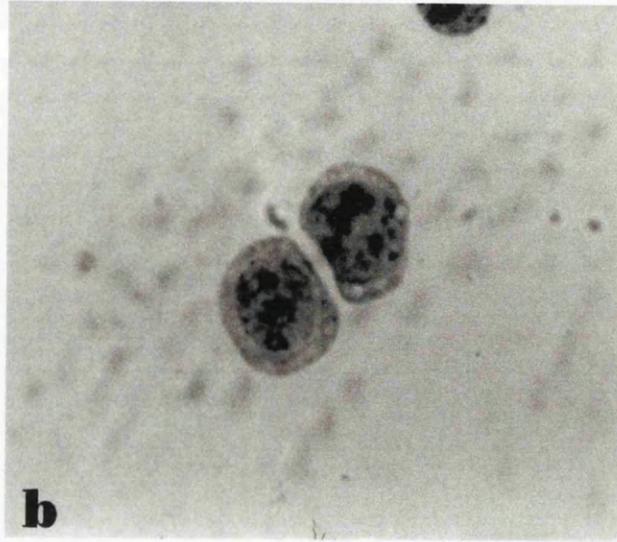
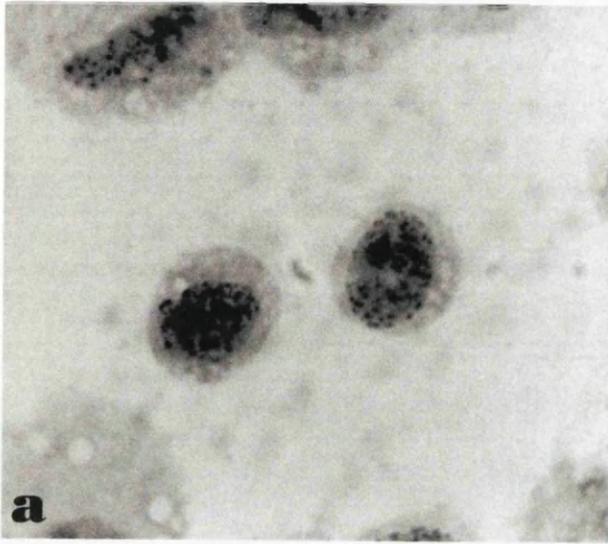


Figure 3.10: Immunolocalisation of NF- κ B subunits and I κ B in adult human microglia. Nuclei of cells isolated from surgical resection material stained with (a) anti-RelA-NLS, (b) anti-c-Rel, (c) anti-RelA (polyclonal) and (d) anti-I κ B α . Preincubation of (e) anti-RelA (polyclonal) and (f) anti-I κ B α antibodies with peptide antigens diminished the staining intensity. All x1280, no counterstaining.



3.4. Discussion.

3.4.1. Summary of findings.

In the present study on inflammatory CNS disease, a nuclear localisation of activated NF- κ B was found to correlate with inflammatory changes and MS lesion activity. In demyelinating lesions strong nuclear staining for all NF- κ B subunits was visualised in the majority of macrophages and a smaller proportion of reactive astrocytes whilst in perivascular inflammatory infiltrates c-Rel was the only NF- κ B subunit found in the nuclei of lymphocytes. I κ B α localisation was similar to that of NF- κ B in MS lesions and CNS infarcts. In control white matter RelA was seen in microglial nuclei whereas I κ B α was immunolocalised in cytoplasm. Immunostaining of isolated adult human microglia showed that both NF- κ B and I κ B co-localise in the nuclei, similar to the findings in microglial cells *in situ*.

3.4.2. Activators and roles of NF- κ B in inflammatory demyelination.

A correlation between lesion activity and the nuclear localisation of the NF- κ B suggests a role for inflammatory mediators in the NF- κ B activation. Pro-inflammatory cytokines IL-1 and TNF α , which have been shown to activate NF- κ B in glial cells *in vitro* (Moynagh *et al*, 1994, Diehl *et al*, 1995), are readily detectable in macrophages and astrocytes in actively demyelinating lesions (Woodroffe & Cuzner, 1993, Brosnan *et al*, 1995). Activation stimuli may also come from ROI generated by the membrane-associated respiratory burst oxidase in microglia and macrophages (Kaul & Forman, 1996). This is supported by the immunolocalisation of RelA in cytoplasm adjacent to the macrophage plasma membrane in active MS lesions and brain infarcts. NF- κ B is sensitive to changes in cellular redox status and stimulation of cells with ROI, especially hydrogen peroxide, results in NF- κ B activation (Schreck *et al*, 1991). However, using the same monoclonal

RelA-NLS antibody, Kaltschmidt *et al.* (1997) demonstrated that over-stimulation of granular cell neurons with ROI inhibited nuclear translocation and resulted in cytoplasmic accumulation of activated RelA. Covalent modification of amino acids is a well-documented effect of ROI (Pacifci & Davies, 1990) and oxidative damage of RelA at supraoptimal doses could inhibit its activity. The observation that NF- κ B was also localised in macrophage nuclei in active MS lesions and the similar distribution of I κ B α supports NF- κ B activation rather than inhibition.

3.4.3. Possible roles of myelin phagocytosis and oxidation in activation of NF- κ B in macrophages.

Phagocytosis and degradation of myelin by macrophages are a hallmark of MS (Cuzner, 1997). As strong NF- κ B staining was observed in myelin-laden foamy macrophages, myelin phagocytosis or the products of lipid peroxidation could be an activating stimulus for NF- κ B. In rat macrophages, receptor-mediated phagocytosis of myelin stimulates the production of TNF α (van der Laan *et al.*, 1996), a NF- κ B-regulated cytokine. In atherosclerosis foamy macrophages are formed through the uptake of oxidised low density lipoprotein. Activation of NF- κ B through the action of oxidised lipoproteins in macrophages and smooth muscle cells is an important step in initiation and development of atherosclerotic lesions (Brand *et al.*, 1997). This can be extrapolated to the pathogenesis of MS as early lesions are characterised by myelin- and oxidised LDL-containing macrophages surrounded by apparently intact myelin (Li *et al.*, 1993, Newcombe *et al.*, 1994). Phagocytosis of myelin in these lesions could activate NF- κ B in macrophages resulting in upregulation of NF- κ B-controlled adhesion molecules, cytokines and chemokines which could attract inflammatory cells to the site leading to further amplification of the inflammatory process and the onset of overt demyelination. Furthermore, predominance of NF- κ B-positive macrophages in CNS infarcts from MS

cases as compared to predominance of astrocytes in cases with degenerative disease (Teraï *et al*, 1996b) may result from influence of inflammatory mediators on NF- κ B activation in macrophages in MS lesions.

3.4.4. Distinct pattern of the NF- κ B activation in lymphocytes in perivascular inflammatory infiltrates.

In the present study p50 and RelA were found predominantly in lymphocyte cytoplasm in perivascular inflammatory infiltrates regardless of the type of pathology examined, which indicates that these subunits are not components of activated NF- κ B complexes in lymphocytes. The inhibition of p50/RelA complexes could be a common characteristic of mononuclear cells transmigrating into the CNS parenchyma as similar observations were made in EAE (Kaltschmidt *et al*, 1994a) and Sindbis virus encephalitis (Irani *et al*, 1997). However, expression of c-Rel in lymphocyte nuclei indicates that this subunit can be differentially regulated in CNS-infiltrating mononuclear cells. Several lines of evidence suggest that c-Rel is important for lymphocyte proliferation and production of interleukin-3 and granulocyte/macrophage colony stimulating factor (Kontgen *et al*, 1995, Gerondakis *et al*, 1996) and can function as a physiological repressor of RelA (Doerre *et al*, 1993). Therefore nuclear localisation of c-Rel could serve to upregulate the production of a distinct set of NF- κ B-controlled cytokines and as a factor preventing excessive activation of RelA in invading inflammatory cells.

3.4.5. Inhibitory I κ B α co-localises with NF- κ B in macrophage nuclei.

Co-localisation of I κ B α with NF- κ B subunits in macrophage nuclei in MS lesions is an unexpected finding. I κ B α generally resides in cytoplasm but could be subject to passive nuclear uptake due to its small size (Zabel *et al*, 1993). Purified human I κ B *in vitro* rapidly dissociates DNA-bound NF- κ B complexes (Zabel & Baeuerle, 1990). Furthermore,

newly synthesised I κ B α accumulating in the nuclei of IL-1 and TNF α stimulated cells has been found to inhibit NF- κ B-dependent transcription and promote active transport of NF- κ B complexes out of the nuclei (Arenzanaseisdodos *et al*, 1995 and 1997). However, the RelA-NLS antibody used in our study is specific for the sequence in NLS which is revealed when I κ B α is not bound to RelA (Kaltschmidt *et al*, 1995b). This raises the questions of whether RelA in macrophages can be regulated by other I κ B proteins. I κ B α has the highest affinity for RelA but other I κ B species, particularly the beta form, can also bind and regulate RelA containing NF- κ B complexes (Beg *et al*, 1993). In this regard, Suyang and colleagues (Suyang *et al*, 1996) reported nuclear localisation of a hypophosphorylated form of I κ B β synthesised in response to NF- κ B activation which was capable of binding NF- κ B but did not block the RelA-NLS sequence or DNA binding of NF- κ B. Alternatively, the nuclear co-localisation of NF- κ B subunits and I κ B α could also be a consequence of autolytic separation of NF- κ B/I κ B α complexes in post-mortem tissue although this is unlikely as similar findings are shown in isolated human microglia. The complexity and high sensitivity of the NF- κ B/I κ B system makes it difficult to assess the activation state of NF- κ B in a particular cell type although nuclear localisation of its subunits is indicative of an active state.

3.4.6. Constitutive NF- κ B activity may facilitate microglial activation.

Nuclear localisation of RelA supported by the cytoplasmic expression of inhibitory I κ B α in resting white matter microglia indicates that NF- κ B is constitutively activated in this cell type but the functional significance of this activity is not clear. In peritoneal macrophages constitutive NF- κ B activity is thought to be responsible for basal expression of the TNF α gene (Collart *et al*, 1990) while in other cells it appears to serve as a transcriptional repressor of NF- κ B-controlled genes. Activation of microglia is a sensitive

indicator of pathological changes in the CNS and involves upregulation of macrophage-like properties and secretion of inflammatory mediators (Cuzner, 1997). Taking into account the specific role of NF- κ B in macrophage activation (Baeuerle & Henkel, 1994) it may be speculated that RelA, a potent transcriptional activator (Schmitz & Baeuerle, 1991), could facilitate microglial activation and response to a variety of pathological stimuli in CNS.

A surprising observation in normal control white matter was the nuclear localisation of I κ B α in the absence of NF- κ B subunits in oligodendrocytes, the myelin-producing cell type which is depleted in MS lesions. One proposed mechanism of oligodendrocyte damage in MS is the cytotoxic action of TNF α (Selmaj & Raine, 1988). Interestingly, over-expression of I κ B α and inhibition of NF- κ B in certain cell lines has been found to result in a higher susceptibility to TNF α -induced apoptosis (van Antwerp *et al*, 1996). It remains to be elucidated whether I κ B α inhibition of the NF- κ B occurs in human oligodendrocytes and whether it can confer a higher susceptibility to TNF α -induced apoptosis in human oligodendrocytes.

3.4.7. The role of NF- κ B in the progression of MS lesions.

Findings in this study identify activated microglia and macrophages as major cell types expressing NF- κ B in MS lesions, indicating a role for this transcription factor in inflammatory demyelination. The results also reveal a complex cell-specific relationship between NF- κ B and its main inhibitor I κ B α in both normal and pathological human CNS tissue. The central position of NF- κ B in the signalling cascade of many pro-inflammatory mediators makes it a suitable target for manipulation of the inflammatory process in MS through modulation of the NF- κ B/I κ B system within macrophages and other immunoeffector cells.

Chapter IV

4. The expression of plasminogen activators, matrix metalloproteases and endogenous inhibitors in multiple sclerosis: comparison of stages in lesion evolution.

4.1. Introduction.

4.1.1. *Proteolysis in inflammatory demyelination: a role for macrophage-derived enzymes.*

Increased proteolytic activity and degradation of tissue proteins are the major features of an inflammatory process. In inflammation of the CNS, proteolysis plays a pivotal role in cell migration across the BBB through degradation of basement membrane and ECM components (Cuzner & Opdenakker, 1999). Proteins of the myelin membrane are also vulnerable to proteolytic attack and incubation of myelin in buffer alone will activate endogenous myelin proteinases and result in significant loss of MBP and MOG (Cuzner & Norton, 1996). Although myelin-associated proteases may contribute to demyelination during the course of MS, it is exogenous acid and neutral proteases that play a major role in myelin degradation. Increased enzyme activity of lysosomal hydrolases and neutral proteases has been described in NAWM and actively demyelinating plaques (Cuzner *et al*, 1975, Einstein *et al*, 1972) whilst the electrophoretic profiles of proteins in both areas reflects the gradual disappearance of myelin and oligodendroglial proteins (Newcombe *et al*, 1982). A correlation between increased enzyme activities and myelin degradation in MS lesions is further strengthened by the concurrent finding of increased amounts of degradation product of some myelin proteins (Itayama *et al*, 1980) as well as free myelin fragments in the CSF (Herndon & Kasckow, 1978), both of which are associated with the exacerbation of disease activity.

Activated microglia and macrophages are the effector cells of myelin damage during the inflammatory process in the CNS (Cuzner, 1997). Net-like disruption and

swelling of myelin lamellae in areas of contact between macrophages and myelin are described in MS lesions (Prineas, 1975). Similarly, disruption of myelin layers and penetration of macrophages have been observed in EAE. The mechanisms by which macrophages damage myelin and the connection with the demyelinating process in MS became apparent after the discovery that macrophages secrete a number of proteases and that this secretion was stimulated by the products of activated lymphocytes (Unkeless *et al.*, 1974, Bowen & Davison, 1973). Cammer *et al.* (1978) showed that incubation of myelin with conditioned medium from cultured activated macrophages results in degradation of MBP in both the presence and absence of plasminogen. Plasminogen-dependent activity is particularly important as small amounts of PAs are capable of initiating a downstream enzymatic cascade involving several classes of enzymes. The activation of MMPs represents the functional end-point of such a cascade and could have wide ranging consequences for the pathogenesis of MS lesions, such as ultrastructural changes in myelin membranes with the loss of protein-lipid associations and generation of encephalitogenic peptides capable of inducing an autoimmune response (Opdenakker & van Damme, 1994).

4.1.2. MMPs: potential roles in CNS inflammation.

MMPs are a family of zinc-dependent proteases that are active at physiological pH. All members share sequence homology and functional characteristics (Massova *et al.*, 1998). According to their substrate specificity MMPs are classified into collagenases, gelatinases, stromelysins and membrane-type MMPs (Woessner, 1991). Their major role is a controlled removal of ECM components in both physiological and pathological conditions. The MMP activity is regulated at several levels, transcriptional, protein synthesis and activation levels and through the action of endogenous MMP inhibitors, all of which may be tissue or cell-specific. Upregulation of MMPs in cells present at the site of tissue damage is stimulated by inflammatory cytokines such as IL-1 and TNF α through

the signalling cascades involving transcription factors AP-1 and NF- κ B (Opdenakker *et al*, 1991, Bond *et al*, 1998). In turn metalloproteases are involved in activation of the precursor of TNF α and IL-1 β (Chandler *et al*, 1997).

MMPs are synthesised and secreted into the extracellular space in zymogen form in which the zinc atom in the active site is blocked by a cysteine in the proenzyme domain. The cysteine-switch mechanism of MMP activation (van Wart & Birkedal-Hansen, 1990) involves removal of cysteine and autocatalytic activation of MMPs. This stepwise activation can be triggered by plasmin, the generation of which is highly focused at sites where both plasminogen and its activators are bound (Opdenakker & van Damme, 1992). Such interactions connect the plasmin system, a cascade of neutral serine proteases, with the MMP cascade leading to the amplification of the latter. As MMPs have the potential to degrade basement membrane and other matrix components leading to extravasation of inflammatory cells from the circulation into tissue, activation of these enzymes in the CNS may constitute an important, if non-specific, pathogenic mechanism for both the disruption of the BBB and of the myelin sheath (Cuzner & Opdenakker, 1999).

Recent data indicate that MMPs may also facilitate regeneration in the CNS through release of growth and angiogenic factors and proteolytic degradation of pro-inflammatory cytokines (Yong *et al*, 1998). PAIs and TIMPs, inhibitors of plasmin and MMPs in the extracellular space, share many regulatory characteristics with their respective enzymes (Opdenakker & van Damme, 1992, Matrisian, 1992). Both are simultaneously upregulated by inflammatory cytokines and in the case of TIMP may not only inhibit active enzyme but also determine the rate of proenzyme conversion (Olson *et al*, 1997). Hence the balance between enzymes and their inhibitors in lesions will determine the net enzyme activities.

4.1.3. Aims of the study.

The activation of the plasmin/MMP cascade in MS lesions may lead to amplification of the inflammatory process and myelin damage, increased antigen presentation of encephalitogenic epitopes and cell infiltration. On the other hand MMPs may also increase the amount of myelinogenic growth factors such as IGFs facilitating remyelination or enhancing migration of oligodendrocytes. The aims of this study are to analyse the cellular expression of three classes of MMPs (collagenase, gelatinases and stromelysin), PAs and their endogenous inhibitors by immunocytochemistry and of MMP-9 by *in situ* hybridisation in MS lesions at different stages of disease activity to evaluate their role in the inflammatory process in MS and as possible targets for therapeutic intervention. In order to correlate MMP-9 production and activation kinetics with lesion activity, the amounts of MMP-9 functional subtypes, i.e. active versus TIMP-complexed and total MMP-9, are determined by ELISA in protein extracts of CNS tissue from cases of MS and normal controls.

4.2. Materials and methods.

4.2.1. CNS tissue.

Clinical data for individual MS and normal control cases are given in Table 2.4a and 2.4b. For the MMP study post-mortem brain and spinal cord tissue from 13 cases of MS and 5 normal controls were examined. The MS samples included 20 blocks containing a plaque and adjacent white matter and 4 blocks containing normal-appearing white matter only. Five normal control blocks were matched as closely as possible to the MS specimens for age and death to freezing time (**Table 4.1**).

TISSUE	AGE	SEX (F/M)	DFT	DURATION OF MS
MS CASES (N=13)	44.1 (22-66)	7/6	38.2 (11-75)	11 (1-22)
NC CASES (N=5)	45.8 (37-60)	0/5	51 h (30-75)	N/A

Table 4.1: The mean age, sex and death to snap-freezing time (DFT) data for MS and normal control cases.

4.2.2. Antibodies and immunocytochemistry.

The characteristics of the antibody panel used in this study and the dilutions for each antibody are given in **Table 4.2**. Single and double immunocytochemical staining was performed as described in Chapter II (Section 2.2.5. and 2.2.6.). Double immunoperoxidase staining was performed with anti-MMP-2 and anti-MMP-9 antisera and monoclonal marker antibodies for astrocytes (anti-GFAP), oligodendrocytes (14E) and microglia/macrophages (EBM-11) on a selection of normal control, NAWM and active MS lesion samples. Controls for double staining included omission of one of the primary antibodies but avidin/biotin and peroxidase blocking steps were not performed. All sections were fixed in cold acetone (10 min, 4°C), with the exception of those for immunostaining of PAs and PAI-1, for which acetone fixation was postponed until after

incubation with the primary antibody. Polyclonal anti-MMP and TIMP-1 antibodies were diluted to give similar final immunoglobulin concentrations. Routine immunocytochemical controls included omission of primary antibodies and non-immune mouse ascites (NS-1, Sigma) and rabbit serum (Dako) applied at the same immunoglobulin concentration as the primary antibodies.

ANTIGEN	ISOTYPE	Ig CONCENT.	DILUTION	SOURCE
MMP-1	Polyclonal	1.1 mg/ml	1:100	Biogenesis Ltd.
MMP-2	“	0.7 mg/ml	1:50	
MMP-3	“	1.2 mg/ml	1:100	“
MMP-9	“	0.6 mg/ml	1:100	“
MMP-9 (Rega 2D9)	IgG1	1.2 mg/ml	1:10	REGA Institute
TIMP-1	Polyclonal	1 mg/ml	1:80	Biogenesis Ltd.
t-PA	IgG1	1 mg/ml	1:10	“
u-PA	IgG1	1 mg/ml	1:20	“
PAI-1 *	IgG1	1 mg/ml	1:10	“

Table 4.2: Immunoglobulin concentration and optimal dilution for the panel of PA and MMP antibodies.

4.2.3. *In situ* hybridisation.

In situ hybridisation was carried out according to the method published by Woodroffe & Cuzner (1993) with the following modifications. The sense and anti-sense MMP-9 human riboprobes were prepared from the vector pB5-92 and labelled with digoxigenin (DIG). The bluescript plasmid containing a 2.3 Kb fragment coding for human MMP-9 was cut with Not I or Eco RI restriction enzymes and the plasmid purified by

phenol-extraction and ethanol precipitation. DIG-labelled sense and anti-sense riboprobes were prepared by transcription on 1µg linearised plasmids, with T3 and T7 RNA polymerases respectively, according to protocols provided by the manufacturer (Boehringer Mannheim, Germany).

Snap-frozen sections (10µm) were mounted on RNAase-free slides (Merck) and fixed in freshly made PBS-buffered 4% paraformaldehyde for 5 min at RT. After washing 3x in PBS, sections were treated with 0.1M triethanolamine/0.25% (v/v) acetic anhydride and rinsed in PBS. Sections were immersed in graded ethanol solutions (70%, 80%, 95% and 100%) for 2 min each, placed in chloroform for 5 min and then in 95% ethanol for another 2 min. Finally, sections were air-dried. The whole procedure was carried out under RNAase-free conditions.

The MMP-9 probes were diluted 1:100 (v/v) in the hybridisation buffer (50% formamide, 10% 5x SSC dextran sulphate, x5 Denhardt's solution and 100µg/ml heat denatured sperm DNA) and then 50µl of the mixture was added per section, covered with the small square of parafilm and incubated overnight at 37°C in a humid sealed container. Post-hybridisation washes were carried out in 2 x SSC for 1 hour at room temperature and in 1 x SSC for 1 hour and for 30 minutes at 37°C. Sections were washed 1x in buffer 1 (100mM Tris, pH 7.5, 150mM NaCl) and blocked with 2% normal sheep serum in buffer 1 for 30 min at RT. DIG label was detected by sheep anti-DIG antibody diluted 1:500 in buffer 1 containing 1% normal sheep serum and the sections were incubated for 3-5h at room temperature in a humid atmosphere. Following two 10 min washes in buffers 1 and 3 (100mM Tris, pH 3.5, 10mM NaCl), the 5-bromo-4-chloro-3-indolyl phosphate (BCIP) with nitroblue tetrazolium (NBT, Sigma) colour substrate solution was applied at 200µl per section and incubated overnight in the dark. The colour substrate solution was made by adding 34µl of NBT solution, 35µl of BCIP solution and 2.4mg levamisole (Sigma) to

10ml of buffer 2 (100mM Tris-HCl, pH9.5, 100mM NaCl) immediately before use.

Sections were subsequently washed in buffer 3 and mounted in Ralmount (Merck).

4.2.4. *In situ* zymography.

This method, used to examine proteolytic activity in tissue sections from normal control and MS cases, is based on a fluorescent-labelled enzyme substrate which is in contact with unfixed tissue sections (Galis *et al*, 1995). Proteolytic cleavage by endogenous enzymes in sections removes the label which results in formation of non-fluorescing areas that correspond to the location of proteolytic activity.

The slide-coating solution for *in situ* zymography was prepared by mixing resorufin-labelled casein (1mg/ml in distilled water, Calbiochem, Nottingham, UK) with 1% agarose in Tris-HCl, pH7.4 (Sigma) 1:1 (v/v) at 37°C. Slides were coated by smearing and the coating was allowed to gel at RT. The quality of coating was monitored under fluorescent microscope. Sections (10µm) from 3 normal control and 4 MS cases were cut on the casein-resorufin coated slides, overlaid with 50µl of zymography buffer (50mM Tris-HCl, pH7.4, 10mM CaCl₂, 0.05% Brij 35) and coverslipped. Slides were incubated at 37°C overnight (~18h) and inspected for substrate lysis every 6h. Lysis was visualised under TRITC-excitation filter as black areas in a red-fluorescing background.

4.2.5. *Preparation of protein extracts for ELISA.*

Thirty snap-frozen blocks of normal control white matter, NAWM and plaque, weighing between 0.5 and 1g wet weight were used (for clinical data on individual cases see Tables 2.4a and 2.4b). Tissue was thawed, finely cut with a razor-blade and resuspended in ice-cold Tris-HCl buffer (100mM Tris-HCl, pH 8.1, 1% Triton X-100, 1mM PMSF, 100mg/ml aprotinin) at 1:10 (w/v). Samples were homogenised by sonication (25 sec) or in Ultra-Turex homogeniser (25 sec at 8000 rpm), passed three times through

No. 19 and 21 needles and incubated on ice for 30 min. The tissue suspension was spun in a fixed-angle centrifuge at 15000 rpm for 45 min at 4°C (J2-21, Beckman, High Wycombe, UK). The supernatant was collected and immediately frozen and the pellet discarded. In samples of active plaques a gradient was formed with the myelin fraction on the top of a clear supernatant. The protein concentrations of all samples were determined by the method of Lowry.

4.2.6. *Lowry method for determination of protein concentration.*

This assay is based on the colour reaction between Cu^{2+} and protein and the subsequent reduction of the Cu^{2+} -treated protein by Folin-Ciocalteu's phenol reagent to form a coloured product which can be measured by spectrophotometry (Lowry *et al.* 1951).

Aliquots of tissue homogenate were kept at -70°C until assayed. The 50 μ l aliquots were diluted 1:20 (v/v) with 0.05M NaOH and the volume was adjusted to 150 μ l with deionised water. After adding 50 μ l of 0.4M NaOH, a 1ml aliquot of freshly prepared Na_2CO_3 - CuSO_4 - Na^+/K^+ -tartarate was added to each sample, vortexed immediately and incubated at RT for 15 min. After the addition of 100 μ l of 1N Folin-Ciocalteu's reagent, samples were vortexed and incubated in dark at RT for further 30 min. Following the incubation period samples were read spectrophotometrically at 750nm. A fresh standard curve was used for each assay and was made up with bovine serum albumin fraction V (Sigma). The range of the standard curve was 5-30 μ g. The protein concentration of samples was expressed in mg per gram of tissue wet weight (**Table 4.3**).

TYPE OF TISSUE	MEAN PROTEIN CONCENTRATION (mg/gram wet weight)
Normal control (n=7)	6.88 mg/ml \pm 0.57
Normal-appearing white matter (n=6)	6.85 mg/ml \pm 0.67
Active lesions (supernatant) (n=5)	8.75 mg/ml \pm 1.00
Active lesions (myelin fraction) (n=5)	8.62 mg/ml \pm 0.46
Subacute lesions (supernatant) (n=8)	7.34 mg/ml \pm 1.20
Subacute lesions (myelin fraction) (n=8)	6.98 mg/ml \pm 0.90
Chronic lesions (n=3)	7.69 mg/ml \pm 0.51

Table 4.3: Mean protein concentration \pm standard deviation in tissue homogenates from normal control and MS cases.

4.2.7. ELISA for MMP-9 and u-PA.

Enzyme-linked immunosorbent assays (ELISA) for determination of the activity and protein concentrations of MMP-9 in CNS tissue homogenates from normal control and MS cases were performed in collaboration with Dr. Roeland Hanemaaijer of the Gaubius Laboratory, TNO, Leiden, The Netherlands.

The presence of active MMP-9 in tissue homogenates was assessed on the basis of the amidolytic activity of modified pro-u-PA used as the MMP-9 substrate (Verheijen *et al*, 1997). Briefly, the 96-well plates were coated with 125 μ l of affinity purified monoclonal mouse anti-human MMP-9 antibody (5 μ g/ml, Fuji Chemical Industries, Toyama, Japan) for 16h at RT. Wells were washed 3x with PBS containing 0.05% Tween 20 (PBS-T, Sigma). Supernatants were diluted five-fold in PBS, and 125 μ l was incubated for 16h at 4°C. Wells were washed 3x with PBS-T and bound MMP-9 was incubated in Tris-HCl buffer (50mM Tris-HCl, pH7.6, 150mM NaCl, 5mM CaCl₂, 1 μ M ZnCl₂ and 0.01% v/v Brij-35) with or without *p*-aminophenylmercuric acetate (APMA) for 2h at 37°C before 15 μ l of modified u-PA (final concentration 5 μ g/ml) was added. The amidolytic activity of

modified u-PA was determined at 37°C with 0.04mM chromogenic substrate S-2444 (Chromogenix, Mölndal, Sweden) by measuring absorbance changes at 405nm at different time intervals.

Activity was expressed in units defined as 1,000 $\Delta A/h^2$ per mg of protein. The chromogenic substrate S-2444 has a maximum absorbance at 405nm. The absorbance is measured every hour and is expressed against h^2 because it is a two-step reaction: firstly MMP-9 activates the modified urokinase and secondly modified urokinase cleaves the substrate producing a coloured reaction. The reaction shows linear dependence on time squared ($r=0.99$) and the unit is defined as 1000 X change in absorbance per unit of time. As tissue extracts were used in this assay, it is necessary to compensate for the amount of protein which gives the tissue concentration of a given enzyme.

The protein concentrations of latent and total MMP-9 and u-PA were determined using a capture ELISA (Koolwijk *et al*, 1996, Hanemaaijer *et al*, 1998). The monoclonal antibodies used in the u-PA assay recognise single chain u-PA and u-PA/PAI-I complexes with comparable efficiency whilst the antibodies used in MMP-9 assay recognise either latent (Amersham) or all three MMP-9 fractions. A similar procedure was used for all three assays. 96-well microtiter plates (Flow Labs, Irvine, UK) were coated overnight with 100 μ l of the capture antibody (monoclonal anti-u-PA or anti-MMP-9, 0.5 μ g/ml in PBS). After washing with PBS-T, the plates were incubated for 1h with 150 μ l 0.1% (w/v) casein in PBS-T to block non-specific protein binding to the plates. After further three washes in PBS-T, 100 μ l of serial dilutions of standard u-PA or MMP-9 (Serono, Aubbone, Switzerland) and protein extracts were added. After incubation for 2h at 37°C for u-PA or overnight at 4°C for MMP-9, plates were washed three times and incubated with 100 μ l of biotinylated anti-u-PA antibody and polyclonal anti-MMP-9 (TNO-B21 or antibody supplied with the Amersham kit) respectively, for 1.5h at 37°C followed by 1h incubation

with 100µl of peroxidase-conjugated avidin (1:25000, Pierce, Rockford, USA). Finally, the plates were washed four times with PBS-T, and 100µl of tetramethylbenzidine substrate was added to the reaction and stopped with 50µl of 2M H₂SO₄ after 15 min incubation at RT. The extinction at 450nm was measured with a multichannel spectrophotometer. The results were expressed in ng enzyme per mg of protein.

4.2.8. Statistics.

All data were analysed using non-parametric statistical tests. ELISA protein concentrations of MMP-9 and u-PA and enzyme activity of MMP-9 in the protein extracts from MS and normal control tissue samples were compared using the Kruskal-Wallis test. This is a non-parametric test used to compare three or more unpaired groups and the p value gives a measure of variance between the medians of the samples. Comparisons between individual groups were made using Dunn's Multiple Comparison Test at a significance level of $p < 0.05$. All data relating to ELISA measurements is shown as the median with the range of minimum and maximum values of the tested sample.

4.3. Results.

4.3.1. Constitutive expression of MMPs but not PAs in control white matter glia.

In normal control white matter expression of t-PA (**Fig. 4.1e**) and u-PA was restricted to blood vessel walls (**Table 4.4**). PAI-1 was expressed in the same location with the exception of a small number of weakly positive glial cell bodies in the brain parenchyma (**Fig. 4.1f**). In some sections t-PA and PAI-1 but not u-PA were visualised along fine axons in white matter whereas in cortical grey matter neuronal cell bodies were weakly immunostained.

The staining patterns in normal control white matter with polyclonal antibodies raised against MMP-1, 2 (**Fig. 4.1d**), 3 and 9 (**Fig. 4.1a**) were qualitatively similar, visualising the cell bodies of microglia, astrocytes and occasionally oligodendrocytes, as determined by double staining with anti-GFAP, EBM-11 and 14E antibodies. In the case of MMP-3 and 9 the basement membrane and endothelial cell layer of blood vessels were also positive. A highly specific monoclonal antibody REGA-2D9 against MMP-9 which gave a single protein band of 92kD in Western blot analysis (Paemen *et al*, 1995) was also utilised. In normal control white matter REGA-2D9 (**Fig. 4.1b**) stained glial cell bodies and blood vessels, largely resembling the pattern observed with the polyclonal antibodies. TIMP-1, a main inhibitor of MMPs, was visualised on both basement membrane of blood vessels and glial cells although its cellular expression levels appeared lower in comparison to that of anti-MMP antibodies (**Fig. 4.1c**).

Normal rabbit serum applied at the same immunoglobulin concentration as the polyclonal reagents gave only weak staining of the small number of microglia in white matter and macrophages in lesions (**Fig. 4.4g and h**). Control mouse ascites fluid applied at the same immunoglobulin concentration as the monoclonal antibodies gave no staining (**Fig. 4.4e and f**). Varying the fixation protocol or incubation time of primary antibody did

not affect the staining pattern of any of the antibodies used in the study.

4.3.2. MMPs and uPA are upregulated in reactive glia in NAWM and early MS lesions.

Low level expression of t-PA and PAI-1 in blood vessel and glial cells was typical of both MS lesion-adjacent and far white matter. A similar expression pattern was seen in early MS lesions, characterised by the presence of ORO-positive macrophages but no apparent demyelination, although there was an increase in numbers of t-PA- and PAI-1-positive mononuclear cells in the perivascular space (**Fig. 4.2c and d**). In contrast, the expression of u-PA was more widespread and in addition to perivascular cells, immunopositive reactive astrocytes were seen throughout NAWM and early lesion parenchyma.

Activated microglia in NAWM immediately adjacent to active lesions and macrophages in early lesions expressed all four MMPs and TIMP-1 (**Fig. 4.2a and 4.4b**). REGA-2D9 also stained cell bodies of small reactive astrocytes in both locations indicating that both MMP-9 and u-PA, the upstream element of the PA/MMP cascade, are expressed in this cell type. Double staining with anti-GFAP and 14E antibodies confirmed astrocyte expression of MMP-9 (**Fig. 4.4a**) and indicated that some oligodendrocytes also express this MMP in NAWM. MMP-3 and 9 were expressed at high levels on blood vessel matrix and mononuclear cells in the perivascular space of early lesions again co-localising with PAs (**Fig. 4.4c**).

4.3.3. Upregulation of PAs in foamy macrophages in active lesions is not mirrored by the upregulation of their inhibitor PAI-1.

In actively demyelinating plaques the marked upregulation of t-PA was visualised in foamy macrophages (**Fig. 4.2e**). The number of positive macrophages ranged from a few cells in perivascular inflammatory infiltrates in group 2 lesions (early active) to the all ORO-positive macrophages in group 3 lesions (late active). Axons in the vicinity of these

cell also showed immunocytochemically detectable t-PA. u-PA was found in both macrophages and hypertrophic astrocytes, although the expression in the latter cell type was more prominent particularly in the border of actively demyelinating lesions (**Fig. 4.2f** and **h**). In contrast, the expression of PAI-1 was confined to blood vessel matrix and cells in the perivascular space but was much lower in macrophages and was not detected in astrocytes in plaque parenchyma (**Fig. 4.2g**). The expression pattern of PA and PAI-1 antibodies in subacute and chronic lesions mirrored that in NAWM although anti-t-PA antibody weakly stained remaining macrophages in the ORO-positive border of subacute lesions. Astrocytes in the gliotic scar expressed only low levels of u-PA.

In actively demyelinating lesions all four MMPs were expressed in foamy macrophages (**Fig. 4.3d, e** and **h**) whilst MMP-3 and 9 (**Fig. 4.3e**) also selectively highlighted variable numbers of tortuous thick axons. These two metalloproteases were also found at the BBB, expressed on the blood vessel matrix and perivascular inflammatory cells of which the majority were macrophages (**Fig. 4.3d**). Staining with anti-collagen IV antibody demonstrated an increase in the amount of collagenous debris around these blood vessels (**Fig. 4.3c**). With regard to lesion activity, there was a general correlation between levels of MMP expression and inflammation, although a significant number of cells predominantly with astrocyte morphology were also positive in chronic lesions. A correlation between TIMP-1 and MMP expression patterns observed in normal control and NAWM was generally borne out in MS lesions at different stages of development (**Fig. 4.3g**). In addition, anti-TIMP-1 staining of collagen debris seen around some blood vessels in active areas was more pronounced in older lesions.

4.3.4. Parenchymal and perivascular cells synthesise MMP-9 in active MS lesions.

In situ hybridisation was performed with a MMP-9-specific probe in order to visualise *de novo* protein synthesis in glial and inflammatory cells. The application of

sense and anti-sense riboprobes to serial tissue sections demonstrated that mRNA for MMP-9 was detectable in parenchymal cells in both normal control (**Fig. 4.5d**) and MS white matter, in general agreement with the immunocytochemical visualisation of the MMP-9 protein. Numerous cells in the parenchyma of active MS lesions contained MMP-9 mRNA, probably representing increased synthesis in both macrophages and reactive astrocytes (**Fig. 4.5a**). In addition, perivascular cells of probable mononuclear origin were strongly positive with the anti-sense probe in active MS lesions (**Fig. 4.5c**), again in line with the immunocytochemistry results.

4.3.5. Caseinolytic activity detected in active MS lesions is restricted to foamy macrophages.

In situ zymography using fluorescent-labelled casein was used to detect proteolytic activity in tissue sections from normal control and MS cases. A distinct pattern of caseinolysis was observed in the parenchyma (**Fig. 4.6c**) and in perivascular infiltrates (**Fig. 4.6d**) within active MS lesions which appeared to be associated with lipid-laden macrophages. Immunofluorescent staining with the human macrophage marker EBM-11 (**Fig. 4.6e**) and ORO histochemistry (**Fig. 4.6f**) confirmed the co-localisation of caseinolytic areas with the foamy macrophages. The extent of caseinolysis detected after the overnight incubation (18h) was similar to that seen at 12h, demonstrating that this proteolytic activity is limited to cellular and/or pericellular areas. Caseinolytic areas were not detected in normal control or NAWM except around occasional cells in the perivascular space.

Antibody	NC white matter n=5	MS white matter n=4	Primary MS lesions n=3	Active MS lesions n= 11	Chronic lesions n=5
t-PA	Blood vessel matrix	Blood vessel matrix, mononuclear cells in cuffs	Endothelial cells, blood vessel matrix and mononuclear cells in cuffs	Foamy macrophages in plaques	Blood vessel matrix, axons and occasional macrophage
u-PA	As for t-PA	As for t-PA	Small reactive astrocytes	Hypertrophic astrocytes and foamy macrophages	Some hypertrophic astrocytes
PAI-1	As for t-PA	As for t-PA	Endothelial cells and mononuclear cells in cuffs	Foamy macrophages in plaques and blood vessel matrix	Blood vessel matrix and occasional macrophage
MMP-2	Microglia and astroglia	Activated microglia	Foamy macrophages	Foamy macrophages	Occasional macrophage
MMP-9	Microglia, astroglia and blood vessel matrix	As for control white matter plus mononuclear cells in cuffs	Foamy macrophages in plaques, mononuclear cells in cuffs and blood vessel matrix	Astrocytes and macrophages in plaque, lymphocytes in cuffs and blood vessel matrix	Astrocyte processes and occasional macrophage
MMP-9* REGA-2D9	Predominantly astroglia	Predominantly reactive astrocytes	Reactive astrocytes in plaques	Predominantly astrocytes with occasional macrophage in plaques	Astrocytes cell bodies and processes
MMP-3	As for MMP-9	As for MMP-9	As for MMP-9	As for MMP-9	As for MMP-9
TIMP-1	Glial cells and blood vessel matrix	As for control white matter	Foamy macrophages in plaques	Macrophages in plaque, blood vessel matrix and collagen debris	Blood vessel matrix and collagen debris

Table 4.4: Staining patterns of PAs, MMPs and inhibitor antibodies on serial sections of normal control white matter and NAWM and MS lesions at different stages of development.

Figure 4.1: Expression of proteases of the matrix degrading cascade in normal control human white matter. Staining of glial cells with (a) anti-MMP-9 (polyclonal), (b) anti-MMP-9 (Rega-2D9), (c) anti-TIMP and (d) anti-MMP-2. Blood vessels stained with (e) anti-t-PA and (f) anti-PAI-1. No counterstaining, all at magnification x512.

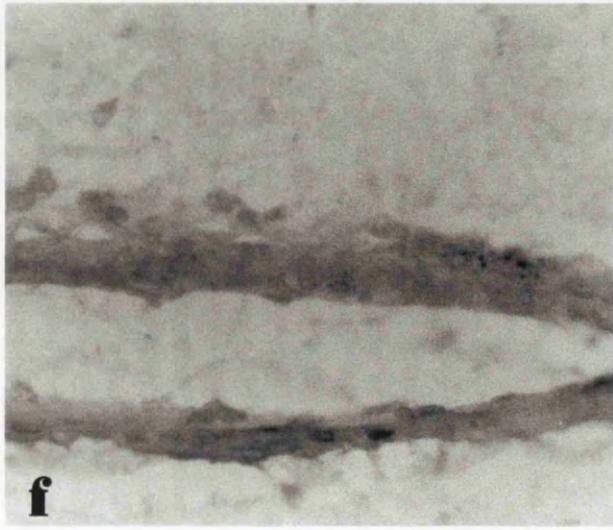
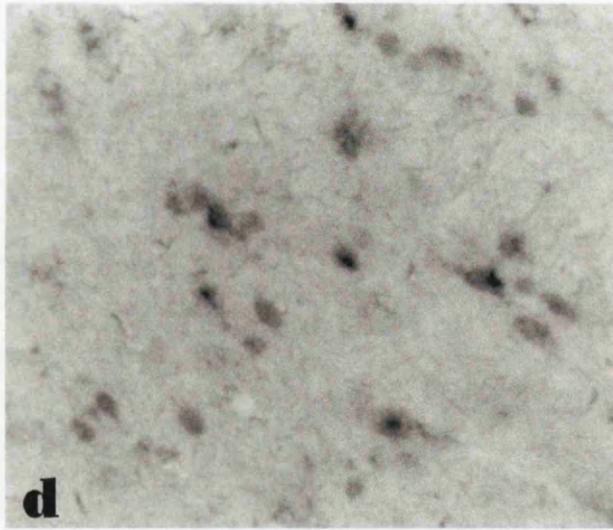
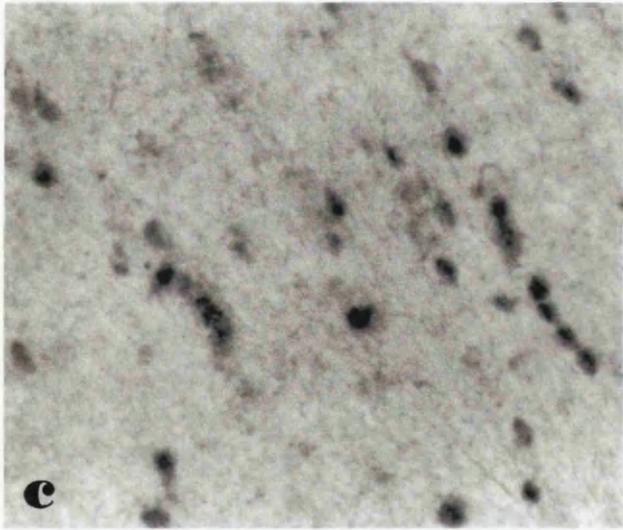
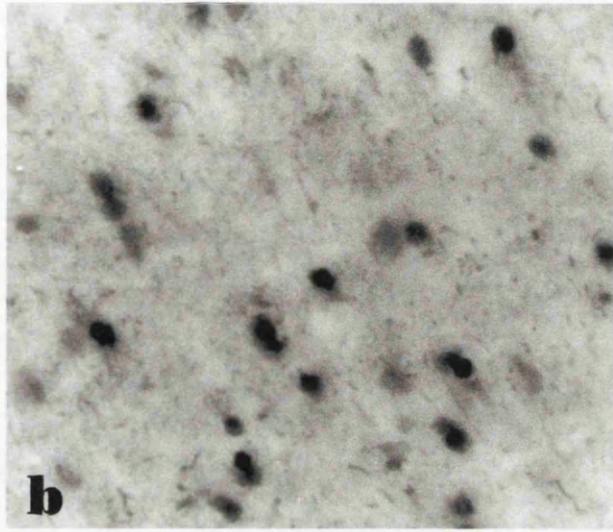
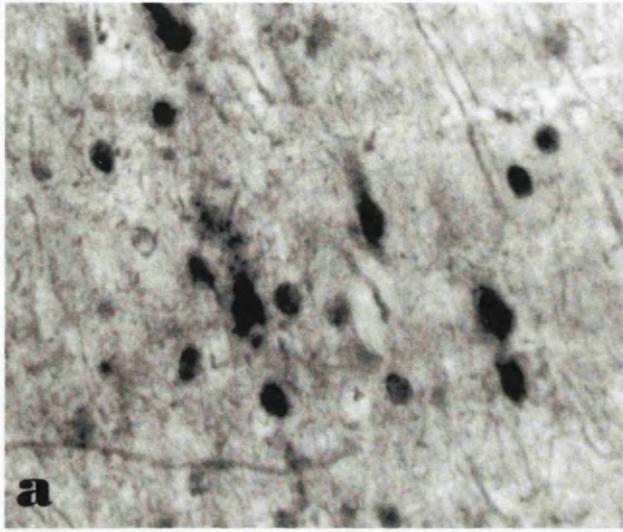


Figure 4.2: Immunocytochemical analysis of t-PA and PAI-1 expression in early and demyelinating MS lesions. A perivascular cuff in an early MS lesions stained with (a) polyclonal anti-MMP-9, (b) anti-CD2, (c) anti-t-PA and (d) anti-PAI-1. (e) Foamy macrophages in the parenchyma of an active MS lesions stained with anti-t-PA and (f) hypertrophic astrocytes in the lesion border stained with anti-u-PA. Macrophages in the same lesion stained with (g) anti-PAI-1 and (h) anti-u-PA. (a-d) at x128, (e-h) atx512.

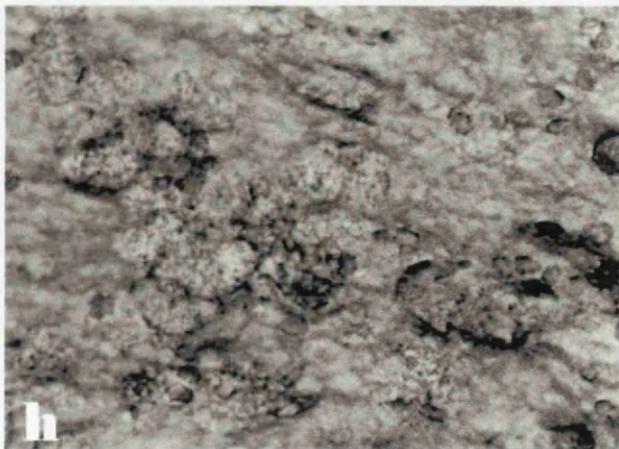
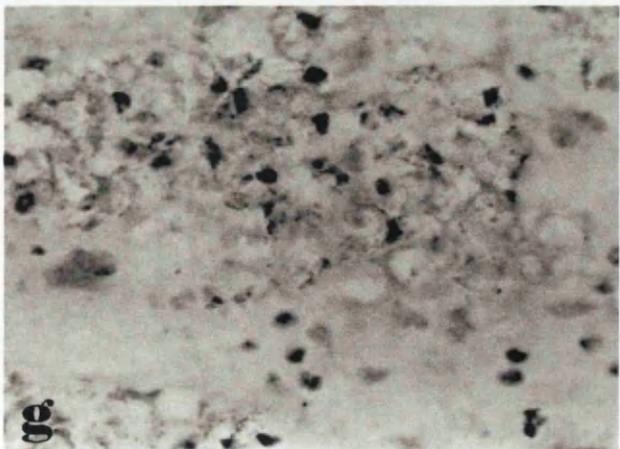
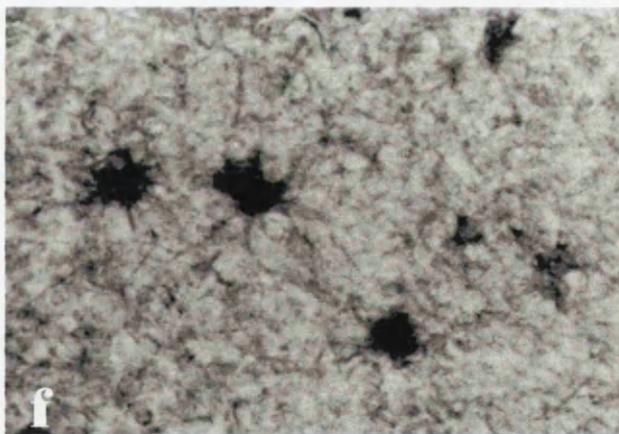
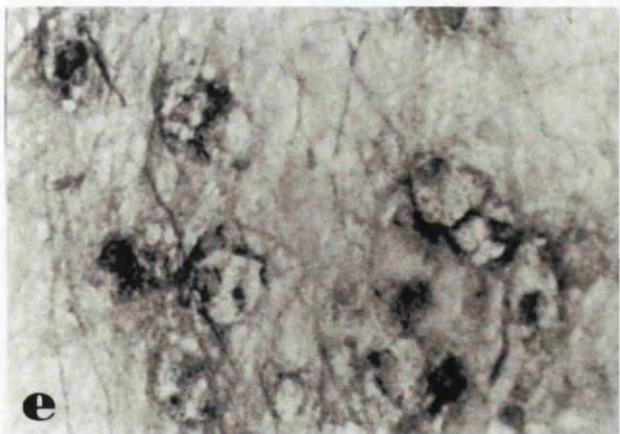
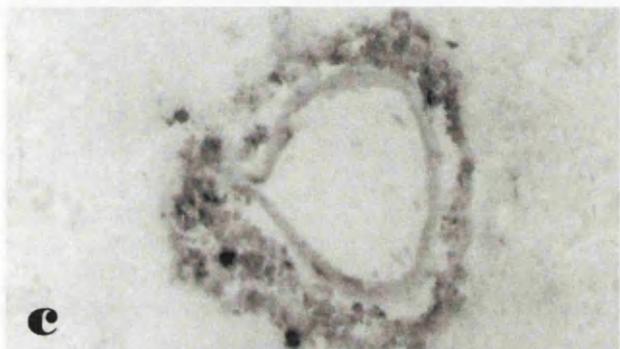
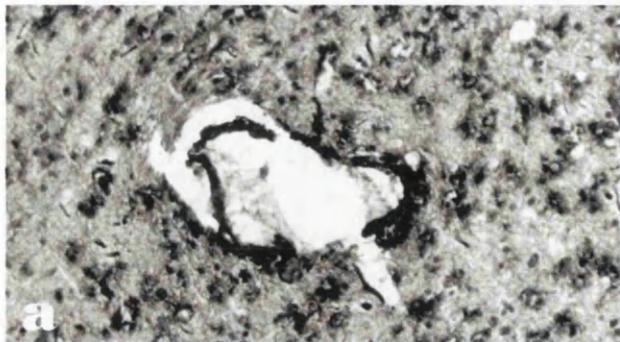


Figure 4.3: Matrix metalloproteinase expression in primary and demyelinating MS lesions. (a) macrophages in the lesion parenchyma stained with EBM-11 and (b) hypertrophic astrocyte stained with anti-GFAP. (c) Staining of blood vessel collagen with the anti-collagen IV antibody. Note the increased amount of collagenous debris in the vicinity of these blood vessels. (d) anti-MMP-3 staining of foamy macrophages in plaque and perivascular spaces. (e) Macrophages and tortuous axons stained with anti-MMP-9 (polyclonal) and (f) hypertrophic astrocytes stained with Rega-2D9. Macrophages stained with (g) anti-TIMP and (h) anti-MMP-1 polyclonal antibodies. All at x512 except (c) and (d) x128.

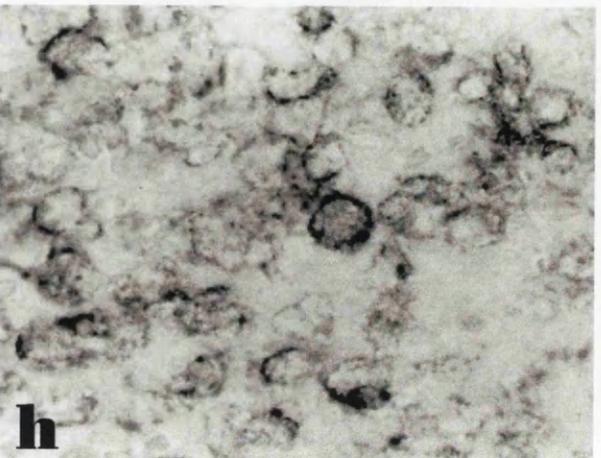
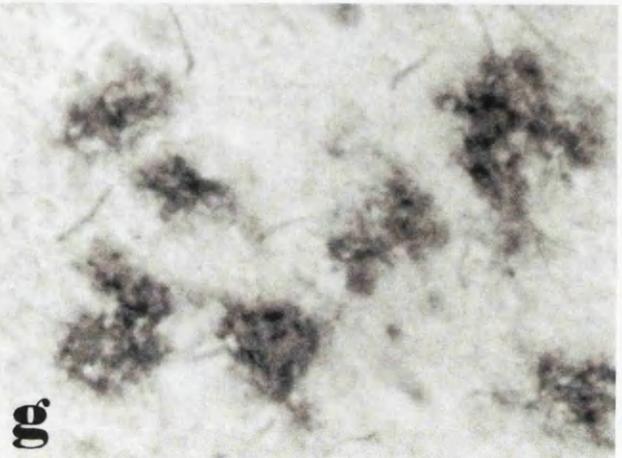
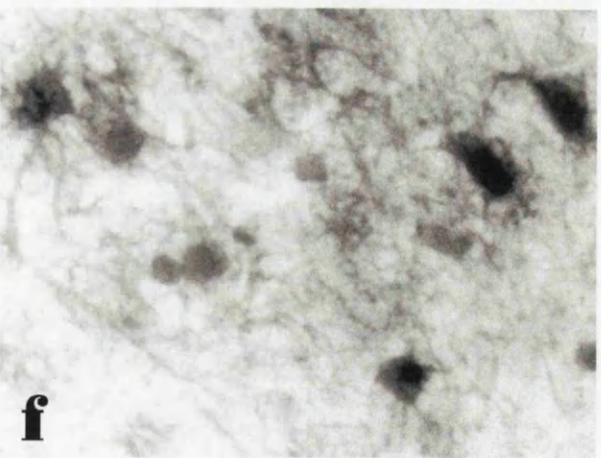
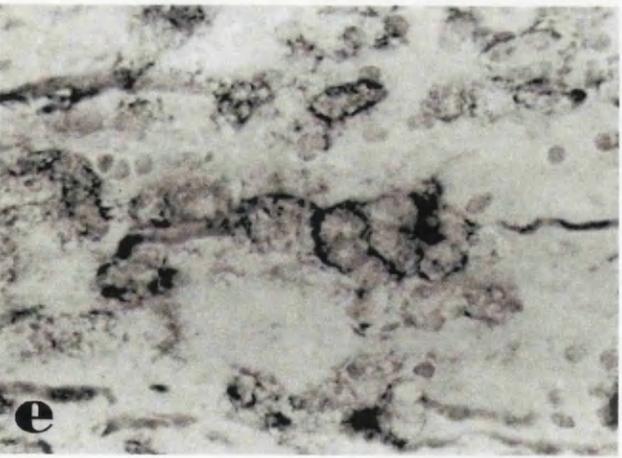
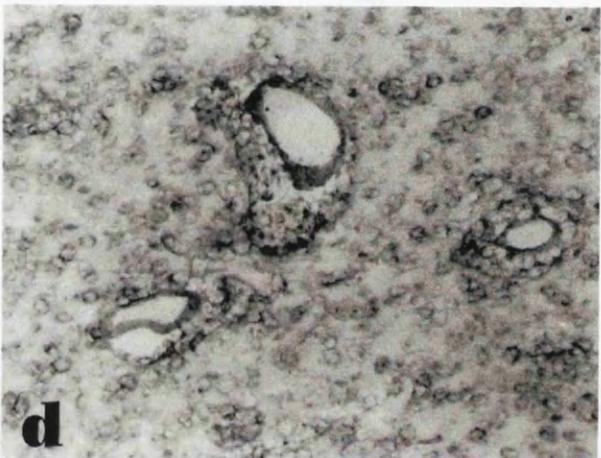
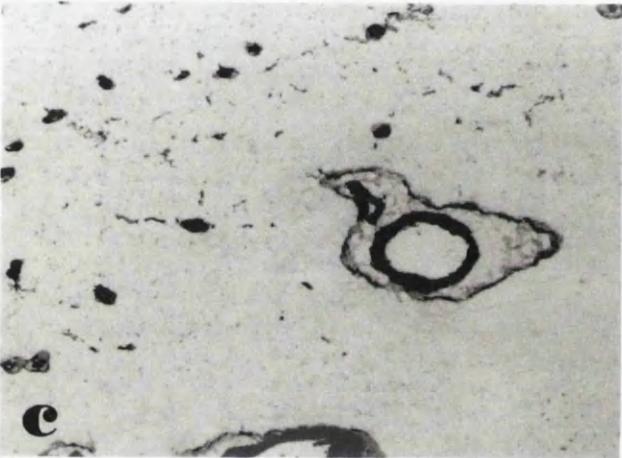
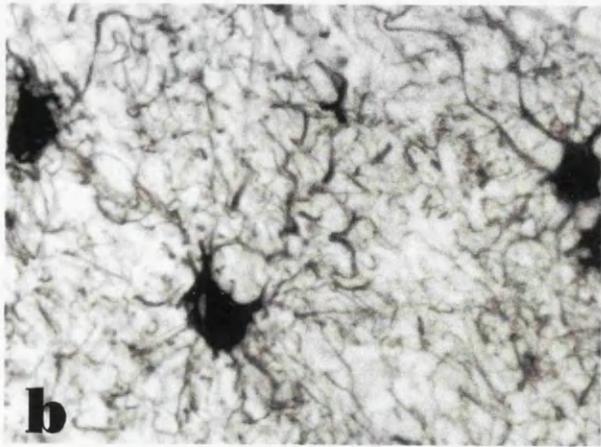
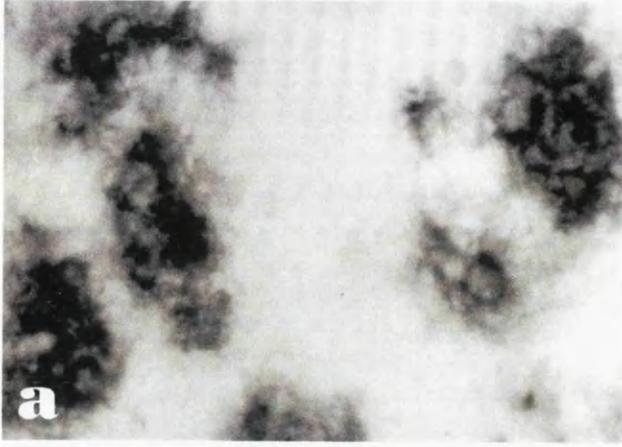


Figure 4.4: Double staining for the MMP-2 and MMP-9. (a) Reactive astrocytes in NAWM stained with anti-GFAP (purple) and anti-MMP-9 (brown) antibodies. (b) Activated microglia in a neighbouring section stained with EBM-11 (purple) and anti-MMP-2 (brown). (c) Low magnification photomicrograph of an inflammatory cuff in primary lesion stained with anti-MMP-9 (purple) and EBM-11 (brown). (d) Neighbouring section stained with EBM-11 (brown); immunostaining with anti-MMP-9 was omitted resulting in the lack of purple stained blood vessel walls and macrophages. **Immunocytochemical controls:** Mouse ascites applied at the same Ig concentration as monoclonal antibodies stained only (e) corpora amylacea in normal control white matter and (f) occasional cells in the lumen of blood vessels. Normal rabbit serum weakly stained (g) glial cells in normal control white matter and (h) macrophages in the parenchyma of active lesions. (a) and (b) at x512, (c-h) at x128.

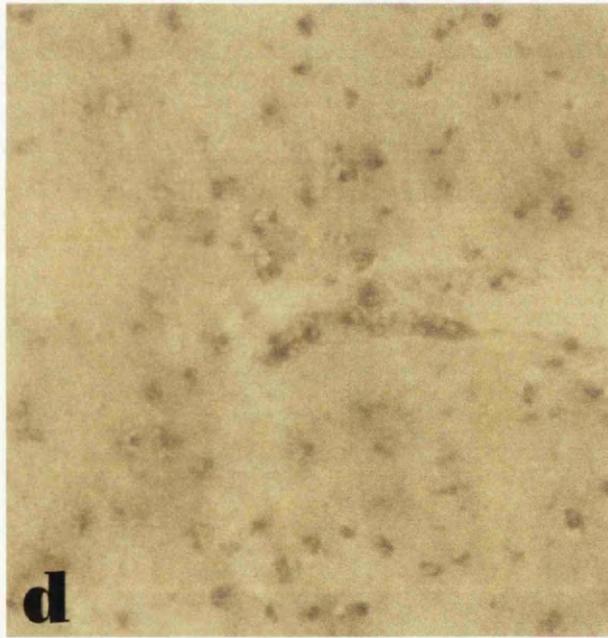
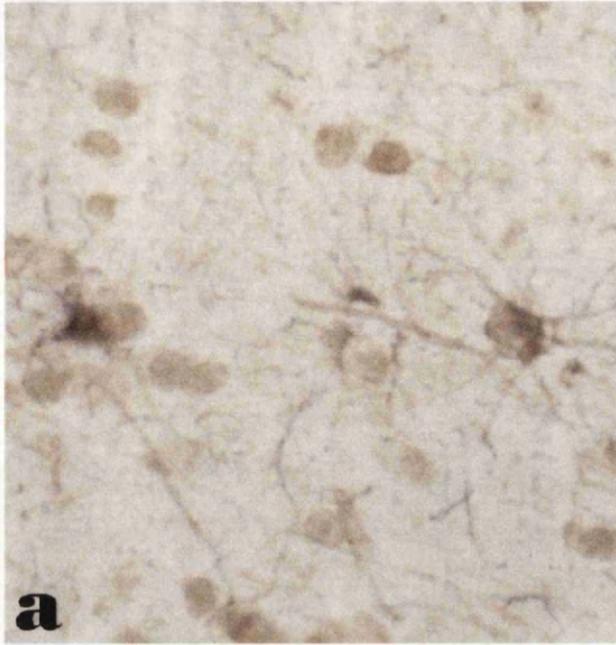


Figure 4.5: *In situ* hybridisation for MMP-9 in active plaque and control white matter. (a) MMP-9 mRNA was localised in cells in both perivascular infiltrates and lesion parenchyma as shown with anti-sense riboprobe whilst (b) staining with the sense probe was markedly weaker. (c) High magnification detail of perivascular inflammatory infiltrate shown in Fig.4.5a. (d) Staining of glial cells in control white matter with anti-sense probe. (a) and (b) at x128, (c) and (d) at x512.

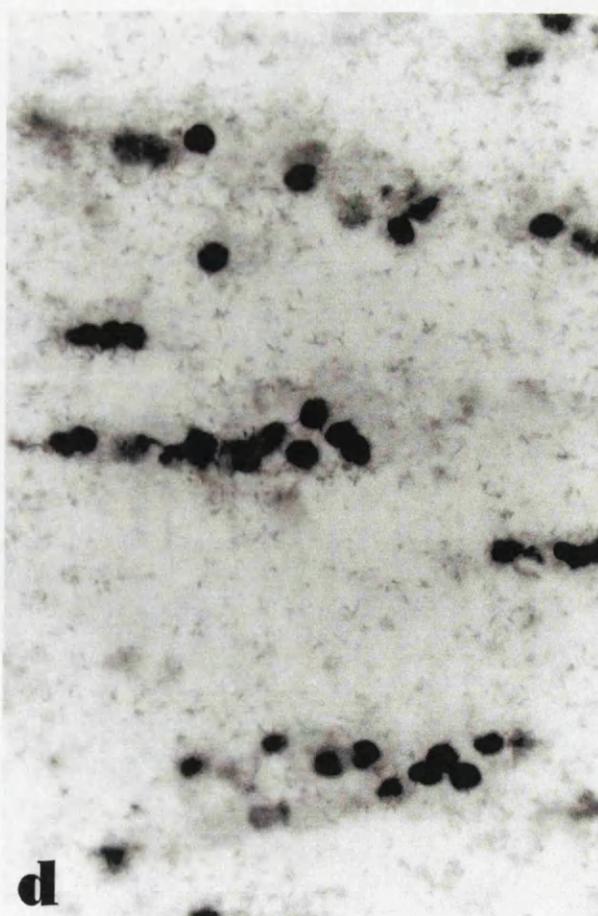
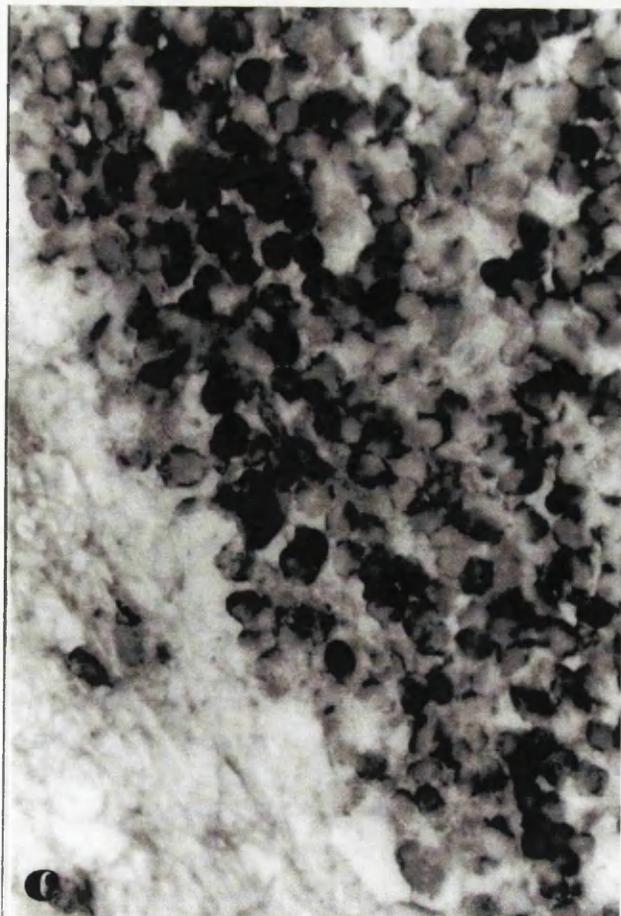
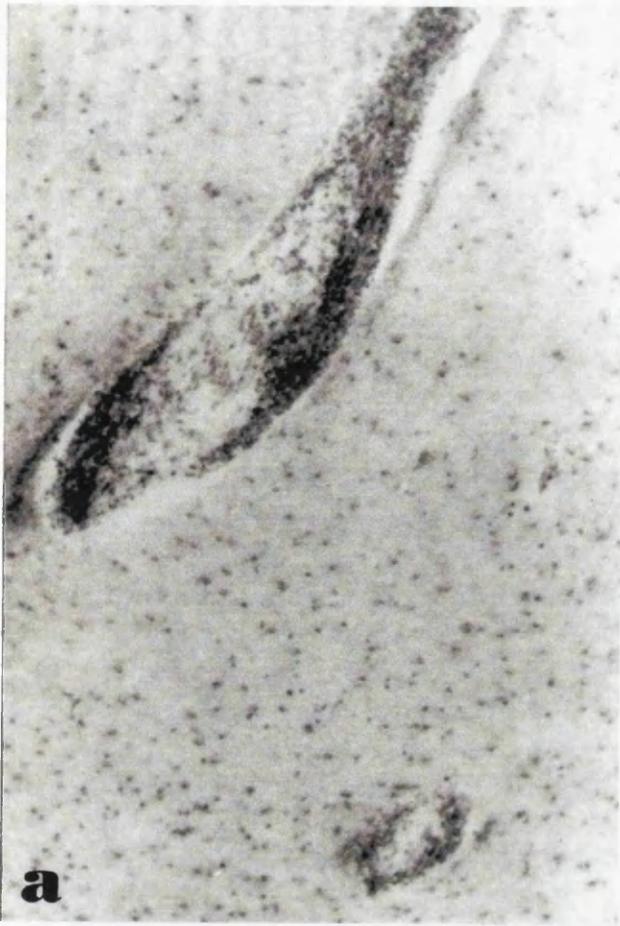
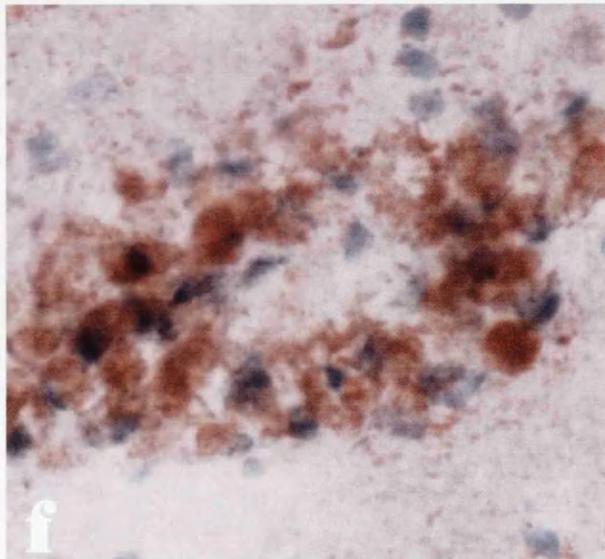
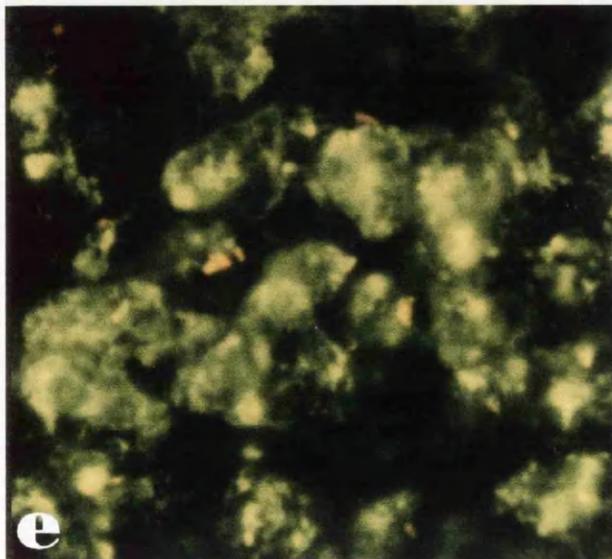
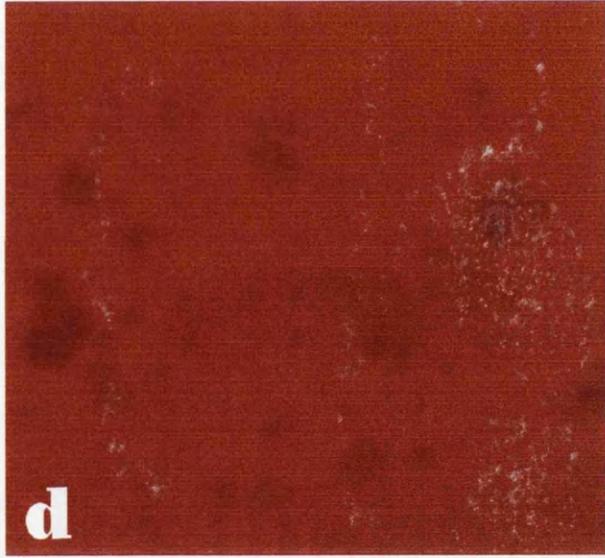
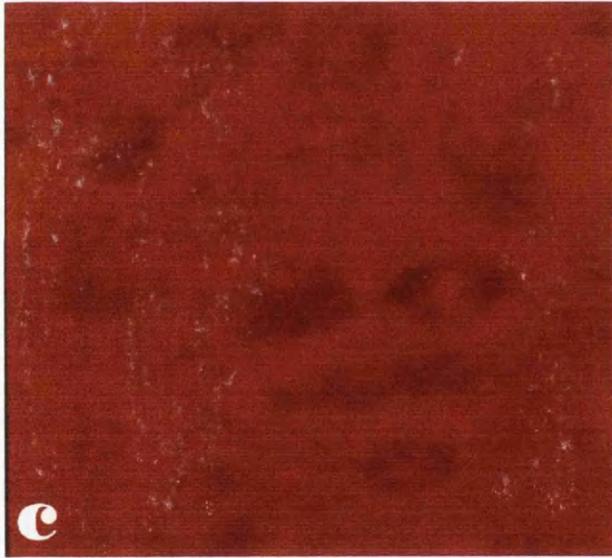
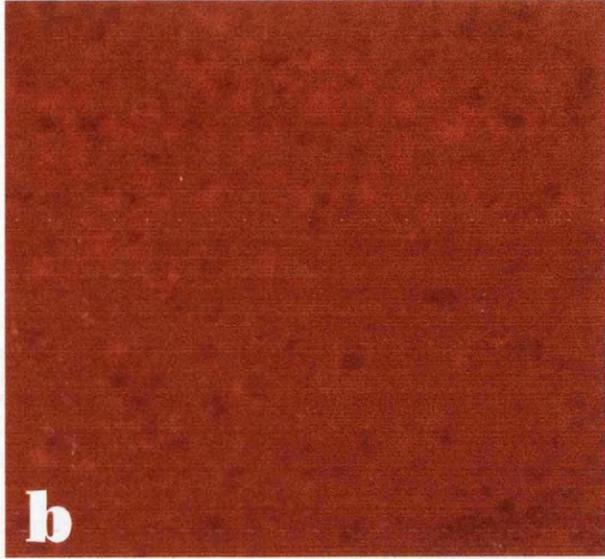
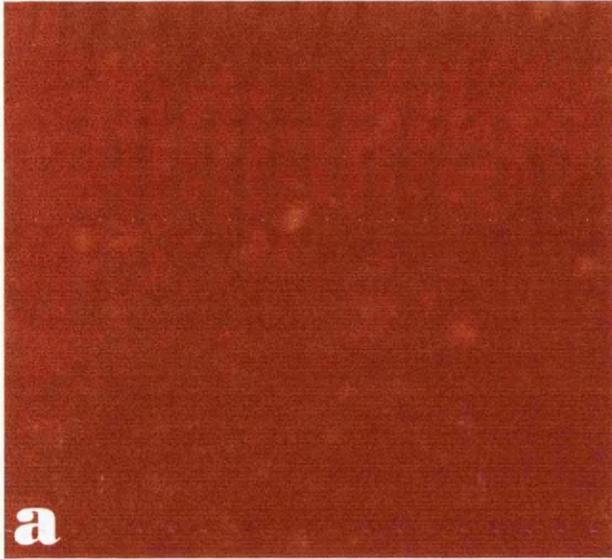


Figure 4.6: Caseinolytic activity in active MS lesions detected by *in situ* zymography. An area in the parenchyma of an active MS lesion (a) before and (b) after overnight incubation. A well-defined caseinolytic areas are visible in the lesion parenchyma. High power detail of (c) lesion parenchyma and (d) perivascular inflammatory infiltrate. Myelin-laden macrophages in perivascular inflammatory infiltrate shown in (d) stained with (e) human macrophage marker EBM-11 and (f) ORO. Caseinolysis is associated with foamy macrophages in the lesions parenchyma and perivascular infiltrates. (a) and (b) x128, (c-f) x512.



4.3.6. Limited amounts of active MMP-9 are detectable in active MS lesions.

The amount of already active and APMA-activated total MMP-9 activity was determined using modified u-PA as the MMP-9 substrate. Small amounts of active MMP-9 were variably present in protein extracts of NAWM and MS lesion samples and did not correlate with the inflammatory lesion activity (**Table 4.5** and **4.6**, **Fig. 4.7a**). In addition, extracts of two normal control white matter samples, both of which were histologically characterised by small perivascular inflammatory infiltrates and increased glial reactivity, also had detectable active MMP-9. The total MMP-9 activity, which reflects the sum of already active and *in vitro* APMA-activated MMP-9 zymogen, was found to be significantly higher in active lesions (8.44 U/mg, range 7.38 – 11.09) compared with normal control (1.92 U/mg protein, range 1.54 – 4.42, $p < 0.05$) (**Table 4.5** and **4.6**, **Fig. 4.7b**). However, the difference in total MMP-9 activity between lesions at different stages of development, i.e. active, subacute or chronic, and NAWM was not statistically significant. Overall, the predominant lack of activated MMP-9 in tissue extracts indicates that this metalloprotease is either present in the latent form or complexed to TIMPs.

4.3.7. MMP-9 and uPA protein concentration is increased in active MS lesions.

A sandwich ELISA which utilised the same capture antibody specific for all forms of MMP-9 and different detection antibodies was used to assess the protein levels of this metalloprotease. This allowed for comparison between the amount of latent MMP-9, representing the rate of production, and total MMP-9 which represents the cumulative sum of latent and TIMP-complexed MMP-9. The results indicate a correlation between histopathological scores and the protein concentration of MMP-9 both between different MS cases and within samples from a single case (**Table 4.5** and **4.6**). The highest amounts of latent and total MMP-9 were found in protein extracts of active lesions (1.72 ng/mg protein, range 0.98 – 3.23, and 8.00 ng/mg, range 7.67 – 8.00) (**Fig. 4.8a**) indicating an

increase in both MMP-9 production and conversion into active enzyme. The amount of complexed MMP-9, which was deduced by subtracting the amount of latent from the total MMP-9, was also significantly higher in active lesions (6.28 ng/mg, range 4.77 – 7.02) compared to normal controls (0.62 ng/mg, range 0.22 – 1.92, $p < 0.01$). As in the case of total APMA-activated MMP-9 the differences in protein concentrations between NAWM and various types of lesions were not statistically significant.

Determination of free and PAI-complexed u-PA revealed a marked increase in protein levels in active MS lesions (Table 4.5 and 4.6, Fig.4.8b). The u-PA concentrations in active MS lesions (0.14 ng/mg, range 0.09 – 0.18) was significantly higher than those in the normal control (none detected, $p < 0.01$) and NAWM (0 ng/mg, range 0 – 0.04, $p < 0.05$) indicating a strong correlation between u-PA protein levels and lesion activity.

Samples	Active MMP-9 U/mg	Total ^a MMP-9 U/mg	Latent MMP-9 ng/mg	Total ^b MMP-9 ng/mg	u-PA ng/mg
NCWM	0 0 - 0.63	1.92 1.54 - 4.42	0.13 0.06 - 0.79	0.73 0.28 - 2.71	0
NAWM	0 0 - 0.83	3.635 2.29 - 9.94	0.39 0.02 - 2.16	1.85 0.72 - 7.45	0 0 - 0.04
AL	0 0 - 0.68	8.44 7.38 - 11.09	1.72 0.98 - 3.23	8.00 7.67 - 8.00	0.14 0.09 - 0.05
SAL	0 0 - 0.93	8.63 1.65 - 14.86	1.16 0.03 - 3.87	7.53 0.59 - 8.14	0 0 - 0.05
CL	0 0 - 0.56	7.24 5.32 - 12.17	0.94 0.14 - 1.86	6.06 4.15 - 8.00	0.07 0 - 0.14
KW test p value	p = 0.994 NS	p = 0.014 *	p = 0.056 NS	p = 0.009 **	p = 0.0008 ***

Table 4.5: Summary of results obtained with ELISA for enzyme activity and protein concentrations of MMP-9 and u-PA in tissue extracts from MS and normal control samples. ^a Total MMP-9 refers to the amount of APMA-activated MMP-9 whereas ^b refers to the total protein concentration of MMP-9 (latent, active and complexed). Data are presented as median (minimum and maximum), p represents a p value of the Kruskal-Wallis (KW) test. Abbreviations: NS - non significant, AL - active MS lesions, SAL - subacute MS lesions, CL - chronic MS lesion.

Samples	Histo. score	Active MMP-9 U/mg	Total ^a MMP-9 U/mg	Latent MMP-9 ng/mg	Total ^b MMP-9 ng/mg	u-PA ng/mg
NAWM-1	0-1	0.51	9.94	1.05	7.45	0
NAWM-2	0-1	0	6.61	2.16	5.56	0
AL-1	3-4	0	11.09	1.72	8.00	0.18
AL-2	3-3	0.44	10.15	3.03	8.00	0.09
SAL	1-2	0.47	9.17	1.00	8.00	0.03

Table 4.6: The MMP-9 and u-PA protein concentrations in samples of NAWM and lesions derived from a single MS case (B444). Histopathological score is based on the number of ORO-positive macrophages in lesion parenchyma and lymphocytes in perivascular inflammatory infiltrates and ranges from 0 (no cells present) to 5 (high numbers). ^a Total MMP-9 refers to the amount of APMA-activated MMP-9 whereas ^b refers to the total protein concentration of MMP-9 (latent, active and complexed). Numeric values represent values obtained with different ELISA and are normalised for the protein concentration in the samples.

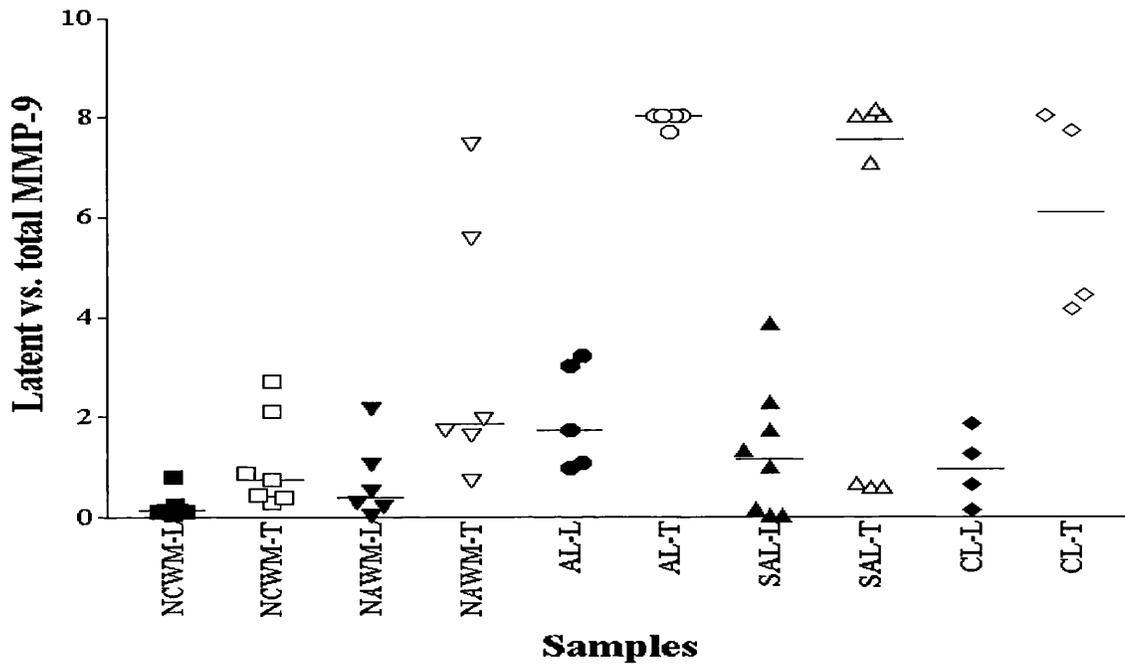


Figure 4.8a: Comparison of the latent (L) and total (T) MMP-9 protein concentrations in normal control, NAWM and MS lesions at different stages of development. The difference between the levels of total and latent MMP-9 represents the concentration of active and TIMP-complexed enzyme. Bars indicate median. For abbreviations see Figure 4.7a.

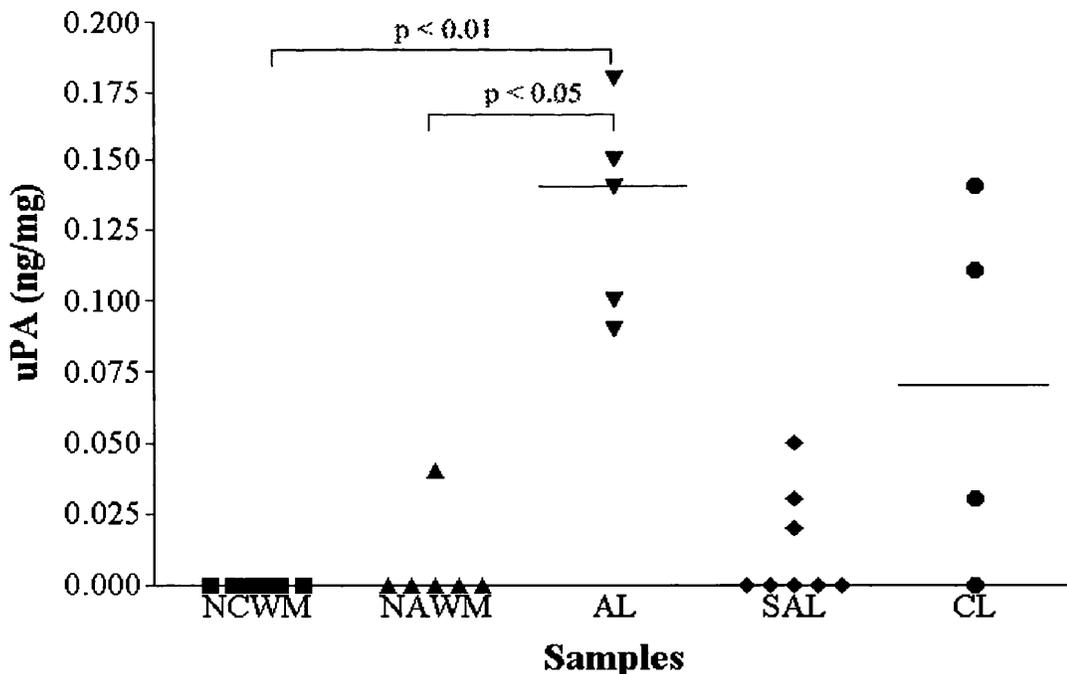


Figure 4.8b: u-PA protein concentration measured by ELISA in normal control and NAWM and MS lesions at different stages of development. Result are given as ng u-PA per mg protein and are presented as individual data point. Bars indicate median and p is the value of Dunn's multiple comparison test. For abbreviations see Figure 4.7a.

4.4. Discussion.

4.4.1. Summary of findings.

Constitutive expression of MMPs was visualised in normal control white matter whereas PAs and PAI-1 were confined to blood vessel endothelium and matrix and were immunocytochemically undetectable in the CNS parenchyma. However, PA-positive mononuclear cells associated with blood vessels were found in NAWM and in the early MS lesions. u-PA expression in NAWM and early lesion parenchyma appeared earlier than that of the t-PA and the co-localisation of u-PA and MMP-9 was seen on reactive astrocytes. In active lesions foamy macrophages expressed both MMPs and PAs whilst hypertrophic astrocytes in the lesion border selectively expressed MMP-9 and u-PA. Caseinolytic activity which was detected only in active lesions appeared to be confined to foamy macrophages. TIMP-1 staining mirrored that of the MMPs although this was less clear-cut as the pathology of lesions became more extensive. In contrast, PAI-1 was absent in astrocytes but was found at low levels in foamy macrophages. ELISA results showed a sharp increase in the concentration of free and PAI-complexed u-PA in active plaques. Similarly, there was a marked but more gradual increase in latent and TIMP-complexed MMP-9 protein concentrations and the total MMP-9 activity.

4.4.2. Expression of the plasminogen cascade in MS: relationship with the lesion activity.

A central role for plasmin in the activation cascade of MMPs is well documented (Opdenakker & van Damme, 1992, Cuzner & Opdenakker, 1999). Plasmin, which is generated by the action of plasminogen activators, initiates the pro-MMP activation by sequential cleavage, and hence might be a rate-limiting step in the cascade. This is well illustrated in PA-deficient mice in which generation of active MMP-9 depends on exogenous source of PAs (Lijnen *et al*, 1998). Furthermore, in cultured macrophages cell surface-associated plasmin enhances the activation of u-PA even in the presence of

inhibitors (Duvaljobe & Parmely, 1994). Such action provides a positive feedback for further plasmin activation and amplification of the MMP cascade. The low level of t-PA and u-PA expression in human control white matter underlines the key role PAs have in triggering the MMP cascade during the pathological processes in the CNS. Upregulation of t-PA and u-PA but not PAI-1 in macrophages and astrocytes in hypercellular plaque borders indicates a window in which the concentration of PAs may exceed that of their inhibitor. The caseinolytic activity detected by *in situ* zymography which was found in macrophages in active lesions points to this cell type as a major source of proteolytic enzymes. The co-localisation of PAs and MMPs suggests that PAs and plasmin promote demyelination in MS through activation of the MMP cascade although MMP-independent effects can not be excluded (Cammer *et al*, 1978). Plasmin-dependent activation of MMPs has been observed in other organ-specific autoimmune diseases such as rheumatoid arthritis and may contribute to the progression of the disease (Norga *et al*, 1996). The concomitant disappearance of immunoreactivity for both PAs and MMPs in the chronic MS lesion further supports a role for their co-ordinate action at the demyelinating stage of lesion development.

u-PA expression was more widespread in both NAWM and lesions and occurred earlier than that of t-PA suggesting that this PA may be the endogenous brain PA responsible for triggering the MMP cascade in early MS lesions. This is further supported by the sharp increase in free and complexed u-PA protein concentrations in active lesions which indicates increased synthesis and conversion of the pro-u-PA. Similarly, in the facial nucleus after the nerve axotomy an early increase in u-PA but not t-PA activity was found (Nakajima *et al*, 1996). In contrast gradual and late upregulation of t-PA in macrophages in active lesions may be related to the opening of the BBB and the infiltration of peripheral mononuclear cells. Furthermore, t-PA is a poor enzyme in the absence of fibrin (Woessner,

1998) and might depend on the increased influx of fibrinogen and fibrin through the damage BBB to promote its proteolytic activity. A cell-specific distribution of PAs in MS lesions may imply a certain degree of functional specialisation in glial cells. u-PA binding to u-PAR forms a unique chemotactic system that promotes cell adhesion and migration (Blasi, 1997) and in monocytes this system is necessary for the migration along the chemotactic gradient (Gyetko *et al*, 1994). It is possible that hypertrophic astrocyte-derived u-PA may form chemotactic gradient towards the lesion edge and attract macrophages to the area of highest inflammatory activity and tissue damage. The role of cell-bound t-PA in that case would be primarily to support pericellular proteolysis particularly as t-PA directly interacts with the ECM components.

The cellular distribution of u-PA and t-PA and differences in expression levels of PAs and PAI-1 may be the consequence of a specific combination of cytokines and local interactions between cells and the ECM. Inflammatory cytokines and macrophage growth factors *in vitro* upregulate production of PAs and their inhibitors in both astrocytes and microglia (Nakajima *et al*, 1992, Hamilton *et al*, 1991). Furthermore, ligand binding to the macrophage scavenger receptor, known to be involved in myelin phagocytosis, induces upregulation of u-PA (Hsu *et al*, 1998), and myelin accumulation in plaque macrophages is likely to increase u-PA and MMP expression (Falcone *et al*. 1993. Galis *et al*. 1995). The effects *in vivo* will depend on the local ECM microenvironment and cellular interaction and may result in preferential upregulation of PAs over that of PAI and initiation of the proteolytic cascade.

4.4.3. *MMPs are constitutively expressed in human CNS.*

The widespread expression of MMPs in the normal CNS confirms in general the preliminary report by Gijbels and Steinman (Gijbels *et al*, 1992), and agrees with reports of MMP expression in non-neural tissue, such as that of MMP-2 in normal arterial samples

(Galis *et al*, 1994). However, gelatinolytic activity has been reported in control brain tissue unaccompanied by immunohistochemical reactivity for MMP-9 and TIMP, and was found to be restricted to endothelial cells (Nakagawa *et al*, 1994). The observations in this study may reflect the wider epitope specificity of the polyclonal antibodies, although non-specific reactivity cannot be ruled out. Equally the monoclonal anti-MMP-9 visualised parenchymal glial cells, providing supportive evidence for constitutive expression of MMPs, which are likely to be present either as pro-enzymes or with active sites blocked by TIMPs. The detection of MMP-9 mRNA in glia in sections of control white matter serial with those used for immunocytochemistry and protein concentrations determined by ELISA provide confirmation of this constitutive expression. This may reflect either species-specific regulation of MMPs as similar constitutive expression was found in rats but not mice (Anthony *et al*, 1998, Pagenstecher *et al*, 1998). Nevertheless, the co-expression of MMPs and their inhibitors in normal control white matter in the absence of catalytic plasmin activity indicates the latent nature of this enzyme cascade in resting glia.

4.4.4. *The role of MMPs in inflammatory demyelination.*

In MS tissue expression of both MMP-9 and t-PA along axons in serial sections was frequently found in active lesions, localising both enzymes to the proximity of periaxonal myelin, a major target of inflammatory attack in MS. The capability of MMPs to cleave myelin proteins has been documented *in vitro* and in animal models of MS (Chandler *et al*, 1995, Chandler *et al*, 1996). The intense MMP-3 and 9 immunostaining of macrophages and activated microglia in the hypercellular zone radiating into the white matter is in agreement with both *in vitro* and *in vivo* evidence that mononuclear phagocytes are the major cell type synthesising these enzymes (Shapiro *et al*, 1991). The cytokines documented as inducing MMP expression in glial and mononuclear cells (Gottschall & Deb, 1996b, Johnatty *et al*, 1997) are also readily detectable in active MS lesions both in

perivascular cells and in activated microglia at the lesion edge (Woodroffe & Cuzner, 1993). It is thus highly probable that the increased CSF levels of these enzymes reported in inflammatory CNS disease originate from macrophages and lymphocytes at the lesions site (Gijbels *et al*, 1992, Leppert *et al*, 1998). The widespread expression of MMP-9 in activated microglia and foamy macrophages as well as astrocytes in the demyelinating lesion may reflect an excess of this metalloprotease over tissue inhibitor. The overall distribution of MMPs in MS lesions does not appear to be specific for MS as similar findings are reported in other neuropathological conditions such as Alzheimer's, cerebrovascular disease and bacterial meningitis (Peress *et al*, 1995, Kolb *et al*, 1998). However, it is feasible that individual MMPs may have specific functions in the pathogenesis of MS. In this study MMP-3 and 9 were found co-localised with PAs at the BBB and deposited on axons indicating that these two MMPs may be mediators of tissue damage in MS. Such a scenario is supported by the findings in rats injected intracerebrally with MMP-9 and plasmin which resulted in opening of the BBB and axonal damage (Armao *et al*, 1997).

4.4.5. MMPs in cell infiltration and BBB damage.

Initial lesions in MS appear to arise around periventricular veins and subsequently spread into the white matter in a form of Dawson's finger. Such lesions are characterised by increased perivascular infiltration of mononuclear cells and vascular wall damage. t-PA- and u-PA-positive mononuclear cells in the blood vessels walls in MS white matter and in early lesions may be the first sign of facilitated entry of leukocytes into the CNS. *In vitro* studies have demonstrated that activation of T-cells, an important component of the inflammatory infiltrate in the MS plaque, induces rapid upregulation of u-PA receptor (Nykjaer *et al*, 1994) whereas adhesion to endothelial cells induces u-PA activity but downregulates PAI-1 (Romanic & Madri, 1994). The presence of MMP-9 protein on blood

vessel collagen and mRNA in infiltrating mononuclear cells indicates that proteolytic enzymes are produced at the BBB in MS lesions and immunolocalise with the ECM components of the vessel wall. Lymphocytes can be stimulated by inflammatory cytokines and chemokines to produce MMP-9 (Montgomery *et al*, 1993) and direct contact between activated T cells and monocytes *in vitro* is considered to be a major pathway for induction of metalloproteinase expression and to a lesser extent of TIMP (Lacraz *et al*, 1994a). Furthermore, Johnatty *et al*. (1997) have demonstrated that the pro-inflammatory cytokines IL-1 and TNF- α may selectively upregulate MMP-9 on CD4-positive but not regulatory CD8-positive lymphocytes thus affecting the transmigration of a specific subset of immune cells. The requirement for MMPs in lymphocyte migration across collagenous basal lamina which anchors endothelial cells to parenchymal surfaces has been demonstrated *in vitro* using the hydroxamic acid inhibitor of MMPs (Leppert *et al*, 1995). In MS the phenomenon of mononuclear cell infiltration is closely linked to the damage and increased permeability of BBB. In NAWM and active plaques immunostaining with anti-collagen IV antibody frequently shows an increase in fibrous and granular debris in the proximity of blood vessels. The evidence for the MMP involvement in BBB damage comes from animal studies in which intracerebral injection of MMP-2 or -9 resulted in increased vascular permeability inhibitable by endogenous and synthetic MMP inhibitors (MunBryce & Rosenberg, 1998a, Rosenberg *et al*, 1998). Plasmin injection in addition causes fibrinoid necrosis of blood vessel walls, a vascular pathology associated with MS (Armao *et al*, 1997). The BBB damage leads to influx of cell and proteins and amplification of the inflammatory process in the brain parenchyma. In MS patients the changes in capillary permeability often precede T₂-weighted MRI evidence of tissue damage and correlate with the increased concentrations of MMP-9 and u-PA in the CSF (Rosenberg *et al*, 1996).

Therefore, the demonstration of t-PA and u-PA-positive mononuclear cells in the

perivascular cuffs of the primary MS lesion and co-localisation with MMP-9 on blood vessel collagen could represent the catalyst for the entry of primed lymphocytes and monocytes which may trigger the conversion of pro-MMPs to their active forms in microglia and astrocytes, causing the damage to the BBB and precipitating the inflammatory demyelinating process in MS and lesion spreading.

4.4.6. Potential MMP roles in tissue regeneration.

Although MMPs are frequently associated with tissue damage, they also take part in many physiological processes such as embryo implantation or tissue reparation during wound healing or bone fracture. Furthermore, in kidneys some forms of therapy which selectively upregulate TIMP-1 and inhibit MMPs lead to fibrosis and impairment of function (Duymelinck *et al*, 1998). Such studies raise the question whether MMPs may play a role in regeneration of the CNS and whether inhibition of MMPs in MS can actually promote scarring and accumulation of ECM proteins which in turn may inhibit oligodendrocyte migration into the lesions. Recently Uhm *et al*. (1998) demonstrated that oligodendrocytes use MMP-9 to extend processes along astrocyte monolayers. MMPs and PAs also mediate neuronal precursor motility and axon outgrowth during CNS development. Further, macrophage plasminogen activity releases matrix bound growth factors such as bFGF, an action dependent on cell-bound u-PA (Falcone *et al*, 1993) and MMPs also remove inhibitory IGF-BPs increasing the bioavailability of potent myelinogenic insulin-like growth factors (Collettsolberg & Cohen, 1996).

It is possible that MMP activity in MS may reflect diametrically opposed functions depending on the phase of the disease; protease activity observed during remission may be derived from oligodendrocytes undergoing a process outgrowth as an attempt to remyelinate axons. Although this is possible at earlier stages in lesion development, the PA and MMP effect on remyelination in MS is limited and probably indirect through an

increase in bioavailability of myelinogenic growth factors and ECM remodelling.

4.4.7. *Conclusions.*

The results from this study highlight the role of MMPs as a causative factor in MS pathology. Immunolocalisation of MMPs and PAs on plaque macrophages coupled to increased total MMP-9 activity as shown by ELISA and *in situ* zymography implies a role in inflammatory demyelination in active MS lesions. Furthermore, immunocytochemistry places MMP-9 and t-PA in close proximity to periaxonal myelin and blood vessel matrix, the major targets of proteolytic attack in MS. This identifies MMPs and their upstream regulators PAs as therapeutic targets and further strengthens the hope that the development of specific enzyme inhibitors might complement the as yet limited arsenal of drugs to treat MS.

Chapter V

5. Insulin-like growth factors and binding proteins in multiple sclerosis plaques: implications for remyelination and inflammation.

5.1 Introduction.

5.1.1. Remyelination in MS lesions.

The major feature of MS lesions is extensive demyelination, a consequence of successive inflammatory attacks which result in chronic gliosed lesions. Remyelination does occur in MS but is incomplete and limited to early stages of lesion development (Prineas *et al*, 1993a). Furthermore, the newly formed myelin is characterised by a decreased thickness to axon diameter ratio and shortened internode distance. This contrasts with EAE and other animal demyelinating models in which remyelination closely follows demyelinating episodes leading to full functional recovery.

There are several possible reasons for the lack of remyelination in the human CNS. In active MS lesions secretory products of inflammatory cells, such as TNF- α , were shown to damage oligodendrocytes and impede remyelination (Selmaj & Raine, 1988). Reparation in the form of astrogliosis and accumulation of extracellular matrix in chronic lesions may form a physical barrier to oligodendrocytes which prevents repopulation and remyelination of lesions (Sobel, 1998). Nevertheless, a proportion of mature oligodendrocytes and their precursors survive and can be found even in chronic lesions (Wolswijk, 1998). These findings point to the possible lack of mediators which promote oligodendrocyte mitogenesis and new myelin synthesis. Growth factors such as IGF, PDGF, EGF and bFGF have profound effects on all aspects of oligodendrocyte development and synthesis of the macromolecular components of myelin membranes. In the context of an inflammatory CNS disease growth factors may promote survival of

oligodendrocytes but may also stimulate immune cells. Therefore, growth factors functioning at an interface between the the inflammatory front and unaffected tissue may tip the balance towards either tissue repair or further tissue damage.

5.1.2. *Insulin-like growth factors and binding proteins in myelination and immunity.*

IGFs are growth factors for many cells in an organism and are closely related to the anabolic hormone insulin (Jones & Clemmons, 1995). Major mitogenic effects of IGFs are mediated through IGF-IR although both insulin and IGF-II also bind to their specific receptors mediating individual effects. Fine regulation of IGFs is achieved through the action of IGF-BPs which form a delivery system from the place of synthesis to target tissues and prolong the half-life of IGFs. IGF-BPs can be both inhibitory and stimulatory for IGF function depending on a number of local factors and may also initiate IGF-independent effects through integrin binding (Collettsolberg & Cohen, 1996).

The interest in the IGF family in MS arises from their role in CNS development and myelination as well as from their effects on cells of the immune system. The IGFs influence proliferation and maturation of oligodendrocyte progenitors (Mozell & McMorris, 1991, McMorris *et al*, 1993, Armstrong *et al*, 1992) and increase myelin production both *in vitro* and *in vivo* (Carson *et al*, 1993, Mozell & McMorris, 1991, Saneto *et al*, 1988). High levels of IGF-I, IGF-II and IGF-IR are detected in developing rodent and human brain and their expression correlates spatially and temporally with the onset of myelination (Bondy, 1991, Lee *et al*, 1992b, Dekeyser *et al*, 1994a). Data from transgenic and gene knock-out animals further support a specific role of IGFs in CNS development and myelination. Mice overexpressing IGF-I transgene were found to have an increased brain size and total myelin content in comparison with other organs and their non-transgenic littermates (Carson *et al*, 1993). Studies in IGF-I knock-out animals have shown that IGF-I gene disruption results in regional brain hypomyelination although the effects

were more indirect affecting primarily neurons (Beck *et al*, 1995, Cheng *et al*, 1998). In the immune system IGF-I stimulates lymphopoiesis (Clark *et al*, 1993), immunoglobulin production (Robbins *et al*, 1994) and mitogen-induced proliferation of human lymphocytes (Kooijman *et al*, 1992a). Rodent monocytes and macrophages respond to IGF-I with increased production of the pro-inflammatory cytokine TNF- α (Renier *et al*, 1996) and phagocytosis and oxidation of lipoproteins (Hochberg *et al*, 1992).

Synthesis of IGFs within the normal adult CNS is low except in the choroid plexus which is the major CNS source of IGF-II. IGF-I is mainly produced in liver and can be delivered from peripheral blood by an active transport mechanism across the blood-brain barrier (Reinhardt & Bondy, 1994). In a number of animal models of CNS disorders such as physical trauma (Garcia-Estrada *et al*, 1992, Walter *et al*, 1997, Li *et al*, 1998) and ischemic injury (Beilharz *et al*, 1995) components of the IGF system are produced locally by glial cells and neurons. Similarly, enhanced IGF-I expression was found in astrocytes in experimental models of demyelination induced by cuprizone (Komoly *et al*, 1992), cryogenic injury (Yao *et al*, 1995b), and transfer of myelin-specific T cells (Liu *et al*, 1994). Astrocyte expression of IGF-I was found to correlate with increased myelin protein mRNA levels and occurrence of remyelination. In addition, IGF-I treatment of both acute demyelinating and chronic relapsing EAE resulted in a decrease of the clinical scores, lesion size and the number of macrophages and T-cells within lesions (Yao *et al*, 1995a, Liu *et al*, 1997, Li *et al*, 1998). The reconstitution of the BBB appeared to be the mechanism underlying such effects. In contrast in the mouse EAE model treatment with an IGF-I/IGF-BP3 complex induced a relapse through expansion of encephalitogenic T-cells (Lovett-Racke *et al*, 1998) providing direct evidence that IGFs can stimulate immune cells during the course of CNS disease. Such effects were only reported for IGF-BP3 complexes but not IGF-I-treated animals which highlights the importance of IGF-BPs as naturally

occurring regulators of IGF function.

5.1.3. Aims of the study.

The cited studies indicate that in MS lesions IGFs and IGF-BPs may be both beneficial as remyelinating agents and detrimental through stimulation of immune cells. In order to address this apparent paradox the cellular distribution of IGFs and their binding proteins have been studied at different stages of MS lesion development by immunocytochemistry.

5.2. Materials and Methods.

5.2.1. Tissue.

Clinical data for each MS and normal control case are given in Chapter 2, Tables 2.4a and 2.4b. For the IGF study the total of 23 snap-frozen blocks containing plaques and/or macroscopically normal-appearing white matter from 14 MS cases and 8 blocks of white matter from 8 normal control cases were used. Samples from MS cases included 5 with active, 7 with subacute and 6 with chronic MS lesions and 5 blocks of far normal-appearing white matter (**Table 5.1**). Seven snap-frozen blocks from 3 cases of cerebral infarcts and 2 brain tumours (meningioma and glioblastoma multiforme) were used, as neurological disease controls to assess the specificity of immunocytochemical findings for MS. Snap-frozen samples of liver and pancreas were used as a positive control tissue.

TYPE OF TISSUE	AGE (RANGE)	SEX RATIO M:F	DFT (RANGE)	DURATION OF MS
NC CASES n=8	49.4 y (22-69)	5:3	36.0 h (8-89)	N/A
MS CASES n=14	54.1 y (34-80)	5:9	24.6 h (9-41)	17.6 y (2-36)

Table 5.1: Age, sex ratio and death to snap-freezing time (DFT) data for MS and normal control cases.

5.2.2. Antibodies.

The list and properties of antibodies used in this study are given in the **Table 5.2** below. Data for the marker and control antibodies are given in Chapter II, Table 2.2.

Antibodies	Antigen	Isotype	Dilution	Source
IGF-I	IGF-I from human plasma	IgG1 κ	1:50	Upstate Biotechnology, Lake Placid, USA
IGF-II	IGF-II conjugated to human α -globulin	IgG	1:20	Serotec, Oxford, UK
Insulin	Human insulin	IgG1	1:20	Sigma, Poole, UK
IGFR-1α	Peptide representing exon 3 of the IGF-IR α subunit	IgG2a	1:100	TCS Biologicals, Bothof Claydon, UK
IGFR-1β	C-terminal peptide (15aa) of the IGF-IR β subunit	IgG3	1:100	“
IGF-BP1	IGF-BP1 from human amniotic fluid	IgG1	1:40	Serotec, Oxford, UK
IGF-BP2	IGF-BP2 from Madin Darby Bovine Kidney cells	Poly	1:1000	Upstate Biotechnology
IGF-BP3	Bovine IGF-BP3	Poly	1:1000	“

Table 5.2: Antibody panel used in IGF study. Poly – polyclonal rabbit antiserum.

5.2.3. Immunocytochemistry.

Single and double immunocytochemical staining was performed as described in Chapter II, Sections 2.2.5. and 2.2.6. For double staining experiments monoclonal antibodies (anti-IGF-I, anti-insulin and anti-IGF-BP1) were detected using FITC-labelled goat anti-mouse secondary antibody (Sigma) whilst polyclonal antisera (anti-GFAP and anti-CNPase) were detected with biotin-labelled goat anti-rabbit secondary antibody and TRITC-labelled avidin. Routine immunocytochemical controls included omission of primary antibodies as well as the application of normal rabbit serum (Dako) and control mouse ascites fluid (Sigma) at the same concentrations as primary antibodies. Antigen blocking was performed for anti-IGF-I and anti-insulin antibodies with recombinant human IGF-I (UBI) and bovine insulin (Sigma), respectively (Chapter II, Section 2.2.7.).

5.3. Results.

5.3.1. IGFs and IGF-IR are expressed in normal control grey matter.

Expression of IGF-I (**Fig. 5.1a**), IGF-BP1 and insulin was detected in small numbers of glial cells in normal control white matter. Similar results were obtained with antibodies against IGF-II (**Fig. 5.1b**), IGF-IR subunits α and β , and IGF-BP2 and 3. Glial cell staining in white matter has not been further characterised. In contrast, in cortical grey matter strong staining of neuronal cell bodies was seen with anti-insulin, IGF-IR (**Fig. 5.1d**) and IGF-BP1 (**Fig. 5.1c**) antibodies whereas at the grey-white matter junction large fibrous astrocytes were moderately immunostained with anti-IGF-I. The matrix of cerebral blood vessel in both white and grey matter was only stained with the anti-insulin antibody although anti-IGF-I visualised astrocyte endfeet surrounding these vessels.

Immunocytochemical controls, including omission of the primary antibody and mouse ascites, were negative. Normal rabbit serum stained glial cells but the staining was markedly weaker than that of the IGF-BP2 and BP3 antisera. Pre-incubation of IGF-I (**Fig. 5.1e** and **f**) and insulin antibodies with recombinant antigens abolished staining in both normal control and MS tissue. In pancreas samples, anti-insulin antibody specifically highlighted β -islets (**Fig. 5.1g**) whereas the rest of the antibodies including IGF-I (**Fig. 5.1h**) weakly stained both endocrine and exocrine areas. Results from the liver samples were inconclusive due to the high background staining.

5.3.2. Oligodendrocytes in NAWM express IGF-BP1 but not IGF-IR.

In NAWM adjacent to and distant from plaques a distinct cellular distribution of IGFs and IGF-BPs became evident which was also observed in both demyelinating lesions and CNS infarcts. Hypertrophic fibrous astrocyte perikarya and processes were immunostained with IGF-I (**Fig. 5.2a**) in both white matter and at the cortical grey and white matter junction. Astrocyte endfeet surrounding white matter blood vessels were also

IGF-I immunopositive. IGF-BP1 was similarly expressed in fibrous astrocytes in NAWM. Distribution of both IGF-I- and IGF-BP1-positive astrocytes correlated with that of GFAP-positive cells (**Fig. 5.2b**). In white matter adjacent to active lesions a number of enlarged oligodendrocyte cell bodies were IGF-BP1-positive. Comparison with the 14E antibody which stains oligodendrocytes and hypertrophic astrocytes, and double staining with an oligodendrocyte-specific anti-CNPase antibody (**Fig. 5.2e and f**), confirmed these findings. IGF-BP1-positive astrocytes and oligodendrocytes were also found in NAWM surrounding chronic lesions.

Co-expression of IGF-II (**Fig. 5.2c**), IGF-R1 α and β , IGF-BP2 and BP3 was visualised on activated microglia and correlated with the distribution of EBM-11-positive cells (**Fig. 5.2d**). A subpopulation of astrocytes in the proximity of active plaque borders was also positive for IGF-IR. Infiltrating mononuclear cells around blood vessel walls expressed only low levels of IGF-II-and IGF-IR. In cortical grey matter IGF-IR and IGF-BP1 were expressed at high levels in neuronal cell bodies and axons. In grey matter adjacent to active MS lesions, astrocytes were strongly stained with anti-insulin and IGF-I antibodies as shown by double staining with anti-GFAP antibody. The same astrocyte population also expressed the β subunit of the IGF-IR.

5.3.3. IGF-II and IGF-IR expression characterise foamy macrophages in active plaques.

In the border of actively demyelinating plaques large hypertrophic astrocytes with several thick processes were strongly stained with the anti-IGF-I antibody (**Fig. 5.3c and e**). Their numbers sharply declined towards the lesion centre where only dense astrocyte processes were visualised. Insulin- and IGF-BP1-positive (**Fig. 5.3f**) astrocytes were found throughout the lesion parenchyma and borders and correlated in distribution with the GFAP-positive cells (**Fig. 5.3d**). Double staining with anti-GFAP antibody showed that IGF-BP1 was confined to astrocyte cell bodies (**Fig. 5.4e and f**). In subacute and chronic

plaques expression of IGF-I and insulin were associated only with astrocyte processes in gliotic scars. In contrast the expression levels of IGF-BP1 appeared to be unchanged and reflected the number of astrocytes within chronic lesions.

The anti-IGF-II antibody (**Fig. 5.4b**) stained foamy macrophages in plaques and perivascular inflammatory infiltrates. IGF-BP2 (**Fig. 5.4d**) and BP3 were similarly expressed by macrophages throughout the lesion parenchyma. Both IGF-IR subunits were detected on foamy macrophages (**Fig. 5.4c**) and a small proportion of hypertrophic astrocytes within the lesion. Although macrophages in inflammatory infiltrates were strongly IGF-II positive other mononuclear cells expressed only low levels of IGF-IR and binding proteins. In subacute and chronic lesions the few remaining macrophages confined to perivascular spaces and lesion borders were positive for IGF-II, IGF-BP2 and BP3.

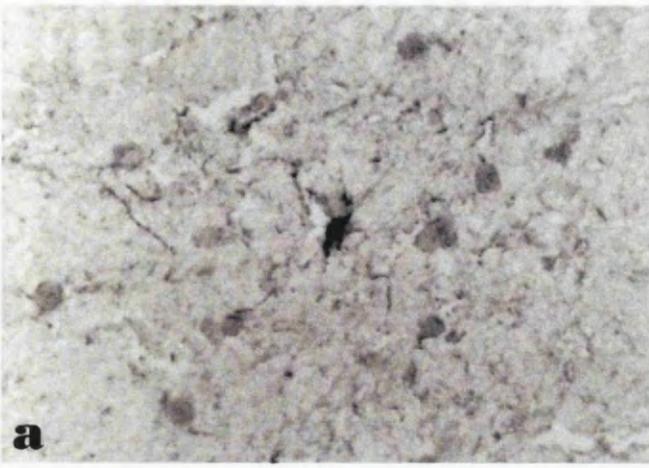
5.3.4. IGF distribution in infarcts and tumours is similar to that in MS lesions.

CNS infarcts and brain tumours were used to assess the specificity of the IGF expression pattern observed in MS lesions. Staining of recent CNS infarcts showed a similar distribution of IGF-I, IGF-II, insulin, receptor subunits and binding proteins to that in MS lesions. Astrocyte cell bodies in penumbra were positive for IGF-I (**Fig. 5.5d** and **e**) and IGF-BP1 whilst insulin expression in astrocytes was lower in comparison with that in MS lesions. Macrophages infiltrating infarcted grey matter were immunostained with antibodies against IGF-II (**Fig. 5.5b** and **f**), IGF-R1 (**Fig. 5.5c**) and IGF-BP2 and 3. In unaffected grey matter but not infarcts, neuronal cells expressed IGF-I and insulin. IGF-I-positive infiltrating macrophages were found in glioblastoma multiforme in addition to positive tumour cells (**Fig. 5.5g**) whilst IGF-II was confined to infiltrating mononuclear cells (**Fig. 5.5h**). The other IGF-I family members showed the same cellular distribution as was seen in infarcts and MS lesions.

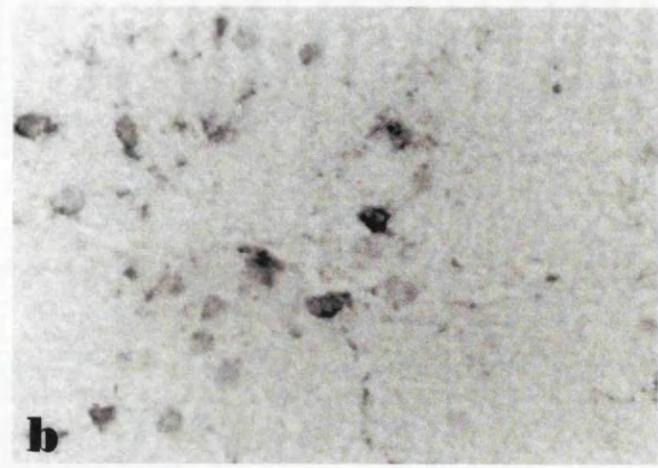
	Normal-appearing white matter (n= 5)	Active lesions (n= 5)	Subacute and chronic lesion (n= 7 and 6)
Histopathology	Reactive glia surrounded by apparently normal myelin.	Partially demyelinated lesion with numerous ORO+ cells and pronounced perivascular infiltration.	Completely demyelinated lesion with no ORO+ cells or limited numbers confined to lesion edge.
α-IGF-I	Hypertrophic astrocyte cell bodies and processes.	Hypertrophic astrocytes predominantly in lesion border.	Astrocyte processes.
α-Insulin	As for IGF-I.	Hypertrophic astrocyte cell bodies and processes.	As for IGF-I.
α-IGF-BP1	Hypertrophic astrocyte and oligodendrocyte cell bodies.	Hypertrophic astrocytes and occasional oligodendrocyte.	Astrocyte processes and cell bodies.
α-IGF-II	Activated microglia.	Foamy macrophages.	Occasional macrophage confined to lesion edge or perivascular space.
α-IGF-BP2/3	As for IGF-II.	Foamy macrophages.	As for IGF-II.
α-IGF-IR	Activated microglia and hypertrophic astrocytes.	Foamy macrophages and a subpopulation of astrocytes.	As for IGF-II.

Table 5.3: Staining patterns of the IGF antibody panel on serial sections of macroscopically normal-appearing MS white matter and MS lesions at different stage of development. In normal control white matter all IGF and IGF-BP antibodies stained only occasional glial cells.

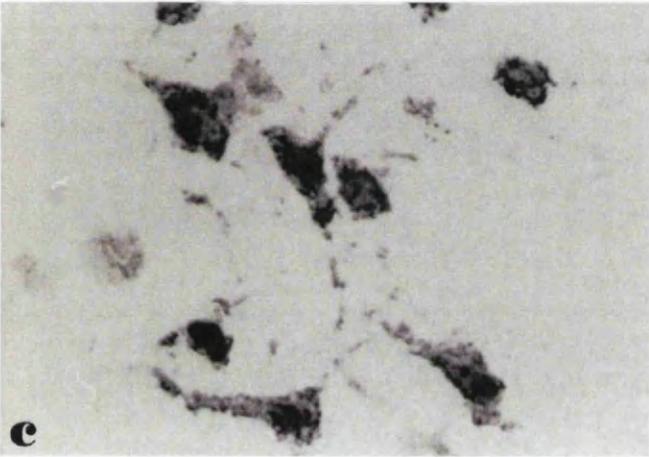
Figure 5.1. Expression of IGFs and IGF-BPs in normal control brain white and cortical grey matter. Immunoperoxidase staining of glial cells in white matter with (a) anti-IGF-I and (b) anti-IGF-II. Neuronal cell bodies in grey matter stained with (c) anti-IGF-BP1 and (d) anti-IGF-IR α antibodies. Staining with the anti-IGF-I antibody on a section of an active MS lesions (e) was completely abolished by blocking with the recombinant antigen (f). On sections of snap-frozen human pancreas (g) anti-insulin antibody stained β -islets whilst (h) anti-IGF-I antibody weakly stained pancreas parenchyma. All at magnification x512 except (e) and (f) at x256; no counterstaining.



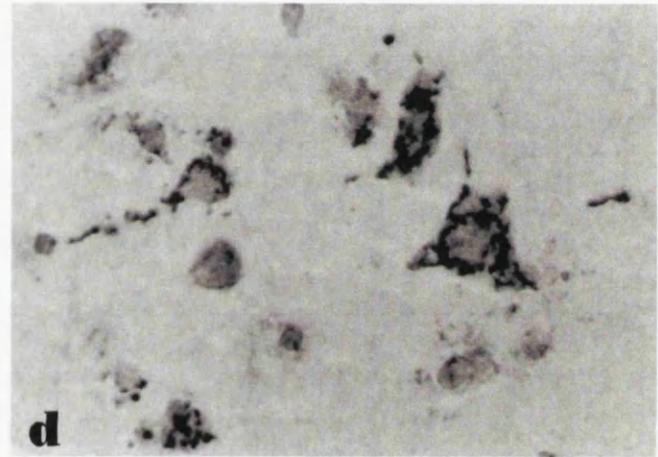
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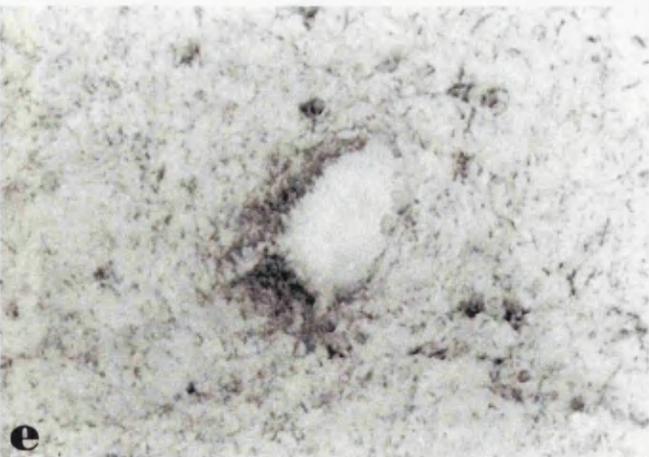
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c



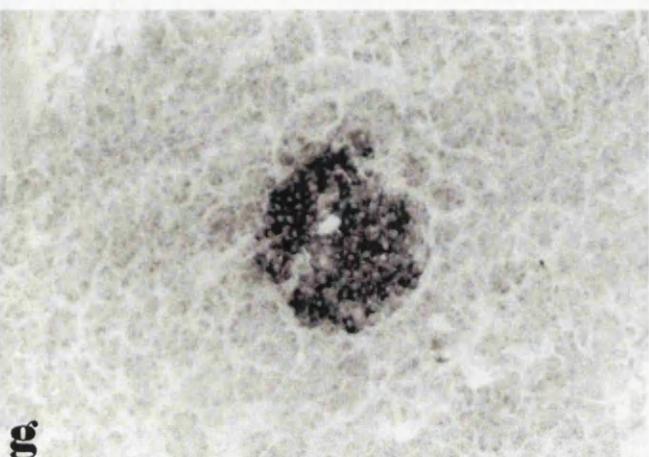
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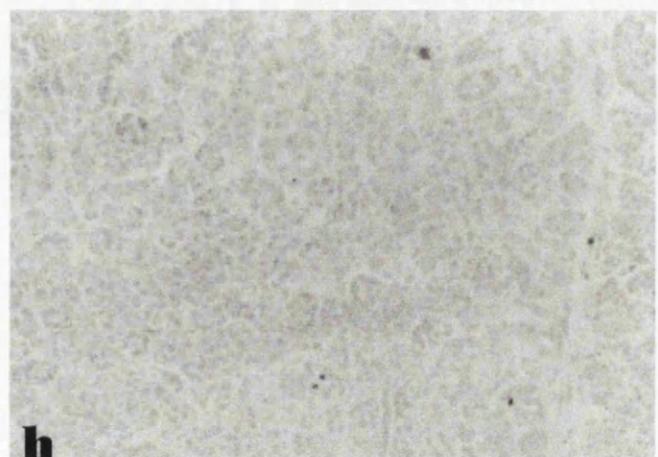
e



f



g



h

Figure 5.2. Serial sections of normal-appearing white and grey matter from an MS case. Immunoperoxidase staining of astrocytes with (a) anti-IGF-I and (b) anti-GFAP and activated microglia with (c) anti-IGF-II and (d) the EBM-11 antibody. Double immunofluorescence staining of oligodendrocytes in white matter adjacent to an actively demyelinating lesion with (e) anti-IGF-BP1 (FITC) and (f) anti-CNPase (TRITC) antibodies confirms the expression of IGF-BP1 in this cell type. (g) hypertrophic astrocytes in grey matter from MS cases immunoperoxidase stained with anti-insulin and (h) neuronal and astrocyte perikarya stained with anti-IGF-IR β antibody. All x512.

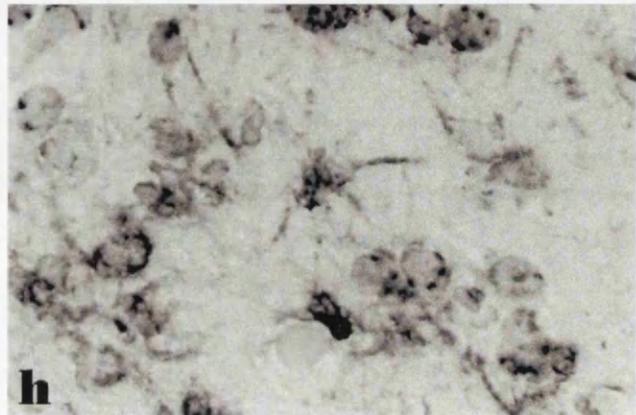
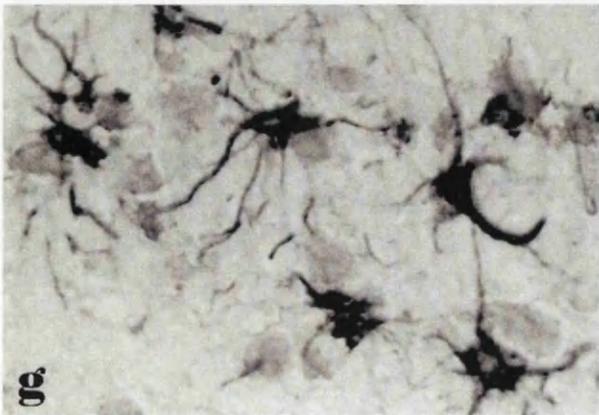
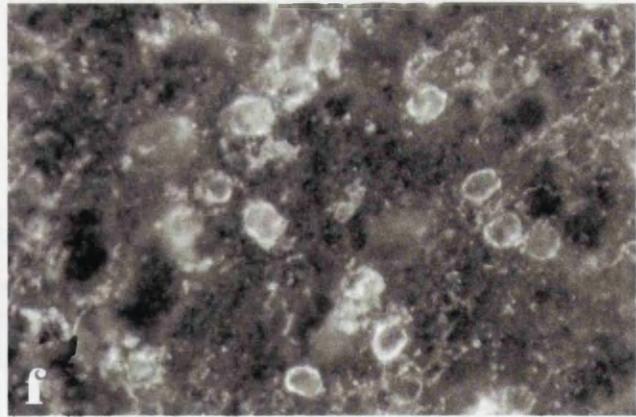
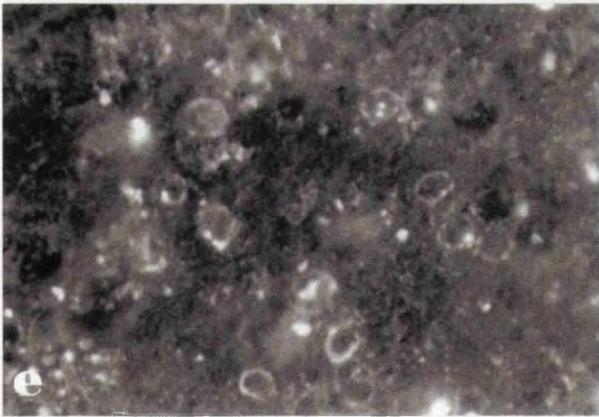
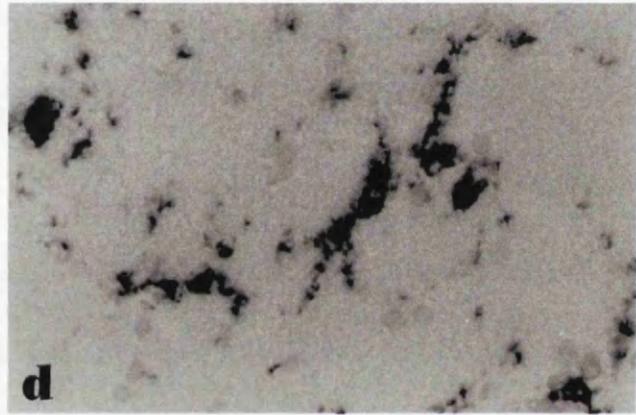
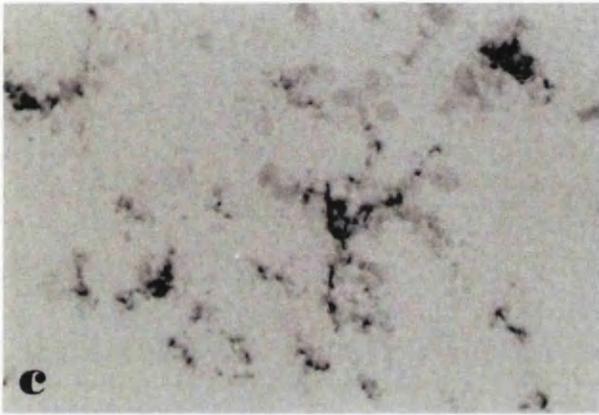
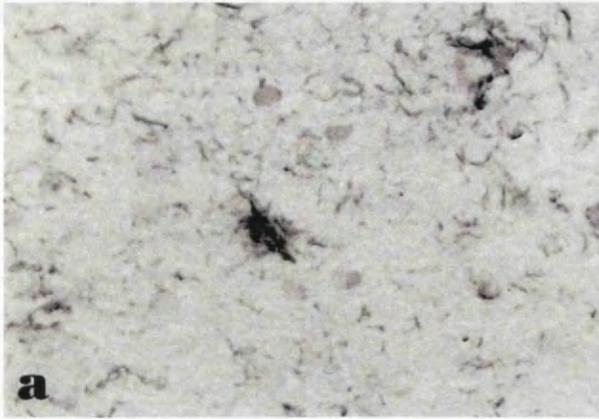


Figure 5.3. Serial sections of an active MS lesion in white matter bordering cortical grey matter. Low magnification photomicrographs showing (a) distribution of EBM-11-positive macrophages in plaque, (b) anti-galactocerebroside staining of the neighbouring section to demonstrate areas of demyelination, (c) distribution of IGF-I- and (d) GFAP-positive astrocytes in plaque border and parenchyma. White, grey matter and plaque areas are designated in Fig. 3B by the capital letters W, G and P, respectively. (e) IGF-I-positive hypertrophic astrocytes in lesion border and (f) IGF-BP1-positive astrocyte cell bodies in lesion parenchyma. All x32 except (e) and (f) x512.

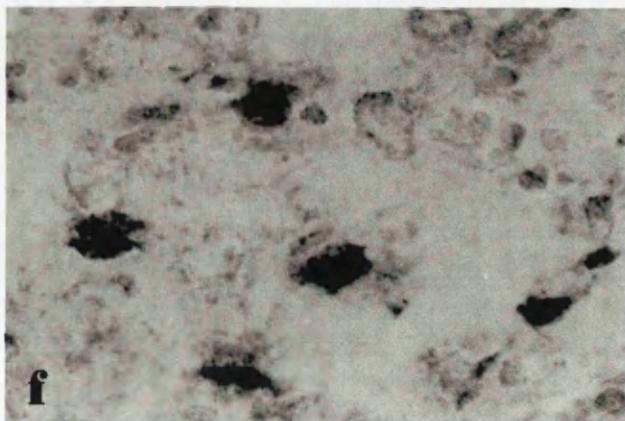
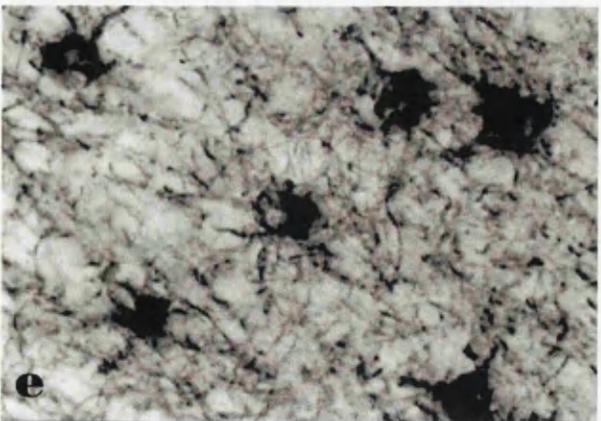
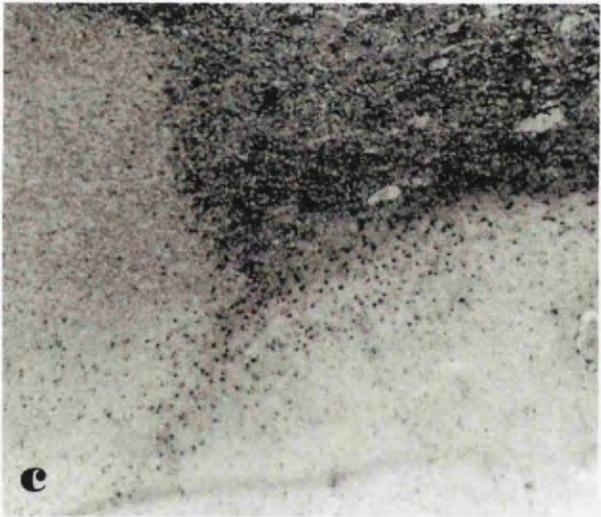
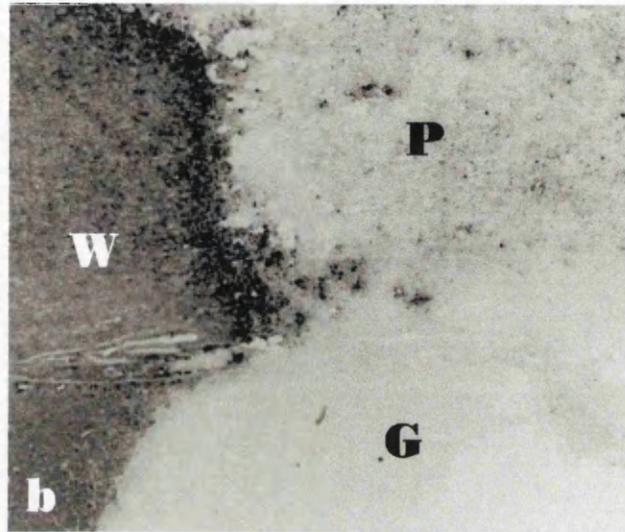
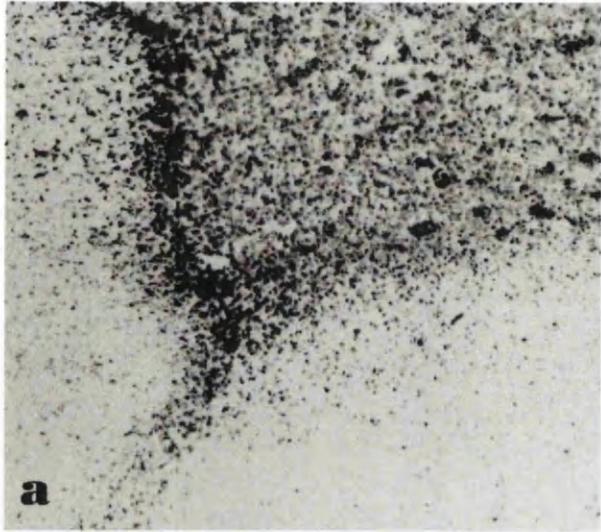


Figure 5.4. Foamy macrophages in the active MS lesion shown in Figure 3. stained with (a) macrophage marker EBM-11, (b) anti-IGF-II, (c) anti-IGF-IR α and (d) anti-IGF-BP2 antibodies. Double staining of astrocytes in active lesion parenchyma shown in Fig. 3 with (e) anti-IGF-BP1 (FITC) and (f) anti-GFAP (TRITC). Double-stained cells are indicated with asterisks. All x512.

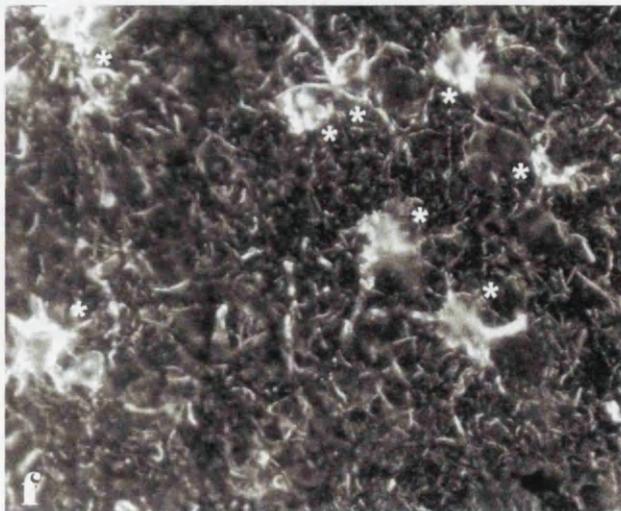
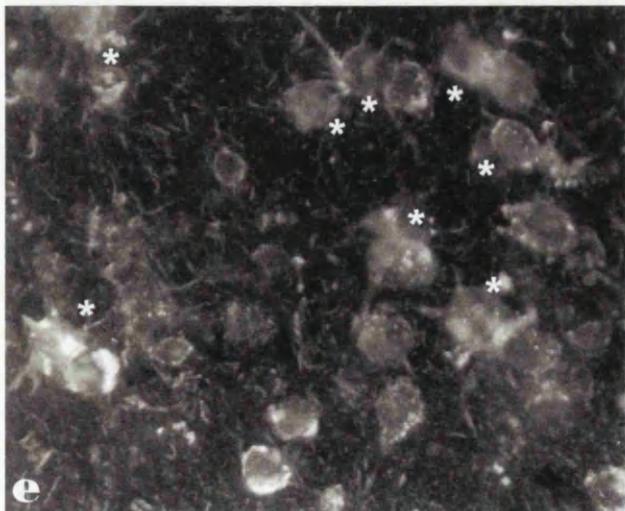
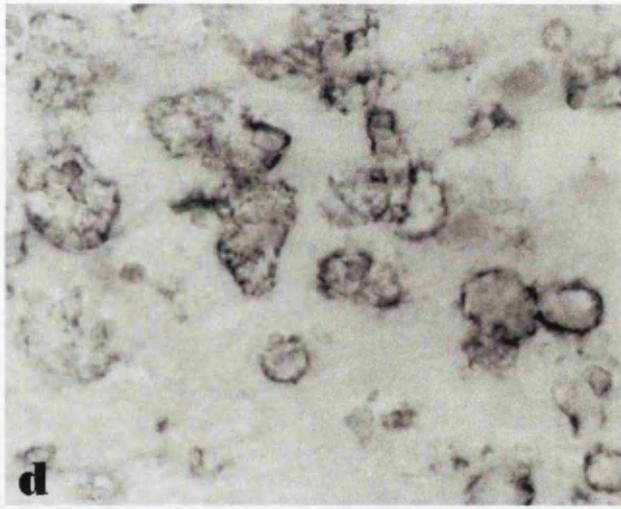
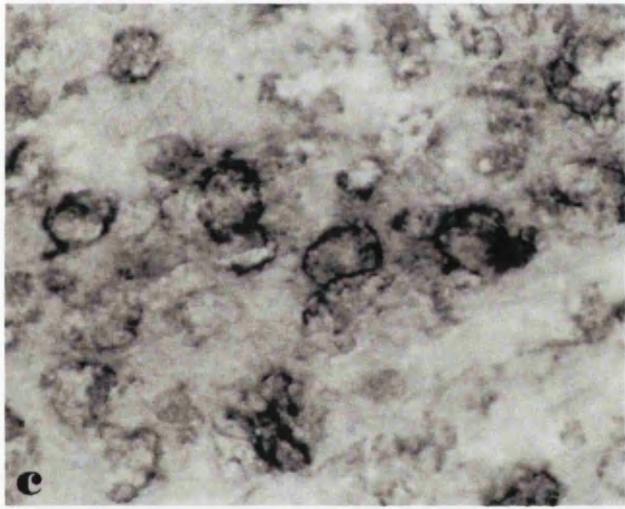
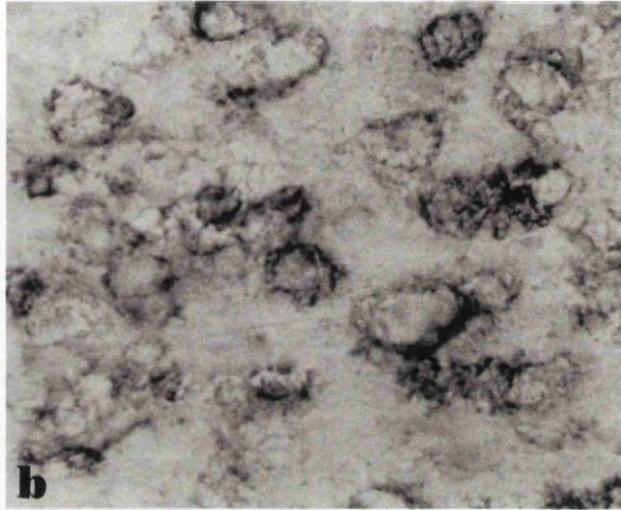
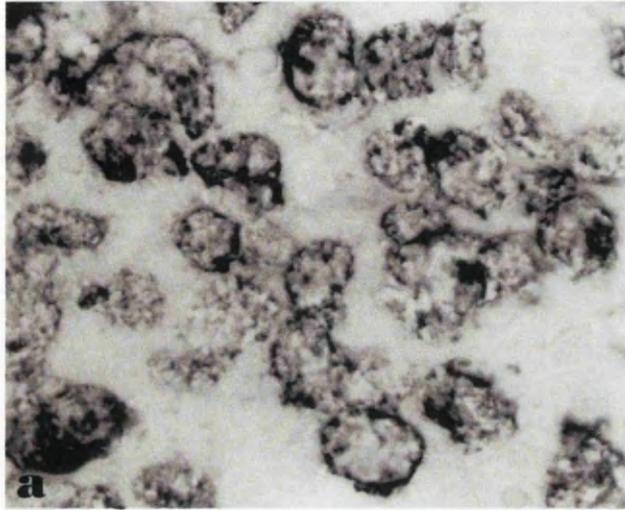
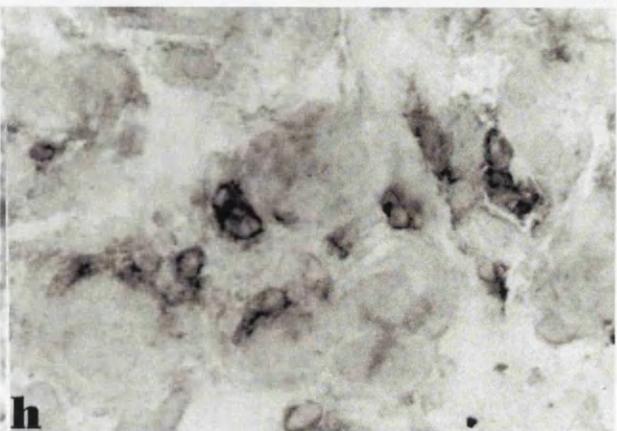
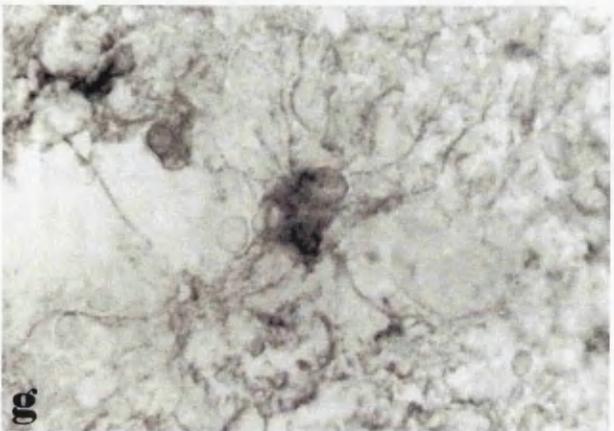
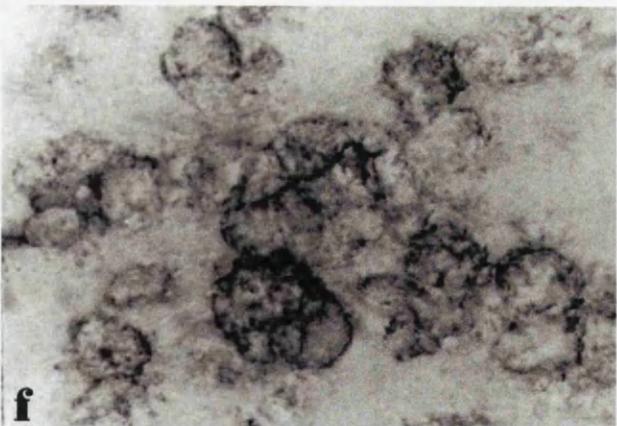
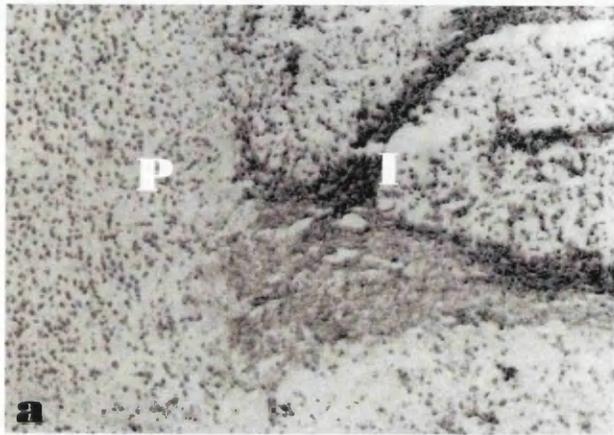


Figure 5.5: The expression of IGFs in brain infarcts and tumours. Low magnification photomicrographs showing (a) distribution of EBM-11-positive macrophages in infarct (capital letter I) and penumbra (P). Macrophages were also highlighted with (b) anti-IGF-II and (c) anti-IGF-IR whilst (d) anti-IGF-I stained reactive astrocytes in the penumbra. High power photomicrographs showing (e) hypertrophic astrocytes stained with anti-IGF-I and (f) foamy macrophages stained with anti-IGF-II. (g) In glioma multiforme anti-IGF-I stained bizarre astrocyte-like tumour cells. (h) Anti-IGF-II expression was confined to infiltrating mononuclear cell in a meningioma. (a-d) x32, (e-h) x512.



5.4. Discussion.

5.4.1. Summary of findings.

The results in this study demonstrate the co-localisation of IGF-I and IGF-BP1 on astrocytes, and IGF-II, IGF-BP2 and BP3 on microglia and macrophages in NAWM and MS lesions. The IGF-IR was present on astrocytes and microglia in plaque and NAWM and neuronal cells in cortical grey matter but appeared to be absent in oligodendrocytes in NAWM. The highest levels of this receptor were found in macrophages in MS lesions and neuronal cells in grey matter. Only minor differences in the cellular distribution of IGFs and IGF-BPs were observed between MS lesions, brain infarcts and tumors.

5.4.2. IGF-I is a part of an early astrocyte response to pathological changes in the CNS.

IGF-I was expressed in fibrous astrocytes in three distinct areas of the brain in MS, at the grey-white matter junction, NAWM and the active plaque border. Because IGF-I is reported to support both astrocyte proliferation and neuronal survival, acute phase gliogenic or neurotrophic roles for IGF-I within MS lesions are implicated. In addition, expression on astrocyte endfeet surrounding cerebral blood vessels in white matter also suggests a role in BBB function.

The astrocyte reaction to pathological changes in the CNS mirrors that of microglia in terms of both sensitivity and intensity (Kraig *et al*, 1991, Mucke *et al*, 1991). Such a reaction, termed astrocytosis, is characterised by astrocyte proliferation, extensive hypertrophy of nuclei, cell bodies and processes and an increase in the cytoskeletal protein GFAP which is a useful immunocytochemical indicator of astrocyte activation. GFAP expression is rapidly upregulated in response to a variety of CNS insults and is not only confined to the site of injury. In EAE simultaneous upregulation of GFAP and IGF-I mRNA suggests that this is a part of a co-ordinated astrocyte response to CNS injury (Liu *et al*, 1994). To a large extent IGF-I immunoreactivity in MS tissue reflected that of GFAP

indicating that upregulation of IGF-I may be a part of the astrocyte reaction to inflammatory demyelination. IGF-I and insulin were shown *in vitro* to promote astrocyte proliferation and migration (Tranque *et al*, 1992, Matsuda *et al*, 1996, Faberelman *et al*, 1996) which are the phenomena underlying astrogliosis. Gliotic scarring is characterised by GFAP accumulation and in MS lesions it limits the potential for remyelination. In organotypic cultures of mouse brain, high doses of insulin were shown to upregulate GFAP mRNA and to increase the number of GFAP-positive astrocytes (Toran-Allerand *et al*, 1991). However, *in vivo* data addressing the question of IGF-I regulation of GFAP expression are limited. Paradoxically, Fernandez *et al*. (1997) have shown that continuous infusion of IGF-I in animals injected with the neurotoxin 3-acetylpyridine significantly decreased the degree of reactive gliosis in the injured area. Furthermore in experimental allergic neuritis (EAN) upregulation of GFAP is not followed by increased IGF-I production in astrocytes, indicating that co-ordinated expression of IGF-I and GFAP may be CNS specific (Bastuardo *et al*, 1995). Therefore, IGF-I and insulin are capable of promoting astrocytosis and/or astrogliosis *in vitro* but the outcome of astrocyte stimulation *in vivo* will depend on IGF interactions with other mediators within MS lesions including other growth factors and cytokines.

5.4.3. Neuroprotective role of astrocyte derived IGF-I.

Beside autocrine effects on astrocyte proliferation and migration IGF-I may also exert paracrine effects on glial and neuronal cells expressing IGF-IR. This situation may arise at the white-grey matter junction where constitutively IGF-I-positive astrocytes are found in the vicinity of IGF-IR-expressing neuronal cell bodies. The neurotrophic roles of IGF-I and insulin are well characterised and astrocyte-derived factors have been shown to promote neurite outgrowth and neuronal survival (Feldman *et al*, 1997). Fibrous astrocytes were markedly stronger stained with anti-IGF-I and anti-insulin antibodies at the interface

between active plaques and grey matter, indicating an increased concentration of IGF-I peptide. Both astrocytes and neurones in this area also expressed IGF-BP1 which could be a suitable transport vehicle for the IGF-I between the two cell types. As IGF-BP1 specifically binds to the fibronectin receptor, $\alpha 5\beta 1$ integrin (Jones *et al*, 1993b), mechanisms of such transport may be receptor-mediated enabling IGFs to be delivered to a specific cell type. Interestingly in injured chick nerve the $\alpha 5\beta 1$ integrin becomes prominently expressed in the vicinity of the lesion (Lefcort *et al*, 1992). Studies on motoneuron regeneration and experimental ischemic lesions further support the neuroprotective role of astrocyte-derived IGF-I (Bastuardo *et al*, 1994, Lee *et al*, 1992a, Gluckman *et al*, 1998). It may be speculated that IGF-I, delivered to neurones via IGF-BP1, may prevent or attenuate the inflammatory injury to neuronal cells during the course of MS. Taking into account the beneficial effects of IGF-I on lesion size in EAE (Liu *et al*, 1997, Yao *et al*, 1995a, Li *et al*, 1998), a high level expression of IGF-I at the interface of cerebral white and grey matter may limit lesion spreading into the grey matter.

5.4.4. IGF-I has the potential to support both remyelination and inflammation.

Immunolocalisation of IGF-I-positive astrocytes within MS lesions is in agreement with other studies that showed specific upregulation of IGF-I in relation to the site of CNS injury (Lee *et al*, 1996, Hammarberg *et al*, 1998, Garcia-Estrada *et al*, 1992). In MS lesions astrocyte-derived IGF-I can stimulate both oligodendrocytes in adjacent white matter and microglia/macrophages in the advancing plaque border with different consequences. Studies in experimental models of demyelination strongly suggest that IGF-I promotes remyelination and diminishes the inflammatory response in the CNS (Liu *et al*, 1997, Yao *et al*, 1995a, Li *et al*, 1998). An increase in the concentration of IGFs can be expected in MS lesions due to enhanced local glial cell synthesis or influx through a damaged blood-brain barrier. In hypoxic-ischemic injury movement of IGF-I from the CSF

into brain via white matter tracts and perivascular spaces is enhanced (Guan *et al*, 1996a). However, a concomitant increase in IGF-BPs could have either a significant inhibitory or stimulatory effect on IGF actions within MS lesions. Although high levels of IGF-I were detected there was no convincing evidence of ongoing remyelination in MS lesions.

There are several possible reasons for the apparent lack of IGF-I-induced remyelination in MS. Oligodendrocytes require multiple growth factors for many functions (Barres *et al*, 1993) and IGF-I may not be sufficient to initiate remyelination. Although oligodendrocytes were found in MS lesions they appear to be quiescent cells and may require competence factors such as PDGF to initiate the cell cycle (Wolswijk, 1998). The lack of detectable IGF-IR on oligodendrocytes may also impair their response to IGF-I. This is in contrast to findings in EAE lesions in which upregulation of IGF-IR mRNA and protein in oligodendrocytes was observed in the vicinity of IGF-I positive astrocytes (Liu *et al*, 1994). However, a sustained downregulation of IGF-IR was observed in rats after hypoxic-ischemic injury implying a role for the local injury-related factors (Beilharz *et al*, 1998). In chondrocytes, the target of inflammatory attack in rheumatoid arthritis, raised IGF-I and insulin concentrations rather than inflammatory mediators were shown to downregulate IGF-IR (Joosten *et al*, 1990, Blount & Crawford, 1994). Furthermore, IGF-I stimulation of activated human T-cells also rapidly downregulates IGF-IR (Schillaci *et al*, 1998). Although the latter two studies indicate that IGF-I plays a pivotal role in regulation of its receptor, repeated oligodendrocyte exposure to inflammatory mediators in MS lesions may also affect the receptor levels and therefore the oligodendrocyte responsiveness to this growth factor.

IGF-BPs generally inhibit binding of IGFs to the IGF-IR thus limiting their bioavailability in target tissue. Oligodendrocytes in NAWM expressed IGF-BP1 on their surface which depending on its local concentration, phosphorylation status and association

with cell surface receptor, either inhibits or stimulates IGF-mediated cell responses. An inhibitory role for IGF-BP1 in the CNS is supported by findings in double IGF-I/IGF-BP1 transgenic mice in which the oligodendrocyte numbers and proteolipid protein levels were markedly diminished in comparison with single IGF-I transgenic littermates (Carson *et al*, 1993). Mixed glial cell cultures from these transgenic animals were significantly less responsive to stimulation of proliferation by IGF-I than cultures from wild-type mice (Ni *et al*, 1997). The underlying mechanism in these studies is likely to be the molar excess of IGF-BP1 over IGF-I which prevents binding to the IGF-IR. In contrast, the hypophosphorylated form of human IGF-BP1, due to its lower affinity for IGFs, can potentiate the mitogenic effects of IGF-I (Elgin *et al*, 1987, Jones *et al*, 1991). Furthermore, IGF-I/IGF-BP1 complexes bound to cell surface integrins may form a local growth factor reservoir which slowly releases IGF-I resulting in enhancement of cell growth and tissue repair (Koistinen *et al*, 1990, Kratz *et al*, 1992, Galiano *et al*, 1996). Limited migratory capacity of oligodendrocytes has been considered as a factor contributing to the lack of remyelination in MS lesions. Significantly, binding of IGF-BP1 to integrins in a fibroblast cell line was found to stimulate cell migration in a monolayer wounding assay (Jones *et al*, 1993b). Although multiple factors determine the effect of IGF-BP1 on IGF actions it is tempting to speculate that IGF-BP1 may increase focal concentrations of IGFs on the oligodendrocyte surface and compensate for the low receptor levels, although in that case co-localisation of IGF-I and IGF-BP1 would be expected.

In actively demyelinating plaques IGF-I and insulin could also be indirectly involved in myelin breakdown through stimulation of macrophages, as both growth factors enhance the phagocytic capacity and production of inflammatory mediators such as TNF- α and ROI (Hochberg *et al*, 1992, Renier *et al*, 1996, Rosa *et al*, 1996). The high expression of IGF-IR by macrophages in plaques and activated microglia in adjacent NAWM supports

such a hypothesis. However, the two main effects of IGF-I treatment in EAE animals were reduced inflammatory infiltration and restoration of the BBB (Liu *et al*, 1997, Yao *et al*, 1995a, Li *et al*, 1998). *In vitro* IGF-I inhibits human monocyte adhesion to endothelium in a dose-dependent manner (Motani *et al*, 1996). In that case IGF-I expression on astrocyte endfeet in BBB may reduce mononuclear cell infiltration and contribute to the restoration of the BBB in MS lesions.

5.4.5. Macrophages may determine the bioavailability of IGFs.

The association of IGF-II expression with macrophage infiltration seen in areas of inflammatory demyelination indicates a role for IGF-II in macrophage differentiation in MS lesions which is mediated through IGF-IR. One of the effects of IGF-I treatment in acute and chronic relapsing EAE was a reduction in numbers and in spreading of macrophages. Antagonistic actions of IGF-II on IGF-I-mediated neuronal survival and protein anabolism have been previously documented in animal models (Koea *et al*, 1992, Guan *et al*, 1996b). Interestingly, IGF-II provides an autocrine enhancement of human trophoblast cell invasiveness into Matrigel, largely by stimulating cell migration (Hamilton *et al*, 1998). It may be speculated that IGF-II, coupled to high expression of IGF receptor, can override a putative inhibitory IGF-I effect on macrophages and support their proliferation and invasion of the NAWM.

Autocrine and paracrine effects of IGFs can be further potentiated through enzymatic degradation of inhibitory IGF-BPs by plasmin and MMPs which decreases the affinity of binding proteins and results in release of free IGFs (Collettsolberg & Cohen, 1996). IGFs and their binding proteins have opposing effects on the components of the MMP/PA proteolytic pathway. IGF-I and insulin increase the serum concentration and the endothelial cell expression of PAI-1 (Nordt *et al*, 1998). In contrast, expression of IGF-BP2 in the KB3.1 human cell line increased proteolysis of IGF-BP3 and subsequently

bioavailability of the IGF-II produced by these cells as a result of amplified expression of t-PA and inhibition of PAI-1 (Menouny *et al*, 1998). As activated microglia and macrophages are a major source of plasminogen activators and MMPs in MS lesions (Cuzner *et al*, 1996, Anthony *et al*, 1997), IGF-BP-proteolytic activity generated by these cells could determine the amount of free IGFs available to IGF receptors on macrophages and other immune or glial cells in MS lesions.

5.4.6. A scenario for IGFs involvement in the pathogenesis of MS.

Data from this study suggest that the major effects of IGFs in MS lesions may be the stimulation of myelin clearance by macrophages and astrogliosis. Although in the early stages of MS lesion development IGFs and in particular IGF-I may stimulate remyelination, repeated inflammatory attacks may diminish the capability of oligodendrocytes to respond to IGFs. This could be further potentiated by the putative antagonistic effects of IGF-II and the competition for growth factors by infiltrating inflammatory cells that express high levels of IGF-IR and proteolytic enzymes capable of cleaving inhibitory IGF-BPs. However, in the light of IGFs being survival factors for oligodendrocytes and neurons and the beneficial effects of IGF-I treatment in EAE models, the increased levels of IGF-I in active lesions may attenuate inflammatory damage to these cell types and the BBB during the course of MS.

Chapter VI

6. Conclusions and further work.

6.1. Interactions between NF- κ B, MMPs and IGFs in MS lesions.

The findings from the studies in this thesis link three systems of mediators, the transcription factor NF- κ B with the effector matrix metalloproteases and insulin-like growth factors, into a hypothetical model for the propagation of demyelination in MS. The increased expression of MMPs and IGFs in active MS lesions was found to correlate with the nuclear immunolocalisation of NF- κ B in activated glial and inflammatory cells. The findings, in the context of previously reported data (**Table 6.1**), suggest that interactions between these mediators may occur in MS lesions fuelling the inflammation and demyelination.

Activation of NF- κ B in a condition of inflammation and stress, primes in both autocrine and paracrine manners the upregulation of cytokines, adhesion molecules and proteolytic enzymes. NF- κ B-mediated upregulation of MMP-9 (Bond *et al*, 1998, Yokoo & Kitamura, 1996) and u-PA gene expression (Reuning *et al*, 1995) in macrophages, astrocytes and lymphocytes appears directly related to the increased protein expression of these enzymes, probably via both direct and indirect transcriptional routes.

Increased proteolytic activity of the plasmin/MMP cascade in MS lesions leads to BBB and myelin damage, increased mononuclear cell infiltration and further amplification of the inflammatory reaction. Proteolytic removal of IGF-BP represents an important mode of IGF delivery to the target tissues and plasmin and several MMPs have been shown to cleave IGF-BPs increasing the bioavailability of IGFs. IGF-II may stimulate macrophages in the hypercellular rim of the lesion to migrate and invade the near white matter as in the case with tumour cells (Edwards & Murphy, 1998). In addition, IGFs and insulin may also

increase production of pro-inflammatory cytokines and proteolytic enzymes through further activation of the NF- κ B (Bertrand *et al*, 1995, Renier *et al*, 1996, Tranque *et al*, 1994).

Although the majority of data in this thesis indicates the existence of a destructive cycle of events in established MS lesions, beneficial effects can not be excluded at the earlier stages in MS lesion development when the capacity for remyelination and oligodendrocyte numbers are preserved. IGFs and insulin are potent stimulators of myelination and survival factors for oligodendrocytes and neurones and may synergise with NF- κ B in promoting anti-apoptotic effects (Bertrand *et al*, 1998). Proteolytic activity of PAs and MMPs may be similarly targeted to oligodendrocytes enhancing process extension and remodelling of extracellular matrix thus promoting remyelination.

Biological mediators involved in autoimmune and inflammatory responses in MS represent attractive targets for therapeutic intervention. However, the complexity and very often dual role of these mediators, well illustrated by the example of IGFs and MMPs which can promote both tissue damage and tissue reparation, emphasises the importance of establishing appropriate conditions for the desired effects. Defining the precise expression profiles of these mediators in relation to a particular stage of MS lesion development or a cell type will help to determine rate-limiting steps in inflammatory cascades which can be useful targets for the therapy of multiple sclerosis.

Levels of interaction	References
NF-κB and Matrix metalloproteases	
Upregulation of PA/MMP production through the action of NF- κ B -controlled cytokines.	Baeuerle & Henkel, 1994
Transcriptional regulation of the u-PA gene.	Reuning <i>et al</i> , 1995
Transcriptional regulation of the MMP-9 gene.	Yokoo & Kitamura, 1996 Bond <i>et al</i> , 1998
NF-κB and Insulin-like growth factors	
Insulin activates NF- κ B in Chinese Hamster Ovary cell line.	Bertrand <i>et al</i> , 1995
IGF-I upregulates the production of TNF α , a NF- κ B dependent gene, in macrophages.	Renier <i>et al</i> , 1996
Matrix metalloproteases and Insulin-like growth factors	
Plasmin and MMPs cleave IGF-BPs increasing the bioavailability of IGFs.	Collettsolberg & Cohen, 1996
PAI-I protects IGF-BPs from proteolytic degradation though direct binding.	Nam <i>et al</i> , 1997
IGF-I upregulates the expression of t-PA in astrocytes.	Tranque <i>et al</i> , 1994
Insulin upregulates the expression of PAI-I in endothelium.	Nordt <i>et al</i> , 1998

Table 6.1: The existing experimental data relating to the specific interactions between NF- κ B, MMPs and IGFs.

6.2. Further work.

In order to extend the findings in this thesis further work should focus on the following aspects of the inflammatory cascades (i) the PA/MMP proteolytic profile of MS lesions (ii) the kinetics of NF- κ B activation and its role in transcriptional regulation of microglial u-PA and MMP-9 and (iii) the role and regulation of IGFs in MS lesions.

Determining the precise expression and activity profile of PAs, MMPs and their inhibitors is necessary to assess the contribution of an individual enzyme at each stage of MS lesion development. ELISA assays can be used to determine protein concentration and activity of different enzyme fractions in protein extracts from NAWM and MS lesions. Availability of monoclonal antibodies specific for latent or active forms of MMPs and PAs makes it possible to relate ELISA data to the enzyme expression in snap-frozen human section from normal control and MS cases. The correlation can be further strengthened by *in situ* RT-PCR to determine the precise cellular location of increased production of an individual MMP or PA.

The dramatic increase in u-PA concentrations in protein extracts of active MS lesions highlights the regulatory role of upstream activators of the plasmin/MMP cascade. The function of u-PA is dependent on interaction with u-PAR and to complement the existing data the protein concentration and distribution of this receptor should also be determined by ELISA and immunocytochemistry, respectively. Furthermore, as microglia and macrophages are a major source of PA and MMP proteolytic activity in MS lesions, u-PA/u-PAR-associated properties of these cells, i.e. proteolysis, adhesion and chemotaxis should be assessed *in vitro* in cultured adult human microglia and monocytes.

NF- κ B is the crucial transcriptional regulator of many inflammatory genes including those for u-PA and MMP-9. The investigation of NF- κ B activity in microglia is hampered by the sensitivity of this cell type to stimuli and the fact that activation of

microglia and NF- κ B occurs during the isolation procedure. Co-culture of microglia on astrocyte monolayers has been shown to induce a resting phenotype in these cells and may be a suitable experimental system to investigate the kinetics of NF- κ B activation. The inhibitory peptides and anti-sense probes to target specific NF- κ B subunits and the DNA-shift assay can be used to investigate the role of inflammatory mediators in the NF- κ B activation in microglia.

IGFs can be used to promote remyelination in MS and clinical trials in motoneurone disease are already in progress. The local effects of IGFs depend on the presence of high affinity IGF-BPs. Proteolysis of IGF-BPs generates low affinity fragments thus enhancing local concentration of IGFs. Using recombinant human IGF-BPs incorporated into gels as substrates for zymography and specific protease inhibitors, the existence and specificity of IGF-BP proteolysis can be demonstrated in protein extracts of MS tissue. Further, the role of IGF-II in microglial migration and proliferation *in vitro* can be assessed in the presence and absence of IGF-BPs and in comparison with other chemotactic mediators including the u-PA/u-PAR system.

7. References

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