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The Role of Chemokines in the Pathology of Multiple Sclerosis and Other Neuroinflammatory Diseases

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ABSTRACT

The recruitment of circulating leukocytes and resident glial cells to sites of CNS inflammation is dependent on the chemokine gradients they encounter and the chemokine receptors they express. Multiple sclerosis (MS), subacute sclerosing panencephalitis (SSPE) and coeliac disease (CD), with associated neurological complications, are neuroinflammatory diseases with different aetiologies, but which share common CNS neuropathological features including large perivascular inflammatory cell infiltrates, microglial hyperplasia and reactive astrocytosis. The results of this study suggest that in SSPE CNS the interferon-γ-inducible α chemokines IP-10 and Mig, predominantly expressed by astrocytes and microglia, play a role in lesion formation. In contrast, the β-chemokine MIP-1α, expressed both by perivascular macrophages and resident microglia, plays a role in the recruitment of inflammatory cells into CD cerebellar tissue. The highest levels of α- and β-chemokine expression were detected in actively demyelinating MS lesions with high levels of inflammation and widespread demyelination. In these lesions, RANTES was predominantly expressed by the endothelium, MCP-1, IP-10 and Mig by reactive astrocytes, and MIP-1β by microglia. These findings suggest not all neuroinflammatory diseases with common pathological features share a common chemokine profile.

The highest level of chemokine receptor expression was also associated with chronic active MS lesions: infiltrating T-lymphocytes predominantly expressing CXCR3 and CCR5, and foamy macrophages within the lesion predominantly expressing CCR3, CCR5 and CCR8. *In vitro* studies confirmed the production of chemokines and the expression of chemokine receptors by isolated rat astrocytes and microglia following

cytokine stimulation. The results of this study suggest chemokines play a critical role in the recruitment of cells to sites of inflammation in the CNS.

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ABBREVIATIONS

ABC	avidin-biotin complex
AGA	anti-gliadin antibody
APC	antigen presenting cells
APS	ammonium persulphate solution
BBB	blood-brain barrier
BCA	bicinchoninic acid
BCIP/NBT	5-bromo-4-chloro-3-indoyl-phosphate/ nitro blue tetrazolium
BME	basal medium eagle
BSA	bovine serum albumin
CCR	β-chemokine receptor
CD	coeliac disease
CFA	complete Freund's adjuvant
CNS	central nervous system
CSF	cerebrospinal fluid
CXCR	α-chemokine receptor
DAB	3,3'diaminobenzidine
DAG	diacylglycerol
DARC	Duffy antigen receptor for chemokines
DEPC	diethyl pyrocarbonate
D-F	death to snap-freezing time
DMEM	Dulbecco's modification of eagles minimum essential medium
DMTr	dimethyltrityl

EAE	experimental autoimmune encephalomyelitis
$EBSS^+$	Earle's balanced salt solution with calcium and magnesium
$EBSS^-$	Earle's balanced salt solution without calcium and magnesium
EtOH	ethanol
GFAP	glial fibrillary acidic protein
HCHO	paraformaldehyde
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
H_2O_2	hydrogen peroxide
ICAM-1	intercellular adhesion molecule-1
ICC	immunocytochemistry
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP_3	inositol 1,4,5-trisphosphate
IP-10	interferon-γ-inducible protein-10
ISH	*in situ* hybridisation
JNK/SAPK	c-jun kinase/stress-activated protein kinase
LARC	liver activation-related chemokine
LFA-1	leukocyte function associated antigen-1
LPS	lipopolysaccharide
LT	lymphotoxin
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein

MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
Mig	monokine induced by interferon-γ
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
M-tropic	macrophage-tropic
MV	measles virus
NAWM	normal appearing white matter
NBS	newborn calf serum
NC	normal control
NK	natural killer cell
NSS	normal sheep serum
ORO	oil red O
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PKC	protein kinase C
PLP	proteolipid protein
PMA	phorbol myristate acetate
pvc	perivascular cuff
RANTES	regulated upon activation, normal T-cell expressed and secreted
RBC	red blood cells
RT	room temperature

RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SDF-1	stromal-cell derived factor-1
SDS	sodium dodecyl sulphate
SIV	simian immunodeficiency virus
SME	subacute measles encephalomyelitis
SSC	sodium saline citrate
SSPE	subacute sclerosing panencephalomyelitis
TARC	thymus and activation-regulated cytokine
TBS	tris buffered saline
TCR	T-cell receptor
TEA	triethanolamine
TNF	tumor necrosis factor
TTBS	tween plus tris buffered saline
T-tropic	T-cell tropic
V-CAM	vascular cell adhesion molecule

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CHAPTER 1

INTRODUCTION

1.1 MULTIPLE SCLEROSIS (MS)

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that results in the formation of large areas of demyelination known as plaques. It has been suggested that autoreactive T-lymphocytes induce an immune attack on the CNS leading to chronic T-cell mediated inflammation, and ultimately to damage of the myelin sheath and the cells responsible for the production and maintenance of myelin, the oligodendrocytes (Storch & Lassmann, 1997). Epidemiological studies, however, suggest that the pathogenesis of this disease is more complicated and is influenced by a combination of genetic and environmental factors (Ebers, 1996; Oksenberg et al., 1996).

1.1.1 AETIOLOGY OF MULTIPLE SCLEROSIS

MS is the most common primary demyelinating disease of the CNS in man in temperate climates. The incidence of MS varies with latitude: at high latitudes (40-60 parallels above and below the equator) the incidence is 30 per 100,000 of the population, this decreases to 10 per 100,000 in lower latitudes and virtually disappears at the equator (Allen & Kirk, 1992).

Genetic studies have shown an increased incidence of MS in monozygotic twins (concordance rate 20-30%), and in siblings and dizygotic twins (concordance rate 4-5%) (Mumford et al., 1994). The ability to respond to an antigen, whether foreign or self, is mainly determined by the human leukocyte antigen (HLA) gene (Oksenberg & Hauser,

1997), in particular the major histocompatibility complex (MHC) class I and II regions of the HLA gene (Oksenberg et al., 1996). HLA class II molecules have two main immune response functions. Firstly, the HLA class II molecules on the surface of antigen presenting cells (APC) bind processed antigen and present it to $CD4^+$ T-lymphocytes, which are then activated and initiate an immune response. Secondly, the HLA class II molecules present on the stromal cells of the thymus help determine the specificity of the immune response through the positive and negative selection of T-cells (Oksenberg et al., 1996). The association between certain haplotypes of MHC and MS was first reported in 1972 (Bertrams et al., 1972), and it has been suggested that MHC class II in particular plays a critical role in determining the susceptibility of an individual to MS (Oksenberg & Hauser, 1997). A predisposition to MS has been associated with the HLA-A3, HLA-B7 and HLA-DR2 alleles (Oksenberg et al., 1996). In particular, a relationship between the DR2 haplotype and susceptibility to MS has been established, and demonstrates that the DR2 allele, or a gene closely linked to the DR2 region, is associated with MS (Oksenberg et al., 1996).

1.1.2 CLINICAL FEATURES OF MULTIPLE SCLEROSIS

MS patients commonly present with limb weakness, ataxia, nystagmus and optic neuritis (Hohol et al., 1995). Onset of disease typically occurs in adults between the ages of 20-40 (Hohol et al., 1995), with a higher disease incidence in females compared to males (approximately 2:1) (Oksenberg et al., 1996). Diagnosis is by high resolution magnetic resonance imaging of the CNS to identify plaques (Stone et al., 1995), and by examination of the cerebrospinal fluid (CSF) to detect an increase in the γ-globulin/albumin ratio and γ-globulin oligoclonal bands (Brosnan & Raine, 1996). The majority of patients

exhibit a classic relapsing-remitting disease course characterised by episodes of clinical worsening followed by remission, although some cases are chronic progressive and do not display the pattern of acute relapse. Most patients ultimately progress to a state of severe disability with neurological impairment (Hohol et al., 1995).

1.1.3 MS LESION CLASSIFICATION

Current literature classifies MS CNS tissue into (i) normal appearing white matter (NAWM) (ii) acute MS lesions (iii) subacute plaques or (iv) chronic silent plaques (Lassmann et al., 1998). NAWM, as its name suggests, shows no apparent phenotypic difference to normal control CNS tissue (Allen & Kirk, 1992). The CNS in both control and NAWM is under immunological surveillance and hence displays some T-lymphocytes associated with small venules. Acute MS lesions are characterised by perivascular cuffs containing lymphocytes and macrophages centred around venules and low levels of demyelination, especially in the periventricular white matter, the optic nerve and tract, the corpus callosum, the brainstem and the upper cervical spinal cord (Brosnan & Raine, 1996). Subacute lesions contain large numbers of lipid-containing macrophages, and large perivascular inflammatory infiltrates (Lucchinetti et al., 1996). Older chronic silent lesions are immunologically silent, in terms of inflammation, and comprise large astroglial scars and demyelination (Raine, 1990) (Section 2.1.1).

1.2 INFLAMMATION IN THE CENTRAL NERVOUS SYSTEM (CNS)

The CNS in MS is characterised by perivascular cuffs of inflammation primarily consisting of T-lymphocytes and macrophages recruited from the circulation, large confluent plaques of demyelination, reactive astrocytosis and microglial hyperplasia.

1.2.1 T-LYMPHOCYTE SURVEILLANCE OF THE CNS

Neuronal function requires a micro-environment which is precisely controlled. The blood-brain barrier (BBB) and the blood-CSF barrier isolate the brain and spinal cord from changes elsewhere in the body which may have an adverse effect. The BBB is composed of endothelial cells, attached to each other by tight junctions, and astrocyte end feet processes, and has restricted permeability (England & Wakely, 1991).

The concept of immune privilege was originally defined as the protection of tissue grafted to certain sites, and was later extended to describe the seclusion of particular areas of the body from immune surveillance. The CNS was originally thought to be an immunologically privileged site separated from the immune reactions of the rest of the body by the BBB (Kajimura et al., 1990). However, a small number of T-cells are present in normal human CNS tissue, suggesting a role for T-lymphocyte surveillance of the CNS in the absence of a sophisticated lymphatic drainage system (Nikcevich et al., 1997). These T-lymphocytes, when triggered by a specific antigen, can induce a local immune response (Gold et al., 1997). In MS it is generally considered that T-lymphocytes penetrate the BBB and, through a cascade of reactions, ultimately cause the formation of plaques of demyelination throughout the white matter. Examples of other tissues that utilise specialised immunological defence mechanisms are the retina, the cornea, the anterior chamber of the eye, the testis and the liver (Streilein, 1995).

1.2.1.1 RECRUITMENT OF T-LYMPHOCYTES INTO THE CNS

The BBB is composed of endothelial cells and surrounding glia which prevent the passage of high molecular weight substances into the CNS (Shibata & Mori, 1988). The crucial event in MS lesion formation is the transendothelial migration of leukocytes. T-cell

transmigration requires the expression of complementary adhesion molecules on the surface of the BBB endothelium, and the production of matrix metalloproteinases (MMPs) to disrupt the BBB and the CNS extracellular matrix (Storch & Lassmann, 1997).

A three-step model for leukocyte transmigration has been proposed based on *in vivo* and *in vitro* investigations: (I) leukocytes are captured and roll along the endothelium (II) the cells are activated and adhere to the vessel wall and (III) transmigrate into the CNS (Pardi et al., 1992; Picker & Butcher, 1992), as shown in Figure 1.

Figure 1 Transmigration of leukocytes into the CNS

Initially the leukocytes weakly attach to the endoluminal side of the BBB via a selectin-mediated process, and roll along the vascular wall enabling the cells to sample the local environment (Bevilacqua & Nelson, 1993). Chemokines, low molecular weight chemoattractant cytokines, activate and mediate the directional migration of leukocytes along their concentration gradient (Karpus et al., 1995) (Section 1.4). Chemokine-

mediated activation of the leukocytes stimulates firm adhesion of the cells to the endothelium through the interaction of β-2 integrins, such as the integrins leukocyte function associated antigen-1 (LFA-1) on lymphocytes and its complementary molecule intercellular adhesion molecule-1 (ICAM-1) on the endothelium (Hynes, 1992). The leukocytes then flatten and transmigrate across the endothelium along chemotactic gradients (Butcher & Picker, 1996). Within the CNS the leukocytes, which can respond to several chemokines, migrate along one chemotactic gradient, then another until they reach their target. Their ultimate position is determined by the chemokine receptors they express and the chemokine gradients they encounter (Campbell et al., 1997) (Section 1.5).

Several groups have examined the phenotype of lymphocytes present in MS lesions, with conflicting results. It has been reported that infiltrating cells around small venules are predominantly $CD4^+$ T-lymphocytes (Compston et al, 1991), other studies report a predominance of $CD8^+$ T-cells (Woodroofe et al., 1986; Esiri et al., 1989), while others have found equal numbers of both $CD4^+$ and $CD8^+$ cells throughout the lesion (Boyle & McGeer, 1990). These discrepancies may reflect differences in disease stage, and may indicate that different lymphocyte subpopulations are involved in the pathogenesis of MS lesion formation, at different stages of disease progression.

It has been suggested that activated T-lymphocytes cross the BBB and are induced to secrete cytokines by their interaction with APC expressing MHC class II, resulting in further impairment of the BBB and recruitment of leukocytes into the CNS (Compston et al, 1991). Most studies suggest this multifocal perivascular inflammation precedes macrophage-mediated myelin loss, and imply myelin degradation is a result of either immunoregulatory molecules, such as cytokines, released by leukocytes or of T-cell antigen-specific mechanisms. However, it should be noted that the detection of myelin

breakdown products in macrophages does not indicate if it is a consequence or a cause of myelin degradation (Lucchinetti et al., 1996), and that myelin breakdown has been detected outside regions of leukocyte infiltration, suggesting demyelination may precede inflammation (Rodriguez & Scheithauer, 1994).

Experimental autoimmune encephalomyelitis (EAE) is an animal model with clinical and pathological similarities to MS. At the height of the disease, paralysis of the animal reflects chronic perivenular inflammation of the CNS. Different immunisation procedures in various animal strains produces varied clinical patterns of EAE. For example, acute EAE is a monophasic inflammatory disease induced in susceptible animals following active immunisation with myelin basic protein (MBP) and complete Freund's adjuvant (CFA); whilst chronic relapsing EAE is a chronic, multi-phasic inflammatory demyelinating disease induced following immunisation with myelin proteolipid protein (PLP) and CFA (Begolka et al., 1998) .

At present, little is known about the mechanisms involved in the down-regulation of inflammation in the CNS in MS during remission. In EAE, T-cell apoptosis, programmed cell death, is the major mechanism of T-cell clearance (Schmied et al., 1993). Apoptosis has been reported in active MS lesions (Bauer et al., 1995). T-cell activation requires two signals: occupancy of the T-cell receptor by peptide complexed with MHC class II molecules, and interaction between CD28 expressed on T-cells and the B7 family of co-stimulatory molecules expressed on the surface of antigen presenting cells (APC) (Nikcevich et al., 1997). It has been suggested that an imbalance of antigen stimulation or a lack of co-stimulation may lead to the apoptosis of T-cells rather than their activation (Storch & Lassmann, 1997).

1.2.1.2 TARGET ANTIGEN OF T-LYMPHOCYTES

The topographical distribution of inflammatory lesions in the CNS in MS is determined by the antigen specificity of the T-cell response (Berger et al., 1997). In EAE activated T-cells recognise components of the myelin sheath as target antigens (Steinman, 1996). Clinical studies have shown that MS patients produce an immune response to antigens such as MBP, myelin oligodendrocyte glycoprotein (MOG), or PLP found in myelin or oligodendrocytes (Steinman, 1996), suggesting myelin and its associated proteins are the key target antigens for T-cells. Some MS patients, however, have inflammation in areas devoid of myelin such as the retina (Birch et al., 1996), suggesting a non-myelin associated antigen may be the target of disease.

In EAE a T-cell mediated inflammatory response in the CNS can be induced by either MBP, MOG, PLP, glial fibrillary acidic protein (GFAP) or S-100β protein (Kojima, et al., 1994; Adelmann et al., 1995; Lorentzen et al., 1995). It is of interest to note that the S-100β protein, an astrocytic protein, is capable of mediating an intense inflammatory response both in the white matter of the CNS and in the retina in rats (Kojima, et al., 1994).

1.2.1.3 CLASSIFICATION OF T-LYMPHOCYTES

Within the CNS T-cells activate resident glial cells and infiltrating leukocytes via the secretion of signal molecules known as cytokines. Cytokines enable communication between cells through their action on high affinity receptors on their target cell (Paul & Seder, 1994). $CD4^+$ T-cells can be divided into T_H1, T_H2 and T_H0 subgroups depending on the cytokines they express (Mossman & Coffmann, 1989). T_H1 helper cells mediate

cellular immune responses, are involved in the development of chronic inflammatory conditions, and secrete cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and lymphotoxin (LT) (Romagnani, 1994). T_H2 cells upregulate IgE production, are prominent in the pathogenesis of allergic diseases and produce interleukin (IL)-4, IL-5 and IL-10 (Abbas et al., 1996). The T_H0 cells produce a combination of T_H1 and T_H2 associated cytokines. Both T_H1 and T_H2 cells are able to interact and down-regulate each other through their secreted cytokine products (Olsson, 1995).

1.2.1.4 TH_1 AND TH_2 CYTOKINE PROFILES IN MS

Cytokines are small, 15-25kDa, secreted soluble proteins which enable communication between cells (Paul & Seder, 1994) (Section 1.3). These cellular signals can be subdivided into those which enhance inflammatory reactions, the pro-inflammatory cytokines predominantly expressed by T_H1 cells, and those which oppose inflammatory reactions, the anti-inflammatory cytokines predominantly expressed by T_H2 cells (Lucas & Hohlfeld, 1995).

Current evidence suggests MS is primarily mediated by T_H1 cells (Hohlfeld et al., 1995), which secrete proinflammatory cytokines. These cytokines stimulate the expression of adhesion molecules on the BBB endothelium and result in leukocyte infiltration into the CNS (Paul & Seder, 1994), as well as the induction of chemokine expression which continues the local inflammatory response by further recruiting and activating inflammatory cells (Schall & Bacon, 1994). Pro-inflammatory cytokines also activate resident glial cells and infiltrating inflammatory cells in MS, and are thought to be the major mediators of tissue damage (Brosnan & Raine, 1996) (Section 1.3.1).

T_H2 cytokines play an important role in the regulation of T_H1 cytokines (O'Garra & Murphy, 1996). T_H2 cytokines can inhibit T_H1 responses (Fiorentino et al., 1991), suggesting they have an immunoregulatory role in the development of MS lesions.

1.2.2 ANTIGEN PRESENTING CELLS IN THE CNS

APC are defined as cells that express MHC class II molecules together with a group of co-stimulatory molecules, and that are able to process and present antigen to T-cells (Weller et al., 1996). Astrocytes phagocytose cell debris, may express MHC class II antigens, and process and present antigen *in vitro* (Hart & Fabry, 1995), but it is not known whether astrocytes serve as APC in MS. Macrophages, microglia and perivascular cells express MHC class II and co-stimulatory molecules in MS, and are considered to be the major APC in the CNS (Hart & Fabry, 1995).

1.2.2.1 MICROGLIA AND HAEMATOGENOUS MACROPHAGES

Microglia, resident brain macrophages, were first described as CNS cells that are distinct from astroglia and oligodendroglia by del Rio-Hortega in 1932. The expression of the surface antigens, leukocyte common antigen (CD45) and the IgG Fc receptor I (CD64) suggest that microglia are derived from the bone marrow (Lassmann et al., 1993). Throughout development of the CNS, amoeboid microglia act as scavengers removing waste material (Oehmichen, 1983), whilst during post-natal development they differentiate into ramified microglia and form an immunoregulatory network throughout the CNS (Streit et al., 1988). Microglia react locally to brain injury by hypertrophy and an increase in expression of MHC class II, suggesting these cells play an important role in the immune response of the CNS (Woodroofe et al., 1986; Hayes et al., 1987). There is also evidence

that microglia are in contact with abnormal myelin internodes, suggesting they may play a role in demyelination in MS (Prineas et al., 1984).

In MS, lymphocytes and macrophages/monocytes are recruited from the circulation into the CNS and form perivascular cuffs of inflammation. In the normal CNS, microglia can easily be identified by their characteristic ramified, branched morphology. However, during an inflammatory response microglia transform into amoeboid macrophage-like cells, and are difficult to distinguish from the haematogenously-derived macrophages (Li et al., 1993).

1.2.2.2 THE ROLE OF MACROPHAGES IN MS PATHOLOGY

Demyelinating MS lesions contain high numbers of lipid-containing macrophages (Bruck et al., 1996). Several reports suggest that infiltrating macrophages play a major role in lesion formation (Esiri & Reading, 1987; Cuzner et al., 1988; Adams et al., 1989), while others suggest that haematogenous macrophages are found primarily in the perivascular cuffs in MS lesions, and that resident microglia are the main phagocytic cell (Ulvestad et al., 1994). EAE studies have shown that if monocytes/macrophages are removed from the circulation then clinical symptoms are reduced in the relapsing-remitting disease, but are not affected in chronic-progressive EAE (Huitinga et al., 1990). These findings suggest that different macrophage-subpopulations may be involved in the pathogenesis of plaque formation at different stages of disease progression.

An increased expression of HLA class II-DR on macrophages and microglia in MS lesions has been reported, suggesting they act as accessory cells in lymphocyte activation at the induction stage of inflammatory demyelination (Woodroofe et al., 1986). Microglial activation, with increased MHC class II expression, occurs at the early stages of EAE

(McCombe et al., 1994). Expression is pronounced on perivascular microglial cells and suggests a role for activated microglia in antigen presentation at the BBB. *In vitro* studies have shown that activated macrophages produce cytokines, proteases, and oxygen radicals which are capable of damaging the oligodendrocyte-myelin complex (Giovannoni & Hartung, 1996), however, it is as yet unknown what initiates this attack *in vivo.*

1.2.2.3 ASTROCYTES

Astrocytes are supporting cells of the CNS, and are involved in the nutritive support of neurones. They have many functions including the uptake of potassium released during neuronal activity and the storage of glycogen (England & Wakely, 1991). Reactive astrocytosis is a prominent feature of MS lesions, and is particularly abundant at the plaque edge (Brosnan & Raine, 1996). Astrocytosis is characterised by astrocyte proliferation and extensive hypertrophy of the cell body and cytoplasmic processes, as well as extensive synthesis of GFAP (Eng et al., 1996). Following an insult astrocytes can form an astrocytic scar to separate affected areas from the rest of the CNS, however this scar may prevent remyelination in MS (Eng et al., 1994).

Astrocytes have been shown to express a wide variety of factors including MHC molecules, adhesion molecules, reactive nitrogen species, and cytokines (Cannella & Raine, 1995). Although *in vitro* experiments have demonstrated IFN-γ-stimulated astrocytes express MHC class II, this role of antigen presentation has not been conclusively demonstrated in MS lesions (Bo et al., 1994). Immunocytochemistry studies have shown that astrocytes express both pro- and anti-inflammatory cytokines (Cannella & Raine, 1995), as well as reactive nitrogen intermediates (Lee et al., 1995). Nitric oxide, particularly in conjunction with reactive oxygen species released by macrophages, could

directly damage oligodendrocytes and the BBB (Brosnan & Raine, 1996). Astrocytes have been shown to express chemokines in the acute phase of MS and activate adhesion molecule expression associated with lymphocyte trafficking (Miyagishi et al., 1997), suggesting a significant role of resident glial cells in the accumulation of inflammatory cells into the CNS in MS.

1.3 CYTOKINES

Cytokines are small, secreted proteins which enable communication between cells (Paul & Seder, 1994). A single cytokine is often produced by more than one cell type, and is capable of different effects on several target cells. Cytokines rarely exert their actions alone, rather they act together and have many feedback mechanisms (Giovannoni & Hartung, 1996).

1.3.1 CYTOKINE EXPRESSION IN MS

Both *in vivo* and *in vitro* studies indicate that cytokines play a role in the induction and regulation of MS pathology (Brosnan et al., 1995). IFN-γ, a pro-inflammatory cytokine, amplifies T_H1 development and inhibits T_H2 proliferation (Fitch et al., 1993). IFN-γ plays an important role in the activation of macrophages (Cavaillon, 1994), the regulation of MHC class II (Woodroofe et al., 1989), the induction of adhesion molecule expression on endothelial cells (Brosnan et al., 1995), and induces the expression of cytokines by resident glial cells (Traugott & Lebonn, 1988; Woodroofe & Cuzner, 1993). Studies have shown IFN-γ is primarily expressed by inflammatory cells in the perivascular cuffs in MS lesions (Woodroofe & Cuzner, 1993), and by some microglia and astrocytes

within the plaque (Traugott & Lebon, 1988). Administration of IFN-γ to MS patients has been reported to increase the number of disease relapses (Panitch et al., 1987).

IL-10, a T_H2 cytokine (Mossmann & Coffman, 1989), is one of the few cytokines known to inhibit the action of IFN-γ-induced macrophage-activation (Fiorentino et al., 1991). In MS lesions this anti-inflammatory cytokine is exclusively expressed by astrocytes (Brosnan et al., 1995), and is commonly seen in astrocyte end-feet near blood vessels, suggesting a role in BBB regulation (Cannella & Raine, 1995).

IL-1 induces the expression of adhesion molecules on the BBB endothelium (Pober, 1988), activates T-lymphocytes (Dinarello, 1989), stimulates astrocyte proliferation (Giulian et al., 1998), and stimulates the secretion of TNF-α and IL-6 by astrocytes (Lee et al., 1993). This pro-inflammatory cytokine is primarily expressed by macrophages within acute and chronic active MS lesions, and by microglia in the adjacent non-demyelinated white matter (Brosnan et al., 1995).

TNF-α is closely related to LT (Brosnan et al., 1995). Both pro-inflammatory cytokines induce the expression of adhesion molecules on endothelial cells and astrocytes (Pober, 1988; Hurwitz et al., 1992). In acute MS lesions TNF-α and LT are predominantly expressed by macrophages, some astrocytes and endothelial cells within the plaque, and by microglia in the adjacent white matter (Cannella & Raine, 1995). Elevated levels of TNF-α have been detected in the CSF and peripheral blood of MS patients before relapse, suggesting a role for this cytokine in disease progression (Rieckmann et al., 1994).

TGF-β blocks macrophage activation (Bogdan et al., 1992) and prevents the adhesion of leukocytes on endothelial cells (Gamble & Vadas, 1988). This anti-inflammatory cytokine is associated with the blood vessel endothelium and surrounding extracellular matrix in MS (Brosnan et al., 1995).

Other cytokines associated with MS include IL-4, IL-6 and IL-12. IL-4 is predominantly expressed by microglia, foamy macrophages and lymphocytes at the edge of actively demyelinating plaques (Brosnan et al., 1995). IL-6, however, is present in all perivascular leukocytes in active MS lesions (Woodroofe, 1995), suggesting a role for this cytokine in lesion development. Levels of IL-12 have been detected in the blood, but not the CSF, of chronic progressive MS patients (Nicoletti et al., 1996), while increased levels of IL-12 mRNA have been detected in acute MS lesions (Windhagen et al., 1995).

1.3.2 CYTOKINE EXPRESSION IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

EAE, an animal model of inflammatory demyelinating disease, is a T_H1 $CD4^+$ cell-mediated autoimmune disease which shares many of the clinical and histological features associated with MS (Bradl & Linington, 1996). Distinct patterns of cytokine expression are associated with lesion development in EAE.

The first cytokines to appear in the CNS during EAE are LT, expressed by lymphocytes, and IL-12, expressed by macrophages, coinciding with the appearance of inflammatory cells shortly before onset of clinical signs of disease (Issazadeh et al., 1995). Later during the acute phase of disease IFN-γ (Reeno et al., 1994) and TNF-α (Issazadeh et al., 1995) are primarily expressed by infiltrating T_H1 lymphocytes and macrophages respectively. TGF-β expression by $CD8^+$ T-cells peaks before onset of recovery (Issazadeh et al., 1995), while IL-10 secretion by T_H2 cells increases dramatically in the recovery phase of disease (Kennedy et al., 1992). Expression of IL-1α by infiltrating and resident macrophages, and IFN-γ by infiltrating T-lymphocytes is associated with the chronic stage of EAE (Kennedy et al., 1992).

The pro-inflammatory cytokines, including TNF-α and IFN-γ, are directly responsible for tissue damage in EAE (Merrill et al., 1992). The encephalitogenic capacity of T-cells correlates with their ability to produce TNF-α (Powell et al., 1990). Over-expression of TNF-α by resident astrocytes or neurons in TNF-α transgenic mice results in chronic infiltration of the CNS by lymphocytes which are autoreactive for myelin, reactive astrocytosis and microgliosis (Probert et al., 1995; Probert et al., 1997). Astrocyte-specific expression of transmembrane TNF, in TNF-α transgenic mice, has been shown to result in a spontaneous neurological disorder manifested by ataxia, seizures, and paralysis and bears histological evidence of chronic CNS inflammation and myelin degeneration (Akassoglou et al., 1997). EAE studies have shown that injecting antibodies against TNF-α or LT into the animals results in a decrease in disease severity (Ruddle et al., 1990), and correlated with a down-regulation of vascular cell adhesion molecule (V-CAM) expression (Barten & Ruddle, 1994). Although expression of TNF-α in the CNS clearly results in a pathology similar to that seen in MS lesions, it is not possible to solely attribute the inflammatory demyelinating response to TNF-α.

Administration of IL-4 (Racke et al., 1994), IL-6 (Gijbels et al., 1995) or TGF-β (Johns et al., 1991) has been shown to suppress disease induction in EAE, suggesting an immunoregulatory role of these cytokines in MS lesion formation (Paul & Seder, 1994). Administration of IFN-γ, however, results in an increase in disease severity (Billiau, 1995). Reports have indicated that IL-12 can re-induce paralysis in Lewis rats following recovery from actively induced EAE (Smith et al., 1997), suggesting IL-12 may contribute to relapse in patients with MS. Inhibiting the activity of pro-inflammatory cytokines is a central theme in many strategies used to control disease in EAE, and MS (Raine, 1995).

1.4 CHEMOKINES

Chemokines (chemoattractant cytokines) are important in the pathogenesis of immune-mediated inflammation in the CNS, and are involved in the activation and directional migration of cells to sites of inflammation (Karpus et al., 1995; Baggiolini, 1998). There is also evidence that chemokines are involved in modulating normal homeostatic mechanisms in the CNS; for example, IL-8 has been shown to enhance the survival of rat hippocampal neurones *in vitro* (Araujo & Cotman, 1993). Chemokines are small, secreted, 8-12kDa, heparin-binding cytokines which mediate the chemotaxis of various cell types including neutrophils, monocytes, T-lymphocytes, basophils, eosinophils and fibroblasts (Oppenheim et al., 1991; Schall, 1991; Van Damme, 1994). During the past few years many new chemokines have been discovered. To date, chemokines can be divided into at least four subfamilies depending on the presence and position of a conserved motif of four cysteine residues: the α-chemokines (CXC chemokines) which have one amino acid residue separating the first two cysteine residues; the β-chemokines (CC chemokines) in which the first two cysteine residues are adjacent; the γ-chemokines (C chemokines) that lack two (the first and third) of the four conserved cysteine residues; and the δ-chemokines (CX_3C chemokines) which have three intervening amino acids between the first two conserved cysteine residues (Schall & Bacon, 1994; Rollins, 1997). The majority of known chemokines are members of either the α- or β-chemokine families. The structural classification of chemokines has been shown to relate to their biological properties (Schall & Bacon, 1994). The α-chemokines act primarily on neutrophils, whilst the β-chemokines are associated with chronic inflammation and primarily act on monocytes and lymphocytes (Vaddi et al., 1997). The structural fold of the polypeptide

chain is very similar in both α- and β-chemokines, suggesting that chemokines share a similar three-dimensional structure (Lodi et al., 1994).

1.4.1 α-CHEMOKINES

α-chemokines can be further subdivided into those which have an ELR amino acid motif (glutamic acid-leucine-arginine) at their N-terminus and those which do not (Taub & Oppenheim, 1994). Those with the ELR motif, including IL-8, are potent neutrophil chemoattractants (Baggiolini, 1998), while the non-ELR α-chemokines, such as the IFN-γ-inducible chemokines monokine induced by IFN-γ (Mig) and IFN-γ-inducible protein-10 (IP-10), chemoattract lymphocytes but not neutrophils (Taub et al., 1993; Liao et al., 1995; Loetscher et al., 1996a). Although the BBB has been shown to express the required adhesion molecules, neutrophil infiltration is not a feature of CNS inflammation (Bell & Perry, 1995; Scott et al., 1996). Most ELR-chemokines are angiogenic whereas non-ELR chemokines are angiostatic (Vaddi et al., 1997).

1.4.1.1 INTERFERON-γ-INDUCIBLE CHEMOKINES

The first indication that chemokines may be part of an immune response was the discovery that recombinant IFN-γ induced IP-10 gene expression in human monocytes (Luster et al., 1985). Although IP-10 was one of the first chemokines to be identified, a functional role in the recruitment of leukocytes to sites of inflammation has only recently been established. Mig was identified by differential screening of a cDNA library prepared from lymphokine-activated macrophages (Farber et al., 1990). The genes for IP-10 and Mig have been mapped to adjacent positions at chromosome 4q21, within 16kb of each

other (Lee & Farber, 1996). These chemokines display approximately 37% amino acid sequence identity, suggesting a close evolutionary relationship (Luster et al., 1985).

The α-chemokines IP-10 and Mig are both dramatically induced by IFN-γ (Luster et al., 1985; Farber, 1990; Farber, 1993) and promote monocyte and T-lymphocyte migration *in vitro* (Taub et al., 1993; Liao et al., 1995). Although IFN-γ appears to be a specific inducer of Mig gene expression, IP-10 gene expression can also be induced by IFN-α, IFN-β, LPS and viruses (Baggiolini, 1998). IP-10 mRNA has been detected in astrocyte and microglial cell cultures (Vanguri, 1994; Vanguri & Farber, 1995), in astrocytes in inflammatory lesions in the CNS in EAE (Ransohoff et al., 1993), and in neurones and astrocytes in the CNS following ischemic injury (Wang et al., 1998), suggesting these α-chemokines may play a role in the accumulation of inflammatory cells into the CNS in MS.

1.4.2 β-CHEMOKINES

β-chemokines are primarily associated with the chemoattraction of lymphocytes, monocytes, basophils and eosinophils to sites of inflammation (Baggiolini & Dahinden, 1994; Taub & Oppenheim, 1994; Baggiolini, 1998), and include RANTES (regulated upon activation, normal T-cell expressed and secreted), macrophage inflammatory proteins (MIPs) and monocyte chemoattractant proteins (MCPs) (Oppenheim et al., 1991). This family of cytokines are also involved in the up-regulation of integrin molecule expression by lymphocytes and facilitate T-cell adhesion to endothelial cells (Lloyd et al., 1996). The expression of multiple chemokines at different stages of lesion formation may, in part, determine the cellular composition of the inflammatory infiltrate.

1.4.2.1 MACROPHAGE INFLAMMATORY PROTEINS

MIP-1 was originally identified as an endotoxin-induced macrophage product with chemoattractant and activating properties (Wolpe et al., 1988). Further purification of MIP-1 identified two distinct proteins, MIP-1α and MIP-1β (Sherry et al., 1988). The mature forms of MIP-1α and MIP-1β are approximately 70% identical in their genetic sequence (Schall et al., 1991), and are primarily chemotactic for subsets of T-lymphocytes and macrophages (Taub et al., 1993; Hayashi et al., 1995). There is also evidence that MIP-1β, but not MIP-1α, increases endothelial adherence by T-cells (Tanake et al., 1993). Chemotaxis assays have shown that MIP-1α is a potent chemoattractant for human blood monocytes, while MIP-1β has a minimal effect on monocyte migration *in vitro* (Uguccioni et al., 1995).

MIP-1α is primarily produced by mononuclear cells, neutrophils, inflammatory fibroblasts and astrocytes (Karpus et al., 1995), whilst MIP-1β expression is primarily associated with monocytes and macrophages (Wilson et al., 1990; Seebach et al., 1995). Both chemokines have been detected in inflammatory diseases including atherosclerosis (Taub & Oppenheim, 1994), EAE (Miyagishi et al., 1997) and MS (Simpson et al, 1998), suggesting these chemokines may play a role in the activation and recruitment of leukocytes to sites of inflammation.

1.4.2.2 MONOCYTE CHEMOATTRACTANT PROTEINS

Monocyte chemoattractant protein (MCP)-1 is the best characterised β-chemokine and is associated with the chemotaxis of monocytes and T-cells both *in vivo* and *in vitro* (Zachariae et al., 1990; Baggiolini et al., 1994; Ransohoff et al., 1996). MCP-1 is produced by a variety of immune and non-immune cells, and is associated with a number

of inflammatory states including rheumatoid arthritis (Villiger et al., 1992), psoriasis (Gillitzer et al., 1993), atherosclerosis (Koch et al., 1993), EAE (Ransohoff et al., 1993; Glabinski et al., 1996) and MS (McManus et al., 1998a; Simpson et al., 1998). Studies have shown that MCP-1 induces the migration of T-lymphocytes through an endothelial cell barrier (Carr et al., 1994), suggesting a role for MCP-1 in the recruitment of inflammatory cells into the CNS during an inflammatory response.

To date five MCP proteins (MCP-1, -2, -3, -4 and -5) have been identified, and have been shown to share approximately 65% amino acid identity (Proost et al., 1996; Garcia-Zepeda et al., 1996; Sarafi et al., 1997). MCP-1, -2, -3, -4 and -5 chemoattract monocytes (Uguccioni et al., 1995; Garcia-Zepeda et al., 1996; Sarafi et al., 1997), T-lymphocytes (Loetscher et al., 1994; Taub et al., 1995) and basophils (Weber et al., 1995; Garcia-Zepeda et al., 1996), suggesting a role in leukocyte chemotactic migration to sites of inflammation. MCP-1, -2 and -3 have been shown to specifically stimulate the directional migration of T-lymphocytes and monocytes *in vivo*, suggesting they may play an important role in immune cell recruitment to sites of antigenic challenge (Taub et al., 1995).

1.4.2.3 RANTES

RANTES expression is associated with T-lymphocytes (Schall et al., 1990; Kuna et al., 1993 and Godiska et al., 1995), stimulated astrocytes (Noe et al., 1996; Barnes et al., 1996) and cytokine-stimulated endothelial cells (Marfaing-Koka et al., 1995). This pro-inflammatory chemokine is a potent chemoattractant for eosinophils, basophils, monocytes and CD45RO/CD4$^+$ T-lymphocytes (Schall, 1992; Kameyoshi et al., 1992; Alam et al., 1993). Recent studies have demonstrated RANTES expression in inflammatory diseases

including atherosclerosis (Taub & Oppenheim, 1994), EAE (Miyagishi et al., 1997) and MS (Simpson et al., 1998).

It is of interest to note that unlike other α- and β-chemokines which are only expressed following cellular activation, RANTES is constitutively expressed by unstimulated T-lymphocytes (Schall et al., 1988), and expression is decreased upon CD3 ligation (Schall, 1991). RANTES is chemotactic for monocytes and memory T-cells, but not neutrophils (Schall et al., 1990). Recent findings suggest that this is one of the first chemotactic signals expressed in the CNS in MS, and is involved in the initial recruitment of leukocytes across the BBB (Simpson et al., 1998).

1.4.3 γ-CHEMOKINES

γ-chemokines possess only cysteines 2 and 4 of the standard chemokine four-cysteine residue motif, but the carboxyl end shares a high proportion of amino acid similarity with the β-chemokines (Kelner et al., 1994). To date lymphotactin is the only known member of this chemokine subfamily, and is a potent chemoattractant for T-lymphocytes, but has no effect on monocyte or neutrophil migration (Kelner et al., 1994; Kennedy et al., 1995).

1.4.4 δ-CHEMOKINES

Fractalkine, also known as neurotactin, is the only known member of the δ-chemokine family, and is predominantly expressed in the heart, lung, and by endothelial cells and microglia in the brain (Pan et al., 1997). Unlike other known chemokines, fractalkine is a type I membrane protein containing a chemokine domain at the amino terminus tethered on a long mucin-like stalk. Fractalkine has been shown to promote

adhesion of monocytes and T-lymphocytes to cells expressing the chemokine, but has no effect on neutrophils (Bazan et al., 1997).

1.4.5 CHEMOKINE EXPRESSION IN MS

It has been demonstrated that the level of MIP-1α is significantly elevated in the CSF of MS patients during relapse and remission compared to non-inflammatory neurological diseases (Miyagishi et al., 1995), suggesting chemokines may play a role in the migration and accumulation of leukocytes to inflammatory lesions in the CNS in MS.

Recent studies have reported the expression of MIP-1α and MIP-β by microglia and haematogenous macrophages, MCP-1 predominantly by astrocytes, and RANTES by perivascular T-cells and astrocytes in actively demyelinating MS lesions (McManus et al., 1998a; Simpson et al., 1998). Expression of MIP-1α is also associated with astrocytes in the MS lesion (Simpson et al., 1998).

In cultured human astrocytes, TNF-α enhances the expression of MCP-1 mRNA (Barna et al., 1994), and pre-treatment with IFN-γ significantly increases TNF-α-stimulated MCP-1 expression (Hayashi et al., 1995). It has been proposed that reactive T-cells infiltrating the CNS are induced to secrete pro-inflammatory cytokines which stimulate chemokine expression by resident glial cells, and which in turn results in further recruitment of inflammatory cells into the CNS in MS (Miyagishi et al., 1997). Circulating PLP-specific $CD8^+$ T-lymphocytes obtained from MS patients have been shown to secrete MIP-1α, MIP-1β, IP-10 and pro-inflammatory MMPs (Biddison et al., 1997), suggesting these cells may contribute to MS pathology.

1.4.6 CHEMOKINE EXPRESSION IN EAE

In the acute phase of EAE, $CD4^+$ T-cells and macrophages predominate over $CD8^+$ T-cells and B-cells, whilst in the chronic phase of disease the numbers of $CD8^+$ T-cells is dramatically increased (Hickey & Gonatas, 1984; Traugott, 1985). Differential expression of chemokines may play a role in the selective recruitment of specific lymphocyte subsets, and may explain the different inflammatory cell profiles during lesion development.

Several reports have indicated that chemokine mRNA expression is upregulated at the onset of EAE, and has been found to immediately follow leukocyte entry into the CNS (Hulkower et al., 1993; Ransohoff et al., 1993; Godiska et al., 1995; Miyagishi et al., 1997), suggesting a role for chemokines in the development or regulation of an inflammatory response. *In situ* hybridisation and immunocytochemistry studies have shown the mRNA expression of RANTES, MIP-1α and MIP-1β by T-lymphocytes located in the perivascular region of the CNS in EAE, suggesting that circulating T-cells which have crossed the BBB are the major sources of these chemokines (Berman et al., 1996; Miyagishi et al., 1997). IP-10 and MCP-1 are expressed by astrocytes adjacent to sites of inflammatory insults, and are associated with onset of disease (Ransohoff et al., 1993). The expression of RANTES is also associated with astrocytes and macrophages/microglia; while MIP-1α and MIP-1β are associated with mononuclear cells around blood vessels (Miyagishi et al., 1997).

CNS production of MIP-1α, but not MCP-1, have been shown to correlate with the development of severe clinical disease (Karpus et al., 1995). Studies have shown that both acute and relapsing paralytic EAE can be prevented by the administration of anti-MIP-1α antibodies (Karpus et al., 1995), suggesting MIP-1α plays a prominent role in the development of disease.

1.5 CHEMOKINE RECEPTORS (CCR)

The majority of chemokines induce a response by a haptotactic mechanism, that is they are immobilised on the surface of the target cell (Rot, 1993). Chemokine receptors can be divided into three subfamilies depending on the ligands they bind: the CXC receptors (CXCR1-4), the CC receptors (CCR1-8) and the orphan receptors (BOB and Bonzo) (Bacon, 1997). Orphan receptors are so called because, as yet, it is not known which ligands they bind. To date, two of the known chemokine receptors bind only one ligand: CXCR1 binds IL-8, and CXCR4 binds stromal-cell derived factor-1 (SDF-1) (Premack & Schall, 1996). The majority of chemokine receptors are shared receptors, that is they are capable of binding more than one ligand (Premack & Schall, 1996).

There is a large degree of homology both between and within CXCR and CCR families: 33% between CXCR1 and CCR1, and 69% between CCR2B and CCR5 (Bacon, 1997). The intracellular and extracellular loops of these seven transmembrane domain receptors are highly conserved, but the amino- and carboxyl-terminals show great variation (Bacon, 1997).

The Duffy antigen receptor for chemokines (DARC) is a non-selective, non-signalling chemokine receptor identical to the Duffy blood group antigen (Horuk et al., 1993). This erythrocyte chemokine receptor may play a role in limiting circulating chemokine levels, thereby maximising the sensitivity of haematogenous leukocytes to locally elevated chemokine concentrations (Makita et al., 1996). It has been reported that pathogens, in particular Herpesviridae, encode chemokine receptor homologues (Olcese et al., 1994; Qin et al., 1996), suggesting these organisms may gain a competitive advantage against host defences by blocking locally produced chemokines.

Figure 2 Chemokine receptors: schematic structural representation

1.5.1 CHEMOKINE-INDUCED CELL SIGNALLING

Chemokines mediate their effects by binding to seven-transmembrane domain high affinity G-protein-coupled receptors (Horuk, 1996) (Figure 2). The βγ subunits of the G-proteins are believed to activate phosphoinositide-specific phospholipase C, thereby generating inositol 1,4,5-trisphosphate (IP-3) and diacylglycerol (DAG) through hydrolysis of phosphoinositide bisphosphate (Bokoch, 1995). The IP-3 binds specific receptors in the plasma membrane and on the endoplasmic reticulum, thereby inducing the release of intracellular calcium. The calcium, in conjunction with DAG, then activates protein kinase C (PKC) which in turn activates a cascade of signal transduction events both within the cytoplasm and nucleus (Bokoch, 1995). Chemokine-receptor signalling is frequently, but

not always, pertussis-toxin sensitive, indicating heterogeneity in the system (Ransohoff & Tani, 1998).

The heterotrimeric G-proteins may also play a role in the activation of the Ras/MAPK (mitogen-activated protein kinase) pathway, while the GTP-binding proteins of the Ras superfamily, such as RhoA, have been implicated in the JNK/SAPK (c-jun kinase/stress-activated protein kinase) and phosphoinositide-3 kinase activation pathway (Crespo et al., 1994; Minden et al., 1995). Whether the activation of Ras/MAPK and/or the small molecular weight GTP-binding proteins is secondary to heterotrimeric G-protein activation, or is a direct consequence of chemokine binding is, as yet, unknown.

As well as the generation of IP-3, the subsequent mobilisation of intracellular free calcium, and the activation of the small GTP-binding proteins, chemokine-receptor binding also stimulates the polymerisation of actin (Newton & Vaddi, 1997). The actin filament network mainly determines the shape of a cell, hence polymerisation, which occurs at the leading edge of the migrating cell, results in the formation of lamellopodia in the direction of the chemotactic gradient (Newton & Vaddi, 1997).

1.5.2 α-CHEMOKINE RECEPTORS

The differential expression of chemokine receptors may explain the localisation and/or the specific recruitment of cells, such as T_H1 or T_H2 lymphocytes, to sites of inflammation (Mackay, 1996). Table 1 shows the known α-chemokine receptors (CXCR) and the ligands they bind (Baggiolini, 1998).

Expression of CXCR1 and CXCR2, the two IL-8 chemokine receptors, is restricted to natural-killer (NK)-like cells within the T-cell lineage (Carr et al., 1994). The majority of chemokine receptors, however, are expressed by many leukocyte subgroups, as

well as epithelia and neuronal cells (Mackay, 1996). To date CXCR2 binds all known ELR-α-chemokines, while CXCR3 binds all known non-ELR-α-chemokines (Liao et al., 1995; Premack & Schall, 1996). CXCR3 is highly expressed on IL-2-activated T-lymphocytes and NK cells (Qin et al., 1998), and displays ligand-specificity for IP-10 and Mig (Loetscher et al., 1996), suggesting these chemokines may play a role in the selective recruitment of activated T-cells to sites of inflammation.

Table 1 α-Chemokine Receptors and their Ligands

Receptor	Ligand
CXCR1	IL-8
CXCR2	IL-8, GRO-α, -β, -γ, ENA-78, NAP-2
CXCR3	IP-10, Mig
CXCR4	SDF-1

CXCR4, also known as fusin, is a specific chemokine receptor in that it binds one known ligand, SDF-1 (Premack & Schall, 1996). SDF-1 has been shown to block entry of the T-cell line-tropic form of HIV-1 *in vitro* (Bleul et al., 1996; Oberlin et al., 1996), suggesting CXCR4 may act as a receptor for HIV-1 infection.

1.5.3 β-CHEMOKINE RECEPTORS

The mechanism(s) by which chemokines induce the directional migration of leukocytes along their concentration gradient are poorly understood. Table 2 shows the known β-chemokine receptors (CCR) and the ligands they bind (Baggiolini, 1998).

Table 2 β-Chemokine Receptors and their Ligands.

Receptor	Ligand
CCR1	MCP-1, -3, MIP-1α, RANTES
CCR2A & 2B	MCP-1, -3, -5
CCR3	Eotaxin, RANTES, MCP-2, -3, -4
CCR4	MIP-1α, MCP-1, RANTES
CCR5	MIP-1α, -1β, RANTES
CCR6	MIP-3α
CCR7	MIP-3β
CCR8	TARC, MIP-1β

CCR1 and CCR2 expression is upregulated in IL-2-activated T-cells and correlates with the ability of these cells to migrate to RANTES and MCP-1 *in vitro* (Loetscher et al., 1996). Chemotaxis assays have shown memory T-lymphocytes migrate to RANTES (Schall et al., 1990) suggesting these cells express the RANTES receptors CCR1, CCR4 and/or CCR5 (Mackay, 1996).

CCR3 is expressed by eosinophils, basophils, T_H2 cells and microglia (Ponath et al., 1996; He et al., 1997). The expression of CCR3 on polarised T_H2 cells, both *in vivo* and *in vitro*, has been shown to correlate with IL-4 production (Sallusto et al., 1998), suggesting a mechanism of leukocyte recruitment during allergic inflammation. CCR5 is expressed by macrophages/monocytes, T_H1 lymphocytes, granulocyte precursors and microglia (Choe et al., 1996; Deng et al., 1996; He et al., 1997). Recent *in vitro* studies have shown enrichment of chemokine receptors, in particular CCR2 and CCR5, on the membrane at the leading edge of migrating T-cells in response to chemokines (Nieto et al.,

1997), indicting that chemokine receptors may act as a sensor mechanism for chemotactic signals. Both CCR3 and CCR5 have been detected on resident glial cells in the CNS of simian immunodeficiency virus (SIV)-infected macaque monkeys (Westmoreland et al., 1998), and have been shown to act as co-receptors for M-tropic HIV entry into microglia.

MIP-3α, also known as liver activation-related chemokine (LARC), was recently identified and found to mediate its chemotactic properties through the chemokine receptor CCR6 (Greaves et al., 1997). MIP-3β, a recent addition to the β-chemokine family, is a pro-inflammatory peptide which exerts its action via CCR7 (Rossi et al., 1997). Both CCR6 and CCR7 have been detected on T-lymphocytes (Greaves et al., 1997), but much remains to be determined about the expression of these receptors.

CCR8, the most recent addition to the CCR family, has been shown to bind thymus and activation-regulated cytokine (TARC) and MIP-1β as ligands (Bernardini et al., 1998). It has been suggested that binding to CCR8 may regulate activation, migration and proliferation of lymphoid cells (Bernardini et al., 1998).

1.5.4 EXPRESSION OF CHEMOKINE RECEPTORS BY T-LYMPHOCYTES

It is generally assumed that chemokines attract lymphocytes to sites of inflammation based on ligand specificity and expression of specific chemokine receptors. *In vitro* studies have shown that a shape change is elicited seconds after an attractant is added to a leukocyte suspension (Baggiolini, 1998). This stimulation results in the formation of lamellipodia following the polymerisation of actin, and in the upregulation and activation of integrins (Baggiolini, 1998), suggesting a role in the adherence and transmigration of leukocytes across the endothelium to sites of chemokine expression.

The migration of T-lymphocytes to the appropriate tissue site is a highly regulated process. Factors which determine whether a T_H1- or a T_H2-response occur at a site of inflammation are largely unknown. Chemokine receptor expression varies considerably in lymphocytes. CCR1, CCR2 and CCR5 are up-regulated by IL-2, whereas other stimulatory conditions, such as exposure to anti-CD3 antibodies, down-regulate receptor expression and chemotaxis (Baggiolini, 1998). Recent studies have reported the preferential expression of the chemokine receptors CXCR3 and CCR5 on T_H1 cells, and the expression of CCR4 and CCR3, on T_H2 cells (Bonecchi et al., 1998), which may, in part, explain the selective recruitment of T-cell subsets to inflammatory sites.

1.5.5 CHEMOKINE RECEPTOR EXPRESSION IN THE CNS IN MS

At present little is known about the expression of chemokine receptors in MS lesions. However, an increased expression of receptors for the chemotactic agonists C5a, IL-8 and FMLP (a bacterial N-formyl peptide) has been reported on foamy macrophages and reactive astrocytes in chronic active MS lesions, with much lower levels detected in chronic-silent lesions (Muller-Ladner et al., 1996), suggesting that an increase in expression of chemokine receptors is restricted to inflammatory lesions, and that lesion-specific mechanisms may regulate receptor expression.

1.5.6 CHEMOKINE RECEPTORS AND HIV

The discovery that chemokine receptors act as co-receptors for HIV infection of a cell has resulted in substantial interest in the cellular distribution of these receptors. T-cell line-tropic (T-tropic) HIV-1 viruses use CXCR4 as a co-receptor (Feng et al., 1996), whereas macrophage-tropic (M-tropic) viruses use CCR3 and CCR5 (Choe, et al., 1996;

Dragic et al., 1996). Mutation of the gene encoding CCR5 provides homozygotes with a strong resistance against M-tropic HIV infection (Libert et al., 1998).

Studies have shown that chemokines are strong inhibitors of HIV replication (Cocchi et al., 1995). The entry of HIV-1 into target cells can be prevented by blocking the CCR3 co-receptor with eotaxin (He et al., 1997). Likewise, MIP-1β has been shown to inhibit the entry of M-tropic HIV-1 strains into target cells by blocking CCR5 (Cocchi et al., 1996).

The SIV-infected macaque, the animal model of HIV (Persidsky et al., 1995), displays increased levels of chemokines associated with the endothelium and/or perivascular macrophages in areas of perivascular inflammatory cell cuffing in the CNS (Sasseville et al., 1996). Recent reports have indicated enhanced expression of CCR3, CCR5 and CXCR4 by perivascular macrophages and multi-nucleated giant cells, CXCR3 by perivascular lymphocytes, and CCR3 and CCR5 by resident glial cells in SIV-infected macaque (Westmoreland et al., 1998). The results of these studies suggest that multiple chemokines and their receptors may play a role in the recruitment of leukocytes into the CNS in HIV and SIV-encephalomyelitis.

1.5.7 REGULATION OF RECEPTOR EXPRESSION

The anti-inflammatory cytokine IL-10 has been shown to upregulate the expression of CCR1, CCR2 and CCR5 in human monocytes (Sozzani et al., 1998). Conversely, expression of CCR2 and, to a lesser extent, CCR1 and CCR5 can be down-regulated in peripheral blood mononuclear cells by bacterial lipopolysaccharide (LPS), IL-1 and TNF-α acting on receptor mRNA stability (Sica et al., 1997). IFN-γ has been shown to inhibit CCR3 and CCR4, and upregulate CXCR3 and CCR1 expression (Sallusto et al., 1998).

These findings suggest that pro-inflammatory signals may decrease chemokine receptor expression to retain leukocytes at the inflammatory site and to prevent their reverse transmigration (Randolph & Furie, 1996).

1.6 SUBACUTE SCLEROSING PANENCEPHALITIS

The measles virus (MV) is a highly contagious pathogen which causes acute measles, and is the aetiological agent that induces subacute sclerosing panencephalitis (SSPE) (Ogata et al., 1997). Complications of measles are uncommon, but can be fatal when the CNS is affected (Gogate et al., 1996). Persistence of the MV in the CNS of otherwise healthy individuals can result in SSPE, or in subacute measles encephalomyelitis (SME) in immunocompromised patients (Gogate et al., 1996). Approximately one per million of the population per year contract SSPE, which is twice as likely to occur in males as in females (Norrby & Kristensson, 1997).

SSPE is a rare, fatal form of encephalitis which results in a general destruction of both the white and grey matter in the CNS, in particular the cerebral cortex and brainstem, and to a lesser extent the cerebellum and spinal cord (Norrby & Kristensson, 1997). Onset of the disease usually occurs 10-15 years after the initial childhood infection (Cosby & Brankin, 1995).

1.6.1 THE PATHOGENESIS OF SSPE

SSPE is a slowly progressive, always fatal form of encephalitis which, like MS, is characterised by perivascular cuffs of inflammation, extensive leukocyte infiltration, demyelination and gliosis within the lesion (Kreth et al., 1982; Esiri et al., 1989; Allen et

al., 1996). The perivascular cuffs consist mainly of MHC class II-positive cells, whilst parenchymal lesions also contain B-cells (Nagano et al., 1991).

At present there is disagreement over the T-cell profile in SSPE, as previously discussed in MS (Section 1.2.1.1). Some groups state that $CD4^+$ T-cells are the major lymphocyte infiltrate in the CNS and are also present in greater numbers in the CSF of SSPE patients (Marrosu et al., 1983; Nagano et al., 1991), whilst others state that $CD8^+$ T-lymphocytes predominate over $CD4^+$ T-cells both in SSPE lesions and in the CSF (Czlonkowska et al., 1986; Esiri et al., 1989).

Cytotoxic T-cell recognition of virus-infected cells play an important role in viral clearance (Doherty, 1985). MV-infected cells express MHC class I, which triggers antigen-recognition in cytotoxic T-lymphocytes, and results in MHC class I-specific cytotoxic T-lymphocyte mediated lysis (Gogate et al., 1996). Lack of MHC class I expression on neurons may result in viral persistence in the CNS as the neurons are not under cytotoxic T-cell surveillance (Gogate et al., 1996). One possible therapeutic treatment in the early stages of SSPE may be to administer IFN-γ to induce MHC class I expression, and stimulate cytotoxic T-cell-mediated lysis of the infected cells (Gogate et al., 1996).

1.6.2 ENTRY OF MEASLES VIRUS INTO THE CNS

It is generally considered that MV enters the CNS across the BBB either indirectly through infected T-lymphocytes, or by direct infection of the endothelial cells (Cosby & Brankin, 1995). In normal CNS, CD46, a membrane co-factor protein, prevents the deposition of complement factors on host cells, thereby preventing lysis of the cells by complement (Liszewski et al., 1991; Loveland et al., 1993). CD46 has been identified as

the cellular receptor for MV (Dorig et al., 1993; Naniche et al., 1993). *In vitro* experiments have shown that some forms of MV down-regulate the expression of CD46 on the cell surface (Schneider-Schaulies et al., 1995). Recent reports have shown that in normal CNS, CD46 is detected at low levels on neurons and astrocytes, however, in SSPE CNS, CD46 is not detected within the lesion, but is detected at normal levels in the adjacent normal appearing white matter (Ogata et al., 1997). As yet, the significance of the down-regulation of CD46 is unknown, but it has been suggested that it could increase the vulnerability of the cell to complement thereby inducing cell lysis (Ogata et al., 1997).

1.6.3 CYTOKINE EXPRESSION IN THE CNS IN SSPE

Cytokines are expressed by infiltrating leukocytes and resident glial cells in the CNS in SSPE, suggesting they may play a role in the pathogenesis of the lesion formation (Nagano et al., 1994). IL-1β expression is associated with astrocytes, macrophages, some reactive microglia and endothelial cells in SSPE lesions (Nagano et al., 1994). IL-1 has been shown to induce reactive astrocytosis *in vitro* (Guilian et al., 1988), a common feature in the CNS in SSPE. Elevated levels of IL-1β were also detected in the CSF of SSPE patients, compared to MS and other neurological diseases (Mehta et al., 1997), indicating this pro-inflammatory cytokine may play a role in the pathogenesis of SSPE.

IL-2 is produced by antigen-activated T-lymphocytes, and plays an important role in the proliferation and differentiation of effector cells (Kroemer & Wick, 1989). This pro-inflammatory cytokine has been detected in lymphocytes in the perivascular cuffs and surrounding tissue in SSPE (Nagano et al., 1994). IL-6, detected in astrocytes and macrophages within the lesion (Nagano et al., 1994), induces differentiation of cytotoxic T-cells in the presence of IL-2 (Uyttenhove et al., 1988).

IFN-γ has been detected in T-lymphocytes, astrocytes, macrophages and endothelial cells within the SSPE lesion (Nagano et al., 1994). It has been suggested that IFN-γ may stimulate microglia to secrete TNF-α (Zajicek et al., 1992). Reports have shown that IFN-γ and TNF-α act synergistically to induce MHC class II expression on resident glia (Beneviste et al., 1989), suggesting these cytokines may contribute to immune responses in the SSPE lesion.

TNF-α has been detected in astrocytes and foamy macrophages within the SSPE lesion, while LT expression was detected in T-cells and macrophages in the perivascular cuffs and parenchymal lesions (Nagano et al., 1994). TNF-α and LT have been implicated in demyelination and growth of the lesion in SSPE, as they are capable of damaging oligodendrocytes directly causing a breakdown of the myelin sheath (Selmaj et al., 1991). TNF-α is able to increase vascular permeability and increase expression of adhesion molecules, suggesting a role for this cytokine in the recruitment of leukocytes from the circulation (Pober et al., 1987; Shijo et al., 1989; Probert, 1997). Expression of chemokines and their receptors are, as yet, uncharacterised in SSPE CNS lesions.

1.7 COELIAC DISEASE (CD)

Coeliac disease (CD), also known as gluten-sensitive enteropathy, is a permanent intolerance to gliadin and is characterised by villous atrophy of the small intestine (Kelly et al., 1990). Flattening of the villi and crypt hyperplasia result in malabsorption of nutrients, hence CD normally presents as weight loss and associated specific deficiency syndromes (Kelleher et al., 1984). The prevalence of CD is 1 per 1000 of the population, although it is becoming evident that a greater number of the population have clinically silent CD (Troncone et al., 1996).

Like MS, CD is immunologically mediated and is influenced by environmental and genetic factors (Troncone et al., 1996). CD is thought to be transmitted in a non-Mendelian manner but, as yet, no single specific HLA gene marker has been found (Cornell, 1996). The majority of CD patients express the haplotype HLA-DQ2, whilst the remainder express HLA-DQ8 (Nilsen et al., 1995). The abnormal immunological response to ingested gluten, in genetically susceptible individuals, can be prevented by strict adherence to a gluten-free diet.

Inflammation is often observed in the lamina propria and surface epithelium of the small intestine in CD (Cornell, 1996) It has been suggested that the binding of gluten to the intestinal mucosa becomes a target for immunological reactions (Strober, 1976). Gluten challenge in CD patients induces a dose-dependent increase in $CD8^+$ T-lymphocytes in the jejunal villous epithelium (Brandtzaeg et al., 1989), suggesting a role for these cells in the CD pathology (Halstensen & Brandtzaeg, 1993).

1.7.1 T-LYMPHOCYTE POPULATIONS IN COELIAC DISEASE

In normal intestinal epithelium the majority of intraepithelial lymphocytes express the $\alpha\beta$ T-cell receptor (TCR) (90%), as opposed to the $\gamma\delta$ TCR (10%) (Spencer et al., 1989; Savilhati et al., 1990). In the jejunal epithelium of CD patients the proportion of lymphocytes expressing $\gamma\delta$ TCR increases (Spencer et al., 1989; Savilhati et al., 1990), and remains high whether the patient is placed on a gluten-free diet or not (Savilhati et al., 1990). Although the role of $\gamma\delta$ cells is, at present, unknown it has been suggested that an increase in T-cells expressing $\gamma\delta$ TCR , along with the gene markers associated with CD, are necessary for the development of CD lesions (Holme et al., 1992).

Immunological assays on T-cell clones isolated from CD mucosa indicated both a T_H1 and T_H0 cytokine profile, with IFN-γ being the major cytokine produced (Nilsen et al., 1995). In peripheral blood mononuclear cell (PBMC) cultures from CD patients, IFN-γ was produced in response to gliadin-derived peptides (Cornell and Mothes, 1993). These findings suggest that IFN-γ may be involved in the pathogenesis associated with CD (Nilsen et al., 1995). It has been suggested that cytokine expression may cause an increase in the permeability of the small intestine epithelium (Madara & Salford, 1989), upregulation of epithelial HLA class II (Sollid et al., 1987), and growth and differentiation of B-cells (McGhee et al., 1989), all of which are evident in the active CD intestinal lesion.

1.7.2 NEUROLOGICAL COMPLICATIONS OF COELIAC DISEASE

It has recently been reported that more than 50% of patients with neurological complications of unknown aetiology have cryptic gluten sensitivity (Hadjivassiliou et al., 1996; Hadjivassiliou et al., 1998). These neurological complications include cerebellar ataxia, dementia, peripheral neuropathy, and vasculitis of the CNS (Cooke, 1976; Collin et al., 1991). Large perivascular inflammatory cell infiltrates have been detected centred around small venules in cerebellar CNS tissue from CD patients with associated neurological complications (Hadjivassiliou et al., 1996; Hadjivassiliou et al., 1998). Cases of rapid, unremitting neurological deterioration resulting in death, and cases of neurological deterioration becoming static have both been reported in CD patients (Hadjivassiliou et al., 1996; Hadjivassiliou et al., 1998).

Sera of patients with untreated CD contain IgA and IgG anti-gliadin antibodies (AGA) (Dieterich et al., 1997). AGA are produced at the site of tissue damage following gluten challenge (Falchuk & Strober, 1974), and may have a role in tissue damage via complement activation (Doe et al., 1973). Recent reports state over 50% of patients with

neurological dysfunction of unknown origin have circulating AGA, suggesting these antibodies may be neurotoxic to the CNS, either directly or indirectly (Hadjivassiliou et al, 1996). AGA, however, are not unique to CD patients, and have been detected in patients with other gastrointestinal disorders (Troncone & Ferguson, 1991). To date, neither a cytokine nor chemokine expression profile have been characterised in the CNS of CD patients with associated neurological complications.

1.8 THE AIM OF THIS STUDY

The aim of this study has been to characterise the expression of chemokines and their receptors in neuroinflammatory diseases of the CNS, and to assess their role in disease pathogenesis.

- The expression of the α-chemokines IP-10 and Mig, and their receptor CXCR3 were examined in multiple sclerosis at different stages of lesion development, in SSPE and CD CNS tissue using immunocytochemical (ICC) analysis.
- Expression of the β-chemokines MIP-1α, MIP-1β, MCP-1 and RANTES was examined at the protein level by ICC and at the mRNA level by non-radioactive *in situ* hybridisation (*ISH*). The chemokine profiles in the three CNS inflammatory diseases were compared.
- The presence of the β-chemokine receptors CCR3, CCR5 and CCR8 in MS CNS were investigated by RT-PCR and Western Blotting, and their cellular localisation detected by ICC and *ISH*.
- Factors which induce chemokine and chemokine receptor expression in rat astrocyte and microglial primary cell cultures were investigated by ELISA, ICC and *ISH*.
- Chemokine receptor expression by peripheral blood mononuclear cells was investigated by ICC.

CHAPTER 2

MATERIALS AND METHODS

2.1 HUMAN CNS TISSUE

2.1.1 MS TISSUE

Table 3 Age, sex, death to snap-freezing (D-F) time data and duration of disease in multiple sclerosis (MS) and normal control (NC) tissue samples

	Age (yr)	**Sex (M/F)**	**D-F (h)**	**Duration of MS (yr)**
MS cases (n=13)	47.6 (29-65)	(3/10)	26 (9-52)	14.25 (7-22)
NC cases (n=6)	43.33 (28-67)	(1/5)	24.33 (9-40)	N/A

The cause of death of the MS patients was bronchopneumonia (11), pulmonary embolism (1) and cerebrovascular accident (1). The cause of death of the control patients was cardiac arrest (2), peritonitis (1), road traffic accident (1), haemorrhage (1) and myocardial infarction and coronary artery thrombosis (1). N/A: not applicable.

Human MS and normal control CNS tissue samples were obtained from the Multiple Sclerosis Laboratory, Institute of Neurology, London (Newcombe & Cuzner, 1993; Appendix 1), as summarised in Table 3. Post-mortem CNS tissue, 1cm^3, was snap-frozen in isopentane cooled on liquid nitrogen, wrapped in tin foil and stored in an airtight container at -70°C. 10μm thick sections were cut in a cryostat (Bright Instruments) at -20°C, and were screened histologically by haematoxylin to assess perivenular

inflammatory cuffing and by oil red O staining to show the extent of recent demyelination. The histological evaluation was scored by two independent observers. To assess the degree of inflammation present, the tissue scored a minimum of 0 if no perivascular infiltrates were observed, and a maximum of 5 if large perivascular inflammatory cuffs were present. To assess the degree of demyelination present, the tissue scored a minimum of 0 if no demyelination was present, and a maximum of 5 if large confluent plaques were present. Based on these observations the MS tissue was categorised into four groups which represent the sequence of events in lesion formation: (i) normal appearing white matter (NAWM), (ii) acute lesions with perivascular inflammation and ongoing demyelination, (iii) chronic active lesions with demyelination and inflammation, and (iv) chronic silent demyelinated plaques (Lassmann et al., 1998). NAWM had a similar phenotypic profile to normal control CNS tissue, and displayed no demyelination (Figure 3a) and few T-lymphocytes associated with small venules (Figure 3b). Acute MS lesions contained a small amount of demyelination (Figure 3c), and perivascular cuffs of T-cells and macrophages centred around venules (Figure 3d). Chronic active lesions displayed hypertrophic astrocytes, lipid-containing macrophages (Figure 3e) and large perivascular inflammatory cuffs (Figure 3f). Chronic silent lesions comprised large demyelinated astroglial scars (Figure 3g), and were immunologically silent (Figure 3h).

Figure 3 MS Lesion Classification

Based on the degree of demyelination, indicated by oil red O staining, and the levels of inflammation, indicated by $CD4^+$ immunostaining, MS CNS tissue was categorised into four groups which represented the sequence of events in MS lesion formation. NAWM (sample 419-29) displayed (a) no demyelination and (b) few T-lymphocytes associated with small venules. (c) Acute MS lesions (sample 414-24) displayed some demyelination and (d) perivascular leukocyte cuffing centred around small venules. (e) Subacute lesions (sample 413-36) contained large numbers of lipid-containing macrophages and (f) large perivascular inflammatory infiltrates. (g) Chronic inactive MS lesions (sample 440-72) contained large demyelinated plaques (indicated by p) with no on-going demyelination and (h) were immunologically silent. (Magnification x220)

Figure 3 MS Lesion Classification

Figure 3 MS Lesion Classification Contd.

2.1.2 SSPE CNS TISSUE

Table 4 Age, sex, death to snap-freezing (D-F) time data and duration of disease in a SSPE tissue sample

	Age (yr)	Sex (F/M)	D-F (h)	Duration of SSPE (yr)
SSPE case (n=1)	20	0/1	4	unknown

The cause of death of the SSPE patient was a heavy viral load in the frontal cortex resulting in acute and fatal encephalitis.

Two blocks of frozen SSPE tissue from one case were obtained from Dr. L. Cosby, The Queen's University of Belfast, Belfast (Table 4). Post-mortem CNS tissue, approximately 1cm^3, was stored in an airtight container at -70°C until required for sectioning. The tissue blocks (10μm sections) were screened histologically using haematoxylin to assess perivascular cuffing. Unlike MS, demyelination was not a prominent feature of the SSPE nor CD CNS tissue used in this study. The CNS in SSPE was characterised by perivenous infiltration with plasma cells and lymphocytes (Figure 4), hypertrophy of astrocytes and proliferation of microglia with marked gliosis.

Figure 4 Immunopathology of SSPE

A prominent feature of SSPE CNS white matter is extensive perivascular $CD4^+$ T-lymphocyte infiltration. (Magnification x450)

Figure 5 Immunopathology of CD

One characteristic feature of CD cerebellar CNS tissue is perivenular $CD4^+$ T-lymphocyte infiltration. (Magnification x190)

Figure 4 Immunopathology of SSPE

Figure 5 Immunopathology of CD

2.1.3 CD CNS TISSUE

Table 5 Age, sex, death to snap-freezing (D-F) time data and duration of disease in a CD tissue sample

	Age (yr)	Sex (F/M)	D-F (h)	Duration of CD (yr)
CD case (n=1)	63	0/1	18	unknown

The cause of death of the CD patient was advanced cerebellar atrophy.

One cerebellum from a patient with CD and associated neurological dysfunction was obtained from Dr. C. Smith, Royal Hallamshire Hospital, Sheffield (Table 5). Large blocks of post-mortem CNS cerebellar tissue were snap-frozen. Smaller blocks were cut (approximately 1cm^3) from the larger block, and the samples stored in an airtight container at -70°C until required for sectioning. The cerebellar white matter in CD was characterised by large perivascular cuffs containing T-lymphocytes and macrophages (Figure 5), and by reactive astrocytes, similar in histology to the MS and SSPE tissue samples used in this study.

2.2 IMMUNOCYTOCHEMISTRY (ICC)

Immunocytochemistry (ICC) is a method employed to visualise the distribution of proteins within a tissue section, or cell preparation, using antibodies raised against the antigen of interest. Several techniques have evolved to visualise the antigen-antibody complex including direct or indirect labelling of the antibody with fluorescent labels or enzyme conjugates, such as peroxidase or alkaline phosphatase. The resultant complex can be viewed using a microscope, and the cellular localisation of the protein determined.

Counterstains are often required to visualise the cellular architecture. These complement the antibody labelling colours and are compatible with the detection system selected.

2.2.1 3 STEP AVIDIN-BIOTIN PEROXIDASE IMMUNOCYTOCHEMISTRY

Materials

- Polysine microscope slides (BDH, UK)
- Slide racks (BDH, UK)
- Glass slide dishes (BDH, UK)
- Fixative: either methanol (-20°C) (Sigma, UK)
  or acetone (4°C) (Sigma, UK)
- 10xPBS stock 0.02M KH_2PO_4 (Sigma, UK)
  0.13M K_2HPO_4 (Sigma, UK)
  1.5M NaCl (Sigma, UK)
  Adjusted to pH7.4 using NaOH
- PBS 10xPBS diluted ten-fold in d.H_2O
- Mouse or rabbit ABC Vectastain Elite Kit™ (Vector Laboratories Ltd., UK)
- 0.2% 3,3' diaminobenzidine tetrahydrochloride (Sigma, UK)
- Ethanol (Sigma, UK)
- Xylene (Sigma, UK)
- DPX mountant (BDH, UK)

Method

Human CNS tissue (10μm) was sectioned at -20°C in a cryostat (Bright Instruments, UK), collected on Polysine microscope slides and stored in an airtight container at -70°C until required. Sections were warmed to room temperature (RT), fixed

for 10min in the appropriate fixative (methanol at -20°C for the CD68 antibody, or acetone at 4°C for the remaining antibodies used in this study), and air-dried for approximately 10min.

The sections were then placed in a humid chamber containing 1x phosphate buffered saline (PBS), blocked for 30min at RT using 50μl of the appropriate serum diluted 3:200 in PBS (normal horse serum for monoclonal antibodies, normal goat serum for rabbit polyclonal antibodies), prior to incubation with the primary antibody for 30min at RT (Table 6). The optimal antibody dilution which gave minimum background staining was selected from a series of antibody titrations (Section 2.2.2).

The sections were washed in PBS, with stirring, three times over 10min. The avidin and biotinylated horse-radish peroxidase macromolecular complex (ABC) method with biotinylated secondary antibody was used as part of the mouse (biotinylated horse anti-mouse secondary antibody used in conjunction with monoclonal primary antibodies) or rabbit (biotinylated goat anti-rabbit secondary antibody used in conjunction with rabbit polyclonal primary antibodies) ABC Vectastain Elite Kit™. The sections were incubated in a humid chamber at RT with 50μl of the appropriate biotinylated secondary antibody diluted 1:200 in PBS for 30min. This step introduced biotin onto the section at the location of the primary antibody.

Following three 5min washes in PBS, the slides were incubated for 30min at RT with 50μl of the Vectastain® Elite ABC reagent. At least 30min before use, the ABC reagent was prepared by diluting equal volumes of reagent A and reagent B 1:100 in PBS. The ABC reagent bound to the biotin residues on the secondary antibody, introducing the peroxidase enzyme onto the sections at the site of the antigen. Following a brief wash in PBS, the sections were placed in the substrate 3,3' diaminobenzidine tetrahydrochloride

(DAB) for 5min (Section 2.2.2) (Figure 6). The DAB reacted with the enzyme to produce an insoluble brown precipitate, thereby enabling the site of antigen to be readily visualised. The sections were counterstained in Mayer's haematoxylin, for 20 seconds, and washed briefly in dH_2O. The sections were dehydrated in a graded series of ethanol (EtOH) (70%, 80%, 95% and 100% for 2min each), cleared in xylene for 2min and mounted in DPX. The slides were examined using interference contrast on an Olympus BX-60 microscope, and photographed using 200ASA Kodak colour print film.

Sections incubated with blocking serum instead of primary antibody were included as a negative control. Isotype specific antibody controls (Sigma, UK) were also included to confirm the specificity of the staining pattern. The IgG preparations were applied to the sections at the same concentrations as the primary antibody, and indicated whether staining was specific for the antigen of interest, or whether it was due to non-specific adsorption of the primary antibody to tissue sites (Section 2.2.3).

Figure 6 Avidin-Biotin Peroxidase Immunocytochemistry

Non-specific binding of the antibody to the tissue was prevented by incubating the section for 30min in serum from the animal in which the secondary antibody used in the investigation was made. The section was then incubated for 30min with the primary antibody directed against the antigen of interest. After washing the slides in PBS, the sections were incubated for a further 30min with the appropriate biotinylated secondary antibody. Following another wash in PBS the sections were incubated for 30min with the Vectastain® Elite ABC reagent, which binds to the biotin residues on the secondary antibody, thereby introducing the peroxidase enzyme onto the sections at the site of the antigen. To enable visualisation of the antibody complex, DAB substrate was added and reacted with the enzyme to produce an insoluble brown reaction product.

Table 6 Source and specificity of antibodies used in immunocytochemistry

Ab Specificity	Isotype	Ig Conc.	Dilution	Source
CD4	IgG_1	50μg/ml	1:100	Sigma
CD8	IgG_1	200μg/ml	1:100	Sigma
CD25	IgG_1	190μg/ml	1:100	Dako
CD68	IgG_2	430μg/ml	1:200	Dako
CCR3	rIgG	-	1:500	SKB*
CCR5	rIgG	-	1:500	SKB*
CCR8	rIgG	-	1:500	SKB*
CXCR3	IgG_1	500μg/ml	1:300	R&D Systems
GFAP	IgG_1	6.5mg/ml	1:250	Sigma
IFN-γ	rIgG	1mg/ml	1:200	PeproTech Ltd.
IP-10	rIgG	1mg/ml	1:200	PeproTech Ltd.
MIP-1α	IgG_{2A}	500μg/ml	1:250	R&D Systems
MIP-1β	IgG_{2B}	500μg/ml	1:250	R&D Systems
Mig	rIgG	1mg/ml	1:200	PeproTech Ltd.
MCP-1	IgG_1	500μg/ml	1:250	R&D Systems
RANTES	IgG_1	500μg/ml	1:250	R&D Systems

All antibodies used in this study were either mouse monoclonal or rabbit polyclonal (r).

*CCR antibodies are, as yet, uncharacterised and were a kind gift from Dr. J. White at SmithKline Beecham, USA.

2.2.2 3,3'DIAMINOBENZIDINE TETRAHYDROCHLORIDE

Materials and Equipment

- 0.2% 3,3' diaminobenzidine tetrahydrochloride (Sigma, UK)
- PBS (Section 2.2.1)
- 30% hydrogen peroxide (Sigma, UK)

Method

To enable visualisation of the antibody complex the peroxidase substrate 3,3'diaminobenzidine (DAB) was used. DAB, a potential carcinogen, was made in the fume cupboard following the manufacturers instructions. 100mg DAB was dissolved in 500ml PBS, and 200µl of 30% hydrogen peroxide (H_2O_2) added. The sections were left in DAB until the required intensity of stain developed (approximately 5min). DAB produced a red/brown precipitate that was insoluble in alcohol, therefore the sections were permanently mounted following dehydration through a graded series of ethanol and clearing through xylene.

2.2.3 ANTIBODY CONTROLS AND TITRATIONS

ICC of both control (Figure 7a) and inflammatory CNS tissue (Figure 7b) in the absence of a primary antibody displayed minimal background staining, indicating the specificity of the antibodies used. Similarly, isotype specific immunoglobulins (Ig) used at the same Ig concentration as the antibodies (Table 6) did not stain the tissue (Figure 7c). No difference in the pattern or staining intensity was seen between MS tissue of different post-mortem delays, suggesting this delay has not affected the immunoreactivity of the cellular proteins.

Figure 7 Antibody controls

Immunostaining of (a) control (sample 480-18) and (b) MS (sample 396-98) CNS tissue in the absence of primary antibody displayed minimal background staining, indicating the specificity of the antibodies used in this study. (c) Similarly, isotype specific immunoglobulins, such as IgG_1 shown here, did not stain the tissue (sample 413-41). Sections were counterstained with haematoxylin. (Magnification x220)

Figure 7 Antibody controls

Antibody titration experiments were used to determine the antibody dilution which provided specific staining of the antigen with minimal background staining. A range of dilutions were selected for each antibody used in this study which encompassed the manufacturers recommended dilution. For example, the CCR5 antibody was titrated using doubling dilutions from 1:60 through to 1:1000 (Figure 8). The optimal antibody dilution of 1:500, as shown in Table 6, was selected as it gave the lowest levels of background staining together with the highest specific antigen staining.

2.3 *IN SITU* HYBRIDISATION

In situ hybridisation (*ISH*) is a technique used to localise cellular DNA and RNA. Oligonucleotide probes, complementary to the sequence of interest, can be labelled with ^{3}H or ^{35}S and detected by autoradiography, or they can be labelled with digoxigenin or biotin and detected by non-radioactive techniques.

2.3.1 NON-RADIOACTIVE *IN SITU* HYBRIDISATION

To avoid accidental RNAse contamination of the sections from sources in the laboratory the *ISH* method described below was carried out under RNAse-free conditions. Disposable sterile gloves were worn throughout the preparation of materials and solutions, and during the *ISH* procedure. As gloves remain RNAse-free only if they do not come into contact with contaminated glassware and surfaces, it was necessary to change gloves frequently throughout the experiment. All solutions were prepared using RNAse-free glassware and were autoclaved. The d.H_2O and PBS solutions used in the procedure were also treated with 0.02% diethyl pyrocarbonate (DEPC) (Sigma, UK), which is a strong,

Figure 8 Antibody titration

Antibody titration experiments were used to determine the optimal antibody dilution (sample 488-110). Titrations with anti-CCR5 antibody were performed using doubling dilutions from 1:60 through to 1:2000, and the optimal antibody dilution selected. (a) The 1:120 dilution of CCR5 was associated with high levels of background staining. (b) This was reduced when a dilution factor of 1:250 was used. (c) A dilution factor of 1:500 displayed minimal background staining with the highest level of specific antigen staining. (d) 1:1000 dilutions of CCR5 had little specific antigen stain. Brown DAB substrate indicates positive staining. Sections were counterstained with haematoxylin. (Magnification x220)

Figure 8 Antibody titration

but not absolute, inhibitor of RNAases. All equipment was baked in an oven overnight (o/n) at 140°C to further ensure no RNAse contamination of the sections.

The following controls were applied to all *ISH* procedures performed in this study. Initial *ISH* using a 30 base oligo-poly-dT probe (R&D Systems, UK) confirmed the presence of intact mRNA in all cell types in both control (Figure 9a) and inflammatory (Figure 9b) CNS tissue. *ISH* using hybridisation buffer alone, in place of digoxigenin-labelled probes, on the section acted as a negative control, showed minimal background staining and confirmed the specificity of the probes used in this study (Figure 9c).

2.3.1.1 PREHYBRIDISATION TREATMENT

Materials

- RNAse-free Polysine microscope slides (BDH, UK)
- slide racks (BDH, UK)
- glass slide dishes (BDH, UK)
- 4% paraformaldehyde (Sigma, UK) in PBS
  The solution was stirred on a hotplate at 60°C in a fume cupboard until the HCHO completely dissolved, sterile filtered and cooled to RT before use.
- PBS (Section 2.2.1) containing 0.02% DEPC (Sigma, UK)
- Triethanolamine (TEA) 0.1M TEA (Sigma, UK)
  0.15M NaCl (Sigma, UK)
  adjusted to pH8.0 with 0.25% acetic anhydride
- Acetic acid (Sigma, UK)
- EtOH (Sigma, UK)

Figure 9 *ISH* controls

ISH using an oligo-poly-dT probe indicated the presence of intact mRNA in all cell types in (a) control (sample 480-18) and (b) MS (sample 413-41) CNS tissue (Magnification x190). (c) *ISH* using hybridisation buffer in place of digoxigenin-labelled probes showed minimal background staining and confirmed the specificity of the probes used in this study (sample 413-64). Purple/black colour indicates positive mRNA staining. (Magnification x220, unless otherwise stated)

Figure 9 *ISH* controls

- d.H_2O containing 0.02% DEPC (Sigma, UK)
- Chloroform (Sigma, UK)
- 20xSSC buffer (Sigma, UK)

Method

Sections (10μm) were collected on RNAse-free Polysine microscope slides and stored in an airtight container at -70°C until required. Sections were warmed to RT, and fixed in freshly prepared sterile 4% paraformaldehyde (HCHO) in PBS for 5min. Following three 5min washes, with stirring, in PBS the sections were washed in 0.1M triethanolamine, adjusted to pH8.0 in a fume cupboard with 0.25% acetic anhydride, for 10min, then rinsed again in PBS. The sections were dehydrated in a graded series of EtOH (70%, 80%, 95% and 100% for 2min each), placed in chloroform in a fume cupboard for 5min to delipidate the sections, returned to 95% EtOH for 2min, air-dried and placed in a humid chamber containing 2x sodium saline citrate (SSC).

2.3.1.2 HYBRIDISATION

Materials

- De-ionised formamide

  10% BioRex Resin (BioRad, UK) in formamide (Sigma, UK) was mixed on a magnetic stirrer under sterile conditions. After 30min the solution was decanted into a sterile bottle using methanol-sterilised filter paper.
- Denatured sheared herring sperm DNA

  1% sheared herring sperm DNA (Boehringer-Mannheim, UK) was boiled in H_2O for 10min, and cooled on ice for 10min before use.
- Hybridisation Buffer

The following components were mixed in a sterile 50ml tube in the order shown:

5ml	de-ionised formamide (Sigma, UK)
2.5ml	20x SSC (Sigma, UK)

The solution was warmed to 50°C, and the following added:

1g	dextran sulphate (Sigma, UK)

The contents of the tube were mixed at 50°C until the polymer dissolved, and the following added:

1ml	50xDenhardt's solution (Sigma, UK)
100µl	denatured sheared herring sperm DNA (Boehringer-Mannheim, UK)
400µl	sterile d.H_2O

The tube was kept at 50°C until required.

- Parafilm (Fahrenheit, UK)
- 20xSSC buffer (Sigma, UK)

Method

Approximately 30µl of the digoxigenin-labelled oligonucleotide probe cocktail (Table 7; Table 8) diluted in hybridisation buffer was added to the sections, and lightly covered with a square of parafilm approximately the same size as the section. Care was taken to ensure no air-bubbles were present on the section. The optimal probe dilution which gave minimum background staining was selected from a series of probe titrations (a similar method as described in Section 2.2.3). The sections were incubated o/n with the probe at 37°C in a sealed humid chamber containing 2xSSC. The sections were washed in 2xSSC for 1h at RT, 1xSSC for 1h at RT, and 1xSSC for 30min at 37°C, with stirring. The slides were then processed for immunological detection.

Table 7 Source and specificity of probes used in *in situ* hybridisation

Probe	Dilution	Source
CCR3	1:500	Synthesised in-house*
CCR5	1:500	Synthesised in-house*
IFN-γ	1:200	R&D Systems, UK
MCP-1	1:400	R&D Systems, UK
MIP-1α	1:400	R&D Systems, UK
MIP-1β	1:400	R&D Systems, UK
RANTES	1:400	R&D Systems, UK

*The chemokine receptor probes were synthesised in-house by Mr A. Fairclough.

Table 8 Sequence of digoxigenin-labelled oligonucleotide probes used in *in situ* hybridisation studies

Probe	Sequence
CCR3	3'dig-AAA GAA CCA GCT CTG TCT CTC CAT CCA CAG 5'
CCR5	3'dig-CGG AGC CCT GCC AAA AAA TCA ATG TGA AGC 5'
IFN-γ	©R&D Systems
MCP-1	©R&D Systems
MIP-1α	©R&D Systems
MIP-1β	©R&D Systems
RANTES	©R&D Systems

2.3.1.3 IMMUNOLOGICAL DETECTION

Materials

- slide racks (BDH, UK)
- glass slide dishes (BDH, UK)
- Buffer 1 0.1M Tris (Sigma, UK)
  0.15M NaCl (Sigma, UK)
  Adjusted to pH 7.5 using HCl
- Buffer 1 containing 2% normal sheep serum (NSS) (Sigma, UK)
- Alkaline phosphatase conjugated sheep anti-digoxigenin antibody (Boehringer-Mannheim, UK)
- Benchtop shaking platform (New Brunswick Scientific, UK)
- Buffer 1 containing 1% NSS (Sigma, UK)
- Buffer 2 0.1M Tris (Sigma, UK)
  0.1M NaCl (Sigma, UK)
  Adjusted to pH 9.5 using HCl
- Sigma fast™ 5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium tablets (0.15mg/ml BCIP/ 0.3mg/ml NBT) (Sigma, UK)
- PBS containing 50% glycerol (Sigma)

Method

Following a 1min wash in buffer 1, the sections were placed in buffer 1 containing 2% normal sheep serum (NSS) for 30min at RT. The sections were then incubated with 100µl of alkaline phosphatase conjugated sheep anti-digoxigenin antibody, diluted 1:500 in buffer 1 containing 1% NSS, in a humid chamber for 3-5h. The sections were washed with shaking in buffer 1 for 10min at RT, followed by a 10min wash with shaking in buffer 2 for 10min, before being incubated with 100µl of the substrate Sigma fast™ BCIP/NBT.

The sections were left o/n at RT to allow the stain to develop, washed in d.H_2O and mounted in PBS/glycerol. The slides were examined on an Olympus BX-60 microscope, and photographed using 200ASA Kodak colour print film.

2.3.2 SYNTHESIS OF OLIGONUCLEOTIDE PROBES

An amidite, a fully protected nucleotide, is the basic building block in probe synthesis. In order to produce an oligonucleotide four different amidites were needed which corresponded to the bases adenine (A), cytosine (C), guanine (G) and thymine (T). The oligonucleotide probes were synthesised by Mr A. Fairclough, Department of Biomedical Sciences, Sheffield Hallam University, based on published methods (Kling, 1997). Oligonucleotide chemokine receptor sequences for CCR3 and CCR5 were obtained from Genebank UK, and an oligo™ program used to design the probes. The reaction cycle of oligonucleotide synthesis consisted of four main steps: (I) deprotection, (II) coupling, (III) capping and (IV) oxidation of the amidite, each stage separated by washes.

The first stage was deprotection of the amidite (Figure 10). This step involved the removal of the dimethyltrityl group (DMTr) to expose the hydroxyl group at position 5 of the 2-deoxyribose unit, and the subsequent activation of the phosphoramidite by tetrazole. The coupling stage resulted in the formation of an internucleotide bond between the 5' hydroxyl group and the phosphorous containing group at the 3' position of the incoming protected nucleotide. The capping step terminated any unreacted 5' hydroxyl groups. Finally, the phosphite linkage was oxidised to phosphate by iodine. These steps were repeated, adding one nucleotide to the chain each time, until an oligonucleotide of the required sequence and length was obtained.

Figure 10 A fully protected amidite

2.3.2.1 LABELLING OLIGONUCLEOTIDE PROBES WITH DIGOXIGENIN

Materials

- Labelling reaction mix:

The following components were mixed in a sterile Eppendorf on ice, in the order shown:

4µl	5x tailing buffer (Boehringer-Mannheim, UK)
4µl	5mM $CoCl_2$ solution (Sigma, UK)
1µl	2.5mM dATP (Boehringer-Mannheim, UK)
1µl	1mM DIG-11-dUTP (Boehringer-Mannheim, UK)
100pM	oligonucleotide
1µl	50U/µl terminal transferase (Boehringer-Mannheim, UK)

d.H_2O to a final volume of 20µl

- 2% glycogen solution (Sigma, UK)
- 0.2M EDTA solution, pH 8.0 (Sigma, UK)
- 4M LiCl (Sigma, UK)
- EtOH (Sigma, UK)

Method

The chemically synthesised single-stranded oligonucleotides were reacted in a final synthesis step with an amino-linker phosphoamidite (Boehringer Mannheim, UK), creating a 3'-terminal aminofunction. To introduce the digoxigenin label at the 3' position, a labelling reaction mix was made up in a sterile eppendorf on ice, as shown above. The tube was incubated at 37°C for 15min, then placed on ice. 1μl glycogen solution was mixed with 200μl EDTA solution, and 2μl of the resultant solution added to the eppendorf to stop the reaction. The digoxigenin-labelled probe was precipitated with 2.5μl LiCl and 75μl prechilled (-20°C) EtOH, for 2h at -20°C. The pellet was washed with 50μl cold ethanol by centrifugation, dried under vacuum and dissolved in 20μl d.H_2O. The probe was stored at -70°C until required.

2.4 PCR ANALYSIS OF CCR EXPRESSION

The expression of CCR mRNA was investigated in control and MS CNS tissue using the reverse transcriptase polymerase chain reaction (RT-PCR). These experiments were performed with the assistance of Mr C. Murdoch, Department of Molecular Biology and Biotechnology, Sheffield University. To prevent contamination of the samples, sterile equipment and disposable, sterile gloves were used throughout the experiment.

2.4.1 RNA EXTRACTION FROM HUMAN CNS TISSUE

Materials

- Sterile 0.5ml Eppendorf (BDH, UK)
- Tri-Reagent (Sigma, UK)

Method

Three 25μm MS or control CNS sections were cut in a cryostat and collected using sterile, chilled forceps into a sterile, chilled Eppendorf. The sections were triturated with 0.5ml Tri Reagent until the sample dissolved. Tri Reagent, a mixture of guanidine thiocyanate and phenol in a monophase solution, simultaneously isolated RNA, DNA and protein on lysis of the tissue sample.

2.4.2 RNA ISOLATION

Materials

- Chloroform (Sigma, UK)
- Sorvall Super T21 Centrifuge (DuPont, UK)
- Isopropanol (Sigma, UK)
- EtOH (Sigma, UK)
- Sterile pure H_2O (Gibco, UK)
- GeneQuant II spectrophotometer (Pharmacia, UK)

Method

0.2ml of chloroform was added to the Eppendorf per ml of Tri Reagent, the tube was capped and shaken vigorously. After standing at RT for 2-15min, the Eppendorf was

centrifuged at 2,415g (Sorvall Super T21 Centrifuge) for 15min at 4°C. The upper aqueous layer was transferred to a sterile Eppendorf, and mixed with 0.5ml isopropanol per ml of Tri Reagent used. After 5-10min at RT the tube was centrifuged at 2,415g for 10min at 4°C. The supernatant was removed and the RNA pellet washed in 1ml 75% ice-cold EtOH per ml Tri Reagent. The sample was vortexed, centrifuged at 1,514g for 5min at 4°C, and air-dried for 10min. The RNA pellet was resuspended in 25μl sterile pure H_2O, and the sample stored at -70°C until required.

To calculate the RNA concentration of the sample, 5μl was added to 995μl d.H_2O and the absorbance at 260nm was recorded using a spectrophotometer. The RNA concentration (μg/μl) was calculated using the equation:

RNA concentration (μg/μl) = absorbance at 260nm x dilution factor x 0.04

2.4.3 REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Materials

- RT Reaction Mix (40μl)
  - 2μl sterile d.H_2O (Gibco, UK)
  - 8μl 5xRT buffer (Gibco, UK)
  - 8μl KCl (Gibco, UK)
  - 16μl dNTPs (10mM) (Gibco, UK)
  - 1μl oligo (dT) primer (Gibco, UK)
  - 1μl AMV reverse transcriptase (Gibco, UK)
  - 4μl RNA template
- No RT Reaction Mix (34μl)
  - 3μl sterile d.H_2O
  - 8μl 5xRT buffer (Gibco, UK)
  - 8μl KCl (Gibco, UK)

10μl dNTPs (10mM) (Gibco, UK)

1μl oligo (dT) primer (Gibco, UK)

4μl RNA template

- Biometra trio-thermoblock (Biometra, UK)

Method

For each RNA sample from the previous RNA extraction experiment, a reverse transcription step was performed to convert the mRNA into cDNA. A reaction was also set up without the reverse transcriptase (RT) enzyme to ascertain if the final RT-PCR product was from genomic DNA or mRNA. The reaction mixes were set up, the contents of the tubes overlayed with mineral oil to prevent evaporation during the experiment, and the tubes incubated in a Biometra trio-thermoblock using the following incubation programme, based on published methods (He et al., 1997):

37.5°C for 60min

99°C for 5min

4°C for ∞

2.4.4 PCR FOR CHEMOKINE RECEPTOR EXPRESSION

Materials

- PCR Reaction Mix (23μl)

12.75μl	sterile d.H_2O (Gibco, UK)
2μl	10x buffer (Gibco, UK)
2.5μl	$MgCl_2$ (Gibco, UK)
0.25μl	Primer A
0.25μl	Primer B
0.25μl	Taq DNA polymerase (Gibco, UK)

5μl cDNA template

- Biometra trio-thermoblock (Biometra, UK)

Method

To investigate if the control and MS CNS samples contained chemokine receptor cDNA, a PCR step was carried out using specific primers for CCR3 and CCR5 (a kind gift from Dr P. Monk, Department of Molecular Biology and Biotechnology, Sheffield University). The CCR3 and CCR5 primer sequences are shown in Table 9.

Table 9 CCR3 and CCR5 primer sequences.

Primer	Sequence
CCR3	5'-TCC TTC TCT CTT CCT ATC-3' 3'-GGC AAT TTT CTG CAT CTG-5'
CCR5	5'-AAT CTT CTT CAT CAT CCT-3' 3'-TCT CTG TCA CCT GCA TAG-5'

A PCR reaction mix was set up, the contents overlayed with mineral oil, and the tube incubated in a thermal cycler using the following incubation programme. The incubation programme used was based on reported methods (He et al., 1997):

95°C for 5min
95°C for 1min
58°C for 1min } 35 cycles
72°C for 1min
72°C for 6min
55°C for 5min
4°C for ∞

2.4.5 VISUALISATION OF PCR PRODUCTS

Materials

- 10xTBE 0.9M Tris Base (Sigma, UK)
  0.9M Boric Acid (Sigma, UK)
  0.025M EDTA (Sigma, UK)
- 2% agarose gel 2g agarose/100ml 1xTBE
  Microwave for 1min
- Loading dye 50% glycerol
  0.2% bromophenol blue
  0.1M EDTA
- Ethidium bromide (Sigma, UK)
- Phi X 174 DNA/ Hae III molecular weight marker (Promega, UK)
- PCR samples

Method

To visualise the products of the PCR reaction the final PCR solution (15μl plus 5μl loading dye) was run on a 2% agarose gel containing 10mg/ml ethidium bromide, at 100V for approximately 50min. Ethidium bromide binds to the DNA helix and fluoresces under UV light, enabling visualisation of the PCR product. A marker (Phi X 174 DNA/ Hae III marker) (72-1353 base pairs) was also run on the gel, to enable the size of the PCR products to be calculated.

2.5 WESTERN BLOT ANALYSIS OF CCR EXPRESSION

In this study Western blotting was used to confirm the antigen specificity of the uncharacterised chemokine receptor antibodies, which were a kind gift from Dr J. White, SmithKline Beecham, USA. Western blot techniques enable detection of antigens in a tissue or cell culture homogenate using electrophoresis to separate the antigens into molecular weight bands and immunochemistry to visualise the target antigen (Laemmli, 1970). To prevent contamination of the samples, sterile equipment and disposable, sterile gloves were used throughout the experiment.

2.5.1 CNS SAMPLE PREPARATION

Materials

- Separating Gel Buffer 1.5M Tris (Sigma, UK)
  14mM SDS (Sigma, UK)
  Adjusted to pH8.8 using HCl
- Water bath (Fahrenheit, UK)
- Sorval TC6 Centrifuge (DuPont, UK)

Method

Three 25μm MS or control CNS sections were cut in a cryostat and collected in a sterile, chilled Eppendorf using sterile, chilled forceps. The samples were suspended in separating gel buffer by syringe homogenisation, and placed in a water bath at 80°C to solubilise the protein. Following centrifugation at 120g for 5min at 4°C, the supernatant was removed and stored at -20°C until required. The amount of protein extracted from the tissue was calculated using the bicinchoninic acid test (BCA) (Sigma, UK).

2.5.2 BCA METHOD OF PROTEIN DETERMINATION

The BCA test is used to determine the amount of protein present in a solution (Smith et al., 1985). Using spectrophotometer readings from a known range of protein concentrations, a standard curve is plotted. The unknown amount of protein in a solution can then be determined based on its absorbance at the same wavelength.

Materials

- Bovine serum albumin (Sigma, UK)
- Protein extracted from CNS samples
- d.H_2O
- Separating gel buffer (Section 2.5.1)
- BCA reagent (Sigma, UK)
- 96 well plate (Becton Dickinson, UK)
- Plate reader (LabSystems, UK)

Method

20µl of bovine serum albumin (BSA) standards (ranging from 0.5-10mg/ml BSA in d.H_2O) or 20µl of supernatant samples were placed in a 96 well plate. Appropriate blanks were included, 20µl d.H_2O or 20µl separating gel buffer. 200µl BCA reagent (0.4ml 4% copper sulphate solution in 20ml BCA) was added to each well. After 30min at RT the absorbance was read at 570nm, and the amount of protein present in the samples calculated.

2.5.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Materials

- Sample Buffer

0.25M	Tris (Sigma, UK)
0.14M	SDS (Sigma, UK)
16%	glycerol (Sigma, UK)
10%	β-mercaptoethanol (Sigma, UK)
2.5mM	bromophenol blue (Sigma, UK)
Adjusted to pH6.8 using HCl	

- SDS Gel

Lower gel (12% acrylamide-bisacrylamide)

3.0ml	1M Tris (Sigma, UK)
3.2ml	Acrylamide-bisacrylamide (30%) (Sigma, UK)
1.62ml	d.H_2O
80µl	SDS (10%) (Sigma, UK)
80µl	freshly prepared APS (ammonium persulphate solution, 10%) (Sigma, UK)
20µl	TEMED (Sigma, UK)

Stacking gel (5%)

2.5ml	0.5M Tris (Sigma, UK)
1.2ml	Acrylamide-bisacrylamide (30%) (Sigma, UK)
6.3ml	d.H_2O
100µl	SDS (10%) (Sigma, UK)
80µl	APS (10%) (Sigma, UK)
20µl	TEMED (Sigma, UK)

- 10xSDS Tank Buffer

0.25M	Tris (Sigma, UK)
1.86M	Glycine (Sigma, UK)
35mM	SDS (Sigma, UK)

- See Blue Molecular Weight marker (19.2-104kDa) (Sigma, UK)
- Coomassie Blue Stain

45.5%	methanol (Sigma, UK)
0.9%	acetic acid (Sigma, UK)
1.5mM	Coomassie blue (Sigma, UK)

- Destain 45.5% methanol (Sigma, UK)
  0.9% acetic acid (Sigma, UK)

Method

The samples were heated in sample buffer (in a 2:1 ratio) in a water bath at 70°C for 10min. A 12% acrylamide-bisacrylamide gel (10cm x 10cm x 1mm) was prepared, 10μl of molecular weight markers or samples added to each lane, and the gel run at 150V for 1h at RT. To visualise the protein bands on the gel, when required, it was placed in Coomassie Blue stain o/n and rinsed in destain until the bands were visible.

2.5.4 WESTERN BLOT

Materials

- PVDF membrane (0.2μm) (BioRad Laboratories, UK)
- Transfer tank (Fahrenheit, UK)
- Towbin Transfer Buffer 0.2M glycine (Sigma, UK)
  0.025M Tris (Sigma, UK)
  2.6mM SDS (Sigma, UK)
  20% methanol (Sigma, UK)
- TBS 0.15M NaCl
  0.01M Tris
  Adjust to pH8.0 using HCl
- TTBS 1L TBS plus 0.5% Tween 80 (Sigma, UK)
- Blocking Solution 1L TTBS plus 5% Marvel milk powder

Method

PVDF membrane (0.2μm) was cut to approximately the same size as the SDS gel ($10cm^2$), and placed in methanol for 10-15sec. The membrane was then placed in transfer

buffer for 30min at RT, as were the sponges, gel and 3μm pore filter paper. One corner of the gel and filter paper were cut to facilitate their orientation. The cassette was assembled (Figure 11), ensuring all components remained saturated in transfer buffer. The cassette was placed in a transfer tank, containing transfer buffer, on a magnetic stirrer and run at 100V for 1h at 4°C. The PVDF paper was then placed in blocking solution for 1h at 37°C, or o/n at 4°C, before undergoing immunological detection.

Figure 11 Assembly of Transfer Cassette

black cassette leaf	→ to cathode
sponge (x2)	
filter paper (x3)	
SDS gel	
PVDF paper	
filter paper (x3)	
sponge (x2)	
grey cassette leaf	→ to anode

2.5.5 IMMUNOLOGICAL DETECTION OF CCR

Materials

- Benchtop shaking platform (New Brunswick Scientific, UK)
- TTBS (Section 2.5.4)
- Rabbit ABC Vectastain Elite Kit™ (Vector Laboratories Ltd., UK)
- 0.2% DAB (Sigma, UK) (Section 2.2.2)

Method

The following procedures were carried out at RT on a benchtop shaking platform, unless otherwise stated. After being washed three times in TTBS over 30min, the blot was

incubated with 30ml of the rabbit polyclonal primary antibodies against CCR3, CCR5 or CCR8 (Table 6) (diluted 1:500 in PBS) for 1h. Following three washes in TTBS over 1h, the blot was incubated for 1h with 30ml goat anti-rabbit biotinylated secondary antibody (part of the rabbit Vectastain Elite ABC™ kit), diluted 1:200 in TBS. The blot was then washed three times in TTBS over 1h, and incubated with the Vectastain ABC complex (diluted 1:100 in PBS) for 1h. Following a brief wash in TTBS, the antigen-antibody complex was visualised by placing the blot in DAB substrate for 5min. The blot was then briefly washed in TTBS, and the PVDF left to dry at RT.

2.6 CELL CULTURE

To assess which factors induce the expression of chemokines and chemokine receptors by glial cells, the following experiments were carried out on primary rat astrocyte and microglial cell cultures.

2.6.1 PREPARATION OF MIXED GLIAL CELL SUSPENSION

Materials

- 30x 30 day old Sprague Dawley rats
- methanol-sterile filter paper (Whatmann UK)
- McIlwain chopper (The Mickle Lab. Engineering Co., Surrey, UK)
- 500ml glass bottle (Fahrenheit, UK)
- 1.25% w/v Trypsin type III in $EBSS^+$ (Sigma, UK)
- 1000 U/ml Collagenase type XI in $EBSS^+$ (Sigma, UK)
- 200 μg/ml DNase type II in $EBSS^+$ (Sigma, UK)
- Earle's balanced salt solution$^+$ (EBSS with Ca^{2+} and Mg^{2+}) (Gibco, UK)

- Newborn calf serum (NBS) (Gibco, UK)
- Earles balanced salt solution$^-$ (EBSS without Ca^{2+} and Mg^{2+}) (Gibco, UK)
- 132μm and 80μm nylon mesh (J. Stanniar & Co., Manchester, UK)
- Sorvall RT 6000D centrifuge (DuPont, UK)

Method

The following experiments were carried out in a lamina flow hood under sterile conditions, unless otherwise stated. Thirty, 30 day old Sprague Dawley rats were killed by cervical dislocation. The brains were removed using sterile scissors and forceps, and placed in a petri dish on ice. The meninges were removed by rolling the brains on methanol-sterile filter paper, and any visible blood vessels were removed using forceps. The tissue was chopped in 0.4mm slices in two planes at 90° on a McIlwain chopper and placed in a sterile 500ml glass bottle. Trypsin type III, collagenase type XI and DNase type II were added to the bottle at 1ml/g of tissue. Earles balanced salt solution$^+$ (EBSS with Ca^{2+} and Mg^{2+}) was added at 7ml/g of tissue. The mixture was incubated at 37°C with constant stirring on a magnetic stirrer for 15min. After this time the mixture was divided into approximately 30ml per 50ml tube. The enzyme digestion was arrested by the addition of 10ml newborn calf serum (NBS), and 10ml EBSS$^-$ (EBSS without Ca^{2+} and Mg^{2+}) to the tubes, and the mixture spun at 13.5g for 10min at 4°C. The supernatant was discarded, and the pellet placed in a sterile 500ml bottle, and the enzyme digestion and centrifugation steps repeated. Following centrifugation, the pellet was resuspended in EBSS$^-$ and filtered through methanol sterilised 132μm and 80μm nylon mesh using a syringe plunger into a sterile petri dish. EBSS$^-$ was added to the resultant mixed glial cell suspension to bring the final concentration to 5ml/g tissue, and the suspension spun at 13.5g for 10min at 4°C.

2.6.2 OPSONISATION OF ERYTHROCYTES

Materials

- $EBSS^-$ (Gibco, UK)
- anti-erythrocyte antibody (Dako, UK)
- $EBSS^+$ (Gibco, UK)
- Sorvall RT 6000D centrifuge (DuPont, UK)

Method

This procedure was carried out one day before the erythrocyte-antibody complex was to be used. Human venous blood was collected by venepuncture in heparinised tubes, and spun at 13.5g for 10min at 4°C. The plasma and buffy coat overlying the red blood cells (RBC) were removed, and the RBC washed twice in $EBSS^-$ by centrifugation at 13.5g for 10min. The RBC were resuspended at 2%v/v in human anti-erythrocyte antibody (1:600) in $EBSS^+$, and incubated with gentle shaking for 30min at RT. The erythrocyte-antibody complex was washed in $EBSS^-$ by centrifugation, the pellet resuspended in $EBSS^+$ at 2%v/v, and stored at 4°C until required.

2.6.3 ISOLATION OF MICROGLIA FROM A MIXED GLIAL CELL SUSPENSION

Materials

- $EBSS^+$ (Gibco, UK)
- $EBSS^-$ (Gibco, UK)
- Percoll Preparation

  Isolation of microglia from a mixed glial cell preparation required Percoll adjusted to a density of 1.086g/ml. This was achieved by adding together:

  62ml Percoll (Pharmacia, UK)

  28ml $d.H_2O$

10ml 10xBME (basal medium eagle) (Gibco, UK)

- Sorvall RT 6000D centrifuge (DuPont, UK)
- 10xBME (Gibco, UK)
- C-DMEM Preparation

  In a laminar flow hood, under sterile conditions the following were added to Dulbecco's modification of Eagle's minimum essential medium (DMEM):

  10% heat-inactivated FCS (Gibco, UK)

  1% penicillin-streptomycin solution (Gibco, UK)

  (5000U penicillin, 5000μg streptomycin solution)

  The FCS was heat-inactivated by placing in a waterbath at 55°C for 30min.

Method

The pellet (from Section 2.6.1) was resuspended in $EBSS^+$, and made up to half the original volume. An equal volume of antibody-coated erythrocytes (Section 2.6.2) was added, and the suspension incubated at 37°C for 30min. Following centrifugation at 13.5g for 10min at 18°C, the pellet was resuspended in $EBSS^-$ at 10ml/g of starting tissue. 25ml of the suspension was carefully layered over 25ml of Percoll, and centrifuged at 53.7g for 20min at 18°C with the brake off. The cloudy middle layer contained astrocytes and oligodendrocytes and was carefully removed using a pipette (Section 2.6.4). The pellet contained rosetted microglia, and was resuspended in $EBSS^-$ to approximately 1ml/g of starting tissue, and divided into 5ml quantities. The erythrocytes were lysed by addition of 40ml ice-cold sterile d.H_2O for 45sec. The tonicity was restored by addition of 4.5ml 10x BME (basal medium eagle). The tubes were centrifuged at 13.5g for 10min at 4°C, and a second lysis step performed. The pellet was resuspended in 3ml C-DMEM, the cells counted on a haemocytometer and then resuspended to $5x10^5$ cells/ml in C-DMEM.

2.6.4 ISOLATION OF ASTROGLIA FROM A MIXED GLIAL CELL SUSPENSION

Materials

- $EBSS^+$ (Gibco, UK)
- Sorvall RT 6000D centrifuge (DuPont, UK)
- C-DMEM (Gibco, UK) (Section 2.6.3)

Method

The mixed astrocyte/oligodendrocyte suspension obtained from 2.6.3 was diluted two-fold in $EBSS^+$ and centrifuged at 53.7g for 20min at RT. The pellet was washed twice in $EBSS^+$ and resuspended in DMEM to give a final cell suspension of $1x10^5$ cells/ml. The astrocyte suspension was placed in a $75cm^3$ flask, and incubated at 37°C.

2.6.5 ISOLATION OF ASTROGLIA FROM NEONATAL RATS

Materials

- methanol-sterilised filter paper (Whatmann, UK)
- Nylon mesh (132μm and 80μm) (J. Stanniar & Co., Manchester, UK)
- C-DMEM (Gibco, UK) (Section 2.6.3)
- 0.25% Trypsin/ 1mM EDTA solution (Gibco, UK)
- Sorvall RT 6000D centrifuge (DuPont, UK)

Method

Twelve brains from one litter of neonatal rats were removed, and the meninges removed by rolling on methanol-sterilised filter paper. After filtration through methanol-sterilised 132μm and 80μm nylon mesh the cells were centrifuged. The pellet was

resuspended in C-DMEM, and plated out into two 75cm^3 flasks. After 3-4 days the cells were removed by trypsinisation: the media was discarded, 2.5ml Trypsin/EDTA added, the flask gently shaken, and the Trypsin/EDTA discarded. This action removed any cells which were not strongly adhered to the flask. A further 2.5ml Trypsin/EDTA was added, and the flask incubated at 37°C. Following a 3-5min incubation, the flask was shaken vigorously to remove the adherent astrocytes from the bottom of the flask. The cell suspension was centrifuged at 13.5g for 5min, adjusted to 5x10^5 cells/ml in C-DMEM, and plated out into 96 well plates.

2.6.6 DETECTION OF CHEMOKINE AND CHEMOKINE RECEPTOR EXPRESSION BY PRIMARY RAT GLIAL CELLS BY ICC

Materials

- 1mg/ml PMA stock solution(Sigma, UK)
- C-DMEM (Gibco, UK) (Section 2.6.3)
- 4% HCHO (Sigma, UK) (Section 2.3.1.1)
- 8 well chamber slides (LabSystems, UK)

Method

Primary rat microglia or astrocyte cell suspensions (400μl) were plated out into 8 well chamber slides at 2x10^5 cells/400μl C-DMEM/well. After 3 days, the media was changed and 4 wells of the 8 well chamber slide stimulated with 20μg/ml PMA (Sigma, UK) (Figure 12). After 24h, the media was discarded and the cells fixed in 4% paraformaldehyde at 4°C for 10min. Chemokine and chemokine receptor expression by

both the stimulated and unstimulated cells was examined by ICC and/or *ISH*, using the methods described earlier (Section 2.2.1 and Section 2.3).

Figure 12 Chamber slide of stimulated/unstimulated rat primary glial cells

2.6.7 DETECTION OF CHEMOKINE AND CHEMOKINE RECEPTOR EXPRESSION BY PRIMARY RAT ASTROCYTES BY ELISA

Materials

- 100μg/ml recombinant rat IFN-γ stock solution (Sigma, UK)
- 100μg/ml lipopolysaccharide (LPS) stock solution (Sigma, UK)
- EBSS$^+$ (Gibco, UK)
- 4% HCHO (Sigma, UK) (Section 2.3.1.1)
- EtOH (Sigma, UK)
- PBS (Section 2.2.1)
- Mouse or rabbit ABC Vectastain Elite Kit™ (Vector Laboratories, UK)
- Vectastain-alkaline phosphatase™ kit (Vector Laboratories Ltd., UK)
- Alkaline Phosphate Substrate Σ104

  2ml diethanolamine (Sigma, UK) was dissolved in 8ml d.H_2O.

  20mg Sigma 104® phosphatase substrate (Sigma, UK) and 20.3mg MgCl (Sigma, UK)was dissolved in 10ml d.H_2O.

The two solutions were mixed together, and used immediately.

- Plate reader (LabSystems, UK)

Method

200μl/well of the astrocyte cell suspension ($5x10^5$ cells/ml) were plated out into a 96 well plate. After 3 days the cells were stimulated with increasing concentrations of IFN-γ (1, 10 or 100 IU/ml) (as shown in Figure 13), or stimulated with increasing concentrations of LPS (0.01, 0.1, 0.5 or 10μg/ml). All experiments were performed in triplicate.

Figure 13 IFN-γ-stimulation of rat primary astrocytes

ELISA detection of	IFN-γ (IU) ← 0 →			← 1 →			← 10 →			←100→		
MCP-1	O	O	O	O	O	O	O	O	O	O	O	O
IP-10	O	O	O	O	O	O	O	O	O	O	O	O
Mig	O	O	O	O	O	O	O	O	O	O	O	O
CXCR3	O	O	O	O	O	O	O	O	O	O	O	O
CCR3	O	O	O	O	O	O	O	O	O	O	O	O
CCR5	O	O	O	O	O	O	O	O	O	O	O	O
CCR8	O	O	O	O	O	O	O	O	O	O	O	O
-ve	O	O	O	O	O	O	O	O	O	O	O	O

After 24h the cells were washed in warm $EBSS^+$, and fixed in 4% paraformaldehyde for 10min. If intracellular staining of chemokines was being investigated the cells were permeabilised in EtOH for 10min at 4°C, if cell surface antigen expression was being examined the cells were not permeabilised. Detection of chemokine and chemokine receptor expression was examined using a similar method described in

section 2.2.1, using 3-step ABC alkaline phosphatase ICC (ABC-AP) (Vectastain-AP™ kit, Vector Laboratories Ltd., Peterborough, UK).

The cells were blocked for 30min at RT with 200μl of the appropriate serum diluted 3:200 in PBS (normal horse serum for monoclonal antibodies, normal goat serum for rabbit polyclonal antibodies). The blocking serum was discarded, and the cells incubated for 30min at RT with 200μl of the primary antibody raised against the antigen of interest (Table 6). Following three 5min washes in PBS, the cells were incubated with 200μl biotinylated secondary antibody, diluted 1:200 in PBS, for 30min at RT (biotinylated horse anti-mouse secondary antibody used in conjunction with monoclonal primary antibodies, or biotinylated goat anti-rabbit secondary antibody used in conjunction with rabbit polyclonal primary antibodies).

Following three 5min washes in PBS, the cells were incubated for 30min at RT with 200μl ABC-AP reagent. Both ABC-AP reagents A and B were diluted 1:100 in PBS, and mixed together at least 30min before use. The ABC-AP reagent bound to the biotin residues on the secondary antibody, introducing the alkaline phosphatase enzyme onto the cells at the site of the antigen.

Following a brief wash in PBS, the cells were incubated with the substrate Sigma 104® for 20min at RT. The Sigma 104® reacted with the enzyme to produce a yellow colour. To determine the optical density of the substrate, and hence the relative degree of chemokine/chemokine receptor expression by the cells, the plate was read in a plate-reader (LabSystems, UK) at 405nm.

2.7 PERIPHERAL BLOOD MONONUCLEAR CELL CYTOSPINS

Materials

- 5ml peripheral venous blood
- Histopaque (Sigma, UK)
- PBS (Section 2.2.1)
- Sorvall RT 6000D centrifuge (DuPont, UK)
- RPMI (Sigma, UK) (containing 10% heat-inactivated FCS (Gibco, UK), and 1% penicillin-streptomycin (5000U penicillin, 5000μg streptomycin solution) (Gibco, UK)
- 100μg/ml LPS stock solution (Sigma, UK)
- 50% Methanol/acetone (Sigma, UK)
- Cytospin centrifuge (Shandon, UK)

Method

The following experiment was performed on peripheral venous blood obtained from two healthy volunteers, on three separate occasions. Each experiment was run in triplicate. Peripheral venous blood (5ml) was collected by venepuncture into an EDTA tube, and diluted 1:1 with PBS. The resultant cell suspension was pipetted over 10ml histopaque in a sterile 50ml tube, and spun at 30.2g for 20min at 18°C with the brake off. The cloudy interface was collected and washed once in RPMI (Boyum, 1968). The pellet was resuspended in 1ml RPMI, the number of peripheral blood mononuclear cells (PBMC) present calculated using a haemocytometer and the resultant cell suspension adjusted to $1x10^6$ cells/ml. 200μl of the suspension was aliquoted into sterile Eppendorfs. The cells were then stimulated with LPS (10μg/ml) for 12h or 24h at 37°C. Unstimulated cells, in

RPMI alone, acted as a negative control. 200μl of the cell suspension was added to slides in a cytospin centrifuge at 11.2g for 3min. The slides were removed, fixed for 5min in 50% methanol/acetone at 4°C, air-dried and stored at 4°C until required. Chemokine receptor expression was examined using the ICC method described earlier (Section 2.2.1).

CHAPTER 3

α-CHEMOKINE AND CXCR3 EXPRESSION IN INFLAMMATORY LESIONS IN THE CNS

3.1 INTRODUCTION

α-chemokines which do not possess an ELR amino acid motif at their N-terminus, such as the IFN-γ-inducible chemokines IP-10 and Mig, are associated with the specific chemoattraction of activated T-lymphocytes (Liao et al., 1995). The CNS in MS, SSPE and CD is characterised by perivascular cuffs of inflammation consisting of T-lymphocytes and macrophages recruited from the circulation, suggesting IP-10 and Mig may play a role in the recruitment of leukocytes expressing the IP-10/Mig chemokine receptor, CXCR3, into the CNS during disease pathogenesis. As these chemokines are both dramatically induced by IFN-γ, the expression of this pro-inflammatory cytokine was also investigated. IP-10, Mig and CXCR3 expression was detected using a three step avidin-biotin peroxidase immunocytochemistry technique (Section 2.2). IFN-γ was detected both at the protein and mRNA level using ICC (Section 2.2) and non-radioactive *ISH* methods (Section 2.3), respectively.

3.2 RESULTS

3.2.1 α-CHEMOKINE EXPRESSION IN INFLAMMATORY LESIONS IN THE CNS: DETECTION BY ICC

ICC is a method employed to visualise an antigen of interest in cells and/or in tissue sections. In this study ICC was used to detect IFN-γ, IP-10, Mig and CXCR3 proteins within the CNS of control, MS, SSPE and CD tissue samples.

3.2.1.1 CONTROL CNS TISSUE

The four control CNS tissue samples used in this study displayed few $CD4^+$ and $CD8^+$ T-lymphocytes associated with the blood vessel endothelium. IFN-γ protein was detected in cells associated with the blood vessel (Figure 14a). Both IP-10 (Figure 14b) and Mig (Figure 14c) displayed weak, diffuse staining of the endothelium and some surrounding glial cells. Although the IP-10 and Mig antibodies were used at the same optimal Ig concentration, a more intense staining pattern for IP-10 than Mig was visible in all tissue samples, suggesting IP-10 is expressed at a greater level than Mig in control CNS. CXCR3 was weakly associated with the blood vessel endothelium (Figure 14d).

IgG subclass specific antibodies, used as negative controls, did not stain the sections. Similarly, substitution of the primary antibody with blocking serum did not result in any visible staining pattern. These findings indicate the specificity of the antibodies used in this study.

3.2.1.2 MS CNS TISSUE

An elevated level of IFN-γ (Figure 15a), IP-10 (Figure 15b), Mig (Figure 15c) and CXCR3 (Figure 15d) ICC staining was observed in the two NAWM CNS tissue samples used in this study. The pro-inflammatory cytokine and chemokines were detected both in

Figure 14 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 in control CNS

(a) IFN-γ, (b) IP-10, (c) Mig and (d) CXCR3 proteins were detected in blood vessel endothelial cells, and some resident glial cells in control CNS tissue (sample 480-18). (Magnification x220)

Figure 14 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 in control CNS

Figure 15 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 in MS NAWM

(a) IFN-γ, (b) IP-10, (c) Mig and (d) CXCR3 proteins were detected in cells associated with the blood vessel endothelium, and some resident glial cells (arrow) in NAWM (sample 419-29). The arrow represents one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 15 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 in MS normal appearing white matter

T-lymphocytes associated with endothelium, and with surrounding glial cells. In NAWM, CXCR3 was associated with glial cells and endothelial-associated cells.

In three acute MS CNS lesion samples, with increased numbers of perivascular leukocytes, expression of IFN-γ (Figure 16a), IP-10 (figure 16b), Mig (Figure 16c) and CXCR3 (Figure 16d) staining was increased and was associated with these infiltrating inflammatory cells.

A distinct profile of α-chemokine expression was observed in three actively demyelinating MS lesions with high levels of inflammation: IFN-γ protein was associated with T-lymphocytes in the perivascular cuffs, macrophages and astrocytes within the plaque (Figure 17a), and was restricted to reactive astrocytes in the adjacent non-demyelinated white matter (Figure 17b). Staining on serial sections with anti-CD68 (to stain macrophages) (Figure 17c) and anti-GFAP (to stain astrocytes) (Figure 17d) confirmed the phenotype of these cells. Antibodies to IP-10 showed this protein was present in the IFN-γ-positive macrophages (Figure 18a) and astrocytes (Figure 18b). Staining with the Mig antibody showed a similar, but less intense, pattern of expression to IP-10, staining both macrophages (Figure 18c) and astrocytes (Figure 18d). The IP-10/Mig receptor CXCR3 was associated with T-lymphocytes within the plaque, and also with astrocytes both within the lesion (Figure 18e) and in the adjacent non-demyelinated white matter (Figure 18f).

Chronic inactive MS lesions are immunologically silent, that is they contain no inflammatory cells such as infiltrating T-lymphocytes or macrophages, and comprise large astroglial scars. IFN-γ (Figure 19a), IP-10 (Figure 19b), Mig (Figure 19c) and CXCR3 (Figure 19d) immunostaining of one chronic silent MS plaque indicated protein levels had

Figure 16 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 in acute MS lesions

An increased pattern of (a) IFN-γ, (b) IP-10, (c) Mig and (d) CXCR3 staining was associated with the increased number of perivascular inflammatory cells, and some surrounding glial cells (arrow) in acute MS lesions (sample 395-71). The arrow represents one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 16 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 in acute MS lesions

Figure 17 Immunocytochemical detection of IFN-γ in subacute MS lesions

In actively demyelinating MS tissue, IFN-γ protein was (a) associated with T-cells in the perivascular cuffs and foamy macrophages (arrow) within the lesion (indicated by p) (sample 444-69), and (b) associated with reactive astrocytes (arrow) in the adjacent non-demyelinated white matter (sample 488-67). Immunostaining on serial sections with (c) anti-CD68 and (d) anti-GFAP antibody confirmed the cellular localisation of IFN-γ protein. The arrow represents one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 17 Immunocytochemical detection of IFN-γ in subacute MS lesions

Figure 18 Immunocytochemical detection of IP-10, Mig and CXCR3 in subacute MS lesions

Immunostaining for IP-10 and Mig displayed a similar pattern of stain as IFN-γ: (a,b) IP-10 and (c,d) Mig protein were predominantly associated with macrophages (arrow) within the actively demyelinating MS lesion (indicated by p) (sample 444-69) and with astrocytes in the adjacent non-demyelinated white matter (sample 488-67). CXCR3 was associated with (e) T-lymphocytes (small arrow) and astrocytes within the lesion (large arrow), and with (f) glial cells in the non-demyelinated white matter adjacent to the MS lesion (large arrow) (sample 488-67). The arrows represent one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 18 Immunocytochemical detection of IP-10, Mig and CXCR3 in subacute MS lesions

Figure 18 Immunocytochemical detection of IP-10, Mig and CXCR3 in subacute MS lesions Contd.

Figure 19 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 expression in chronic inactive MS lesions

(a) IFN-γ, (b) IP-10, (c) Mig and (d) CXCR3 protein were very weakly associated with the blood vessel endothelium in chronic silent MS lesions (sample 440-88). (Magnification x220)

Figure 19 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 expression in chronic inactive MS lesions

returned to control levels, in that expression was weakly associated with the blood vessel endothelium.

3.2.1.3 SSPE CNS TISSUE

The CNS in the two SSPE CNS tissue samples used in this study were characterised by large perivascular cuffs of inflammation, and reactive astrocytes in the white matter. Overall, SSPE tissue displayed a similar, though not as intense, pattern of α-chemokine staining to chronic active MS lesions. IFN-γ protein was detected in T-lymphocytes in the perivascular cuffs, and was also present in astrocytes in the white matter adjacent to the small blood vessels (Figure 20a). Both IP-10 (Figure 20b) and Mig (Figure 20c) protein were detected in perivascular leukocytes and adjacent reactive astrocytes. As in the MS tissue, IP-10 staining was more intense than Mig, suggesting IP-10 may be expressed at a higher level than Mig in inflammatory lesions in the CNS in SSPE. CXCR3 expression gave a similar pattern of staining to the MS CNS samples. The CXCR3 protein was associated both with perivascular T-lymphocytes and with the α-chemokine-positive reactive astrocytes (Figure 20d).

3.2.1.4 CD CNS TISSUE

The CNS in CD was characterised by perivascular cuffs containing infiltrating T-lymphocytes, and reactive astrocytes. T-lymphocytes were also present in the surrounding parenchyma. Unlike MS and SSPE CNS tissue samples, no α-chemokine protein was detected in CD CNS tissue (Figure 21a,b). The experiments were repeated in triplicate, and gave the same results.

Figure 20 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 expression in SSPE CNS

(a) IFN-γ (Magnification x450), (b) IP-10 and (c) Mig protein were associated with perivascular lymphocytes (small arrow) and reactive astrocytes (large arrow) in the CNS in SSPE. (d) CXCR3 was associated with perivascular T-lymphocytes (small arrow) and the α-chemokine-positive astrocytes (large arrow). The arrows represent one example of a positively staining cell, several others are visible on the section. (Magnification x220, unless otherwise stated)

Figure 20 Immunocytochemical detection of IFN-γ, IP-10, Mig and CXCR3 expression in SSPE CNS

Figure 21 Immunocytochemical detection of IP-10 and Mig expression in CD cerebellar CNS tissue

Neither (a) IP-10 nor (b) Mig were detected in the cerebellum in CD. (Magnification x220)

3.2.2 *IN SITU* HYBRIDISATION

ICC is a method employed to visualise a protein using an antibody detection system, but this method does not define if a cell is synthesising the protein of interest or if the protein has been adsorbed by the cell. One technique to visualise which cells are expressing the mRNA of the protein of interest is non-radioactive *ISH*.

3.2.2.1 *ISH* STAINING FOR IFN-γ

To confirm the cellular source of IFN-γ, *ISH* was carried out on serial sections from the subacute MS blocks which displayed the highest level of IFN-γ protein staining, and on control tissue. As yet, *ISH* probes for IP-10 and Mig are not commercially available, hence experiments to confirm the cellular source of these chemokines were not performed. Control CNS showed a similar pattern of staining as the ICC experiments with IFN-γ mRNA associated with some cells resembling T-cells surrounding blood vessels (Figure 22a), further confirmation that the oligo anti-sense probes do not bind non-specifically to CNS tissue. Actively demyelinating MS lesions showed IFN-γ mRNA was predominantly expressed by T-lymphocytes in the perivascular cuff (Figure 22b).

Figure 22 *ISH* investigation of IFN-γ mRNA expression in control and actively demyelinating MS CNS

(a) IFN-γ mRNA was expressed by T-cells associated with the blood vessel endothelium in control CNS (sample 480-18) (Magnification x190). (b) IFN-γ mRNA was predominantly expressed by T-lymphocytes in the perivascular cuff in actively demyelinating MS CNS tissue (sample 488-67). Purple/black precipitate indicates positive mRNA staining. (Magnification x220, unless otherwise stated.)

Figure 22 *ISH* investigation of IFN-γ mRNA expression in control and actively demyelinating MS CNS

Table 10 Summary of IP-10, Mig and CXCR3 protein distribution in three subacute MS lesions, and in two SSPE and one CD CNS tissue sample

		Inflammatory cuff		Lesion	
Chemokine/ Receptor	Disease	Leukocytes	Endothelium	Macrophages/ Microglia	Astrocytes
IP-10	MS	+	+	+++	+++
	SSPE	+	-	++	++
	CD	-	-	-	-
Mig	MS	+	-	++	++
	SSPE	+	+	+	+
	CD	-	-	-	-
CXCR3	MS	+++	+	++	++
	SSPE	+++	+	+	++
	CD	-	-	-	-

Key: -: no staining associated with the cells

+: weak staining

++: strong staining

+++: all cells stained strongly positive

The table above summarises the chemokine and chemokine receptor staining pattern seen in CNS tissue from three neuroinflammatory disease states, and reflects the scores of two independent observers.

3.3 DISCUSSION

Although MS, SSPE and CD, with associated neurological complications, are neuroinflammatory diseases with different aetiologies, the CNS in each of these diseases displays similar pathological features, such as large perivascular cuffs containing inflammatory cells recruited from the circulation. The α-chemokines IP-10 and Mig are involved in the activation and directional migration of activated T-lymphocytes to sites of inflammation (Karpus et al., 1995; Baggiolini, 1998), and may play an important role in the pathogenesis of these diseases. Table 10 summarises the results obtained in this study, showing the expression of IP-10, Mig and their specific chemokine receptor CXCR3 in the three neuroinflammatory states. The results of this study indicate neither IP-10 nor Mig play a role in the recruitment of inflammatory cells into the cerebellum in CD, suggesting leukocyte infiltration into the CNS is mediated by another mechanism. Both SSPE and actively demyelinating MS CNS tissue, however, displayed an intense pattern of α-chemokine staining.

Control and NAWM CNS tissue displayed low levels of IP-10 and Mig associated with endothelial cells, some T-lymphocytes and, in NAWM, resident glial cells, suggesting these α-chemokines may play a role in the normal traffic of T-cells across the BBB. An increased expression of IP-10 and Mig protein was observed in acute MS lesions. The highest level of α-chemokine expression was observed in subacute MS lesions and in SSPE CNS tissue, whilst immunologically silent chronic inactive lesions displayed dramatically lower levels. This transient expression of IP-10 and Mig during MS lesion formation reflects the amount of inflammation observed in the four lesion types, and suggests these chemokines may play a role in the recruitment of leukocytes from the circulation into the CNS in MS.

The pro-inflammatory cytokine IFN-γ has been shown to play an important role in the activation of macrophages (Cavaillon, 1994), the regulation of MHC class II expression (Woodroofe et al., 1989), the induction of adhesion molecule expression on endothelial cells (Brosnan et al., 1995), the induction of cytokine expression (Woodroofe & Cuzner, 1993), and the induction of chemokine expression by resident glial cells (Luster et al., 1985; Farber, 1990; Farber, 1993). IFN-γ has been shown to significantly increase MCP-1 expression by murine glial cells (Hayashi et al., 1995), suggesting a role for this cytokine in stimulating chemokine expression in resident glial cells in neuroinflammatory lesions. IFN-γ mRNA has been detected in T-lymphocytes in the perivascular cuffs (Woodroofe & Cuzner, 1993), and in microglia and astrocytes in chronic MS lesions (Traugott et al., 1988), and has been detected in the CSF of MS patients (Horwitz et al., 1997). In SSPE lesions, IFN-γ has been detected in endothelial cells, T-lymphocytes, astrocytes and macrophages (Nagano et al., 1994). This study's findings that IFN-γ protein is associated with the blood vessel endothelium, infiltrating T-lymphocytes and resident glial cells in MS and SSPE CNS tissue confirm these reports.

Both IP-10 and Mig are dramatically induced by IFN-γ (Luster et al., 1985; Farber, 1990; Farber, 1993). Elevated levels of these α-chemokines have been demonstrated in a number of disease processes including renal failure (Gomez-Chiarri et al., 1996), adult respiratory distress syndrome (Neville et al., 1995), stroke (Wang et al., 1998) and EAE (Ransohoff et al., 1993; Godiska et al., 1995; Glabinski et al., 1997), all pathological conditions characterised by leukocyte accumulation. Within the CNS, IP-10 has been detected in astrocytes adjacent to infiltrated inflammatory leukocytes in both murine EAE (Ransohoff et al., 1993; Glabinski et al., 1995) and following occlusion of the middle cerebral artery in spontaneously hypertensive rats (Wang et al., 1998). In actively

demyelinating MS lesions and in SSPE tissue used in this study, astrocytes and macrophages were found to express IP-10 and Mig. These findings are in agreement with reported studies which have shown IP-10 mRNA and protein expression in both astrocytes and microglial IFN-γ-stimulated cell cultures (Vanguri et al., 1994; Vanguri & Farber, 1995). In this study the cells were identified as astrocytes and macrophages by the co-localisation of the chemokine and staining for GFAP and CD68 in cells of identical morphology on serial sections using ICC.

IP-10 and Mig share a number of biological activities including the promotion of T-cell chemotaxis *in vitro* (Taub et al., 1993; Liao et al., 1995), inhibition of tumor cell growth in mouse models of disease (Arenberg et al., 1996; Sgadari et al., 1996), and inhibition of angiogenesis induced by ELR-containing chemokines (Strieter et al., 1995). Despite their similarities, there is evidence that the activities of IP-10 and Mig are not identical. In contrast to IP-10, Mig has been reported to have no effect on monocyte chemotaxis but specifically chemoattracts activated T-lymphocytes (Liao et al., 1995). In this regard Mig resembles lymphotactin, which is also a T-cell specific chemoattractant.

IP-10 and Mig show reciprocal desensitisation on human T-cells (Liao et al., 1995) suggesting they share a common receptor. This was confirmed by the cloning of the IP-10/Mig receptor CXCR3 which is highly expressed on activated, but not resting, T-lymphocytes and mediated chemotaxis in response to IP-10 or Mig, but not to other chemokines (Loetscher et al., 1996). This study reports the expression of CXCR3 by T-lymphocytes in the perivascular cuff, and suggests that the α-chemokines IP-10 and Mig selectively recruit activated lymphocytes from the circulation to sites of CNS inflammation.

IP-10 has also been shown to induce concentration-dependent migration of a C6 glial cell line and rat microglia (Wang et al., 1998; Cross & Woodroofe, 1999). The effect of IP-10 on the C6 glial cells was chemokinetic, that is to say the cells were activated by IP-10 and increased their random movement (Wang et al., 1998). The current investigation shows the expression of CXCR3 by astrocytes, which immunostain positively for IP-10 and Mig, suggesting that these chemokines are secreted by glial cells and may interact with receptors on their surface in an autocrine or paracrine manner to maintain cell function and/or further stimulate glial cell activation and movement.

In culture, astrocytes can be stimulated to express MHC, present antigens to T-cell lines, and to produce a number of inflammatory cytokines (Fontana et al., 1984; Frei et al., 1985; Freiz et al., 1985; Beneviste et al., 1990). It has been proposed that in demyelinating lesions, astrocytes may produce chemokines in response to cytokines expressed by inflammatory cells in the perivascular cuffs (Ransohoff et al., 1993), and that in response to the chemokines produced by astrocytes, mononuclear cells migrate into the parenchyma where demyelination occurs (Miyagishi et al., 1997).

While the CNS in MS, SSPE and CD, with associated neurological complications, exhibit similar pathological characteristics, the mechanisms of cell recruitment to these sites of inflammation appears to differ. Like MS and SSPE, the CNS in CD is characterised by large perivascular cuffs containing inflammatory cells recruited from the circulation. However, unlike MS and SSPE, IP-10 and Mig do not appear to contribute to leukocyte recruitment in CD CNS. These findings suggest that the activation and directional migration of inflammatory cells to sites of inflammation in the cerebellum of CD patients is not influenced by these non-ELR α-chemokines, but by another mechanism.

In contrast to CD CNS tissue, high levels of both IP-10 and Mig were detected in SSPE and actively demyelinating MS tissue. The results of this study suggest the following sequence of events: (i) Activated perivenular T_H1-lymphocytes and adjacent glial cells express IFN-γ. (ii) This pro-inflammatory cytokine induces the expression of IP-10 and Mig in surrounding glial cells. (iii) The local production of α-chemokines is involved in the selective recruitment of further activated T-cells and resident glial cells expressing the IP-10/Mig receptor, CXCR3, to sites of inflammation.

Although intracerebral injection of IFN-γ into rodents does not result in demyelination (Sethna & Lampson, 1991), the use of anti-IFN-γ antibodies in EAE enhances resistance to disease (Lublin et al., 1993), suggesting a role for IFN-γ in MS lesion formation. Based on these findings, one possible therapeutic treatment of MS and SSPE may be to neutralise the actions of IFN-γ, thereby preventing IFN-γ-induced chemokine expression by resident glial cells.

CHAPTER 4

β-CHEMOKINE EXPRESSION IN INFLAMMATORY LESIONS IN THE CNS

4.1 INTRODUCTION

β-chemokines are associated with chronic inflammation, and are primarily involved in the activation and selective chemoattraction of monocytes and T-lymphocytes (Baggiolini & Dahinden, 1994; Lloyd et al., 1996; Baggiolini, 1998). Therefore, this study investigated the protein and mRNA expression of the β-chemokines MIP-1α, MIP-1β, MCP-1 and RANTES, to determine their involvement in leukocyte recruitment to inflammatory lesions of the CNS using ICC (Section 2.2)and *ISH* (Section 2.3) techniques.

4.2 RESULTS

4.2.1 β-CHEMOKINE EXPRESSION IN INFLAMMATORY LESIONS IN THE CNS: DETECTION BY ICC

4.2.1.1 CONTROL CNS TISSUE

Immunostaining of four control CNS tissue blocks for the chemokines MIP-1α (Figure 23a) and MCP-1 (Figure 23b) displayed weak staining associated with blood vessel endothelial cells and a few microglia in the surrounding parenchyma. Weak, diffuse staining of RANTES was associated with the endothelium (Figure 23c). MIP-1β protein, however, was expressed by endothelial cells and at low levels by microglia throughout the white matter of control samples (Figure 23d).

Figure 23 β-chemokine expression in control CNS tissue

Immunostaining of control CNS tissue (sample 413-34) for (a) MIP-1α, (b) MCP-1, and (c) RANTES indicated the chemokine proteins were weakly associated with endothelial cells. (d) MIP-1β was expressed at low levels by the blood vessel endothelium and by microglia (arrow) throughout the white matter. The arrow represents one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 23 β-chemokine expression in control CNS tissue

4.2.1.2 MS CNS TISSUE

Expression of MIP-1α, MIP-1β and MCP-1 in two MS NAWM tissue blocks displayed a similar chemokine staining pattern to control CNS tissue. However, an increased RANTES expression was associated with endothelial cells (Figure 24a), suggesting this chemokine may be involved in the early stage of MS lesion formation. Two acute MS lesions, with a small number of inflammatory cells around venules, expressed enhanced staining of RANTES associated with the endothelium (Figure 24b), MCP-1 associated with some astrocytes (Figure 24c), and MIP-1β associated with macrophage-like cells surrounding the blood vessel and in the adjacent parenchyma (Figure 24d).

Four CNS blocks from actively demyelinating lesions, with high levels of inflammation, displayed a complex pattern of chemokine staining. Within the plaque, MCP-1 protein was detected in cells displaying the morphological characteristics of macrophages and astrocytes (Figure 25a). In the adjacent non-demyelinated white matter surrounding the lesion, MCP-1 protein expression was restricted to reactive astrocytes (Figure 25b). Staining on serial sections with anti-CD68 (Figure 25c) and anti-GFAP antibodies (Figure 25d) confirmed the phenotype of these cells. Anti-RANTES antibody indicated the presence of this protein in endothelial cells, lymphocytes in the perivascular cuff, and astrocytes within the lesion (Figure 25e). Staining with anti-$CD4^+$ T-cell antibody on a serial section confirmed the cellular source of RANTES (Figure 25f). No enhanced RANTES staining was detected in the adjacent apparently normal white matter. MIP-1α was detected in macrophage and astrocyte-like cells (Figure 25g), whilst MIP-1β was expressed by CD68 positive cells within the plaque, and by microglia in the neighbouring non-demyelinated white matter (Figure 25h). The MIP-1α and MIP-1β

Figure 24 β-chemokine expression in NAWM and acute MS CNS tissue

(a) Elevated levels of RANTES were associated with the endothelium in MS NAWM (sample 396-98). (b) Immunostaining of acute MS tissue (sample 413-54) indicated RANTES expression associated with the endothelium (arrow head), perivascular leukocytes (small arrow) and glial cells (large arrow), (c) MCP-1 was associated with some astrocytes (arrow), and (d) MIP-1β was associated with perivascular macrophages and microglia (arrow). The arrows represent one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 24 β-chemokine expression in NAWM and acute MS CNS tissue

Figure 25 β-chemokine expression in chronic active MS CNS tissue

(a) MCP-1 protein was associated with macrophages (small arrow) and astrocytes (large arrow) within actively demyelinating MS lesions, and (b) was restricted to reactive astrocytes (large arrow) in the adjacent non-demyelinated white matter (sample 413-41). The cellular localisation of MCP-1 was confirmed using (c) anti-CD68 and (d) anti-GFAP antibodies on serial sections. (e) RANTES protein was detected in endothelial cells (arrow head), perivascular T-lymphocytes (small arrow) and adjacent astrocytes (large arrow) within MS lesions (sample 413-41). (f) Staining with anti-CD4 T-cell antibody confirmed the cellular source of RANTES. (g) MIP-1α protein was associated with macrophages (small arrow) and astrocytes (large arrow) within the actively demyelinating MS lesion (sample 413-41). (h) MIP-1β protein was associated with macrophages (small arrow) within the chronic active MS lesions, and microglia (large arrow) in the adjacent non-demyelinated white matter. The arrows represent one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 25 β-chemokine expression in actively demyelinating MS CNS tissue

Figure 25 β-chemokine expression in actively demyelinating MS CNS tissue Contd.

antibodies were used at the same optimal Ig concentration, and a more intense stain for MIP-1β was evident. This may reflect a greater expression of MIP-1β in the CNS in MS lesions. The intensity of chemokine staining was greatest in the perivascular inflammatory site and gradually decreased from the lesion centre out into the adjacent white matter.

One chronic inactive plaque which was demyelinated and had no inflammation displayed weak immunostaining of glial cells by RANTES (Figure 26a), with MIP-1β also weakly associated with microglia throughout the tissue(Figure 26b). No immunostaining for MIP-1α (not shown) nor MCP-1 (Figure 26c) was visible.

It has been reported by Ulvestad et al. (1994) that antibody binding to the Fc receptor on microglia can result in non-specific staining. However, this only occurred in the absence of blocking serum, which was included in all the staining procedures carried out in this study. Also, isotype specific control included in this study gave negative staining, confirming the specificity of the chemokine staining pattern.

4.2.1.3 SSPE CNS SAMPLES

Unlike MS lesions, neither MIP-1α (Figure 27a), MIP-1β (Figure 27b), MCP-1 (Figure 27c) nor RANTES (Figure 27d) were expressed above control levels in two SSPE CNS tissue blocks. To confirm this result the experiment was performed in triplicate. The incubation time with the primary antibody was also increased, to see if it had an effect on β-chemokine staining. However, there was no detectable expression of the β-chemokines MIP-1α, MIP-1β, MCP-1 nor RANTES in the CNS in SSPE.

Figure 26 β-chemokine expression in chronic silent MS CNS tissue

In chronic inactive MS lesions (a) RANTES was associated with some glial cells (arrow), and (b) MIP-1β protein was associated with microglia (arrow). (c) MCP-1 protein was not present. The arrows represent one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 26 β-chemokine expression in chronic silent MS CNS tissue

Figure 27 β-chemokine expression in SSPE CNS tissue

Neither (a) MIP-1α, (b) MIP-1β, (c) MCP-1, nor (d) RANTES were detected in SSPE CNS tissue. (Magnification x220)

4.2.1.4 CD CNS TISSUE

As in the SSPE tissue samples, neither MIP-1β (not shown), MCP-1 (Figure 28a) nor RANTES (not shown) were detected above control levels in CD CNS. MIP-1α, however, was detected in perivascular macrophage-like cells and in microglia in the surrounding parenchyma (Figure 28b). Staining on serial sections with CD68 confirmed the cellular source of this chemokine (Figure 28c).

4.2.2 β-CHEMOKINE EXPRESSION IN INFLAMMATORY LESIONS IN MS: DETECTION BY *ISH*

ISH studies were carried out on control CNS tissue and on serial sections from MS blocks displaying a strong pattern of β-chemokine ICC staining, to confirm the cellular source of the chemokines. Control tissue was mostly negative for chemokine mRNA expression, except for low levels of MIP-1β mRNA associated with cells resembling microglia (not shown). Tissue from actively demyelinating MS CNS tissue displayed a distribution of chemokine mRNA closely resembling the pattern of immunostaining as described previously (Section 4.2.1.2). MIP-1β mRNA was expressed by cells within the plaque, and corresponded to the ICC staining of CD68 positive cells, with many of the positive cells located in the perivascular cuffs (Figure 29a), whilst MCP-1 mRNA was distributed predominantly in cells with large pale nuclei and astrocytic morphology, and in a few macrophage-like cells (Figure 29b). This is further evidence for higher levels of chemokine expression adjacent to the endothelium than in the surrounding white matter.

Figure 28 *β-chemokine expression in CD CNS tissue*

Neither MIP-1β, (a) MCP-1, nor RANTES were present in the cerebellum in CD. (b) MIP-1α protein was associated with perivascular macrophages (small arrow), and with macrophage-like cells (large arrow) in the surrounding white matter. (c) Immunostaining with anti-CD68 antibody on serial sections confirmed the cellular localisation of MIP-1α. The arrows represent one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 28 β-chemokine expression in CD CNS tissue

Figure 29 β-chemokine expression, detection by *ISH*

ISH studies indicated MIP-1β mRNA was predominantly expressed by (a) perivascular macrophages in chronic active MS lesions. (b) *ISH* for MCP-1 mRNA expression indicated the chemokine was predominantly expressed by astrocytes and macrophages in actively demyelinating MS lesions, confirming ICC studies (Magnification x450). (Magnification x220, unless otherwise stated)

Figure 29 β-chemokine expression, detection by *ISH*

Table 11 Distribution of β-chemokine staining in four subacute MS lesions, and in two SSPE and one CD CNS tissue sample

		Inflammatory cuff		Lesion	
Chemokine	Disease	Leukocytes	Endothelium	Macrophages/ Microglia	Astrocytes
MIP-1α	MS	+	+	+	+
	SSPE	-	-	-	-
	CD	+++	-	+++	-
MIP-1β	MS	+++	+	+++	-
	SSPE	-	-	-	-
	CD	-	-	-	-
MCP-1	MS	+++	+	+++	+++
	SSPE	-	-	-	-
	CD	-	-	-	-
RANTES	MS	+	++	-	+
	SSPE	-	-	-	-
	CD	-	-	-	-

Key: -: no staining associated with the cells

+: weak staining

++: strong staining

+++: all cells stained strongly positive

The table above summarises the β-chemokine staining pattern seen in CNS tissue from three neuroinflammatory disease states, and reflects the scores of two independent observers.

4.3 DISCUSSION

β-chemokines are associated with chronic inflammation, and are important in the pathogenesis of immune-mediated inflammatory diseases of the CNS (Taub & Oppenheim, 1994; Baggiolini, 1998). The binding of a chemokine to a receptor on a target cell surface is important for both activation of the cell and stimulation of cell migration, while binding to the extracellular matrix-associated glycosaminoglycans may provide a chemokine gradient along which a cell can migrate (Liao et al., 1995; del Pozo et al., 1997). As stated earlier MS, SSPE and CD, with associated neurological complications, share common CNS pathological features, such as inflammatory infiltrates recruited from the circulation. Therefore to determine the role of β-chemokines in these neuroinflammatory diseases, the present study examined the expression of the β-chemokines MIP-1α, MIP-1β, MCP-1 and RANTES. The results of these studies are summarised in Table 11.

In this study, endothelial and microglial cell-associated chemokine expression was detected in control and NAWM CNS tissue, suggesting a role for β-chemokines in the normal trafficking of T-lymphocytes across the BBB. Expression of the β-chemokines investigated in this study reflected the degree of inflammation present in the MS tissue, suggesting a role for the chemoattractant cytokines in the specific recruitment of circulating leukocytes into the CNS during disease progression.

Chemokines are a component of CNS inflammation during the course of EAE, a $CD4^+$ T-cell mediated animal model of MS (Hulkower et al., 1993; Ransohoff et al., 1993; Godiska et al., 1995; Glabinski et al., 1996; Glabinski et al., 1997). MCP-1 primarily chemoattracts monocytes/macrophages, but has also been shown to stimulate T-lymphocyte migration (Mantovani et al., 1993; Carr et al., 1994). MCP-1 mRNA has been detected in the spinal cord in acute phase EAE (Hulkower et al., 1993) and localised in

astrocytes both during EAE in SJL/J mice and in actively demyelinating MS lesions (Ransohoff et al., 1993; McManus et al., 1998a). Increased expression of MCP-1 mRNA and protein have been detected in mouse astrocytes following mechanical injury to the brain (Glabinski et al., 1996), as well as in macrophages and endothelial cells (Berman et al., 1996). The results of this study, which suggest MCP-1 is expressed by astrocytes and macrophages within actively demyelinating MS lesions, are in agreement with reported EAE and MS studies (Ransohoff et al., 1993; Glabinski et al., 1996; Calvo et al., 1996; Glabinski et al., 1997; McManus et al., 1998a). Primary glial cell cultures from BALB/c and SJL/J mice have shown MCP-1 to be produced by cytokine-stimulated astrocytes, with little or no MCP-1 detected in stimulated microglia (Jiang et al., 1992), which supports this study's findings that astrocytes and not microglia express MCP-1 in non-demyelinated white matter surrounding the MS lesion. In this study, the cell populations were identified as astrocytes or macrophages by their nuclear morphology, by co-localisation of the chemokine and staining for GFAP and CD68 in cells of identical morphology on serial sections using ICC techniques, and by the distribution of hybridisation products within these cells using non-radioactive *ISH* techniques.

MCP-1 has been reported to upregulate the expression of adhesion molecules on human monocytes (Shyy et al., 1993; Hayashi et al., 1995), implying a role in adhesion and diapedesis during the transendothelial migration of inflammatory cells across the BBB. The results of the present study suggest that MCP-1, produced by resident glial cells in MS CNS tissue, continues the local inflammatory response by forming chemotactic gradients within the CNS. The infiltrating mononuclear phagocytes have the ability to produce cytokines that may further modify the activation state of the astrocytes resulting in MCP-1 production which may then cause further inflammatory cell recruitment. It has

been suggested that astrocyte expression of MCP-1 may by governed by TNF-α, a product of activated macrophages (Glabinski et al., 1996).

Astrocytes occupy a unique position at the BBB and are thought to contribute to local inflammatory reactions within the CNS. As well as MCP-1, astrocytes are also capable of expressing RANTES when co-stimulated with TNF-α, IL-1β and IFN-γ *in vitro* (Barnes et al., 1996), suggesting astrocytes play a crucial role in the recruitment of leukocytes from the circulation during an inflammatory response. The present study demonstrates RANTES expression is associated with astrocytes surrounding blood vessels, as well as endothelial cells and T-lymphocytes in actively demyelinating MS lesions. Other studies have reported the expression of RANTES by antigen-activated T-lymphocytes (Schall et al., 1990; Kuna et al., 1993; Godiska et al., 1995), by astrocytes infected *in vitro* with Newcastle disease virus (Noe et al., 1996), and by HUVECs in the presence of TNF-α and IFN-γ (Marfaing-Koka et al., 1995). RANTES has been shown to preferentially chemoattract $CD4^+$ memory T-lymphocytes (Schall et al., 1993; Taub et al., 1994). The present study shows a diffuse pattern of chemokine staining associated with the endothelium, and suggests RANTES is released from its cellular source and binds to the extracellular matrix. RANTES may form a chemotactic gradient and recruit inflammatory cells from the circulation into the CNS and the MS lesion. The results of this study suggest that infiltrating lymphocytes are also stimulated to produce RANTES, although the precise mechanism of stimulation of both the endothelium and T-cells is, as yet, unclear. MMPs have been identified in astrocytes, microglia and inflammatory cells in active MS lesions (Cuzner et al., 1996). These proteins have the potential to degrade basement membrane and other matrix components, suggesting both MMPs and

chemokines are important in leukocyte migration across the BBB (Goetzl et al., 1996; Biddison et al., 1997).

RANTES and MIP-1β expression have been reported to correlate with the appearance of inflammatory cells in the CNS in rat brain stab injury (Ghirnikar et al., 1996), suggesting a role for both these chemokines in inflammatory events in traumatic brain injury. MIP-1β protein was associated with reactive astrocytes and macrophages at the site of injury (Ghirnikar et al., 1996). The present study shows MIP-1α and MIP-1β expression associated with macrophages and microglia, both within the MS lesion and in the surrounding white matter, with MIP-1α, and not MIP-1β, also weakly staining astrocytes. As was observed in chronic active MS lesions, MIP-1α expression was also associated with macrophages in CD CNS samples. These findings correlate with other studies which have reported diffuse immunostaining of MIPs around the cell bodies of monocytes and macrophages in meningoencephalitis-infected mice (Wilson et al., 1990; Seebach et al., 1995), and suggest these chemokines may play a role in the recruitment of T-lymphocytes and macrophages to the site of inflammation in MS and CD CNS tissue (Taub et al., 1993; Hayashi et al., 1995).

Both MIP-1α and MIP-1β can trigger uropod formation and the redistribution of cell adhesion molecules, such as ICAM-1, following adherence of T-lymphocytes to either endothelial cell adhesion molecules or extracellular matrix proteins (Biddison et al., 1997). Reports have indicated that the β-chemokines MIP-1α, MIP-1β and RANTES are associated with a T_H1 immune response (Schrum et al., 1996), suggesting MS, like the animal model EAE, is also predominantly a T_H1-mediated disease.

The findings of this study suggest that β-chemokines MIP-1α, MIP-1β, MCP-1 and RANTES do not contribute to SSPE pathology, and that other mechanisms, such as

the α-chemokines IP10 and Mig , are involved in the recruitment of inflammatory cells and resident glial cells to sites of CNS inflammation in this disease. In contrast, expression of MIP-1α in CD cerebellar tissue by resident and perivascular infiltrating macrophages may play a role in the recruitment of leukocytes into the CNS in this disease. Reports have indicated MIP-1α preferentially chemoattracts activated $CD8^+$ T-lymphocytes (Taub et al., 1993). Immunostaining of the CD cerebellar tissue used in this study, however, showed equal numbers of $CD4^+$ and $CD8^+$ T-cells present, suggesting other mechanisms, in conjunction with MIP-1α expression, are involved in inflammatory cell recruitment into the CNS.

In MS tissue, the endothelium and perivascular cells may be stimulated, by T_H1 cytokines, to produce RANTES which chemoattracts mononuclear cells from the circulation into the CNS. Resident glial cells may be stimulated to continue the local inflammatory response: astrocytes expressing MCP-1, RANTES and MIP-1α, and macrophages expressing MIP-1β, MIP-1α and MCP-1.

CHAPTER 5

β-CHEMOKINE RECEPTOR EXPRESSION IN INFLAMMATORY LESIONS IN THE CNS IN MS

5.1 INTRODUCTION

The results of this study have shown that chronic active MS lesions display the highest level of β-chemokine expression (Chapter 4). Table 12 summarises the ligand specificity of the β-chemokine receptors of interest in this study. Within the CNS leukocytes, which can respond to several chemokines, migrate along one chemotactic gradient, then another until they reach their target. Their ultimate position is determined by the chemokine receptors they express and the chemokine gradients they encounter (Campbell et al., 1997).

Table 12 β-chemokine receptor ligand specificity

Receptor	Ligand
CCR3	RANTES, MCP-2,-3, -4
CCR5	RANTES, MIP-1α, -1β
CCR8	TARC, MIP-1β

Specific chemokine receptors may be expressed by the different cell types being recruited. Leukocytes may also express different chemokine receptors at different times: one chemokine receptor may be involved in activation and directional migration of the leukocyte from the circulation, while another may play a role in the directional migration

of the leukocyte within the tissue to the site of inflammation. RT-PCR (Section 2.4), ICC (Section 2.2) and *ISH* (Section 2.3) experiments were performed to investigate the expression of CCR in chronic active MS lesions compared to control CNS.

5.2 RESULTS

5.2.1 DETECTION OF CCR BY RT-PCR

To determine if CCR3 and/or CCR5 were expressed in actively demyelinating MS lesions and/or control CNS tissue, RNA samples from the two tissue types were reverse transcribed and subjected to 35 cycles of PCR amplification. Two approaches were used to minimise artefacts due to potential genomic contamination. Firstly, the RT-PCR reaction was run in the absence of reverse transcriptase. Secondly, a parallel PCR was run using primers of the housekeeping gene β-2 microglobulin (B2M). No PCR products were detected when the reverse transcriptase was omitted (Figure 30, 31). The B2M PCR product was found in both control and MS CNS tissue (Figure 30, 31). A PCR band for CCR3 (Figure 30a) and CCR5 (Figure 30b) were both visible in control CNS tissue and had a size of 1050 base pairs, consistent with the predicted size of the product. A PCR band for CCR3 (Figure 31a) and CCR5 (Figure 31b) was also visible in the MS tissue samples, suggesting the chemokine receptors are expressed both in control and MS CNS tissue.

Figure 30 Detection of CCR3 and CCR5 expression in control CNS tissue samples by RT-PCR

Both (a) CCR3 and (b) CCR5 were detected in control CNS tissue, and had a size of 1050 base pairs, consistent with their predicted size. Further evidence for the specificity of the reaction product was the detection of an identical PCR product in RNA extracted from a plasmid containing the CCR insert (CCR3 PuC/ CCR5 PuC). Detection of the housekeeping gene β-2 microglobulin (B2M), and no visible band in the negative control lane (Neg) indicated the absence of genomic contamination.

Figure 31 Detection of CCR3 and CCR5 expression in actively demyelinating MS CNS tissue samples by RT-PCR

(a) CCR3 and (b) CCR5 were also detected in chronic active MS lesions.

Figure 30 Detection of CCR3 and CCR5 expression in control CNS tissue samples by RT-PCR

Figure 31 Detection of CCR3 and CCR5 expression in actively demyelinating MS CNS tissue samples by RT-PCR

5.2.2 PHENOTYPE OF CELLS EXPRESSING CCR3, CCR5 AND CCR8

Although β-chemokine receptor expression was detected in actively demyelinating MS CNS tissue by RT-PCR (Section 5.2.1), the phenotype of the cells expressing CCR could not be determined by this technique. To investigate which cells the β-chemokines were acting upon, the expression of the chemokine receptors CCR3, CCR5 and CCR8 in actively demyelinating MS sections, were investigated by ICC and confirmed by *ISH* studies.

5.2.2.1 CCR DETECTION BY ICC

The β-chemokine antibodies used in this study were a kind gift from Dr. J. White, SmithKline Beecham, USA. The most intense pattern of β-chemokine receptor expression was detected in actively demyelinating MS lesions, correlating with the highest level of β-chemokine expression. CCR3 was associated with foamy macrophages within the plaque and with some microglia and astrocytes in the adjacent normal appearing white matter (Figure 32a). Immunostaining with antibodies against CCR5 and CCR8 displayed a similar pattern of staining. Both CCR5 (Figure 32b) and CCR8 (Figure 32c) were associated with foamy macrophages within the MS lesion, and with some microglia in the adjacent apparently normal white matter. CCR5, however, was also associated with infiltrating T-lymphocytes in the inflammatory cuff.

5.2.2.2 CCR DETECTION BY *ISH*

To confirm which cells were expressing the β-chemokine receptors, *ISH* experiments were performed using probes synthesised in-house by Mr A. Fairclough. The results of these experiments confirmed that foamy macrophages within the chronic active

Figure 32 β-chemokine receptor expression in chronic active MS lesions: detection by ICC

(a) CCR3, (b) CCR5 and (c) CCR8 were predominantly expressed by foamy macrophages within chronic active MS lesions (small arrow). Expression of CCR5 was also associated with T-lymphocytes in the perivascular cuff (large arrow). The arrows represent one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 32 β-chemokine receptor expression in chronic active MS lesions: detection by ICC

Figure 33 CCR3 and CCR5 mRNA expression in actively demyelinating MS lesions

ISH experiments confirmed (a) CCR3 and (b) CCR5 were associated with foamy macrophages within the actively demyelinating MS lesion (small arrow), with CCR5 also being expressed by perivascular T-lymphocytes (large arrow). The arrows represent one example of a positively staining cell, several others are visible on the section. (Magnification x220)

Figure 33 CCR3 and CCR5 mRNA expression in actively demyelinating MS lesions

MS lesion were predominantly expressing CCR3 (Figure 33a) and CCR5, with CCR5 also been detected on perivascular T-lymphocytes (Figure 33b). The pattern of *ISH* staining clearly resembled the ICC results obtained in Section 5.2.2.1.

5.2.3 DETECTION OF CCR3, CCR5 AND CCR8 IN MS CNS BY WESTERN BLOTTING

The CCR antibodies used in this study were a gift from Dr J. White, SmithKline Beecham, and were uncharacterised. To confirm protein had been successfully extracted from the CNS tissue, an SDS gel was run (Figure 34a). To confirm the antibodies recognised their specific CCR, and did not cross-react with other antigens, Western blotting experiments were performed as described in Section 2.5.

Actively demyelinating MS lesions are characterised by extensive astrocyte hypertrophy and GFAP synthesis. To act as a positive control, Western blotting for GFAP was included. These experiments indicated the highest levels of GFAP were present in chronic inactive MS CNS tissue compared to control and other MS CNS tissue (Figure 34b). The highest levels of CCR3 (Figure 34c) and CCR5 protein (Figure 34d) were also detected in actively demyelinating MS lesions, confirming previous ICC and *ISH* studies (Sections 5.2.2.1 and 5.2.2.2). The bands detected in this study had a molecular weight of approximately 35kDa. Recent papers have reported the molecular weight of chemokine receptors as 35kDa (Bernardini et al., 1998), confirming the specificity of the antibodies used.

Figure 34 Detection of GFAP, CCR3 and CCR5 in control and MS CNS tissue by Western Blotting

(a) An SDS gel was run to confirm the extraction of protein from the MS and control CNS tissue. (b) The highest levels of GFAP were detected in chronic inactive MS lesions, associated with extensive astrocyte hypertrophy and GFAP synthesis. A 35kDa band was predominantly detected in actively demyelinating MS CNS tissue, confirming the specificity of the (c) CCR3 and (d) CCR5 antibodies used in this study, and supporting previous findings.

Figure 34 Detection of GFAP, CCR3 and CCR5 in control and MS CNS tissue by Western Blotting

Lane 1 NAWM
Lane 2 acute MS CNS
Lane 3 subacute MS CNS
Lane 4 chronic inactive MS CNS
Lane 5 control CNS

Lane 1 NAWM
Lane 2 acute MS CNS
Lane 3 subacute MS CNS
Lane 4 chronic inactive MS CNS
Lane 5 control CNS

Lane 1 control CNS
Lane 2 chronic inactive MS CNS
Lane 3 subacute MS CNS
Lane 4 acute MS CNS
Lane 5 NAWM

Lane 1 control CNS
Lane 2 NAWM
Lane 3 acute MS CNS
Lane 4 subacute MS CNS
Lane 5 chronic inactive MS CNS

Table 13 Distribution of chemokine receptor staining in four actively demyelinating MS lesions

	Inflammatory cuff		Lesion	
Chemokine receptor	Leukocytes	Endothelium	Macrophages/ Microglia	Astrocytes
CCR3	-	-	+++	+
CCR5	+++	-	+++	-
CCR8	+	-	+++	-

Key: -: no staining associated with the cells

+: weak staining

++: strong staining

+++: all cells stained strongly positive

The table above summarises the chemokine receptor staining pattern seen in actively demyelinating MS CNS tissue, and reflects the scores of two independent observers.

5.3 DISCUSSION

Leukocyte migration is essential for immune surveillance of the CNS, and for targeting immune cells to sites of antigenic challenge. Activated and memory T-cells express higher levels of some adhesion molecules which facilitate their binding to inflamed endothelium (Qin et al., 1998), but this does not fully explain the selective recruitment of inflammatory cells. The β-chemokines MIP-1α, MIP-1β, MCP-1 and RANTES induce

monocyte chemotaxis *in vitro*, but they vary both in their ability to attract and activate neutrophils, lymphocytes, basophils and eosinophils, and in their monocyte chemotactic potency (Combadiere et al., 1996). The selective action of chemokines may be explained by the different chemokine receptor profile expression of the specific target cells.

Given that chemokine receptors play a role in leukocyte migration, attention has recently focused on identifying relevant chemokine receptors on T-lymphocytes. Expression of CCR3, the receptor for RANTES and MCPs, has been detected on polarised T_H2 cells, both *in vivo* and *in vitro* (Sallusto et al., 1998), suggesting a mechanism of leukocyte recruitment during allergic inflammation. CCR5, the receptor for RANTES, MIP-1α and MIP-1β, has been reported to be associated with activated and memory T_H1 cells (Bleul et al., 1997), which correlates to findings that memory and activated T-cells, but not naive T-lymphocytes, respond to RANTES, MIP-1α and MIP-1β in chemotaxis assays (Schall et al., 1990). CCR5 and CXCR4 are generally reciprocally expressed on T-cells (Bleul et al., 1997), suggesting their expression facilitates T-cell positioning in inflamed tissues. CCR8, the receptor for MIP-1β, is also expressed by activated T-lymphocytes (Bernardini et al., 1998). This study reports expression of CCR5 by infiltrating T-lymphocytes, and suggests MS, like its animal model EAE, is predominantly a T_H1-mediated immune response.

Chemokines have been demonstrated to influence the differentiation of naive T-lymphocytes into either T_H1 or T_H2 cells: MIP-1α induces differentiation into T_H1 cells, while MCP-1 induces differentiation into T_H2 cells (Sallusto et al., 1998). IL-2 has been shown to play a role in upregulating CCR1, CCR2 and CCR5 expression by T-lymphocytes, whilst stimulation with other molecules, such as anti-CD3 and/or anti-CD28 antibodies have been shown to downregulate receptor expression and chemotaxis (Qin et

al., 1998). These findings suggest that T-cells may migrate in response to chemokines following IL-2-mediated proliferation (Qin et al., 1998).

It is increasingly apparent that chemokine receptor expression is not limited to cells of the immune system but includes a wide variety of cells including neurons and resident glia (Horuk et al., 1997; Rottman et al., 1997). In addition to chemokine receptor expression in perivascular leukocytes, this study reports constitutive expression of CCR3, CCR5 and CCR8 by glial cells, morphologically compatible with astrocytes and microglia, in both control and MS CNS tissue. The observation that chemokine receptors are expressed by glial cells in control CNS contributes to our understanding of chemokine receptor distribution in normal CNS tissue, although more research is required to understand the role played by chemokines and their receptors in the normal CNS.

Expression of C5a, IL-8 and FMLP receptors by astrocytes and microglia have been reported in MS lesions (Muller-Ladner et al., 1996). The detection of chemokine receptors on glial cells is not unique to MS CNS tissue. Macaque monkeys infected with SIV have been shown to express CCR3 and CCR5 on glial cells (Westmoreland et al., 1998). Also, human and rat microglial cell cultures have been shown to migrate along chemokine gradients in chemotaxis assays, suggesting they express chemokine receptors (Cross et al., 1998). Studies have shown that human microglial and astroglial cell cultures express CCR3 and CCR5 (He et al., 1997).

The results of this study suggest leukocytes expressing CCR5, and to a lesser extent CCR8, are chemotactically recruited into the CNS in MS. The findings that astrocytes and microglia also express these receptors, as well as CCR3, suggests that chemokines secreted by glial cells may interact with their corresponding receptors to maintain normal cell function and/or mediate communication between glia (Westmoreland et al., 1998).

CHAPTER 6

INDUCTION OF CHEMOKINE AND CHEMOKINE RECEPTOR EXPRESSION BY RAT PRIMARY GLIAL CELLS AND PERIPHERAL BLOOD MONONUCLEAR CELLS

6.1 INTRODUCTION

Chemokines and their receptors are important for the selective attraction of specific subsets of leukocytes. As shown earlier, chemokines are predominantly expressed by resident glial cells, as well as infiltrating leukocytes, in inflammatory lesions in the CNS (Chapter 3,4). Chemokine receptors have also been shown to be expressed by resident glial cells (Chapter 5). To confirm the type of cells expressing chemokine receptors, and to determine which factors stimulate and/or regulate chemokine and chemokine receptor expression, the induction of these peptides was investigated in rat glial cell cultures *in vitro* using ELISA (Section 2.6), ICC (Section 2.2) and *ISH* (Section 2.3) techniques.

6.2 RESULTS

6.2.1 INDUCTION OF MIP-1β EXPRESSION BY RAT MICROGLIA

As shown in Chapter 4, microglia predominantly expressed MIP-1β in chronic active MS lesions. Expression of MIP-1β by primary rat microglial cell cultures, stimulated with 20μg/ml PMA, was investigated using *ISH*. Immunostaining with an anti-CD68 antibody confirmed the cells were of the macrophage phenotype (Figure 35a). *ISH*

Figure 35 MIP-1β expression by rat microglia, detection by *ISH*

(a) Immunostaining with anti-CD68 antibody confirmed the phenotype of the cells as microglia. (b) *ISH* with oligo-poly-dT confirmed the presence of intact mRNA in all cells. (c) Unstimulated microglia expressed low levels of MIP-1β mRNA, compared to (d) PMA-stimulated glial cells. (Magnification x220)

Figure 35 MIP-1β expression by rat microglia, detection by *ISH*

using an oligo-poly-dT probe confirmed the presence of intact mRNA in the microglial cell cultures (Figure 35b). Unstimulated cells expressed low levels of MIP-1β mRNA above negative control levels (Figure 35c). PMA-stimulated microglial cells, however, displayed a marked increase in MIP-1β mRNA expression (Figure 35d).

6.2.2 INDUCTION OF MCP-1, MIP-1α AND RANTES EXPRESSION BY RAT ASTROCYTES

As shown in Chapter 4 MCP-1, MIP-1α and RANTES were expressed by astrocytes in actively demyelinating MS lesions. Primary rat neonatal astrocytes were stimulated with 20μg/ml PMA, and the expression of these β-chemokines investigated by ICC. MCP-1 (Figure 36a), MIP-1α (Figure 36c) and RANTES (Figure 36e) expression by rat astrocytes were all dramatically induced by PMA stimulation.

6.2.3 INDUCTION OF IP-10 AND MIG EXPRESSION BY RAT ASTROCYTES

As shown in Chapter 3, IP-10 and Mig were expressed by astrocytes in chronic active MS lesions. These chemokines are known to be dramatically induced by IFN-γ. Chamber slides of primary neonatal rat astrocytes were stimulated with IFN-γ, and the expression of IP-10 and Mig investigated using ICC. Stimulated rat astrocytes were found to express higher levels of IP-10 (Figure 37a) and Mig (Figure 37c) protein compared to unstimulated glial cells (Figure 37b,d).

Figure 36 MCP-1, MIP-1α and RANTES expression in PMA-stimulated rat astrocytes, detection by ICC

PMA stimulation (20μg/ml) of rat astrocytes induced a higher expression of (a) MCP-1, (c) MIP-1α and (e) RANTES protein compared to unstimulated astrocyte production of (b) MCP-1, (d) MIP-1α and (f) RANTES. (Magnification x220)

Figure 36 MCP-1, MIP-1α and RANTES expression in PMA-stimulated rat astrocytes, detection by ICC

Figure 36 MCP-1, MIP-1α and RANTES expression in PMA-stimulated rat astrocytes, detection by ICC Contd.

Figure 37 Induction of IP-10 and Mig production in IFN-γ-stimulated rat astrocytes

The pro-inflammatory cytokine IFN-γ (10IU/ml) induced the expression of (a) IP-10 and (c) Mig in rat astrocytes, when compared to unstimulated expression of (b) IP-10 and (d) Mig. (Magnification x220)

Figure 37 Induction of IP-10 and Mig production in IFN-γ-stimulated rat astrocytes

6.2.4 CHEMOKINE RECEPTOR EXPRESSION BY GLIAL CELL CULTURES: DETECTION BY ICC

In chronic active MS lesions, CXCR3 (Chapter 3) and CCR3 (Chapter 5) were detected on reactive astrocytes. Unstimulated rat astrocytes expressed low levels of (Figure 38a) CXCR3 and (Figure 38b) CCR5. Expression of both CXCR3 (Figure 38c) and CCR3 (Figure 38d) was shown to be increased following 24h stimulation with IFN-γ. Stimulation with LPS, however, resulted in a marked decrease in expression of CCR3 (Figure 38e), but increased CXCR3 expression (Figure 38f).

6.2.5 CHEMOKINE AND CHEMOKINE RECEPTOR EXPRESSION BY RAT ASTROCYTES: DETECTION BY ELISA

Rat astrocytes were cultured in 96 well plates for 24h in the presence of IFN-γ (1-100 IU) or LPS (0.1-1μg/ml). Following these treatments the cells were examined for chemokine or chemokine receptor expression by ELISA, and the results statistically analysed by Anova one-way analysis. A significant decrease in IP-10 expression was detected following stimulation with 10 IU IFN-γ ($P \leq 0.05$) (Figure 39a). IFN-γ, however, had no statistically significant effect on Mig expression by rat astrocytes (Figure 39b). These results, in contrast to general opinion, suggest that at high concentrations the pro-inflammatory cytokine IFN-γ decreases IP-10 production by astrocytes, and has no effect on Mig expression.

In contrast to previous findings (Section 6.2.4), the expression of CXCR3 by rat astrocytes was not significantly affected by changes in IFN-γ concentration. (Figure 39c). Both CCR3 (Figure 39d) and CCR5 (Figure 39e) expression decreased as the concentration of IFN-γ increased ($P \leq 0.01$ at 100IU IFN-γ), suggesting the pro-

Figure 38 CXCR3 and CCR3 expression by rat astrocytes

Both (a) CXCR3 and (b) CCR5 were expressed at low levels by unstimulated rat astrocytes. An increased expression of (c) CXCR3 and (d) CCR3 receptor expression was induced following 24 hour IFN-γ-stimulation (10IU/ml). Stimulation with LPS (100μg/ml), however, resulted in a (e) decrease in CXCR3 expression, and (f) an increase in CCR3 expression, compared to unstimulated astrocytes. (Magnification x190)

Figure 38 CXCR3 and CCR3 expression by rat astrocytes

Figure 38 CXCR3 and CCR3 expression by rat astrocytes Contd.

Figure 39 ELISA investigation of α-chemokine expression and chemokine receptor expression in IFN-γ-stimulated (0-100 IU) rat astrocytes

The effect of IFN-γ on chemokine and chemokine receptor expression by rat astrocytes is shown below (mean ± SE) (n=6). Significant changes from pre-treatment levels are indicated [$P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***)]. (a) Stimulation of rat astrocytes with IFN-γ resulted in a significant decrease ($P \leq 0.05$) in IP-10 expression. IFN-γ had no significant effect on (b) Mig nor (c) CXCR3 expression. This pro-inflammatory cytokine, however, down-regulated both (d) CCR3 ($P \leq 0.01$) and (e) CCR5 ($P \leq 0.01$) expression by rat astrocytes. In contrast, IFN-γ stimulated a significant upregulation in CCR8 expression ($P \leq 0.01$).

Figure 39 ELISA investigation of α-chemokine expression and chemokine receptor expression in IFN-γ-stimulated (0-100 IU) rat astrocytes

a IP-10 expression by IFN-γ-stimulated rat astrocytes

b Mig expression by IFN-γ-stimulated rat astrocytes

c CXCR3 expression by IFN-γ-stimulated rat astrocytes

Figure 39 ELISA investigation of α-chemokine expression and chemokine receptor expression in IFN-γ-stimulated (0-100 IU) rat astrocytes Contd.

d CCR3 expression by IFN-γ-stimulated rat astrocytes

e CCR5 expression by IFN-γ-stimulated rat astrocytes

f CCR8 expression by IFN-γ-stimulated rat astrocytes

inflammatory cytokine plays a role in arresting the migration of astrocytes once they have reached their target, by downregulating receptor expression. The astrocytes, however, showed a significant increase in CCR8 expression ($P \leq 0.01$, following stimulation with 100IU IFN-γ) (Figure 39f), suggesting a potential mechanism of astrocyte recruitment to sites of inflammation.

Overall, expression of the β-chemokines MIP-1α (Figure 40a), MCP-1 (Figure 40b) and RANTES (Figure 40c) by rat astrocytes was not significantly affected by increasing concentrations of LPS. In general, CXCR3 (Figure 40d), CCR3 (Figure 40e) and CCR5 (Figure 40f) expression decreased as the levels of LPS increased. However, at low levels of LPS stimulation (0.01μg/ml) receptor expression was significantly elevated. These findings indicate that chemokine receptor expression by astrocytes can be regulated and may play a role in the migration of resident glial cells within the CNS during an inflammatory insult.

6.2.6 CYTOSPINS

The CNS in MS is characterised by large perivascular cuffs containing leukocytes recruited from the circulation. For chemokines to recruit specific cells to sites of inflammation, requires the expression of complementary chemokine receptors by these cells. *In vivo* investigations have shown CXCR3, CCR5 and CCR8 are predominantly expressed by infiltrating leukocytes in actively demyelinating MS lesions (Chapters 3,5). These haematogenous cells have also been shown to express the proinflammatory cytokine IFN-γ (Chapter 3). To confirm these findings, and to investigate which factors may play a

Figure 40 <u>β-chemokine and chemokine receptor expression in LPS-stimulated rat astrocytes</u>

The effect of LPS stimulation on (a) MIP-1α, (b) MCP-1, (c) RANTES, (d) CXCR3, (e) CCR3 and (f) CCR5 is shown below (mean ± SE) (n=6). Significant changes from pre-treatment levels are indicated [$P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***)]. LPS had no significant effect on chemokine expression by rat astrocytes. However, at low levels of LPS stimulation (0.01μg/ml), expression of CXCR3, CCR3 and CCR5 by rat astrocytes was significantly elevated ($P \leq 0.05$).

Figure 40 β-chemokine and chemokine receptor expression in LPS-stimulated rat astrocytes

a MIP-1α expression by LPS-stimulated rat astrocytes

b MCP-1 expression by LPS-stimulated rat astrocytes

c RANTES expression by LPS-stimulated rat astrocytes

Figure 40 β-chemokine and chemokine receptor expression in LPS-stimulated rat astrocytes Contd.

d CXCR3 expression by LPS-stimulated rat astrocytes

e CCR3 expression by LPS-stimulated rat astrocytes

f CCR5 expression by LPS-stimulated rat astrocytes

role in controlling chemokine receptor by circulating leukocytes *in vivo*, receptor expression by PBMC following cytokine stimulation was examined *in vitro*.

To confirm PBMC had been isolated from human peripheral venous blood cytospin slides were immunostained with antibodies against $CD4^+$ (Figure 41a), $CD8^+$ (Figure 41b) and CD68 (Figure 41c) (Section 2.7). The positive staining observed indicated the isolation of circulating T-lymphocytes and macrophages.

6.2.6.1 IFN-γ EXPRESSION BY PBMC

ISH experiments demonstrated IFN-γ is predominantly expressed by T-lymphocytes in the perivascular cuffs in actively demyelinating MS lesions (Chapter 3). Stimulation of PBMC with 20μg/ml PMA for 4h at 37°C showed a marked increase in IFN-γ expression (Figure 42), suggesting that infiltrating leukocytes contribute to the local inflammatory response by secreting this pro-inflammatory cytokine.

6.2.6.2 CHEMOKINE RECEPTOR EXPRESSION BY PBMC

In chronic active MS lesions, CXCR3, CCR5 and to a lesser extent CCR8 were detected on perivascular leukocytes as well as on resident glial cells (Chapters 3,5). The purpose of this study was to investigate which factors may be involved in the regulation of CCR expression. PBMC were isolated and stimulated with 100IU IFN-γ for 24h at 37°C. The majority of unstimulated PBMC did not express CXCR3 (Figure 43a), but did express CCR3 (Figure 43c) and CCR5 (Figure 43e) at low levels, and CCR8 (Figure 43g) at higher levels. Expression of CXCR3 by IFN-γ-stimulated PBMC was increased (Figure 43b), as was CCR3 (Figure 43d) and CCR5 (Figure 43f). CCR8 expression, however, decreased in the presence of IFN-γ (Figure 43h).

Figure 41 Phenotypic characterisation of PBMC

To confirm the phenotype of the cells present in the PBMC sample, the cells were stained with (a) anti-$CD4^+$, (b) anti-$CD8^+$ and (c) anti-CD68 antibodies (Magnification x450). (Magnification x220, unless otherwise stated)

Figure 41 Phenotypic characterisation of PBMC

Figure 42 Induction of IFN-γ expression by PBMC following PMA-stimulation (20μg/ml) (Magnification x190)

Unstimulated PBMC

PMA-stimulated PBMC

Figure 43 Chemokine receptor expression in IFN-γ-stimulated (100IU) PBMC

(a) Few unstimulated PBMC expressed CXCR3. (b) Receptor expression was upregulated following stimulation with 100 IU IFN-γ. Unstimulated PBMC expressed low levels of (c) CCR3 and (e) CCR5. Again, (d) CCR3 and (f) CCR5 receptor expression was upregulated following stimulation with IFN-γ. (g) CCR8, however was expressed at high levels by resting PBMC, and (h) expression was down-regulated following IFN-γ-stimulation.

Figure 43 Chemokine receptor expression in IFN-γ-stimulated PBMC

Figure 43 Chemokine receptor expression in IFN-γ-stimulated PBMC Contd.

6.3 DISCUSSION

The control of leukocyte and resident glial cell migration to sites of antigenic challenge in the CNS in MS is dependent on the combined actions of chemokines and their receptors. Chemokines and chemokine receptors have been shown to be expressed by resident glial cells in actively demyelinating MS lesions (Chapter 3,4,5), astrocytes predominantly expressing CXCR3, CCR3, CCR5, CCR8, MCP-1, IP-10, Mig and to a lesser extent RANTES and MIP-1α, and microglia predominantly expressing CCR3, CCR5 and MIP-1β. In this study, *in vitro* studies were performed to confirm these *in vivo* findings.

It has been suggested that chemotactic factors secreted by reactive astrocytes, such as IP-10 and MCP-1, may be responsible for the infiltration of haematogenous cells into the CNS (Ransohoff et al., 1993; Eng et al., 1996; Ghirnikar et al., 1996; Ransohoff et al., 1996; McManus et al., 1998a). Both *in vivo* and *in vitro* experiments have shown reactive astrocytes are a cellular source for MCP-1 (Ransohoff et al., 1993; McManus et al., 1998a), RANTES (Noe et al., 1996), IP-10 (Glabinski et al., 1997), and MIP-1α (Murphy et al., 1995).

The present study confirmed, by ICC but not by ELISA, that astrocytes can be induced *in vitro* to express MCP-1, RANTES, MIP-1α, IP-10 and Mig. With the exception of IP-10, which demonstrated a decrease in expression following IFN-γ stimulation, no significant changes in chemokine expression by astrocytes were detected by ELISA. The opposing ICC and ELISA results may be explained by a loss of cells during the ELISA procedure. Further experiments are required to confirm cell number before and after the technique. The ELISA method used in this study did not block endogenous alkaline phosphatase activity. However, a negative control omitting the primary antibody

was included in all ELISA experiments. This reading, which reflected endogenous alkaline phosphatase activity, was subtracted from all other plate readings.

The ICC findings suggest pro-inflammatory cytokine-stimulation of astrocytes *in vivo* induces chemokine expression, and these chemokines continue the inflammatory response by activating both leukocytes and resident glial cells, and by playing a role in the selective recruitment of these cells to sites of inflammation.

Reactive astrocytosis and microglial hyperplasia are prominent features of inflammatory lesions in the CNS in MS. Macrophages have been reported to be a cellular source of MIP-1β (Wilson et al., 1990; Seebach et al., 1995; Ghirnikar et al., 1996; Miyagishi et al., 1997). This study shows an increase in MIP-1β mRNA expression by PMA-stimulated rat microglial cells, providing further evidence of the role of glial cells in the accumulation of inflammatory cells into the CNS.

Previous studies have identified astrocytes expressing CXCR3, CCR3 and CCR5 (Chapter 3, 5; He et al., 1997; Westmoreland et al., 1998). This study reports an increase in CCR8 expression by astrocytes following IFN-γ stimulation, suggesting this pro-inflammatory cytokine may increase the migratory response of astrocytes to MIP-1β chemotactic gradients. Stimulation of astrocytes with IFN-γ or LPS, however, resulted in a decrease in CCR3 and CCR5 receptor expression, suggesting receptor expression may be down-regulated to retain migrating astrocytes at sites of inflammation, and possibly to limit excessive recruitment (Sica et al., 1997).

PMA-stimulated PBMC displayed an increase in IFN-γ expression. ICC investigations indicated that IFN-γ increased expression of IP-10 and Mig by rat astrocytes, confirming *in vivo* findings (Chapter 3; Glabinski et al., 1997). Combining these results, one can speculate that haematogeous leukocytes infiltrate the CNS and,

following antigen recognition, secrete the pro-inflammatory cytokine IFN-γ, which in turn induces chemokine production by astrocytes. The expression of IP-10 and Mig results in the additional recruitment of cells expressing the receptor CXCR3 to sites of CNS inflammation. Chemotaxis assays have demonstrated that both microglial and astroglial cell cultures migrate along an IP-10 chemotactic gradient (He et al., 1997; Cross et al., 1999), indirectly confirming the expression of CXCR3 by CNS resident glial cells reported here.

Following activation, T-lymphocytes acquire effector functions, such as cytokine production, and different migratory capacities (Mackay, 1993; Romagnani, 1994). The results of this study indicate that CXCR3, CCR3, CCR5 and CCR8 are expressed by PBMC, and that expression is increased, with the exception of CCR8 whose expression is decreased, following stimulation with IFN-γ. These findings correlate with previous reports that CXCR3 is present on the majority of peripheral blood T-cells, in particular on T_H1 and T_H0 cells (Sallusto et al., 1998). The responsiveness of these cells to IP-10 and Mig may favour the migration of T_H1 and T_H0 cells to sites of inflammatory reactions dominated by IFN-γ production (Kaplan et al., 1987). In contrast to CXCR3, reports indicate that CCR3 is selectively expressed on T_H2 cells, eosinophils and basophils (Sallusto et al., 1997; Uguccioni et al., 1997). This sharing of chemokine receptors between T_H2 and effector cells is likely to represent a mechanism for the generation of allergic reactions (Sallusto et al., 1998).

CCR5 is expressed in peripheral blood macrophages and T-cells (Raport et al., 1996), while CCR8 is expressed my human monocytes (Tiffany et al., 1997). Reports have shown that CCR5 is preferentially expressed on T_H1 compared to T_H2 cells, and expression is increased upon activation of T-lymphocytes (Sallusto et al., 1998). In

support of this study, expression of CCR3 and CCR5 in monocytoid U937 cells has been shown to be increased following IFN-γ stimulation (Zella et al., 1998). The results of this study show that chemokine receptor expression by PBMC is dependent on the activational state of the cells.

The differential expression of chemokine receptors by astrocytes and PBMC may play a role in the selective migration of leukocytes and resident glial cells to sites of inflammation in the CNS. Chemotactic factors secreted by astrocytes and microglia are responsible for the accumulation of inflammatory cells into the CNS. Inhibiting receptor function may be a possible therapeutic target for MS, and possibly other inflammatory conditions in the CNS, such as CD or SSPE.

CHAPTER 7

DISCUSSION

Chemokines have been shown to stimulate leukocyte-endothelial attachment by up-regulating integrin expression on leukocytes, both *in vivo* and *in vitro* (Ransohoff & Tani, 1998). This family of chemoattractant cytokines has also been shown to play a prominent role in the activation and directional migration of cells, both resident glia and leukocytes, to sites of inflammation in the CNS (Karpus et al., 1995; Baggiolini, 1998). The ultimate position of these cells within inflammatory lesions is determined by the chemokine gradients they encounter, and the chemokine receptors they express (Campbell et al., 1997).

Several groups have shown that chemokines are an important component of CNS inflammation during the course of EAE (Hulkower et al., 1993; Ransohoff et al., 1993; Godiska et al., 1995; Glabinski et al., 1996; Miyagishi et al., 1997), MS (McManus et al., 1998a; Simpson et al., 1998), and following ischemic injury (Wang et al., 1998). The purpose of this study was to investigate and compare the role of chemokines in three neuroinflammatory diseases: MS, SSPE and CD with associated neurological complications, and, if chemokines were detected, to determine which factors may be responsible for the induction of chemokine and chemokine receptor expression.

Pro-inflammatory cytokines have been shown to stimulate adhesion molecule expression by the BBB endothelium (Paul & Seder, 1994), to activate resident glial cells (Brosnan et al., 1996), and to induce chemokine expression (Schall & Bacon, 1994; Barnes et al., 1996; McManus et al., 1998b), suggesting a role in the activation and

recruitment of cells to sites of inflammation. In particular, IFN-γ has been shown to play an important role in the activation of macrophages (Cavaillon, 1994) and the induction of cytokine expression, such as TNF-α, by glial cells (Barnes et al., 1996). IFN-γ has been detected in perivascular T-cells (Woodroofe & Cuzner, 1993) and, to a lesser extent, in resident glial cells (Traugott et al., 1988) in chronic active MS lesions. This pro-inflammatory cytokine has been also been detected in endothelial cells and macrophages in SSPE CNS lesions (Nagano et al., 1994). The present study confirmed these findings, demonstrating IFN-γ mRNA was predominantly expressed by perivascular T-lymphocytes, with some mRNA expression associated with resident glial cells, both in SSPE and chronic active MS lesions. These results suggest that, following activation, perivascular T-cells are induced to secrete IFN-γ, and that this pro-inflammatory cytokine contributes to the local inflammatory response.

The α-chemokines IP-10 and Mig are both dramatically induced by IFN-γ (Luster et al., 1985; Farber, 1990; Farber, 1993). The present study demonstrated that in chronic active MS lesions and SSPE CNS tissue, expression of both IP-10 and Mig proteins were associated with reactive astrocytes and macrophages. These cell types also immunostained positively for IFN-γ protein. *In vitro* ICC studies indicated IFN-γ-stimulated rat astrocytes displayed an increased expression of IP-10 and Mig. Cumulatively, these findings suggest the pro-inflammatory cytokine IFN-γ is secreted by perivenular T-cells and stimulates surrounding astrocytes and macrophages to secrete the α-chemokines IP-10 and Mig in MS and SSPE CNS lesions. α-chemokine expression was not detected in CD cerebellar tissue, suggesting neither IP-10 nor Mig play a role in CD pathogenesis.

In vitro studies have shown IFN-γ, in conjunction with other cytokines, is also capable of inducing β-chemokine expression, such as MCP-1, in glial cells (Barnes et al.,

1996). Elevated levels of β-chemokines have been detected in inflammatory CNS lesions: MCP-1 mRNA was detected in the spinal cord during acute EAE (Hulkower et al., 1993), localised in astrocytes during EAE in mice (Ransohoff et al., 1993), and predominantly detected in astrocytes in subacute MS lesions (McManus et al., 1998a; Simpson et al., 1998). RANTES expression has been detected in antigen-activated T-lymphocytes (Schall et al., 1990; Kuna et al., 1993; Godiska et al., 1995), in astrocytes infected with Newcastle disease virus (Noe et al., 1996), and in post-mortem MS CNS tissue by PCR detection (Hvas & Bernard, 1998).

The expression of RANTES and MIP-1β was reported to correlate with the appearance of inflammatory cells in the CNS in rat brain stab injury, with MIP-1β associated with reactive astrocytes and macrophages at the site of injury (Ghirnikar et al., 1996). A recent study has reported a dose- and time-dependent induction of MIP-1α and MIP-1β expression by human foetal microglia following IL-1β, TNF-α or LPS stimulation (McManus et al., 1998b). Other studies have also reported diffuse immunostaining of MIPs associated with monocyte and macrophage cell bodies in mice with experimental listeria meningoencephalitis (Wilson et al., 1990; Seebach et al., 1995).

The present study confirmed the cellular source of β-chemokine expression in actively demyelinating MS lesions, and showed that chemokines were expressed by infiltrating leukocytes and resident glial cells in a highly regulated and specific pattern (Figure 44): MCP-1 and MIP-1α were predominantly expressed by astrocytes, MIP-1β by macrophages, and RANTES by blood vessel endothelial cells, perivascular leukocytes and astrocytes within the plaque. In the non-demyelinated white matter adjacent to the lesion, astrocytes stained positive for MCP-1 and microglia for MIP-1β.

Figure 44 Chemokine and chemokine receptor expression in actively demyelinating MS lesions

Reactive astrocytosis and microglial hypertrophy are prominent features of CNS inflammation (Woodroofe et al., 1986; Hayes et al., 1987; Brosnan & Raine, 1996). Both glial cell types are capable of chemokine production (Ransohoff et al., 1993; Ghirnikar et al., 1996; Noe et al., 1996; McManus et al., 1998a; Simpson et al., 1998), suggesting they may be stimulated to secrete chemoattractant cytokines and contribute to the inflammatory response. *In vitro* studies have shown that astrocytes are a cellular source of MCP-1 (Eng et al., 1996), RANTES (Noe et al., 1996), and MIP-1α (Murphy et al., 1995). The present study confirmed these observations. Rat astrocyte cultures were induced to express these chemokines following PMA-stimulation. Similarly, this study demonstrated an increase in MIP-1β mRNA expression by PMA-stimulated rat microglia, confirming reports that macrophages are a potential source of MIP-1β (Wilson et al., 1990; Seebach et al., 1995; Ghirnikar et al., 1996; Miyagishi et al., 1997).

These findings suggest perivascular cells are stimulated to secrete RANTES and IFN-γ, and these signals play a role both in the recruitment of circulating leukocytes into the CNS in MS, and in the activation of glial cells. Resident glial cells may then continue the local inflammatory response, secreting both α- and β-chemokines. In addition to a chemoattractant role, it has been proposed that the interaction of chemokines and their receptors may also play a role in maintaining normal cell function and in enabling communication between glial cells (Westmoreland et al., 1998).

Elevated levels of MIP-1α have been detected in the CSF of patients with MS (Bennetts et al., 1997), while administration of anti-MIP-1α antibodies have been shown to prevent EAE (Karpus et al., 1995). These reports suggest a prominent role for MIP-1α in MS disease progression. Similarly, MIP-1α appears to play a role in leukocyte recruitment in CD. In CD cerebellar tissue MIP-1α protein was localised in macrophages

both in the perivascular inflammatory cuffs and in the adjacent white matter. Of the chemokines investigated in this study, only MIP-1α was detected in CD CNS. *In vitro* studies have shown MIP-1α preferentially chemoattracts $CD8^+$ T-lymphocytes (Taub et al., 1993). In this study, however, equal numbers of $CD4^+$ and $CD8^+$ T-lymphocytes were detected in CD inflammatory cuffs. These findings suggest MIP-1α may play a role in the recruitment of inflammatory cells into the cerebellum of CD patients with associated neurological complications, and that another as yet unknown mechanism may also contribute to disease pathology.

In contrast to MS CNS tissue, SSPE samples displayed little, if any, pattern of β-chemokine staining, suggesting that, of the chemokines investigated in this study, only the α-chemokines IP-10 and Mig play a role in the CNS inflammatory response in SSPE. IP-10 and Mig specifically chemoattract activated T-lymphocytes, suggesting infiltrating T-cells play a prominent role in the pathogenesis of SSPE.

The recruitment of leukocytes from the circulation into the CNS is dependant on the expression of adhesion molecules on the BBB endothelium, and CNS chemotactic signals (Butcher & Picker, 1996). Following activation, T-lymphocytes acquire different migratory capacities (Mackay et al., 1993; Romagnani, 1994). The results of this study demonstrated the chemokine receptors CXCR3, CCR3, CCR5 and CCR8 were expressed by PBMC and, following stimulation with IFN-γ, receptor expression was increased, with the exception of CCR8 which had decreased expression. These findings suggest that differential chemokine receptor expression may contribute to the selective recruitment of leukocytes into the CNS.

Reports have indicated CXCR3 and CCR5 are preferentially expressed on T_H1 lymphocytes, while CCR3 is selectively expressed on T_H2 cells (Raport et al., 1996;

Sallusto et al., 1997; Uguccioni et al., 1997; Sallusto et al., 1998). CCR8 has also been shown to be expressed by activated T-cells (Bernardini et al., 1998). Current evidence suggests MS is primarily mediated by T_H1 cells, which secrete pro-inflammatory cytokines (Hohlfeld et al., 1995). In the present study, both CXCR3 and CCR5 were predominantly expressed by perivascular leukocytes in MS lesions, confirming MS is a predominantly T_H1-mediated inflammatory disease (Figure 44). Recently, a common deletion mutation in the gene for the MIP-1α receptor CCR5 has been described (Bennetts et al., 1997). Homozygotes for the mutation fail to express this receptor. A group of unrelated relapsing-remitting MS patients were screened for this mutation, but the CCR5 deletion mutation was not found to be protective against MS (Bennetts et al., 1997), suggesting CCR5 is not an essential component in MS.

The expression of chemokine receptors is not limited to haematogenous leukocytes, but has been shown to include a number of CNS cells (Muller-Ladner et al., 1996; Horuk et al., 1997; Rottman et al., 1997; He et al., 1997; Westmoreland et al., 1997). In MS, expression of C5a, IL-8 and FMLP receptors is associated with astrocytes and microglia (Muller-Ladner et al., 1996). CCR3 and CCR5 have been detected on glial cells in macaque monkeys infected with SIV (Westmoreland et l., 1998), while microglial and astrocyte cell cultures have also been shown to express CCR3 and CCR5 (He et al., 1997). The present study confirms the expression of CXCR3, CCR3, CCR5 and CCR8 by resident glial cells in MS CNS tissue, and suggests a mechanism for the recruitment of glial cells to sites of inflammation (Figure 44).

CXCR3 was detected on perivascular leukocytes and surrounding astrocytes in both MS and SSPE CNS tissue. Current evidence suggests CXCR3 is selectively expressed by activated T-lymphocytes (Loetscher et al., 1996). To date, there are no

reports of IP-10/Mig receptor expression by resident glial cells. *In vitro* migration assays, however, have clearly demonstrated a concentration-dependent stimulation of microglia and C6 glial cell migration to IP-10 (Wang et al., 1998; Cross & Woodroofe, 1999), and indirectly confirm the expression of CXCR3 by glial cells.

In summary, upregulation of adhesion molecule expression by the BBB, chemotactic signals by resident CNS glial cells, and chemokine receptor expression by circulating leukocytes are important in the selective recruitment of haematogeous inflammatory cells to sites of inflammation in the CNS. The selective expression of CCR5 and CCR3 by T_H1 and T_H2 cells respectively provides a mechanism for the migration of specific T-cell subsets during an inflammatory response. The different chemokine profiles detected in MS, SSPE and CD CNS tissue samples contribute to our understanding of the mechanism of cell recruitment, both haematogenous inflammatory cells and resident glial cells, to sites of CNS inflammation.

Cytokines are considered to play an important role in immunological diseases, such as MS. Several therapeutic strategies involving disruption of the cytokine network have evolved, not all with beneficial results (Billiau, 1995). In EAE, administration of IFN-γ was found to have a beneficial effect, while in a clinical trial involving a small number of MS patients, systemic treatment with IFN-γ was associated with relapses at a higher frequency than before treatment (Panitch et al., 1987; Billiau, 1995). A current, and more successful, treatment for MS is the administration of IFN-β (Paty & Li, 1993). IFN-β opposes the action of IFN-γ, and has been shown to be effective in the treatment of relapsing-remitting MS (Paty & Li, 1993).

Immunity to EAE has been induced following vaccination with naked DNA encoding the CC chemokines MIP-1α or MCP-1 (Youssef et al., 1998). This is a novel

mechanism of expressing antigen *in vivo* in order to elicit a humoral and cellular immune response. Administration of MIP-1β naked DNA vaccine was found to significantly aggravate EAE, while RANTES naked DNA had no significant effect on disease progression (Youssef et al., 1998). Anti-inflammatory therapies based on blocking chemokine receptors have also been shown to be effective in murine models of arthritis (Gong et al., 1997), and suggest that blocking chemokine receptors with truncated chemokines may provide a potential therapeutic treatment for neuroinflammatory diseases such as MS, SSPE and CD.

FUTURE WORK

As this study has demonstrated, the interaction of chemokines with their corresponding chemokine receptors may play a major role in the activation and directional migration of specific cells, both circulating leukocytes and resident glia, to sites of inflammation in the CNS in MS. Due to restricted supplies of cerebellar tissue from CD patients with associated neurological complications and SSPE cases, this study was unable to conclusively characterise chemokine profiles in these neuroinflammatory diseases. Therefore, it would be beneficial to continue investigating both chemokine and chemokine receptor expression in SSPE and CD, along with other neuroinflammatory disease states such as Alzheimer's disease, and to expand the number of chemokines investigated to include neurotactin, IL-8 and GRO-α.

This study has reported the expression of a limited number of chemokine receptors in MS lesions. To fully characterise chemokine receptor expression, future work will include other chemokine receptors, such as CCR4. It is hoped that the combined results of these studies will provide an insight into the chemokine receptor profile of perivascular T-lymphocytes, T-cells which have migrated into the surrounding white matter, and resident glial cells. Future work may also reveal a correlation between chemokine receptor expression and T-cell subtypes infiltrating MS CNS tissue. Continuing investigations into chemokine receptor expression by glial and T-cells, both *in vivo* and *in vitro*, and assessing which factors may contribute to the regulation of their expression may provide useful insights into mechanisms of preventing the actions of chemokines, thereby inhibiting the recruitment of cells to sites of inflammation.

CHAPTER 8

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PUBLICATIONS RELEVANT TO THE THESIS

Simpson, J.E., Newcombe, J., Cuzner, L. and Woodroofe, M.N. (1998) Expression of monocyte chemoattractant protein-1 and other β-chemokines by resident glial cells in multiple sclerosis lesions. Journal of Neuroimmunology, 84, 238-249.

Published Abstracts

Hadjivassiliou, M., Simpson, J.E., Woodroofe, M.N., and Grunewald, R. (1998) Investigation of the mechanism of neural damage in patients with gluten sensitivity and neurological dysfunction. Neuropathology and Applied Neurobiology (Abstract Booklet for the 96th meeting of the British Neuropathological Society), 1.

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APPENDIX 1

Age, sex, death to snap-freezing (D-F) time data and duration of disease in multiple sclerosis (MS) and normal control (NC) tissue samples

The following data is from tissue used in the studies reported in this thesis. ORO and cuffing was scored on a scale of 0 to 5 for ORO and haematoxylin staining. 0 is what would be expected in normal control white matter. The first figure is the score for ORO-positive macrophages to show the extent of recent or on-going demyelination; the second figure is the score for perivenular inflammatory cuffing obtained from haematoxylin staining. The sections were cut in duplicate immediately before, in the middle and after serial section.

Key:

C	cervical	P	parietal
Cer	cerebellum	Po	pole
D	subcortical	Q	MS plaque
DD	disease duration	R	right
DFT	time between death and sample snap-freezing	SC	spinal cord
F	frontal	Sv	subventricular
G	grey matter	T	transverse
L	left	Th	thoracic
MS	multiple sclerosis	Tr	trigone
NC	normal control	V	ventricular
O	occipital	W	white matter

APPENDIX 1

Age, sex, death to snap-freezing (D-F) time data and duration of disease in multiple sclerosis (MS) tissue samples

Block Code	Tissue Type	ORO, cuffs	Age (Y)	Case	DD (Y)	M/F	Cause of Death	DFT (h)
387-16	MSW FVR	0,2	53	MS	22	F	Pulmonary embolism	31
387-27	MSW OVL	0,1	53	MS	22	F	Pulmonary embolism	31
395-43	MSQ Pons	0,2	58	MS	14	M	Not known	52
395-44	MSQ CerR	0,2	58	MS	14	M	Not known	52
395-71	MSQ OPoR	1,2	58	MS	14	M	Not known	52
396-95	MSQ OVL	2,3	37	MS	10	F	Bronchopneumonia	24
396-98	MSW FSvL	0,0	37	MS	10	F	Bronchopneumonia	24
397-31	MSQ TSvR	0,0	65	MS	14	F	Bronchopneumonia	30
397-34A	MSW&G FR	0,0	65	MS	14	F	Bronchopneumonia	30
397-45	MSQ SCTL	4,2	65	MS	14	F	Bronchopneumonia	30
400-68	MSQ PVR	5,1	29	MS	7	F	Bronchopneumonia	14
400-71	MSQ TVL	3,0	29	MS	7	F	Bronchopneumonia	14
405-8	MSQ PonsL	2,2	47	MS	7	F	Cerebrovascular accident	16
405-40	MSQ OSvR	0,0	47	MS	7	F	Cerebrovascular accident	16
408-12	MSQ SCCT	0,1	59	MS	20	F	Bronchopneumonia	13
413-32	MSW&G CerR	0,1	59	MS	20	F	Bronchopneumonia	13
413-36	MSQ Pons	5,3	59	MS	20	F	Bronchopneumonia	13
413-41	MSQ FSvR	2,0	59	MS	20	F	Bronchopneumonia	13
413-54	MSQ FVL	0,0	59	MS	20	F	Bronchopneumonia	13
413-55	MSQ TSvL	4,3	59	MS	20	F	Bronchopneumonia	13
414-24	MSQ TrSvR	0,2	60	MS	-	F	Bronchopneumonia	24

APPENDIX 1 Contd.

Age, sex, death to snap-freezing (D-F) time data and duration of disease in multiple sclerosis (MS) tissue samples

Block Code	Tissue Type	ORO, cuffs	Age (Y)	Case	DD (Y)	M/F	Cause of Death	DFT (h)
414-34	MSQ TRVL	1,1	60	MS	-	F	Bronchopneumonia	24
414-35	MSQ OVL	0,0	60	MS	-	F	Bronchopneumonia	24
414-49	MSQ PSvR	2,4	60	MS	-	F	Bronchopneumonia	24
419-29	MSW&G Pons	0,2	46	MS	11	M	Bronchopneumonia	51
440-63	MSQ PVR	0,2	43	MS	18	M	Bronchopneumonia	48
440-72	MSQ TSvR	0,2	43	MS	18	M	Bronchopneumonia	48
440-76	MSW FVR	1,3	43	MS	18	M	Bronchopneumonia	48
440-80	MSQ PVL	0,4	43	MS	18	M	Bronchopneumonia	48
440-88	MSQ OSvL	1,3	43	MS	18	M	Bronchopneumonia	48
440-90	MSQ OVL	1,3	43	MS	18	M	Bronchopneumonia	48
444-5	MSQ SCCT	3,3	29	MS	8	F	Bronchopneumonia	11
444-13	MSQ SCThT	3,3	29	MS	8	F	Bronchopneumonia	11
444-48	MSQ Cer	4,2	29	MS	8	F	Bronchopneumonia	11
444-53	MSQ Cer R	2,4	29	MS	8	F	Bronchopneumonia	11
444-69	MSQ OVR	3,5	29	MS	8	F	Bronchopneumonia	11
488-67	MSQ PVL	5,2	47	MS	20	F	Bronchopneumonia	9
488-68	MSQ PVL	5,0	47	MS	20	F	Bronchopneumonia	9
488-84A	MSQ FVL	5,2	47	MS	20	F	Bronchopneumonia	9
488-91	MSQ PSvR	5,2	47	MS	20	F	Bronchopneumonia	9
488-97	MSQ PSvL	4,3	47	MS	20	F	Bronchopneumonia	9
488-110	MSQ OVPoL	5,2	47	MS	20	F	Bronchopneumonia	9
488-123	MSQ OVL	3,4	47	MS	20	F	Bronchopneumonia	9

APPENDIX 1 Contd.

Age, sex, death to snap-freezing (D-F) time data and duration of disease in normal control (NC) tissue samples

Block Code	Tissue Type	ORO, cuffs	Age (Y)	Case	DD (Y)	M/F	Cause of Death	DFT (h)
393-12	NCW OVR	0,1	37	NC	-	M	Road traffic accident	40
408-21	NCW OVL	0,0	39	NC	-	F	Peritonitis	9
413-64	NCW&G CPR	0,1	62	MS	20	F	Bronchopneumonia	13
480-18	NCW FPoVR	0,1	28	NC	-	M	Cardiac arrest	26
480-19	NCW FVR	0,2	28	NC	-	M	Cardiac arrest	26
481-22	NCW TrVR	0,1	40	NC	-	M	Cardiac arrest	22
504-20	NCW OVR	0,0	49	NC	-	M	Myocardial infection and coronary artery thrombosis	11
504-45	NWOSvR	0,1	49	NC	-	M	Myocardial infection and coronary artery thrombosis	11
523-31	NCW PVR	0,0	67	NC	-	M	Haemorrhage	38
523-32	NCW PSvR	0,0	67	NC	-	M	Haemorrhage	38
523-33	NCW&G PSvR	0,0	67	NC	-	M	Haemorrhage	38

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