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The *in vitro* effects of chemokines on microglia: implications for multiple sclerosis

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Abbreviations

ANOVA	Analysis of variance
AP-1	Activator protein 1
BBB	Blood Brain Barrier
BCA	Bicinchoninic
BSA	Bovine serum albumin
CCR	CC chemokine receptor
CJD	Creutzfeld Jacob Disease
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocytes
CXCR	CXC chemokine receptor
DAB	Diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
EA	Erythrocyte opsonised with antibody
EAE	Experimental autoimmune encephalomyelitis
EBSS	Earle's balanced salt solution
ECM	Extracellular matrix
ENA-78	Epithelial cell derived neutrophil activating protein 78
GCP-2	Granulocyte chemotactic protein
GFAP	Glial fibrillary acidic protein
GRO α/ β	Growth related peptide
HLA	Human leukocyte antigen
HTLV	Human T lymphocyte lymphotrophic retrovirus
ICAM	Intercellular adhesion molecule
ICC	Immunocytochemistry
ICE	Interleukin 1 converting enzyme
IFN	Interferon
IL-1	Interleukin 1
IL-8	Interleukin 8
IP-10	Interferon inducible protein 10
LFA1	Lymphocyte function associated antigen 1

LPS	Lipopolysaccharide
MBP	Myelin basic protein
MCP1	Monocyte chemoattractant protein 1
MDC	Macrophage derived chemoattractant
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma interferon
MIP1α/β	Macrophage inflammatory protein 1 α/β
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
NAP2	Neutrophil activating peptide 2
NO	Nitric oxide
NOS	Nitric oxide synthase
PAI	Plasminogen activator inhibitor
PBMC	Peripheral blood mononuclear cells
PF-4	Platelet factor 4
PGE-2	Prostaglandin E2
PLP	Proteolipid protein
PMA	Phorbol 12-myristate acetate
RANTES	Regulated upon activation, normal T cell expressed and secreted
RT-PCR	Reverse transcriptase polymerase chain reaction
SCM-1	Single cysteine motif-1
SDF-1	Stromal cell derived factor 1
SLC	Secondary lymphoid tissue chemokine
TARC	Thymus and activation related chemokine
TBE	Tris borate EDTA
TCA3	T cell activation gene 3
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumour necrosis factor
tPA	Tissue-type plasminogen activator
TRITC	Tetramethyl Rhodamine Isothiocyanate
uPA	Urokinase-type plasminogen activator

uPAR	Urokinase plasminogen activator receptor
VCAM	Vascular cell adhesion molecule

Abstract

Microglia, the resident macrophages of the central nervous system (CNS), are the primary cell to respond to injury in the brain, both in inflammation e.g. in multiple sclerosis (MS), and in trauma. Microglia can be activated *in vitro* by proinflammatory cytokines such as IFNγ and TNFα and respond by secretion of cytokines, chemokines, proteases, nitric oxide and superoxide radicals

As MS is a putative autoimmune disease of the CNS, chemokines (chemotactic cytokines), secreted by many cell types including T cells and macrophages in MS lesions, have been postulated as key players in the orchestration of the inflammatory response by recruitment and activation of inflammatory cells.

As potential mediators of microglial cell recruitment to sites of injury, the ability of microglia to migrate in response to α and β chemokines was assessed. All chemokines tested demonstrated the ability to induce migration and changes in the distribution of filamentous actin in adult rat microglia and a human microglial cell line, CHME3, *in vitro*. This study indicates that chemokines secreted by activated T cells in the CNS may attract microglia to areas of central nervous system inflammation where they could exert their well documented effects.

Proteases are also found in close proximity to MS plaques in increased amounts as agents of myelin breakdown in MS. Matrix metalloproteinases (MMPs) are zinc-dependant enzymes, capable of degrading extracellular matrix proteins and are known to be produced by microglia. The control of MMP activity by plasmin, as well as the balance between MMP and levels of their natural inhibitors, TIMPs, may determine lytic or anti-lytic activity in MS. The *in vitro* control of MMPs 2 and 9, TIMPs 1 and 2 and urokinase type plasminogen activator (uPA) by microglia was examined in response to several chemokines using ELISA and zymography techniques. The chemokines tested were all found to significantly increase the secretion of MMPs and TIMPs by CHME3 cells after 24 hours stimulation. The chemokines, MCP1, MIP1β and Fractalkine were also shown to increase MMP9 secretion by rat microglia with little or no effect on MMP2. Interestingly, MCP1, MIP1α/β and RANTES significantly decreased the secretion of uPA by CHME3 cells which may indicate an increase in uPAR expression. These results suggest that chemokine mediated control of MMP activity in the breakdown of the blood brain barrier could allow further recruitment of immune cells into the CNS and also cause demyelination of axons by lysis of myelin basic protein.

Since chemokines, as well as cytokines, are possible candidates for microglia activation, microglia were tested in their response to several chemokines by measurement of nitrite production, superoxide secretion and Fc receptor expression. The majority of the chemokines tested were able to increase superoxide and nitrite production as well as Fc receptor expression with the exception of the β chemokine, RANTES, whereby only CHME3 cells produced a significant increase in superoxide production and Fc receptor expression above unstimulated levels suggesting a difference in chemokine receptor expression or a difference in the binding affinity and signal transduction in different cell types.

The present study has led to a further understanding of the possible chemokine control of microglial migration, proteinase production and effector function in CNS diseases, as well as their well documented role in recruitment of lymphocytes. Targetting these chemokines in MS patients, with specific antibodies or use of synthetic inhibitors to block their receptors may lead to downregulation of the immune response and a decrease in the severity of the disease.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1 Cells of the central nervous system

The cell types of the central nervous system (CNS) include neuronal and non neuronal cell types, or glial cells. Glial cells include microglia, astrocytes and oligodendrocytes. In the mammalian CNS, astrocytes and oligodendrocytes outnumber neurons (Perry, 1994). The myelin sheaths which surround and insulate nerve axons are synthesised by oligodendrocytes. The functions of astrocytes are numerous and include production of growth factors, metabolism of neurotransmitters and maintenance of homeostasis (Savchenko *et al*, 1997). The major cytoskeletal protein of astrocytes is glial fibrillary acidic protein (GFAP) which allows the identification of these cells by immunocytochemistry. Microglia are the resident phagocytic cells of the CNS.

1.1.1 Microglia

It was once thought that the CNS was an immunologically privileged site, based on observations that the CNS was not able to reject allografts. However it has now become clearer that the CNS is not defenceless and that microglia, the resident brain macrophages, provide the brain's immune defence (Gremo *et al*, 1997, Carson and Sutcliffe, 1999, Kreutzberg, 1996). Del Rio-Hortega, in 1932 established the basic characteristics of microglial cells using silver staining methods and light microscopy (Theele and Streit, 1993). Their existence was questioned by some scientists until the advent of antibodies which allowed reproducible visualisation of these cells in tissue

sections. Multiple forms of this cell type have now been characterised and termed amoeboid, ramified and reactive microglia (Thomas, 1992). Amoeboid microglia are present in developing brain tissue and are typical of macrophages whereas ramified microglia are found in normal adult tissue and possess a small cell body with many branches (Thomas, 1992, Perry and Gordon, 1988). Reactive microglia arise in adult tissue after injury or infection and are characterised by their lack of ramified processes e.g. following facial nerve transection, experiments show retraction of these long delicate branches into the oval reactive form (Streit and Graeber, 1993). These parenchymal microglia differ from another subset of macrophage like cells found in the perivascular space in close association with the vascular endothelium and the blood brain barrier (Hickey and Kimura, 1988). These cells are capable of antigen presentation and could be considered as a distinct macrophage population which undergo constant replacement by bone marrow precursor cells.

1.1.2 Distribution of microglia

Studies of microglia *in situ* using various specific antibodies has allowed their distribution to be identified. Microglia in the cortex of rats were shown to be fairly uniformly distributed but with higher densities in the frontal lobe. The lowest densities of microglia were found in the cerebellum and medulla oblongata and overall the density was lower in the gray matter than the white matter (Savchenko *et al*, 1997). The numbers of microglia in normal appearing white matter of the human brain have been estimated at 13-15% of all cells (Hayes *et al*, 1987). Although densities vary throughout the brain, the distribution within particular structures are normally highly regularly spaced, each with its own territory and when more microglia are present, the territory of each is smaller (Perry, 1994).

1.1.3 The origin of microglia

The origin of microglia is not conclusive although evidence suggests that microglia have more in common with peripheral macrophages than with other cells of the CNS (Perry, 1994, Altman, 1994). It is believed that cells of bone marrow origin, colonise the CNS and this colonisation is finished in the early postnatal period. These amoeboid microglia, later develop into the adult ramified microglia (Leong and Ling, 1992, Ling and Wong, 1993). An important difference between parenchymal microglia and other macrophage populations, is that most macrophages have a relatively short lifespan whereas parenchymal microglia can live as long as the organism (Carson and Sutcliffe, 1999). Resident tissue macrophages are involved in homeostatic control and defence of the organism against infection and injury and brain microglia share these functions.

1.1.4 Markers expressed by microglia

Microglia, like other macrophages, express a wide range of surface and cytoplasmic markers which make them distinct. Hayes *et al*, 1988 used a panel of monoclonal antibodies to characterise isolated human and rat adult microglia. Both rat and human microglia were found to express the complement receptor C3b which is involved in the binding of complement opsonised particles. Adult human (Williams *et al* 1992) and neonatal rat microglia expressed macrophage cytoplasmic antigens (EBM-11 and ED1 antibodies respectively). Complement receptor C3b was also found to be expressed and intensely stained in amoeboid microglia in the early postnatal rat although the staining intensity diminished in the late postnatal period as the cells became ramified (Ling *et al*, 1990). Human microglia express MHC (major histocompatibility complex) class II antigens (Hayes *et al*, 1987) which is an important molecule in the presentation

of antigen to T cells in the immune response. This is in contrast with rat microglia which express little or no MHC II constitutively although expression can be induced by stimulation with interferon gamma (IFNγ) (Woodroofe *et al*, 1989). Markers for non-specific esterase are often used to identify monocytes which is also present in amoeboid microglia but not in the adult ramified microglia (Hayes *et al*, 1988, Thomas, 1992). Other commonly used markers for microglia expressed on both the ramified and amoeboid forms are the plant lectins, B4 isolectin and agglutinin-120. These lectins bind to the microglia recognising D- galactose and within the brain parenchyma, do not label neurons, astrocytes or oligodendrocytes showing their selectivity for microglia (Thomas, 1992).

Fc receptors are found on developing and adult rodent microglia (Perry and Gordon, 1988) and adult human microglia (Hayes *et al* 1988) and like the complement receptors, are important in binding of antibody opsonised particles. Microglia, like other macrophages are phagocytic which is proven by their ability to ingest antibody coated erythrocytes (Hayes *et al*, 1988, Hall *et al* 1997) and the ability of amoeboid microglia to phagocytose opsonised and non opsonised latex beads (Bocchini *et al* 1988).

1.1.5 The role of microglia in the normal CNS

During development of the CNS, immature microglia invading the tissue are thought to phagocytose dying cells and selectively eliminate certain axonal projections in the cerebral cortex (Thomas, 1992) as well as stimulating astrocyte proliferation by the secretion of interleukin 1(IL-1) (Giulian *et al* 1988a+b) It is more difficult to elucidate the homeostatic function of ramified microglia in the adult CNS. One proposal is that they play an important role in 'fluid cleansing' of extracellular fluid in their microenvironments due to observations on their high levels of motility and pinocytosis in

living cerebral cortical cultures (Booth and Thomas, 1991). This is thought to contribute to tissue maintenance by the removal of cellular metabolites.

1.1.6 The effects of Interferon γ on microglia

Cytokines found in the normal and diseased CNS have a wide range of effects on microglia demonstrated mainly by *in vitro* experiments. MHC class II expression can be greatly increased on rat microglia by stimulation with IFNγ (Woodroofe *et al*, 1989). This effect on rat microglia has been shown to be inhibited by interferon beta (IFNβ) although it had no effect on basal levels of MHC class II expression (Hall *et al*, 1997). The upregulation of MHC class II by IFNγ was also confirmed in experiments using human microglia (Williams *et al*, 1992). The Fc receptor expression by rat microglia can also be upregulated by IFNγ (Woodroofe *et al*, 1989, Hall *et al*, 1997) and IFNβ (Hall *et al* 1997).

1.1.7 Microglia in CNS disease

It is apparent that microglia contribute to a wide range of CNS pathologies and are often the first cell type to respond to an insult, although they can be the targets of infection for example in HIV infection of the CNS (Dickson *et al*, 1993). They appear to be detrimental in many diseases but can also be beneficial to repair. Giliad and Giliad (1995), describe evidence of the accumulation of nerve growth factor by microglia but not astrocytes, which may be important after CNS trauma for nerve cell survival. A model of facial nerve axotomy in the rat has been used extensively to study the response of microglia to injury. Microglia proliferate at the site of injury within 24 hours and produce macrophage colony stimulating factor (M-CSF) just 24 hours after the injury. One week after axotomy a small proportion of microglia in the vicinity express MHC

class II which was seen to last for up to 15 weeks (Streit and Graeber, 1993). Microglia produce a similarly rapid response to inflammation within the CNS. Microglia isolated from inflamed rat brain, 7 days after induction by a viral infection, showed an upregulation of MHC II (Sedgewick *et al*, 1991). In an experimental glioma model produced in rats, there was a massive infiltration into the tumour by activated microglia (Morioka *et al*, 1992). Furthermore, amoeboid microglia in culture have been shown to be cytotoxic to tumour cells by production of tumour necrosis factor alpha (TNFα) (Frei *et al*, 1987).

Microglia are implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis, due to observations on their state of activation in tissue sections and upregulation of cell surface markers including MHC II and Fc receptors (McGeer *et al*, 1993) Furthermore, they have been shown to accumulate on Alzheimer's plaques *in vitro* (Joshi and Crutcher, 1998). N9 cells, a murine microglial cell line has been demonstrated to bind to beta amyloid fibrils, the major components of senile plaques, through the expression of a scavenger receptor, SRA (El Khoury *et al*, 1998) this is proposed as a trigger for the secretion of neurotoxins. A mouse model of Parkinson's disease demonstrated an increased number of MHC class II expressing microglia associated with the induced neurodegeneration, which were less branched in morphology when compared to control brain (Kurkowska-Jastrzebska *et al*, 1999). Giese *et al* (1998), found that accumulation of microglia as well as cellular expression of prion proteins (PrP) *in vitro* was necessary for the neurotoxicity of prion protein in mouse brain cultures. A chemical (L-Leucine Methyl Ester) that selectively kills microglia, blocked the neurotoxicity of PrP. Activated microglia were also shown to be present *in vivo* in prion infected mice with the time course of activation and distribution closely resembling the pattern of PrP deposition. Immunocytochemical

studies on CNS tissue from the human prion disease, Creutzfeld-Jacob disease (CJD), also showed a high density of microglia associated with PrP deposits although microglia did not appear to be involved in PrP processing (Muhleisen *et al*, 1995). More recent studies have shown that the activated microglia expressing increased MHC II associated with spongiform degeneration, show cytoplasmic vacuolation suggesting they may also be targets of CJD (Eitzen *et al*, 1998). Microglia have also been shown to be present at an increased density in the temporal and frontal cortex of chronic schizophrenics possibly indicating changes in the architecture of the cortex (Radewicz *et al*, 1998).

1.1.8 The role of microglia in MS

The most widely studied CNS disease in relation to the involvement of microglia is multiple sclerosis (MS) and also the animal model of MS, experimental autoimmune encephalomyelitis (EAE) (Benveniste, 1997). Microglia appear to play a key role in the pathogenesis of MS due to their rapid activation upon a number of insults, their inducibility in the expression of MHC class II molecules, their motility and phagocytic abilities and close proximity to perivascular cuffs (Gehrmann *et al*, 1995).

Several groups have described the distribution of macrophages and microglia in MS plaques (Woodroofe *et al*, 1986, Esiri and Reading, 1987, Li *et al* 1993). The macrophages in the active MS lesion often have ingested myelin fragments and stain strongly for MHC II, suggesting that the myelin processing may be responsible for activation of these cells. Microglia are present in an activated state throughout the normal appearing white matter which surround active plaques. In some cases, microglia in normal appearing white matter are stained by oil red O, demonstrating the presence of neutral lipids, resulting from the degradation of myelin (Li *et al*, 1993) This suggests that microglia are involved in very early myelin processing events, which may then lead on to

activation of specific immune responses. Demyelination occurs as the lytic dissolution of myelin from axons close to macrophages. Myelin phagocytosis by macrophages is brought about by attachment of myelin debris to clathrin coated pits on macrophages of monocytic and microglial lineage (Hartung, 1995, Raine, 1994). This process also occurs in the experimental model of EAE in rabbit, mouse and guinea pig (Raine, 1994). A review by Smith *et al*, 1999, suggests that damage to myelin by macrophage secreted proteases, externalising the phosphatidyl serine component of the myelin, would allow the scavenger receptors present on activated microglia, which have a high affinity for phosphatidyl serine, to interact with this ligand for phagocytosis to occur. The importance of macrophages infiltrating the CNS in EAE was demonstrated by eliminating macrophages in the liver and spleen of rats before the appearance of any clinical signs, which markedly suppressed the appearance of clinical signs compared to control animals (Huitinga *et al*, 1990).

1.1.9 The products of activated microglia

Many of the effects of microglial cells can be attributed to the large array of substances they can synthesise and secrete, in response to a wide variety of stimuli. A summary by Minghetti and Levi, 1998, lists some of the products which include growth factors, cytokines, complement factors, prostanoids, proteolytic enzymes and free radicals. The list is ever increasing and should now include chemokines (Hayashi *et al*, 1995, Calvo *et al*, 1996, Peterson *et al*, 1997).

Cytokines released by activated microglia include: Tumour necrosis factor α (TNFα) (Sawada *et al*, 1989), Interleukin 1α (IL-1α) (Yao *et al*, 1992), Interleukin 1β (IL-1β), Interleukin 6 (IL-6) (Woodroofe *et al*, 1991, Raine, 1994, Gehrmann *et al*, 1995, Lukas and Hohlfeld, 1995) TGF-β1 (Kiefer *et al*, 1998) as well as Interleukin 1β

converting enzyme (ICE) which is responsible for processing IL-1 to its mature biologically active form (Yao and Johnson, 1997). Microglia are also found to express the β chemokines monocyte chemotactic protein 1 (MCP1), macrophage inflammatory proteins 1 α and β (MIP1α and MIP1β) (Hayashi *et al*, 1995, Calvo *et al*, 1996, Peterson *et al*, 1997, McManus *et al*, 1998b) and the interferon inducible, α chemokine, IP-10 (Ren *et al*, 1998). More recent studies have shown microglial expression of interleukin 12 (IL-12) (Aloisi *et al*, 1997, Suzumura *et al*, 1998) and interleukin 18 (IL-18) (Conti *et al*, 1999). These two cytokines synergise in the induction of IFNγ production by T helper 1 cells (Conti *et al*, 1999). IL-18 also stimulates the *in vitro* production of IL-1β by astrocytes (Culhane *et al*, 1998). IL-1β produced by microglia has been shown to induce astrocytic proliferation and neovascularisation in brain trauma (Gehrmann *et al*, 1995), TNFα has a cytotoxic effect on myelinating oligodendrocytes (Gehrmann *et al*, 1995, Banati *et al*, 1993) and is expressed by microglia in demyelinating lesions (Dickson *et al*, 1993). The effect of IL-6 treatment of microglia using a microglial cell line (N11), showed that chronic exposure to IL-6 augmented the LPS induced production of TNFα and IL-6 (Di Sianto *et al*, 1996) suggesting a role in the orchestration of futher cytokine production. Over-expression of human IL-6 in the CNS in transgenic mice also increased the production of TNFα, IL-1β and IL-6 in the CNS, after LPS injection intraperitoneally or intracerebroventricularly (Di Santo *et al* 1996). Findings by Woodroofe and Cuzner (1993) using *in-situ* hybridisation techniques, also show production of IL-6 and TNFα as well as IFNγ in perivascular inflammatory cuffs from postmortem MS brain tissue. Ren *et al* (1999) have shown that there is differential expression of TNFα in microglia isolated from different regions of the rat CNS, with detection of high levels from microglia isolated from the hippocampus and

suggest that microglial gene expression may depend on the interaction with other neural cell types in the microenvironment. Expression of TGF-β1 by microglia was observed during the recovery phase of EAE which may serve to downregulate activated T cells and contribute to recovery (Kiefer *et al*, 1998).

1.2 Multiple Sclerosis

Multiple Sclerosis (MS) is a demyelinating disorder affecting the white matter of the brain and spinal cord. The disease is characterised pathologically by inflammation and demyelination of axons within the CNS giving rise to symptoms ranging from double vision to paraplegia. Most cases present between 20 and 40 years of age (Kurtzke, 1993). MS is now widely accepted as an autoimmune disorder (Raine, 1994) for which there is no known cure. The disease follows a characteristic relapsing and remitting course and recovery from each episode of demyelination is usually incomplete leading to progressive clinical deterioration. Some patients follow a rapidly progressive course while others may survive for over 20 years with only minor disability.

1.2.1 Epidemiology of MS

MS is most prevalent in populations living furthest from the equator e.g. the prevalence is particularly high in Northern Europe with 1 in 800 of the population in the UK being affected (Kurtzke, 1993). Within the United Kingdom, there is also a striking difference in the prevalence between the North of Scotland and Southern England with regions in Northern England having a higher prevalence than that found in the Southern regions (Compston, 1997). It is also interesting to note that studies on migrants moving from Northern Europe to Southern Africa as adults, took with them the high prevalence rate of their country of origin whereas those migrating below the age of 15 years took on

the frequency of native Africans (Compston, 1997). This suggests that the disease process is established in childhood. MS is also more common in females than in males with a ratio of 2:1 irrespective of ethnicity (Compston, 1997). Studies on MS in pregnancy show a slight decrease in relapse rate during pregnancy when compared with non pregnant periods of the same patients and control women (Runmarker and Anderson, 1995). The reasons for this are undefined although it may be due to the selective immunosupression during pregnancy to allow the foetus to survive as an allograft. Studies on experimental autoimmune uveitis (EAU) show that pregnant animals are less susceptible to induction of EAU or experience a less severe course (Agarwal *et al*, 1999).

1.2.2 Histology of MS

Patients with MS show numerous demyelinated plaques in the brain and spinal cord often closely related to venules. Histological observations generally show myelin breakdown and phagocytosis by macrophages. There is perivascular cuffing with inflammatory cells including T cells, B cells and macrophages in the acute plaque. As myelin breakdown subsides, a reactive gliosis is established leading to a chronic plaque. (Adams *et al*, 1989).

1.2.3 Animal models of multiple sclerosis

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the CNS that is commonly used as an animal model for MS (Bradl and Linington, 1996, Hulkower *et al*, 1993, Huitinga *et al*, 1990). It can be induced in a number of genetically susceptible animals, including primates by injection of an emulsion of homogenised myelin, whole brain homogenate, myelin basic protein (MBP) or proteolipid protein

(PLP) in adjuvant (Owens and Sriram, 1995, Hulkower *et al*, 1993, Huitinga *et al*, 1990). In the Lewis rat, sub-cutaneous injection of homogenised myelin induces an acute paralytic disease about 10-12 days after sensitisation (Hulkower *et al*, 1993, Huitinga, *et al* 1990). There is full recovery usually over the next few days but in some animals, mainly females, a short relapse may occur (Hulkower *et al* 1993). Since EAE is a T cell mediated disease, it may also be induced by passive transfer of MBP or PLP reactive T cells. Changing the immunisation protocol results in the chronic form of EAE in which animals enter a series of relapses and remissions similar to MS. CNS tissue of sensitised animals shows perivascular inflammation with lymphocytes and macrophages/monocytes being the major cell types. Breakdown of the blood brain barrier can also be observed. The observation that MS is an autoimmune disease stems partly from the similarities with EAE, which a is T cell mediated disease, and it is proposed that MS is also T cell mediated (Sriram and Rodriguez, 1997). Although similar, there is a major difference between EAE and MS and that is, EAE is known to be a T cell mediated autoimmune disease as it can be induced with specific antigens whereas in MS, the inducing antigen remains undefined. Nevertheless, much understanding of MS has come from studying EAE which remains the closest animal model. Theiler's murine encephalomyelitis virus (TMEV) induced demyelinating disease in mice, is also considered a good model for MS as infection leads to a chronic demyelinating disease with similarities to MS (Oleszak *et al*, 1995).

1.2.4 Viruses and multiple sclerosis

Epidemiological evidence suggests that MS involves an infectious agent (Kurtze, 1993) encountered probably between the ages of 5-15 years (Calder *et al*, 1989). This may involve molecular mimicry where the immune system is presented with stretches of

amino acids that resemble 'self'. For example, Adenovirus Type 2 contains amino acid sequences similar to those in the crucial encephalitogenic fragment of myelin basic protein (MBP). MBP is the major constituent of the myelin sheath and is produced by oligodendrocytes. A number of different viruses have been proposed as the causative agents in MS including: rabies, measles, mumps, rubella, herpesviruses, chimpanzee cytomegalovirus and retroviruses, however no definitive viral agent has been found as yet and it may be that different viruses are involved in different patients. This would depend on their genetic background (Monteyne *et al*, 1998), since a genetic predisposition also determines a person's susceptibility to MS (Kurtze, 1993).

1.2.5 Genetics of MS

There is no single known factor responsible for the cause of MS, although family and twin studies have shown that genetic factors exert a significant influence of autoimmune disease predisposition (Theofilopoulos, 1995). Individuals with a greater genetic loading (i.e. with an affected parent) have been shown to have a lower age of onset and increased susceptibility suggesting that MS is at least in part, under genetic control (Sadovnick *et al* 1998). Concordance rates for monozygotic and dizygotic twins with MS range from 20-30% and 3-4% respectively (Ebers *et al*, 1995). Although concordance in monozygotic twins with MS is lower than expected for a purely genetic disease, suggesting environmental factors may be involved, the role of genomic imprinting (ie gene silencing by methylation) and non-identity of immune repertoires cannot be ruled out (Theofilopoulos, 1995).

It is widely accepted that a genetic association exists between MS and the HLA region in Caucasian populations and this has been shown to be the single most prevalent susceptibility locus in genome wide genetic screens in familial MS (Sawcer *et al*, 1996,

Haines *et al* 1996, Ebers *et al* 1996). This genetic association with the HLA region has been demonstrated to be due to the HLA-DR2 allele in familial and sporadic cases, however a minority of familial cases do not segregate with HLA-DR2, suggesting that multiple genetic loci are involved (Haines *et al*, 1998).

A number of additional putative MS susceptibility loci have been identified, including the β chain of the T cell receptor (Theofilopoulos, 1995), myelin basic protein (Tienari *et al*, 1992, Barcellos *et al*, 1997) and Intercellular adhesion molecule 1 (ICAM-1) (Mykco *et al*, 1998). Also, genetic polymorphisms have also been shown to affect the clinical course of MS, whereby, patients with both IL-1RA allele $^{2+}$ and IL-1β allele^{2-} had an increased rate of disease progression (Schrijver *et al*, 1999).

1.3 The blood brain barrier

The blood brain barrier (BBB) separates the CNS environment from the systemic blood circulation to maintain homeostasis essential for optimal brain functioning. Under normal circumstances it protects the CNS from water soluble, blood borne components. The BBB is formed by endothelial cells, astrocytes, perivascular microglia and a basal lamina (De Vries *et al*, 1997). The endothelial cells of the CNS are held together by tight junctions formed by proteins in the tight junction complex and also the adherens junction complex. The proteins in the tight junction complex are zonula occludens 1 (ZO1) and occludin, and the adherens junction complex contains vascular endothelial cadherin and vinculin (Perry *et al*, 1997). Astrocytes project their end feet to the endothelial cells and are embedded in the basal lamina along with the perivascular macrophages. The BBB also has a metabolic barrier provided by the constitutive expression of the enzymes, monoamine oxidase A and B and catechol-o methyltransferase which are involved in the degradation of neurotransmitters and some blood borne compounds that may have

entered the CNS (de Vries *et al*, 1997). The transport of hydrophilic essential substances such as glucose, across the BBB is by selective carrier mechanisms and diffusion of compounds depends on their lipid solubility (de Vries *et al*, 1997).

1.3.1 Breakdown of the blood brain barrier

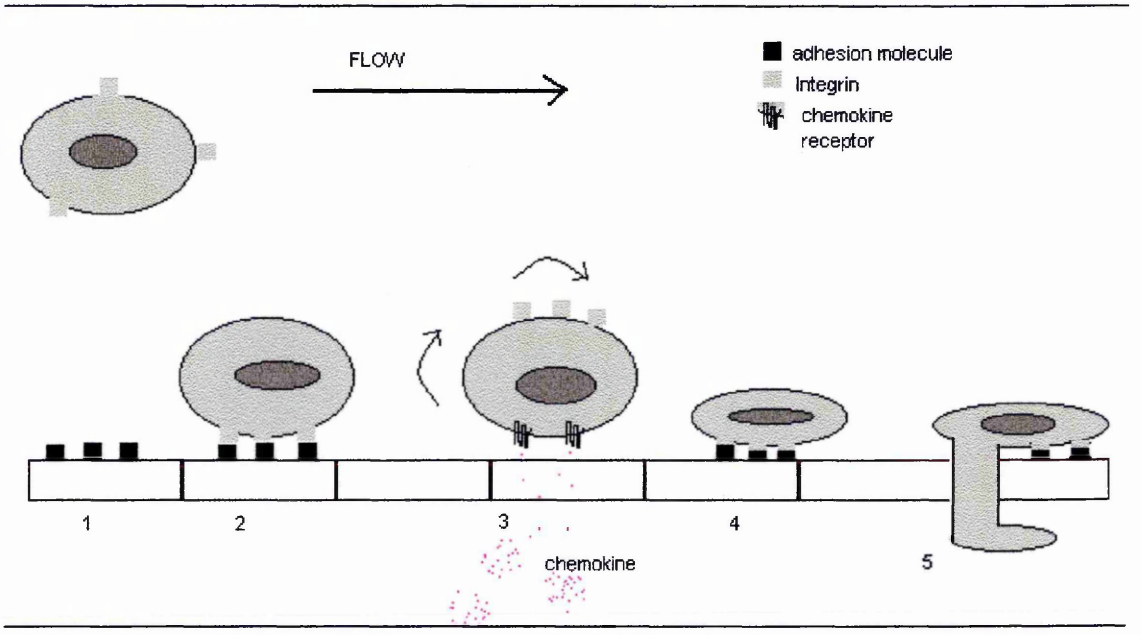
When inflammation occurs in the CNS, as in MS, the BBB can be changed significantly with the influx of inflammatory cells and plasma proteins. The cytokines released by inflammatory cells in MS, TNFα, IL-1β and IL-6 can lead to an increase in the permeability of endothelial tight junctions. Free radicals, which are produced by activated macrophages and microglia have also been shown to increase the permeability of the BBB (Hallenbeck, 1996, de Vries *et al*, 1997). Increased permeability of the BBB is also caused by the effects of proteases including the matrix metalloproteinases and serine proteases (Perry *et al*, 1997, Hallenbeck, 1996). Under normal conditions, small numbers of leukocytes cross the BBB for immune surveillance of the CNS. In the inflammatory response the expression of adhesion molecules such as ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) is upregulated on the endothelium, allowing greater influx of cells expressing the correct ligands (Archaelos and Hartung, 1997).

1.3.2 Cell migration into the CNS

Autoreactive T cells recognising MBP circulate in the blood of MS patients and upon encountering antigen they may become activated (Hartung, 1995). Activated self reactive T cells cross the BBB and initiate an immune response within the CNS. Slow flowing leukocytes attach weakly to the endothelium through the actions of selectins. Adhesion molecule expression by endothelial cells is greatly upregulated by cytokines

generated in the course of an inflammatory response. Interaction with the endothelium is strengthened on encountering specific chemoattractant or activating factors. In the final step, T cells change in morphology and are able to migrate through the endothelium in response to chemotactic signals (Figure 1.1) such as complement component C5a, platelet activating factor and chemokines, the chemotactic cytokines (Hartung, 1995).

Figure 1.1 The multistep model of leukocyte transmigration.



1. Endothelial cells are activated by inflammatory cytokines to increase expression of adhesion molecules e.g. ICAM-1, VCAM-1 and P selectins.
2. Circulating leukocytes attach via selectins.
3. Attatched leukocytes then roll along the endothelium.
4. Leukocyte integrin activation occurs through G protein linked chemokine receptors

The leukocyte endothelium interaction is strengthened by LFA-1/ICAM-1 and VLA4/VCAM-1 pairings, leading to firm adhesion, flattening and transmigration. Once

in the CNS autoreactive T cells undergo local reactivation if they encounter antigen presenting cells that display two signals, namely, processed respective autoantigen on their surface with MHC class II gene products and the costimulatory molecules B7-1 (CD80) or B7-2 (CD86). In MS lesions activated microglia express MHC II as well as B7 (Windhagen *et al*, 1995, Benveniste, 1997). Autoantibodies directed to MBP antigens may also cross the BBB once opened by activated T cells, or they may be produced locally by B cells that have been activated to do so by cytokines released by T helper (Th) cells. These autoantibodies activate the complement cascade and this leads ultimately to demyelination of axons by antibody and complement mediated phagocytosis. The complement component C5a also acts as a chemoattractant for inflammatory cells.

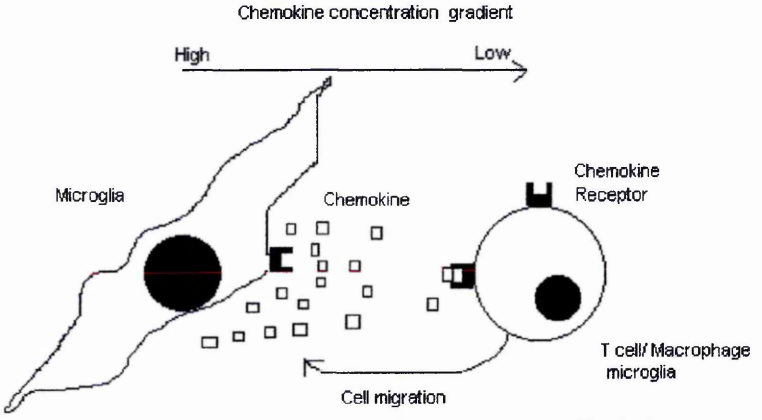
1.3.3 The role of macrophages in the pathogenesis of MS plaques

Macrophages and microglia, are involved in the demyelination process in MS. Primed Th cells, on entering the CNS are further activated by antigen presenting cells e.g. macrophages (Perry 1994, Hartung 1995), microglia (Hartung 1995, Gehrmann *et al* 1993, Hickey and Kimura 1988, Gehrmann *et al* 1995) and astrocytes (Hartung 1995, Fontana *et al* 1984). Th1 cells release IFNγ and TNFα as well as other factors which activate macrophages and microglia which then release inflammatory mediators such as proteases, TNFα, reactive oxygen species, nitric oxide (NO) metabolites and activated complement components (Hartung 1995). These molecules act at short range to damage the myelin sheath. Furthermore, activated macrophages and microglia participate in late 'cleaning up' events by phagocytosis, following inflammation in the CNS

It is hypothesised that microglia are able to migrate to areas of inflammation under the influence of chemokines and once at the site of damage, they become activated

and recruit other microglia and inflammatory cell types to the site of inflammation by release of chemokines such as Monocyte chemotactic protein 1 (MCP1), Macrophage inflammatory protein 1α (MIP1α) and MIP1β (Hayashi *et al,* 1995, Calvo *et al,* 1996, Peterson *et al*, 1997). (See figure 1.2).

Figure 1.2 Schematic diagram of possible mechanism of cell recruitment and activation in the central nervous system by microglial secretion of chemokines at the site of CNS inflammation.



1.4 Chemokines

Recruitment of leukocytes in inflammation is a complex process and involves adhesive interactions with the endothelium and secretion of cytokines. Chemokines are a rapidly expanding superfamily of chemotactic cytokines of which the majority were described in the last decade (Oppenheim *et al*, 1991, Schall, 1991, Baggiolini *et al*, 1994, Schall and Bacon, 1994, Miller and Krangel, 1992, Taub and Oppenheim, 1994, Petrek *et al*, 1995, Taub, 1996). They are low molecular weight proteins (7-14 kD in their monomeric form) and most are around 70 amino acid residues in length. Many of the

chemokines form dimers, trimers and tetramers upon secretion (Taub, 1996) although the monomers have been determined as the major species at physiological concentrations and are the functional ligands (McFadden and Kelvin, 1997) Studies on MIP1α, showed the high molecular weight aggregates of MIP1α to disaggregate under assay conditions and function as monomers (Graham *et al*, 1994).

1.4.1 Chemokine Subfamilies

Chemokines are divided into 4 groups according to a conserved cysteine motif and their chromosomal location. The first two groups to be characterised, the CXC or α chemokines and C-C or β chemokines, have 4 conserved cysteine residues. In the C-C chemokines, the first two cysteines are adjacent whereas they are separated by an intervening amino acid in the CXC chemokines. X ray crystallography has shown the monomeric form of CXC and CC chemokines to have a similar three dimensional structure (McFadden and Kelvin, 1997). More recently, the third group, 'C' or γ chemokines have been described, similar in structure to the C-C and CXC chemokines, but they lack two of the four cysteine residues. There are only two members of this group to date namely murine lymphotactin (Kelner *et al*, 1994) and its human homologue single cysteine motif-1 (SCM-1) (Yoshida *et al*, 1995). The most recent addition to the chemokine subgroups is the CX_3C chemokine (Bazan *et al*, 1997) of which the only one discovered to date has been named fractalkine (also known as neurotactin, Pan *et al*, 1997) and has been found to be membrane bound with a 'mucin like stalk' on top of the molecule. It has been found to be upregulated on the endothelium, induces adhesion of monocytes and T cells *in vitro* and can be cleaved and released from cell membranes as a shed glycoprotein that chemoattracts monocytes and T cells *in vitro* (Schall, 1997). Fractalkine expression has also been shown in the CNS by neurons and microglia

(Harrison *et al*, 1998). Lungkine is the most recently discovered CXC chemokine and was found to be expressed in mouse lung epithelial cells (Rossi *et al*, 1999).

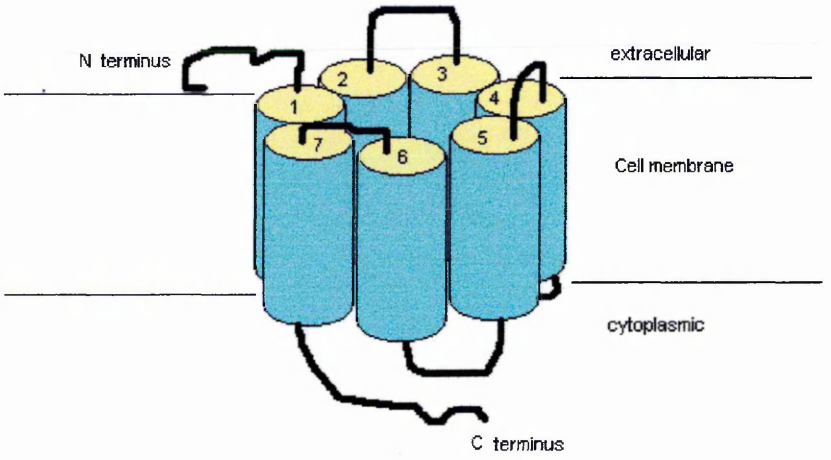
In humans, the genes for CXC chemokines have been mapped to chromosome 4q12-21 and the C-C chemokines, 17q11-21. Lymphotactin is located on mouse chromosome 1 and the human homologue, SCM-1 is also mapped to chromosome 1 (Petrek *et al*, 1995). Many of the CXC chemokines possess an ELR amino acid motif (glutamic acid-leucine-arginine) near the N terminus. These include interleukin-8 (IL-8), neutrophil activating peptide-2 (NAP-2), epithelial cell derived neutrophil activating protein-78 (ENA-78), growth related peptide α (GROα/β), GROβ and granulocyte chemotactic protein-2 (GCP-2), and all induce neutrophil chemotaxis *in vitro* and *in vivo*. The non ELR containing CXC chemokines including stromal cell derived factor-1 (SDF-1), monokine induced by gamma interferon (MIG), interferon inducible protein-10 (IP-10) and platelet factor-4 (PF-4) are not able to chemoattract neutrophils unless the ELR motif is inserted into the amino acid sequence (Taub, 1996). Because of their chemotactic properties on immune cells, chemokines are clearly involved in leukocyte trafficking. They have been extensively studied *in vitro* for their potent chemoattractant activities for various white blood cell populations although their chemotactic potential is not limited to immune cells e.g. IL-8 is chemotactic for melanoma cells *in vitro* (Ming Wang *et al*, 1990). In general CXC chemokines chemoattract neutrophils (with the above exceptions) whereas C-C chemokines attract mainly monocyte and lymphocyte populations. The β chemokines also have specificity for T cells subsets where MIP1α preferentially attracts CD8+ T cells and MIP1β preferentially attracts CD4+ T cells (Taub *et al*, 1993), even though the MIP proteins share 70% homology. Leukocyte trafficking is now only a small part of the discovered roles played by chemokines. Other known biological functions include angiogenesis and haematopoiesis (Taub, 1996).

Stimulation of angiogenesis by CXC chemokines appears to depend on the presence of the ELR motif and in fact the non ELR CXC chemokines, IP-10 and MIG inhibit the angiogenic response (Keane *et al*, 1998). As a group, these molecules function in the recruitment and activation of leukocytes and other cells at sites of inflammation and therefore are important inflammatory mediators.

1.4.2 Chemokine Receptors

As the array of discovered chemokines has expanded, so has the discovery of their receptors. The chemokine receptors are a family of seven transmembrane spanning glycoproteins (Figure 1.3) that are structurally related, the lowest amino acid similarity between 2 receptors being 30%.

Figure 1.3. Schematic diagram of chemokine receptor CCR1 (Adapted from Bertini *et al*, 1994).



The receptor traverses the cell membrane seven times to form 6 loops.

The structure is based upon the distribution of their conserved sequences and the known structure of the seven transmembrane receptor for rhodopsin. The N termini of the receptors are extracellular and the C termini intracellular with the seven hydrophobic domains passing as α helices through the membrane, forming 6 loops (Murphy, 1996).

The chemokine receptors are mainly divided into α and β chemokine receptors but there is also the Duffy antigen on erythrocytes (see table 1.1) which is a promiscuous chemokine binding protein, with α and β chemokine ligands (Murphy, 1996, Premack and Schall, 1996, Wells *et al*, 1998). The fourth type of chemokine receptor are the virally encoded receptors found in the gamma herpesviruses and these have multiple shared ligands (Premack and Schall, 1996).

The β chemokine receptors are located on human chromosome 3 whereas the α chemokine receptors, CXCR1, CXCR2 and CXCR4 are clustered on chromosome 2 (Premack and Schall, 1996). There is also now identified, a receptor for the CX_3C chemokine, fractalkine and as both chemokine and receptor are membrane bound, it is thought they may act as adhesion molecules as well as mediators of leukocyte chemotaxis (Imai *et al*, 1997, Wells *et al*, 1998). Some receptors identified to date, appear to bind only one chemokine whereas others e.g. CCR3 and CCR4 bind many different chemokines. This suggests that in the inflammatory response, cells expressing a particular receptor may respond to many different chemokines expressed at the inflamed site. The fact that some chemokines can bind to more than one type of receptor may have come about by the use of chemokine receptors by viruses to infect cells and the overlapping ligand specificities may reflect host adaptation to viral infection (Premack and Schall, 1996). Displacement experiments *in vitro* have shown that receptors show a preference for one chemokine over another. CCR1 was transfected into a human embryonic kidney cell line and was shown to bind the chemokines, MCP1, MIP1α/β and

Table 1.1 Chemokine receptor families. (Taken from Wells *et al*, 1998)

α Chemokine receptors	
RECEPTOR	LIGAND
CXCR-1	IL-8 GCP 2
CXCR-2	IL-8, GROα β γ, ENA-78, NAP-2
CXCR-3	IP-10, MIG
CXCR-4	SDF-1β
CXCR-5	BCA-1
β Chemokine Receptors	
RECEPTOR	LIGAND
CCR-1	MIP1α, β, MCP-3, RANTES, MCP-1
CCR-2	MCP-1-5
CCR-3	Eotaxin, RANTES, MCP-3, MCP-4
CCR-4	MIP-1α, RANTES, MCP-1, TARC, MDC
CCR-5	MIP-1α, β, RANTES
CCR-6	MIP-3α
CCR-7	MIP-3β, SLC
CCR-8	I-309
CCR-9	CC chemokines
Erythrocyte chemokine receptors	
RECEPTOR	LIGAND
DARC (Duffy antigen)	RANTES, MCP-1, IL-8, GRO, NAP-2
CX3C chemokine receptor	
RECEPTOR	LIGAND
CX3CR-1	Fractalkine

RANTES. All the chemokines tested were able to displace radiolabelled MIP1α from receptors even though MIP1α was the chemokine that bound with the highest affinity (Neote *et al*, 1993).

It may seem impossible to believe that a chemokine could cause directed migration *in vivo* with such a confusing array of chemotactic signals but it is thought that leukocytes can prioritise their responses. The proposed mechanism for this, is receptor crosstalk with an agonist hierarchy, and was demonstrated by chemotaxis experiments where the migration of neutrophils in response to IL-8 was suppressed by the presence of a different chemoattractant peptide, f-met-leu-phe (fMLP) (which binds to a different receptor), but IL-8 could not suppress migration of neutrophils towards fMLP (Campbell *et al*, 1997). Furthermore, neutrophils have been shown to migrate up a concentration gradient to one chemoattatractant and then migrate down that concentration gradient to migrate towards a distant chemoattractant showing 'multistep navigation' of cells in a complex environment (Foxman *et al*, 1997).

1.4.3 Chemokine receptor signalling

Binding of the ligand to its receptor on a cell causes the release of intracellular second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, via G protein complexes. This in turn causes an increase in intracellular free calcium within the cell, resulting in reorganisation of the actin cytoskeleton, formation of focal adhesions and pseudopod extension, which ultimately leads to movement of the cell. Chemokine signalling also involves activation of the GTP binding proteins, Rac and Rho (Premack and Schall, 1996). Rac is involved in actin reorganisation events such as membrane ruffling and Rho activation induces formation of stress fibres and focal adhesions (Machesky and Hall, 1996).

The suggestion of chemokine receptors being linked to G proteins is confirmed by experiments showing Pertussis toxin sensitivity of chemokine signalling *in vitro* (Youngs *et al,* 1997, Cross *et al,* 1997).

1.4.4 Chemokines in disease.

Chemokines have been implicated in a range of acute and chronic pathological inflammatory conditions. There is a complex cascade of inflammatory events in many of these disorders mediated by various leukocytes expressing chemokine receptors attracted into the inflamed site by the effects of chemokines (Baggoilini, 1998). A number of CC and CXC chemokines including IL-8, ENA-78, MCP1, MIP1α and RANTES, have been found in joint fluid in rheumatoid arthritis (RA). RA is characterised by joint inflammation and experimentally induced joint inflammation in animals could be reduced by immunisation with anti MIP1α antibodies (Strieter *et al,* 1996). Upregulation of IL-8 and MCP1 expression in histological sections of lung and broncheolar lavage fluid has been found in a number of respiratory disease states including idiopathic pulmonary fibrosis, active sarcoidosis and bronchial carcinoma, with increased numbers of monocytes and neutrophils being present (Taub and Oppenheim, 1994). An increase in chemokines that chemoattract eosinophils, MIP1α, RANTES and MCP3 have also been identified in bronchoalveolar lavage fluids from asthma sufferers (Keita and Gleich, 1996). Chemokines are also thought to be involved in atherosclerosis as high levels of MIP1α, MIP1β and RANTES have been found in atherosclerotic lesions (Taub and Oppenheim, 1994) and *in vitro* studies demonstrate that minimally modified low density lipoprotein induces MCP1 production in endothelial and smooth muscle cells, components of the arterial wall (Edgington, 1993). The acute and chronic inflammatory conditions involving high levels of chemokine expression also include gout, glomerular

nephritis, endometriosis and psoriasis (Petrek *et al*, 1995). Diseases associated with chemokine involvement also include many CNS conditions.

1.4.5 Chemokines in CNS disease

Because of the role of chemokines in inflammatory conditions, it is now well documented that they also have a role in inflammation in the CNS (Ransohoff *et al*, 1996, Ransohoff and Tani, 1998). Increased cerebrospinal fluid (CSF) levels of MIP1α have been detected in patients with the neurological inflammatory disorders, neuro-Behcets disease, HTLV-1 associated myelopathy and aseptic meningitis, compared to controls with non inflammatory neurological diseases (Miyagishi *et al*, 1995). Cerebral ischaemia is followed by leukocyte accumulation and following occlusion of the middle cerebral artery in the rat, levels of IP-10 mRNA were elevated with a peak level at 6 hours suggesting a role in leukocyte recruitment after stroke (Wang *et al*, 1998). MCP1 and GROα have also been found to be expressed in response to cerebral ischaemia, consistent with a role in neutrophil and macrophage chemoattraction to lesions (Ransohoff and Tani, 1998). The inflammation that occurs after CNS trauma may also be due to the expression of chemokines, as demonstrated by brain stab wound injury in the rat which showed increased MIP1β and RANTES expression 3 days after injury and correlated with the influx of monocytes and lymphocytes (Ghirnikar *et al*, 1996). MCP1 expression was found just 3 hours after mechanical CNS injury before the appearance of inflammatory leukocytes and co-localised to reactive astrocytes, which may be involved in the monocyte recruitment following trauma (Ransohoff and Tani, 1998).

MIP1α expression by glial cells in the human brain has been shown to be present in patients with schizophrenia but not in patients with other neuropsychiatric disorders including manic depressive illness and cerebral vascular dementia (Ishizuka *et al*, 1997)

which may account for the increased density of microglia in the temporal and frontal lobe of chronic schizophrenics (Radewicz *et al*, 1998). Others have shown increased MIP1α expression in neurons and MIP1β expression in reactive astrocytes in Alzheimer's disease brain compared to control brain, as well as elevated IP-10 expression by some reactive astrocytes (Xia and Hyman, 1999).

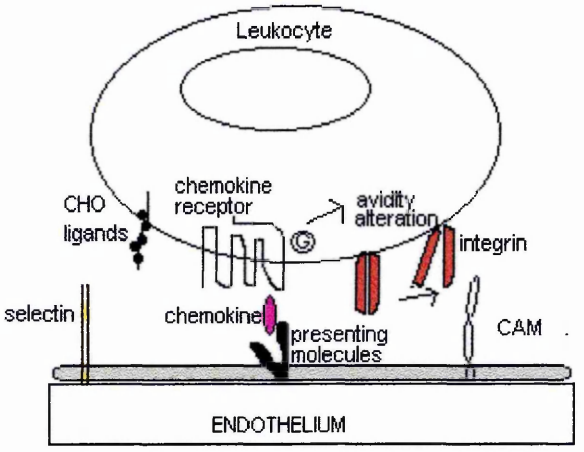
1.4.6 Chemokines in multiple sclerosis and experimental autoimmune encephalomyelitis

Inflammatory cell recruitment into the CNS is a major feature of MS and chemokines are thought to be major chemoattractants in the CNS, due to the discovery of their expression and *in vitro* activities on CNS cell types. T cell migration into the CNS has been suggested to be due to chemokines activating integrin-mediated adhesion of leukocytes to the endothelium (Karpus *et al*, 1995). This suggestion is supported by observations that chemokines bind to glycosaminoglycan sub-populations on endothelial cells which may present the chemokine molecules to selectin bound leukocytes (Witt and Lander, 1994, Taub, 1996) (see figure 1.4).

One of the characteristics of EAE is mononuclear cell infiltration into the CNS and studies on mice by Karpus *et al*, (1995) show that MIP1α but not MCP1 production correlates with increasing disease severity. However, at the height of clinical disease in a rat EAE model induced by guinea pig spinal cord myelin, MCP1 was shown to be expressed by endothelial cells, perivascular infiltrates and astrocytes and the expression was considerably reduced following recovery (Berman *et al*, 1996). Glabinski *et al* (1997) showed a dramatic increase in chemokine mRNA and protein expression of MCP1, IP-10, GROα, RANTES and MIP1α during relapse in chronic EAE in mice. MCP1, IP-10 and GROα were expressed by astrocytes surrounding inflammatory lesions

and RANTES and MIP1α expression by leukocytes was identified at sites of inflammation.

Figure 1.4. Chemokine receptor interactions.



The presentation of chemokine to a specific receptor on a selectin bound leukocyte induces a series of intracellular signals. Binding to the chemokine receptor, linked to a G protein is believed to activate integrin adhesiveness. These signals also induce the release of enzymes which may facilitate transmigration and chemotaxis through the BBB. (Presenting molecules = glycosaminoglycans, CHO = carbohydrate, G = G protein, CAM = cell adhesion molecule.)

Active EAE lesions induced in rats, showed a dramatic increase in expression of the chemokines, RANTES, MIP1α and MIP1β at the peak of clinical disease, predominantly being expressed by infiltrating T cells but macrophages/amoeboid microglia and astrocytes were also found to express MIP1β and RANTES (Miyagishi *et al*, 1997).

Elevated levels of MIP1α have also been detected in the CSF of MS patients correlating with relapse of the disease (Miyagishi *et al*, 1995) as well as an increase in RANTES, MIG and IP-10 in the CSF of MS patients when compared to normal controls

(Sorensen *et al*, 1999). Studies by Hayashi *et al*, (1995) showed LPS induced MIP1α production from microglial cells and that MCP1 and another chemokine, T cell activation gene-3 (TCA 3) induced chemotaxis of microglia *in vitro*. Their findings also suggest that microglia and astrocytes differentially produce chemokines in the CNS and that astrocytes and T cells may recruit and activate microglial cells by production of C-C chemokines. Simpson *et al* (1998) have shown RANTES expression on the blood vessel endothelium in active MS plaques and that MCP1 is expressed by astrocytes and macrophages in the chronic MS lesion. MIP1α and MIP1β were shown to be expressed by astrocytes and microglia respectively. Similarly, cells resembling reactive astrocytes distributed throughout the demyelinated lesion in active demyelinating MS lesions were strongly stained for MCP1, although no positive staining on infiltrating macrophages or lymphocytes was apparent (Van Der Voorn *et al*, 1999).

In vitro studies have shown that myelin proteolipid protein (PLP) specific CD8+ cytotoxic T lymphocyte (CTL) lines express increased amounts of MIP1α and MIP1β in response to stimulation with the appropriate peptide/MHC complex (Biddison *et al*, 1997) and their production may promote the inflammatory response in MS by further recruitment of inflammatory cells and resident macrophages/microglia. Human foetal microglia have previously been shown to migrate *in vitro* to the chemokines MIP1α, MIP1β and MCP1 (Peterson *et al*, 1997) as well as the classical chemoattractant C5a (Yao *et al*, 1990), also produced during the inflammatory process.

1.4.7 Chemokine expression by microglia *in vitro*

Chemokine expression by microglia has been examined on freshly isolated microglia and microglial cell lines. Sun *et al* (1997) examined the *in vitro* mRNA expression of MCP1, MIP1α, RANTES and IP-10 by microglial cell lines, immortalised

by culturing freshly isolated mouse glial cells in macrophage colony stimulating factor (M-CSF) and although only MCP1 was sometimes detected in resting cells, all the other chemokines were expressed after lipopolysaccharide (LPS) stimulation. MIP1α mRNA expression by a microglial cell line (BV-2) was also found to be induced by phorbol 12 myristate 13 acetate (PMA) as well as LPS, although the proinflammatory cytokines TNFα and IL-1β had no effect (Murphy *et al*, 1995). LPS induction of MIP1α expression by mouse primary microglia has been confirmed, but the microglial cultures did not express MCP1 either before or after LPS stimulation in contrast with primary astrocyte cultures which expressed MCP1 following stimulation (Hayashi *et al*, 1995). Conversely, Calvo *et al* (1996) showed MCP1 mRNA expression in unstimulated rat microglia which was upregulated by LPS and also by IL-1β, TNFα and IL-6, however these differences may be accounted for by species differences. Human microglia *in vitro* expressed the β chemokines, MIP1α, MIP1β and MCP1 and expression was shown to be increased when cells were stimulated with LPS, TNFα and IL-1β with LPS having the most pronounced effect (Peterson *et al*, 1997) The α chemokine, IL-8 has also been found to be expressed by human microglia following LPS and IL-1β stimulation and to a lesser extent with TNFα stimulation (Ehrlich *et al*, 1998).

1.4.8 Chemokine receptor expression in the normal and diseased CNS

The response of cells to chemokines is dependent on their expression of the appropriate receptor. Many chemokine receptors have been found to be expressed in the brain under physiological and pathological conditions including CXCR2, CXCR3, and CXCR4 expression on neurons and CCR-3 and CCR-5 on microglia (Xia and Hyman, 1999). Microglia and astrocytes in culture have also been shown to constitutively express

CXCR4 although only microglia responded in chemotaxis experiments to the ligand, SDF-1 (Tanabe *et al*, 1997). Microglia also express CX3CR1, the receptor for fractalkine which is thought to be involved in signalling from neurons to microglia, as neurons are the predominant cell type expressing fractalkine in the CNS and CX3CR1 was upregulated on microglia following facial nerve axotomy in the rat (Harrison *et al*, 1998).

T helper (Th) cell subsets have been shown to differentially express chemokine receptors. Th1 cells expressed mainly CXCR-3 and CCR-5, whereas Th2 cells expressed mainly CCR-4 (Bonecchi *et al* 1998, Sallusto *et al*,1998). Other receptors identified in SIV infected macaque CNS tissue include, CXCR4 expression by glial cells and CXCR-3 expression by infiltrating lymphocytes (Westmoreland *et al*, 1998).

Chemokine receptor expression has been examined in many CNS diseases and in particular in AIDS, due to the discovery that the HIV virus utilises chemokine receptors to infect microglia. Microglia are the targets in HIV infection of the brain and the response of the migroglia to viral infection is thought to stimulate the release of neurotoxic substances such as reactive nitrogen species leading to the symptoms of AIDS dementia (Balter, 1996). HIV viruses infecting macrophages are known as macrophage tropic viruses and mainly utilise CCR5 as a co receptor for entry into cells (He *et al*, 1997). CCR3, expressed by microglia, was also shown to be a co receptor for HIV infection in primary brain cultures as the CCR3 ligand, eotaxin and the CCR5 ligand, MIP1β as well as an antibody against CCR3 inhibited 70-80% of microglial infection (He *et al*, 1997). Sheih *et al* (1998) reported that CCR5 was the major co receptor for HIV in primary cultures of adult microglia with antibodies to CCR3 having no effect in reducing the levels of infection. The difference in results was explained by the fact that other cell types present in the brain cultures used by He *et al*, may have

affected the results and also that the maturation state of the microglia may affect the levels of receptor expression.

Reports on chemokine receptor expression in the CNS has since led on to studies of other disorders such as Alzheimer's disease, where CCR3 and CCR5 are upregulated on some of the reactive microglia surrounding amyloid deposits (Xia and Hyman, 1999). In MS, chemokines are thought to play an important role in the recruitment of inflammatory cells. Recently, MIP1α and MCP1 receptors were discovered on the endothelium on the abluminal surface of microvessels and although their function is unclear, it is thought that they may prevent chemokines from being diluted, augmenting the intensity and duration of the response at the site of inflammation (Andjelkovic *et al*, 1999).

1.5 Matrix metalloproteinases

Degradation of the extracellular matrix (ECM) by proteinases is important in many physiological processes in the CNS including tissue remodelling in development and cell migration. There are four classes of proteinases divided into two subgroups: these are the aspartic and cysteine, acid proteinases and the serine and metallo proteinases which are optimally active at neutral pH.

The matrix metalloproteinases (MMPs) are a family of zinc dependant enzymes capable of degradation of ECM proteins. MMPs, of which there are at least 18 members to date, can be further divided depending on their size and substrate specificity, into collagenases, gelatinases, stromelysins and membrane type MMPs (Yong *et al* 1998). The MMPs and their substrates are listed in Table 1.2.

Table 1.2 Matrix metalloproteinase groups and their substrate specificities. Adapted from Chandler *et al*, 1997.

MMP sub-group	Member	Main substrates
Collagenases	Interstitial collagenase (MMP1)	fibrillar collagens
	Neutrophil collagenase (MMP8)	fibrillar collagens
	Collagenase 3 (MMP13)	fibrillar collagens
	Collagenase 4 (?)	unknown
Gelatinases	Gelatinase A (MMP2)	Gelatin, type IV, V collagens, fibronectin
	Gelatinase B (MMP9)	Gelatin, type IV, V collagens, fibronectin
Stromelysins	Stromelysin 1 (MMP3)	Laminin, non fibrillar collagen, fibronectin
	Stromelysin 2 (MMP10)	Laminin, non fibrillar collagen, fibronectin
	Matrilysin (MMP7)	Laminin, non fibrillar collagen, fibronectin
	Stromelysin 3 (MMP11)	α1 proteinase inhibitor (serpin, serine proteinase inhibitor)
MT-MMPs	MT1-MMP (MMP14)	Pro MMP2, collagens, gelatin
	MT2-MMP (MMP15)	Pro MMP2, collagens, gelatin
	MT3-MMP (MMP16)	Pro MMP2, collagens, gelatin
	MT4-MMP (MMP17)	Pro MMP2, collagens, gelatin
Others	Metalloelastase (MMP12)	Elastin
	Enamelysin (?)	unknown
	Xenopus MMP (?)	unknown
	? (MMP19)	Aggrecan

1.5.1 Structures of matrix metalloproteinases

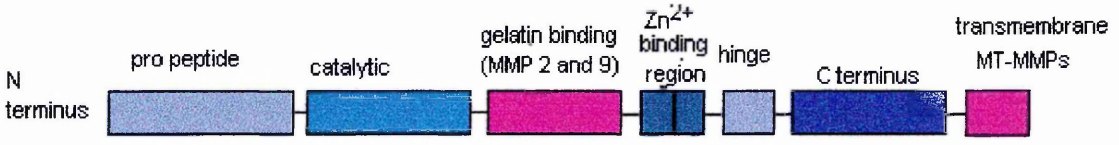
The MMP family members share many similarities in structure and biochemistry. The cloning of several family members has shown that they share amino acid sequence homologies. The catalytic domains of MMPs contain two zinc atoms which are required to maintain their three dimensional conformation and activity (Figure 1.5). Most are secreted as inactive zymogens which require cleavage, proteolytically or by treatment with mercurial compounds such as P-aminophenylmercuric acetate (APMA) The active enzymes are tightly controlled by naturally occurring inhibitors called Tissue Inhibitors of Metalloproteinases (TIMPs). The potential activities of MMPs are tightly controlled in several ways (Yong *et al*, 1998):

a) at the level of gene expression;

b) activation of the zymogen;

c) production of specific protease inhibitors.

Figure 1.5 The domain structure of the matrix metalloproteinases Adapted from Massova *et al* (1998)



The schematic diagram shows the basic domain structure. The catalytic domain has an insertion of fibronectin like repeat in MMPs 2 and 9 and MT-MMPs have a transmembrane region.

The N terminus pro peptide region contains approximately 80 amino acids and is found in all MMPs. The C terminus domain is shared by all MMPs, except MMP7 (matrilysin), and is similar in sequence to the hemopexin family of glycoproteins (Massova *et al*, 1998). In the case of the collagenases and stromelysin 1, the hemopexin like domain is essential for binding to native collagens and in all of the MMPs it is involved in initial interactions with their specific inhibitors, the TIMPs (Murphy and Knauper, 1997). The C terminus is linked by a hinge region which is shorter in collagenases than in other MMPs (Yong *et al*, 1998).

The MT-MMPs are the only members of the MMP family that are localised on the cell surface by the presence of a membrane spanning domain and are involved in the cleavage and activation of pro MMP2 (Sato *et al*, 1994). MMP14 was first cloned from human placenta and has also been detected in a mouse fibroblast cell line and human lung tumours (Sato *et al*, 1994) as well as in white matter microglia in human brain tissue (Yamada *et al*, 1995a).

The gelatinases contain a gelatin binding domain which is an insert of three tandem repeats of fibronectin type II like modules in the catalytic domain. This allows high affinity binding to collagen type I (Goetzl *et al*, 1996) and to denatured type IV and V collagens (Murphy and Knauper, 1997).

1.5.2 Control of matrix metalloproteinase expression

Most MMPs are not constitutively expressed and their production is regulated by cytokines in a complex network which also regulates the production of their inhibitors (Opdenakker and Van Damme, 1994). Johnatty *et al* (1997) demonstrated the regulation of pro-MMP9 production by the chemokines MIP1α, MIP1β and RANTES in human peripheral blood lymphocytes. In most cases the stimuli induce the expression of

activator protein 1 (AP1) transcription factors which bind to promoters controlling transcription. The promoter regions of the collagenases, stromelysins and MMP9 contain the AP1 binding site whereas MMP2 (and MMP11) does not, and it is suggested that it may be constitutively expressed for this reason (Yong *et al* 1998). The AP1 binding site is located between -65 and -79 base pairs upstream from the transcriptional start site and transcriptional activity in response to cytokines is thought to also involve interactions with other cis acting sequences located in the promoter sequences (Benbow and Brinkerhoff, 1997).

1.5.3 Activation of matrix metalloproteinases

The inactive pro enzymes contain a pro peptide region which is bound by a cysteine residue to the zinc atom in the active site. Disruption of this so called 'cysteine switch' is essential for activation, forming a partially active intermediate form, from which the pro peptide may then be cleaved (Birkedal-Hansen, 1995). Cleavage of the pro peptide may be by autocatalysis or by the actions of other MMPs or proteases. Activation of MMP2 occurs at the plasma membrane where it is cleaved by MT-MMP. Other MMPs may be cleaved by the serine protease, plasmin, itself secreted as a pro enzyme requiring activation (Vassali and Pepper, 1994). MMPs may also be activated by bacterial proteases and also by reactive nitrogen species which interact with the cysteine switch (Maeda *et al*, 1998). MT-MMPs require removal of a pro peptide for activation and this is catalysed by the serine protease, furin (Sato *et al*, 1996).

1.5.4 Inhibition of matrix metalloproteinase activity by tissue inhibitors of metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) are a family of four, so far identified, naturally occurring inhibitors ranging from 21-28 kDa in size. TIMPs 1, 2 and 4 are secreted and TIMP3 is predominantly associated with the extracellular matrix. TIMPs contain 12 highly conserved cysteine residues forming six disulphide bonds which are essential in maintaining the correct 3D structure of the molecule (Willenbrock and Murphy, 1994). The six loops formed by the disulphide bonds are divided into two distinct domains termed the C terminal domain and the N terminal domain. The 3D structure of stromelysin 1 bound to TIMP1 has been elucidated by X ray crystallography and showed that the wedge shaped TIMP slots with its edge into the active site cleft of the enzyme, occupying the entire length of the active site (Gomis-Ruth *et al*, 1997). MMPs may be regulated by binding to TIMPs in 1:1 non covalent complexes. Active MMPs bind to TIMPs to regulate proteinase activity and TIMPs also bind the pro forms of the gelatinases (MMP2 and 9), controlling the rate at which physiological factors activate MMPs. The complex formed by TIMP2 and pro MMP2 has been shown to be important in allowing pro MMP2 to associate with MT-MMPs (Birkedal-Hansen, 1995). All active MMPs can also be inhibited by α_2 macroglobulin (Cawson, 1998).

1.5.5 The Extracellular matrix of the CNS

The extracellular matrix (ECM) of the CNS is a complex meshwork of proteins and proteoglycans that provide structural support and influence many biological activities such as cell adhesion and migration (Carbonetto, 1984). It comprises of up to one fifth of the volume of the CNS and consists mainly of proteoglycans (Sobel, 1998). Versican, a product of glial cells in the adult CNS, is one of the largest members of a chondroitin

sulphate proteoglycans family, abundant in the CNS white matter, which can be broken down to glial hyaluronic acid binding protein (GHAP) by MMPs. GHAP is lost in chronic MS plaques (Sobel, 1998). A critical role is played by activated macrophages. including microglia in the brain, in their ability to regulate ECM turnover, especially in inflammation (Gottschall *et al*, 1995). MMPs expressed by microglia, endothelial cells and infiltrating cells are involved in remodelling of the ECM following CNS injury. Breakdown of the BBB in MS with leukocyte infiltration, causes plasma components, not normally associated with the CNS, to become part of the CNS ECM therefore the ECM is altered during the course of the disease. This alteration in ECM deposition in the CNS may form a 'scaffold' for leukocyte and microglial migration within the parenchyma and is likely to depend upon protease secretion (Sobel, 1998). The ECM components themselves are thought to influence MMP secretion by macrophages, influencing their own breakdown, e.g. exposure of human alveolar macrophages exposed to denatured collagens in culture, stimulated the expression of interstitial collagenase (Shapiro *et al*, 1993).

1.5.6 Matrix metalloproteinases produced by microglia

Production of MMPs by microglia has been studied *in vitro* and has shown that rat brain microglia constitutively produce the highly controlled MMP9 which can be upregulated by treatment of the cells with bacterial LPS (Gottschall *et al*, 1995). Colton *et al* (1993) also found that microglia produced MMP9 as well as MMP2 by analysis of culture supernatants. Microglia have been shown to constitutively express MT-MMP 1 with only a slight increase in expression in Alzheimer's disease brain (Yamada *et al*, 1995a).

1.5.7 Matrix metalloproteinases and tissue inhibitors of metalloproteinases in CNS disease

There is growing evidence that MMPs are involved in many CNS pathologies, in particular, the gelatinases. Studies on human CNS tissue from stroke patients, dying at time intervals from 2 hours to several years after the ischaemic event, were carried out by analysing the gelatinase levels in protein extracts. This revealed that MMP9 was increased maximally in the cases dying after 2-4 days whilst MMP2 activity was maximally increased in cases dying at longer post stroke intervals (Clark *et al*, 1997). Both gelatinases persisted for many months after the event and since microglia are also locally abundant, it is postulated that microglia are the major cell type contributing to the gelatinase expression. Anthony *et al* (1997) also demonstrated the presence of MMP9 positive macrophages in infarcted tissue although the predominant cell type expressing MMPs 2 and 9 were infiltrating neutrophils. Microglial activation was noted but little MMP2 or matrilysin staining in these cells was observed in the cases examined. A study on MMPs in cerebral aneurysms also demonstrated their importance in the pathological process in haemorrhage and showed MMP9 localisation in focal areas within aneurysms (Bruno *et al* 1998).

The gelatinases and other MMPs have long been implicated in tumour invasion and metastasis and they are overexpressed in tumours of the CNS, although it is difficult to define whether the MMPs are expressed by the tumour cells or other tumour associated cells e.g. macrophages (Rooprai and McCormick, 1997). CSF analysis of patients with malignant astrocytomas or brain metastases showed that it contained pro MMP9 which was not detected in control patients and it was suggested that this could be used for early diagnosis of brain tumours (Friedberg *et al*, 1998).

MMPs are also associated with Alzheimer's disease, as microglia expressing MMP2 are closely associated with amyloid β deposits. It is not clear whether microglia contribute to its deposition or prevent deposition by degrading amyloid precursor protein derived from damaged neurons (Yamada *et al*, 1995b). Other groups have recently shown that metalloproteinases expressed by microglia can degrade amyloid β in cultures, but the enzymes may not be members of the matrix metalloproteinase family (Qiu *et al*, 1997, Mentlein *et al*, 1998)

1.5.8 Matrix metalloproteinases and tissue inhibitors of metalloproteinases in MS and EAE

There is much evidence for the involvement of MMPs and TIMPs in MS. MS pathology is characterised by BBB breakdown, myelin loss and recruitment of inflammatory cells, all of which are thought to involve the actions of proteases. The importance of MMP production in MS is that it has a possible role in microglial migration to the inflamed site and MMPs have also been shown to cleave myelin basic protein (MBP) *in vitro*, which is the major constituent of the myelin sheath (Proost *et al*, 1993). MMP9 is capable of digestion of MBP, a major component of the myelin sheath (Gijbels *et al* 1993) creating MBP fragments and at least one major encephalitogen was found to be released (Proost *et al*, 1993). MMP9 is thought to contribute to breakdown of the BBB and has been detected at elevated levels in serum from MS patients, compared to normal healthy controls (Lee *et al*, 1999). Serum levels were found to be higher during clinical relapse. MMP9 is also elevated in CSF samples from MS patients compared to healthy controls that had undetectable MMP9 levels in their CSF, although within the MS group, levels were found to be comparable between relapses and clinically stable periods of the disease (Leppert *et al*, 1998).

Immunohistochemical studies on MS and EAE brain have detected immunoreactivity for MMPs 2, 3, 7, and 9, although only MMPs 2 and 7 were observed in normal human brain by Anthony *et al* (1997), whereas Cuzner *et al* (1996) observed MMP2 and 9 in normal brain with increased expression by macrophages and microglia in active MS plaques. Furthermore, injection of MMP2 into rat brain has been shown to cause leakage and destruction of the BBB (Chandler *et al*, 1997). MMP7 was found to be strongly upregulated in animals with EAE, coincident with the peak of clinical disease (Clements *et al*, 1997, Kieseier, 1998). MMP 9 has been shown to exhibit strong positive staining around CNS blood vessels in active demyelinating MS lesions (Cossins *et al*, 1997) and in inflammatory lesions in EAE induced by adoptive transfer (Kieseier *et al*, 1998).

The levels of both MMPs and TIMPs are important in the balance between lytic and anti-lytic activities and their distribution in CNS pathology must be considered. Studies on an SWR mouse model of EAE showed an increase in the expression of both MMP genes and TIMP1 by RNase protection assays on total RNA extracted from brain and spinal cord (Pagenstecher *et al*, 1998). Inflammatory lesions containing the MMP expressing mononuclear cells were surrounded by astrocytes that expressed the TIMP 1 gene and it is suggested that this may confine the extent of tissue destruction and retard the migration of the inflammatory cells throughout the brain parenchyma (Pagenstecher *et al*, 1998). The beneficial effects of TIMP expression in MS, apart from MMP inhibition, may also be due to their growth promoting activities demonstrated *in vitro* (Hayakawa *et al*, 1992, Hayakawa *et al*, 1994) which could be important in remyelination. Despite the links between MMP9 and demyelination, it has also been discovered that oligodendrocytes stimulated with phorbol esters synthesise MMP9, which is utilised for process elongation. This is important in the developing CNS and this

may also have implications for attempts at remyelination in remission phases of MS (Uhm *et al*, 1998). The levels of TIMP 1 and 2 have been assessed in patients with relapsing remitting MS and compared to healthy controls and patients with other CNS pathologies, with an inflammatory response. Mean TIMP 1 levels were lower in MS patients than in patients with other CNS inflammatory conditions but higher than healthy controls, whereas TIMP 2 levels were higher in MS patients than both control groups (Lee *et al*, 1999). With the knowledge that TIMP 1 preferentially binds to MMP9 and that MMP9 levels are increased, this may suggest that the ineffective inhibition of MMP9 activity contributes to an overall lytic activity in MS.

1.5.9 Matrix metalloproteinase inhibition as a treatment for MS

The correlation between MMP activities and the pathology of the MS lesion has led to a growing amount of research on inhibitors of MMP activity as potential therapies. Suppression of actively induced EAE in Lewis rats was observed in 9 out of 10 animals after treatment with the MMP inhibitor, Ro31-9790, whereas 9 out of 10 of the group of animals receiving vehicle alone showed clinical signs of EAE (Hewson *et al*, 1995). An anti-rheumatic drug, D penicillamine, was found to effectively inhibit MMP2 and 9 activity in the CSF of MS patients and also decreased the severity of relapse in a chronic relapsing EAE induced in Biozzi mice, when treated after the first episode (Norga *et al*, 1995). MS patients suffering acute relapse, when treated with the steroid, methylprednisolone, show reduced leakage of the BBB and analysis of MMPs and TIMPs in the CSF showed a decrease in MMP9 levels after steroid treatment and an increase in TIMP levels, suggesting that this may be the mechanism of steroid action (Rosenberg *et al*, 1996). Another drug used in the treatment of MS, Interferon β-1b, was shown to decrease the *in vitro* migration of activated T lymphocytes through fibronectin

coated membranes and reduce their ability to degrade fibronectin. Treatment of the T cells with IFN β-1b also reduced their MMP9 activity and it is proposed that the decrease in cell migration was due to the effects on MMP9 activity (Stuve *et al*, 1996). The mechanism of action for IFN β-1b is thought to be by the reduced expression of IL-2 receptor on the cell surface. IL-2 increases MMP expression by T cells and enhances migration *in vitro* which can be reduced by pre-treatment with IFN β-1b (Leppert *et al*, 1996).

1.5.10 The plasmin/ plasminogen activator system in matrix metalloproteinase activation

The control of MMP activity in part, is by proteolytic cleavage of the proenzyme of which the serine protease plasmin, plays a major role. Plasmin itself is secreted as the proenzyme, plasminogen, which is cleaved to its active form by the plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). tPA is the major circulating activator whereas uPA operates as a major cell surface activator when bound to its receptor, uPAR (Chapman, 1997). Plasminogen activators themselves are also regulated, similarly to MMPs by natural inhibitors, PAIs. tPA and PAI-1 have been found to be expressed by infiltrating mononuclear cells in MS white matter and primary lesions with a lack of tPA expression in control brain (Cuzner *et al* 1996). The expression of both enzyme and inhibitor emphasises the tight control of enzyme activity and the complexity of the activation pathways. Rat microglia in culture are a source of both uPA and plasminogen itself and stimulation with LPS was found to increase plasminogen secretion and decrease uPA secretion into conditioned medium (Nakajima *et al* 1992a,b). uPAR is also expressed on the surface of microglia and is upregulated in the MS lesion (Washington *et al*, 1996). The expression of plasminogen, uPA and uPAR by microglia are thought to have several implications in the development

of MS lesions. As well as their role in proteolysis and activation of MMPs, they also play a complex role in cell migration and/or adhesion (Blasi, 1997, Chapman, 1997). uPAR also functions as a receptor for vitronectin (Wei *et al* 1994) and the receptor is distributed at sites of focal adhesions in fibroblasts and at the leading edge of migrating monocytes (Gyetko *et al*, 1994). uPA itself is a chemotactic factor for leukocytes, endothelial cells and macrophages, requiring binding to uPAR (Blasi, 1997) An epithelial cell line was found to exhibit a three fold enhancement of migration by binding of exogenous uPA to uPAR expressed by the cells (Busso *et al*, 1994).

1.6 Nitric oxide in CNS physiology/pathology

Nitric oxide (NO) is formed from a reaction between molecular oxygen and L-arginine catalysed by nitric oxide synthase (NOS). It is highly reactive and is rapidly oxidised to nitrite and then to nitrate.

$$\text{L-arginine} + O_2 \rightarrow \text{L-citrulline} + NO^{\cdot}$$

There are three isoforms of NOS, all of which can be found in the CNS, these are endothelial (eNOS), constitutive (cNOS) and inducible iNOS (Rang *et al*, 1995). cNOS is found in neurons and is important in nerve transmission. It is calcium dependant and produces relatively small amounts of NO compared to iNOS which operates via a calcium independent mechanism (Smith *et al*, 1999).

iNOS is not constitutively expressed, as its name suggests and the induction of this isoform by cytokines *in vitro* has been reported in human astrocytes (Chao *et al*, 1995) and microglia (Ding *et al*, 1997). Additionally, iNOS mRNA was detected by Northern blot experiments in mRNA extracts of total human brain from MS patients and

was absent in control brain extracts from patients who had no neurological disease (Bagasra *et al*, 1995). The authors also reported that cells of the monocyte/macrophage lineage in MS brain sections expressed iNOS implicating NO, possibly macrophage derived, in MS pathology. There appears to be conflicting evidence as to whether nitrate measurement in CSF samples from MS patients is a good marker for diagnosis. Johnson *et al* (1995) reported an increase in nitrate levels in the CSF of MS patients whereas others (Ikeda *et al*, 1995, de Bustos *et al*, 1999) found no correlation between CSF nitrate in MS patients compared to normal controls or patients with non inflammatory neurological disorders.

Evidence suggests that the role of NO in CNS pathologies may be damaging. Minghetti *et al*, (1996) demonstrated that LPS induced PGE_2 release by cultured rat microglia could be increased by addition of an inhibitor of NOS or decreased by the addition of a compound that spontaneously releases NO. This may suggest a neuroprotective role for NO, although PGE_2 also has proinflammatory activities such as increasing vascular permeability. NO can have a wide variety of effects on cellular systems by altering protein structure and hence function. Several groups have reported on the ability of microglia derived NO to cause neuronal and oligodendroglial damage *in vitro*. Co-incubation of activated rat microglia with neurons resulted in neurotoxicity which was inhibited by the addition of an NO inhibitor (Boje and Arora, 1992). Similar results were also reported by Chao *et al* (1992), who showed that a ratio of 1 microglial cell to 200 neuronal cells was required to impair neuronal cell function. Merrill *et al* (1993) demonstrated the ability of IFNγ stimulated rat microglia to mediate cytotoxicity of oligodendrocytes, and this was significantly reduced by inhibitors of NOS. This suggests an important role for NO in demyelination in MS. Furthermore, MBP mRNA expression by an oligodendrocyte cell line was shown to be significantly inhibited by the

addition of S- nitroso, N- acetly- DL- penicillamine (SNAP), a chemical that spontaneously releases NO into cell cultures (Mackenzie-Graham *et al*, 1994).

Further evidence to suggest an involvement of NO in MS is the detection of iNOS in MS sections, with higher accumulations in areas containing plaques than areas without pathology (Hooper *et al*, 1997). The same group also showed that PLP induced acute EAE in mice was prevented by daily immunisation with an inhibitor of iNOS induction (D609) and that 24 hours after the last treatment, EAE developed. MMPs can also be activated by reactive nitrogen species which interact with the cysteine switch mechanism exposing the active site of the enzymes (Maeda *et al*, 1998). The expression of iNOS has been shown to be increased six fold in patients with AIDS dementia compared to seronegative controls (Adamson *et al*, 1996).

1.7 Superoxide in CNS pathology

Superoxide (O_2^-) is produced by macrophages in respiratory burst and itsproduction from molecular oxygen is catalysed by the membrane bound NADPH oxidase (Smith *et al*, 1999). This highly reactive species is commonly used as a microbicidal agent by macrophages but is also heavily implicated in CNS demyelination. Microglia are known to produce superoxide *in vitro* in response to PMA stimulation (Colton and Gilbert, 1987) which is increased by first priming the cells with cytokines such as TNFα (Hu *et al*, 1995) or IFNγ (Woodroofe *et al*, 1989, Hu *et al*, 1995). The priming effects of these cytokines on microglial superoxide production was found to be downregulated by addition of the anti inflammatory cytokine, TGFβ to cell cultures. (Hu *et al*, 1995). It has also been shown that phagocytosis of myelin by macrophages triggers the production of reactive oxygen species whereas phagocytosis of latex beads did not produce the same effect (van der Goes *et a,l* 1998).

Superoxide can combine with the highly reactive NO to form peroxynitrite with a half life of milliseconds (Beckman and Koppenol, 1996) and it is believed that many of the effects attributed to NO may be caused by formation of peroxynitrite and other agents derived from it such as peroxynitrous acid (Smith *et al* 1999). High intracellular levels of SOD provide some protection from superoxide and NO which competes with superoxide for SOD (Smith *et al*, 1999). Hartung *et al* (1988), however showed suppression of an EAE model in rats when treated with the hydrogen peroxide scavenger, catalase or superoxide dismutase. A peroxynitrite scavenger, uric acid, completely protected mice from PLP induced EAE when given a daily dose of 20mg, whereas all controls that did not receive uric acid became severely paralysed (Hooper *et al*, 1997). The study of superoxide and nitric oxide production by activated microglia, is important as their highly reactive nature is likely to cause tissue damage in the inflammatory response in the CNS.

Aims of this thesis

The overall aims of this thesis were to investigate the possible roles of the chemokines, present in multiple sclerosis plaques, on microglia *in vitro*. Previous research on chemokine function has mainly focused on their chemotactic effects on lymphocytes therefore we aimed to determine other effects on microglia as well as chemotaxis. The postulated functions of microglia include proteinase secretion, free radical production and phagocytosis, thus the roles of chemokines in modulation of microglial functions were of interest and will provide further knowledge on specific areas to target in the treatment of MS.

Chapter 2

ISOLATION OF MICROGLIA AND IMMUNOCYTOCHEMISTRY

2.1 Introduction

2.1.1 Isolation of microglia from rat CNS tissue

The most widely used method of microglia isolation is dependant on the survival and different adherence under culture conditions of the various CNS cell types and was first described by McCarthy and de Vellis (1980). This method is lengthy and it can take 7-10 days in culture before microglia cultures can be obtained. A different method was devised by Hayes *et al*, (1988) which exploits the expression of Fc receptors by microglia. In this method, tissue is dissociated mechanically with or without enzyme digestion to generate a mixed cell population containing the microglia. Opsonised erythrocytes are then used to 'rosette' the microglia which can be pelleted by density gradient centrifugation, removing contaminating astrocytes, oligodendrocytes and neurons. The erythrocytes are then removed by hypotonic lysis to achieve very pure cultures of microglia. Adult microglia isolated by Hayes *et al* (1988) lacked the non specific esterase activity, present in neonatal microglial cultures. As microglia isolated from 30 day old rats are more comparable to adult microglia than the neonatal microglia which may better represent amoeboid microglia, the 30 day old microglia have been used for the majority of experiments in this thesis. A more recent method has been developed by Sedgwick *et al*, (1991) which employs enzyme digestion, density gradient centrifugation followed by flow cytometry to select $CD45^{low}$ cell populations that were shown to be microglia. CD45 is a leukocyte common antigen and cells expressing high

levels of this in the populations of cells isolated were found to be inflammatory leukocytes and represented only a small population of the total percentage of cells.

Due to the relatively small number of microglia that are isolated from rat CNS and the lack of proliferation in culture, it was necessary to use a human microglial cell line as a possible comparison to results obtained from the primary rat microglia.

2.1.2 CHME3, microglial cell line

CHME3 cells (a kind gift from Prof. M.Tardieu, Universitè Paris Sud, Paris, France), are a human foetal microglial cell line derived from CNS tissue acquired from 8 week old embryos after elective abortion, from which microglia were isolated using a shaking and adherence method and transformed with a plasmid containing cDNA encoding the SV40 large T antigen to confer immortality. The CHME3 clone were found to express the markers, CD68/EBM-11, CD68/Ki-M7 and displayed non specific esterase activity. The cells did not constitutively express MHC II but the expression could be induced by treatment with IFNγ (100IU/ml) for 18 hours (Janabi *et al*, 1995). Transformed cells were also found to express IL-6 and display the macrophage characteristics of adherence and phagocytosis (Janabi *et al*, 1995).

2.1.3 **Aims**

1. To isolate pure cultures of neonatal and adult rat microglia and astrocytes to study *in vitro*.
2. To assess the purity of these cultures by immunocytochemistry using cell type specific antibodies.
3. To culture a microglial cell line for use as a comparison to rat microglia in future experiments.
4. To identify whether chemokines have any effect on proliferation of the cell line.

2.2 Materials and Methods

2.2.1 Culture media

Microglia, astrocytes, peritoneal macrophages and the cell line CHME3 were cultured in Dulbecco's Modified Eagles Medium (DMEM, Life Technologies, Paisley, U.K) supplemented with 10% heat inactivated foetal calf serum (Life Technologies), 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies). This complete media was then termed cDMEM. Cells were maintained at 37°C, 5%CO_2/95% air in a humidified environment.

2.2.2 CHME3 cell culture

CHME3 cells were grown in 75cm^2 flasks in cDMEM and passaged every 2-3 days. Briefly, after rinsing with phosphate buffered saline (PBS) (Life Technologies), cells were removed by the addition of 2ml of trypsin/EDTA (Life Technologies) for approximately 5 minutes followed by tapping the flask to remove any remaining adherent cells. Cells were then transferred to 50ml centrifuge tubes in cDMEM and centrifuged at 100g for 10 minutes. Cell pellets were resuspended in cDMEM and replated into new flasks. Cells were used between passages 3 and 12.

2.2.3 CNS Tissue

CNS tissue was obtained following cervical dislocation of 30, 30 day old Wistar rats of both sexes (purchased from Sheffield University, Field Laboratories). Cerebrae were dissected out and the meninges and large visible blood vessels removed by rolling on methanol sterilised filter paper (Whatman, U.K) in a lamina flow hood. CNS tissue

was also obtained from 20-50, 2-3 day old Wistar rats following decapitation, and the meninges removed as above. Tissue was used for isolation of microglia and astrocytes.

2.2.4 Enzymes

Stock solutions of 10X concentrated collagenase, DNase I and trypsin (Sigma, U.K) were prepared in Earles Balanced Salt Solution with calcium and magnesium ($EBSS^+$, Sigma) as shown below and filter sterilised by passing through a 0.22 μm filter (INC Flow Labs, U.K). Enzymes were stored in 10ml aliquots at -20°C.

Enzyme	**Concentration**
Collagenase type XI	1000U/ml
Deoxyribonucleate 5'-oligonucleotidohydrolase (DNase I), type II	200 μg/ml
Trypsin type III	1.25% w/v

2.2.5 Haemagglutination titration

A haemagglutination assay was performed to identify the concentration of rabbit anti human erythrocyte membrane antibody (Dako, U.K) required to cause agglutination of erythrocytes. The sub-agglutinating concentration was then chosen to opsonise erythrocytes used to isolate microglia. Approximately 10 ml of human peripheral blood was obtained by venipuncture into EDTA tubes and centrifuged at 180g (Sorvall RT 6000D centrifuge, DuPont, UK.) for 10 minutes at 4°C. A dilution series of rabbit anti human erythrocyte membrane antibody was prepared in duplicate in $EBSS^+$ to give final volumes of 250μl in a 96 well round bottomed microtitre plate.

Ab concentration										
1/ 50	1/ 100	1/ 200	1/ 300	1/ 400	1/ 500	1/ 600	1/ 700	1/ 800	1/ 1600	0

To each well, 5µl of erythrocytes, packed by centrifugation, was added to give a 2% v/v erythrocyte concentration. The plate was then incubated at room temperature for 30 minutes before being examined over a light box to select the sub-agglutinating dose. The dose of antibody required was determined as 1/600.

2.2.6 Opsonisation of erythrocytes (EA)

Approximately 10 ml of human peripheral blood was obtained by venipuncture into EDTA tubes. Blood was centrifuged at 180g (Sorvall RT 6000D centrifuge, DuPont, U.K, used throughout the procedures) for 10 minutes at 4°C. The plasma and buffy coat layers were removed and the erythrocytes resuspended in EBSS without calcium and magnesium (EBSS-) to 50ml. The cells were then centrifuged at 180g for 10 minutes at 4°C, resuspended in EBSS- the and the washing process repeated.

A 2% v/v suspension of erythrocytes in $EBSS^+$, was then incubated with rabbit anti-human erythrocyte antibody (Dako, UK) at a 1/600 dilution which was the optimal concentration of antibody determined by agglutination titration. After 30 minutes at room temperature with constant mixing on a rotator, the cells were washed twice and resuspended in $EBSS^+$ at 2% v/v and stored at 4°C for up to 24 hours.

2.2.7 30 day old rat microglia preparation

All manipulations were carried out under sterile conditions in a lamina flow hood. CNS tissue from 30 day old rats was chopped in two 90° planes on a McIlwain chopper

(The Mickle Lab. Engineering Co., Surrey, U.K) set at 0.4mm thickness, placed in a sterile pre-weighed 500ml bottle and the weight of tissue determined. 1ml/g of CNS tissue, of the stock enzymes trypsin, collagenase and DNase and 7ml $EBSS^+$ per gram of tissue were added and incubated at 37°C with continuous stirring on a magnetic stirrer for 15 minutes. 1ml newborn calf serum (NCS) per gram of tissue was then added to stop the reaction followed by dilution to twice the original volume with ice cold EBSS-. The cell suspension was then centrifuged at 4°C, 180g for ten minutes. The pellet was resuspended and the enzyme digestion step repeated as above. The digestion was stopped again as before and the digested tissue pelleted again, by centrifugation. The pellet was resuspended and filtered through fine methanol sterilised nylon meshes of 132 and 80μm pore sizes (J. Stanniar and Co., Manchester, U.K) by forcing through the suspension with a sterile 10ml syringe plunger (Figure 2.1).

2.2.8 Neonatal rat microglia preparation

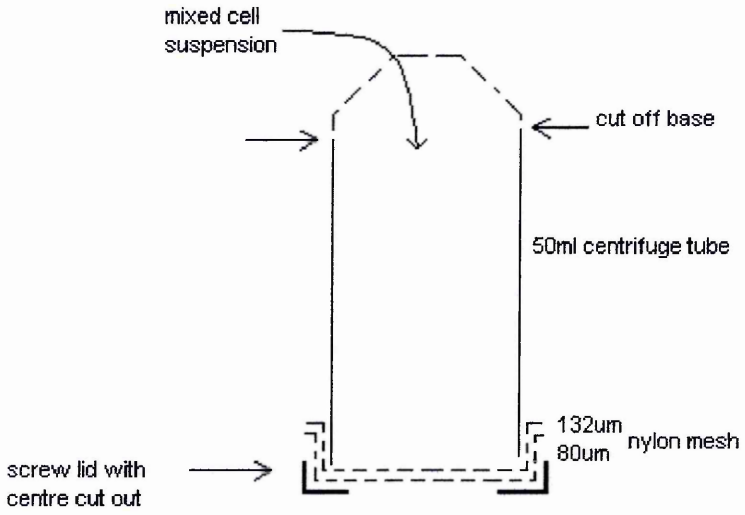
CNS tissue from 2-3 day old rats was filtered immediately through the methanol sterilised mesh as above, without enzyme digestion as it is not required, and the amount of tissue was estimated. Both 30 day old and neonatal cell suspensions were then processed as below.

2.2.9 Isolation of microglia from mixed glia suspensions

Mixed glial suspension was made up to 5ml/g starting tissue in $EBSS^+$ and mixed with an equal volume of 2% EA. This was incubated at 37°C for 30 minutes. Cells were then pelleted by centrifugation (180g for 10 minutes at 4°C) and resuspended in EBSS- to 10ml/g starting tissue. 25 ml of cell suspension was carefully layered onto equal volumes of Percoll (62ml Percoll (Sigma), 10ml 10X Basal medium eagle (BME) (Life

Technologies) 28ml sterile distilled water, giving a final density of.1.086g/ml) in 50ml tubes.

Figure 2.1 A schematic diagram of a sieve made from a 50ml tube



The sieve was made out of a 50ml centrifuge tube with a piece of 132 and 80 μm pore nylon mesh over the end. Tissue is forced through to give the mixed glial suspension (diagram adapted from Woodroofe and Cuzner, 1995).

These cell suspensions layered on density gradients, were centrifuged at 500g for 20 minutes at 18°C, with the brake off. Figure 2.2 shows the distribution of cells after density gradient centrifugation.

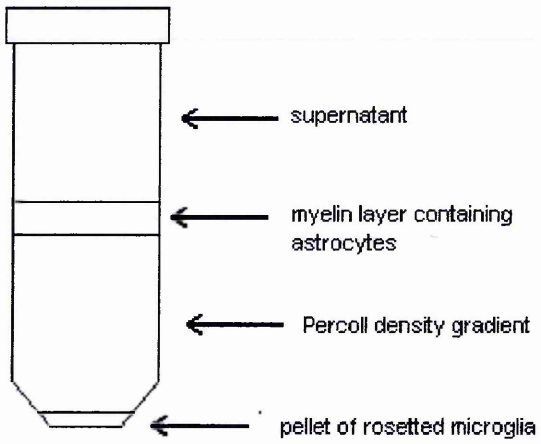
Cell pellets were pooled and resuspended to 1ml/g starting tissue in EBSS- and divided into 5ml quantities in 50ml tubes. Erythrocytes were lysed by addition of 40ml ice cold sterile distilled water for 45 seconds followed by the addition of 4.5ml 10X BME (Life Technologies) to restore tonicity. Cells were then pelleted at 180g for 10 minutes at 4°C and the lysis step repeated. Cells were then resuspended to 2ml in

DMEM for counting in a haemocytometer. Cells were diluted to the required density in cDMEM and placed into culture or in DMEM without foetal calf serum and used directly in chemotaxis assays.

2.3 Isolation of astrocytes

Astrocytes were isolated from 30 day old Wistar rats following the same method as for microglial cell isolation up to and including separation on density gradients. The layer of myelin containing the astrocytes (Figure 2.2) was removed and layered onto Percoll prepared as above and then diluted 1:1 with EBSS and centrifuged again at 500g for 20 minutes at 18°C with the brake off. Pellets were then resuspended in cDMEM and placed into 75cm^2 culture flasks. Non adherent cells were washed away after 24 hours in culture.

Figure 2.2. Distribution of cells after density gradient centrifugation.



2.4 Isolation of peritoneal macrophages.

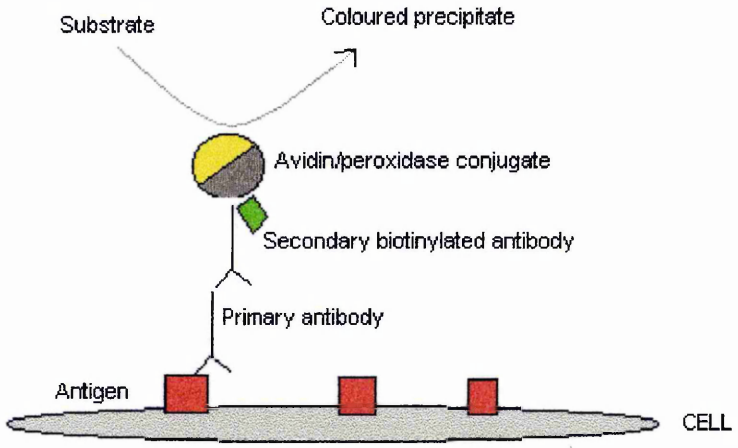
Rat peritoneal macrophages were isolated from adult Wistar rats by peritoneal lavage with 50ml of ice cold EBSS-. These were pelleted by centrifugation at 180g for 10 minutes at 4°C, washed in EBSS-, and resuspended in 2ml cDMEM. Cells were then counted using a haemocytometer and plated into 96 well plates at a concentration of $5x10^5$/ml in cDMEM (200µl/well).

2.5 Immunocytochemistry (ICC) on isolated cells

Cells were plated into 8 well chamber slides (Gibco BRL, U,K) at densities of $2.5x10^5$/ml, $1x10^5$/ml and $0.5x10^5$/ml for microglia, astrocytes and CHME3 cells respectively. 400µl of cell suspensions were added per well. Microglia and astrocytes were maintained in culture for 3-7 days and CHME3 cells were cultured overnight, prior to fixing.

Immunocytochemistry was used in order to identify microglia and other cell types in cultures using cell type specific antibodies. Visualisation of the resulting antigen/antibody complex involves labelling the antibody with an enzyme such as peroxidase. The enzyme then produces a colour change on addition of a specific substrate. The technique may also employ the use of a biotinylated secondary antibody against the Fc portion of the primary antibody which binds avidin-peroxidase conjugate as in the Vectastain Elite ABC Kit (Vector laboratories, U.K) used in the following experiments. Figure 2.3 shows how antigens are visualised by this method.

Figure 2.3. Avidin biotin peroxidase complex method for Immunocytochemical detection of an antigen expressed by a cell.



Cells grown in 8 well chamber slides were fixed at 4°C in the appropriate fixative for the antigen under study for 2 minutes(see Table 2.2). The 30 day old rat microglial cells were all fixed in 4% paraformaldehyde prior to the experiment for convenience.

Table 2.1 Antibodies used for cell characterisation:

Antibody	Concentration	Purchased from	Specificity
OX 42	1:100	Serotec	rat macrophages
αGFAP	1:100	Sigma	astrocytes
ED1	1:100	Serotec	rat macrophages
*αGC	neat supernatant	_	oligodendrocytes
CD68/ EBM-11	1:100	Dako	human macrophages

* Anti GC (galactocerebroside) antibodies were obtained from the cell supernatant of a hybridoma cell line (a kind gift to Dr N Woodroofe from Dr B Rantsch

Table 2.2 Fixatives used for specific antigens under study in immunocytochemistry experiments.

Antibody	Fixative
Anti-GFAP	Acetone
ED1	Acetone
OX42	Ethanol
Anti-GC	Acetone
CD68/EBM-11	5%acetic acid/95%ethanol

All fixatives were used at 4°C.

After fixing, slides were then washed in phosphate buffered saline (PBS) (Sigma). The plastic walls of the chamber slides were removed and wells were covered in 1.5% blocking serum (Normal horse serum, from the kit) in PBS for 30 minutes, to prevent non specific binding of antibodies. Cells, except for the control well, were then incubated with 50µl of primary antibody (Table 2.1) diluted in buffer (0.1% BSA in PBS) for 30 minutes and then washed for 10 minutes with three changes of PBS. Control wells were incubated in 0.1% BSA in PBS alone. Slides were then incubated with biotinylated horse anti mouse secondary antibody at 1:200 dilution, (from the kit) in buffer with 100µl of blocking serum per 5ml of diluted antibody, for 30 mins.

Slides were washed in buffer again for 10 mins and then incubated for 30 mins with Vectastain Elite ABC reagent, prepared 30 minutes prior to use. Cells were then washed in PBS for 10 mins and incubated in 0.2% 3,3'diaminobenzidine tetrahydrochloride (DAB) substrate (Sigma Ltd, UK) containing H_2O_2 (40µl/100ml DAB) for 2-7 mins until the desired staining intensity developed. Slides were finally washed in tap water and mounted in Immumount (Shandon,UK). For ICC on astrocytes,

FITC labelled avidin (1/150) (Vector Laboratories, U.K) was used instead of the ABC reagent for 30 minutes. Following this incubation, cell were washed and mounted in Immumount (Shandon,UK).

Cell staining using DAB substrate was observed by light microscopy on the Olympus BX60 microscope and colour photographs taken at various magnifications on Kodak colour film (200 ASA). FITC staining of astrocytes was observed by fluorescence microscopy and images captured by CCD colour video camera (JVC).

2.6 MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) assay.

The MTT assay is used to quantify cell numbers. MTT is a water soluble tetrazolium salt and is converted from a yellow solution to a purple insoluble formazan by cleavage of a tetrazolium ring by dehydrogenase enzymes present in the mitochondria of living cells. When solubilised in isopropanol, the amount of formazan produced can be measured spectrophomometrically and can be used to show the differences in cell numbers in cultures. MTT assays were performed on CHME3 cells following cytokine stimulation for 24 hours to see if this had an effect on cell proliferation.

2.6.1 MTT assay method

CHME3 cells were plated into 96 well plates at a concentration of $1x10^5$/ml (200µl/well) and cultured for 24 hours. Cells were then stimulated in triplicate with human recombinant tumour necrosis factor α (TNFα) (Peprotech, U.K) (0.1, 1 and 10ng/ml), interleukin-1β (IL-1β) (Peprotech, U.K) (0.1, 1 and 10ng/ml) and IFNγ (Life technologies, U.K) (1, 10 and 100 IU/ml). The chemokine, MCP1, was also included at 1, 10 and 100ng/ml. After stimulation for 24 hours, the supernatant was removed and 50µl of MTT (Sigma) (1mg/ml in Hanks Balanced salt solution (Life technologies)) was

added to each well and the plate was incubated for two hours at room temperature. 100µl of acidified isopropanol (20ml isopropanol + 25µl concentrated HCl) was then added and left for a further 20 minutes before the absorbance was read at 570nm on a Titertek plate reader.

2.7 Bicinchoninic acid (BCA) assay

This is a simple assay to determine total protein content which can be related to cell numbers in a 96 well plate. Protein reduces alkaline Cu (II) to Cu (I) in a concentration dependant manner to form a purple complex which can be read spectrophotometrically. The BCA assay was used to assess whether the chemokines exert an effect on cell proliferation.

2.7.1 BCA assay method

CHME3 cells were plated in to 96 well plates as above and stimulated with the human chemokines MCP1, MIP1α, MIP1β, RANTES, IL-8, and IP-10 (a kind gift from Professor D Taub, NIH, Maryland USA) at 10, 20 and 50ng/ml for 24 hours. The supernatant was removed and the plate stored at -20°C to break open the cell membranes. After approximately 24 hours, plates were thawed and 50µl of lysis buffer was added (50mM Tris HCl, 0.1% Triton-X-100, pH 7.5) per well for 30 minutes at room temperature. Lysed cell supernatants were then removed, replicates pooled and then centrifuged (10,000g for 10 minutes in a micro centaur benchtop centrifuge) to remove cell debris. 20µl of pooled supernatants were then plated in duplicate into clean 96 well plates followed by 200µl of BCA reagent (20ml BCA + 400µl 0.4% $CuSO_4$) for 30 minutes at room temperature, followed by measuring the absorbance at 570nm on a Titertek plate reader.

2.8 Results

2.8.1 Microglial cell isolation from Wistar rat CNS tissue

Microglia isolated from 30 day old Wistar rats and 2-3 day old Wistar rats were counted and the yield was calculated following each isolation. Typical cell yields have been included in the following tables. Higher yields were obtained from neonatal rat brains than from adult rats.

Table 2.3 Examples of typical yields obtained from microglia isolations from 30 day old Wistar rats.

No. of animals used	**weight of tissue obtained (g)**	Total no. of microglia isolated ($x10^5$)	**Yield ($x10^5$) /g starting tissue**
30	31	47	1.5
15	14	44	3.1
15	16	26	1.6
30	30	15.5	0.5
15	14	42	3
10	9	19	2.2

Table 2.4 Examples of typical yields obtained from 2-3 day old Wistar rats

No. of animals used	No. Microglia obtained ($x10^5$)	**Microglia yield /brain ($x10^5$)**
40	44	1.1
50	33	0.6
25	58.5	2.3
25	46.5	1.9
32	26	0.8

2.8.2 Immunocytochemistry on cells in culture to identify cell specific markers

Microglial cells stained positively with antibodies against OX-42, which is an antibody against the complement receptor for C3b, and ED1 a macrophage marker but were negative for GFAP, which was used to identify contaminating astrocytes and anti GC which was used to identify contaminating oligodendrocytes. (Figure 2.4a-d and Figure 2.5a-d)

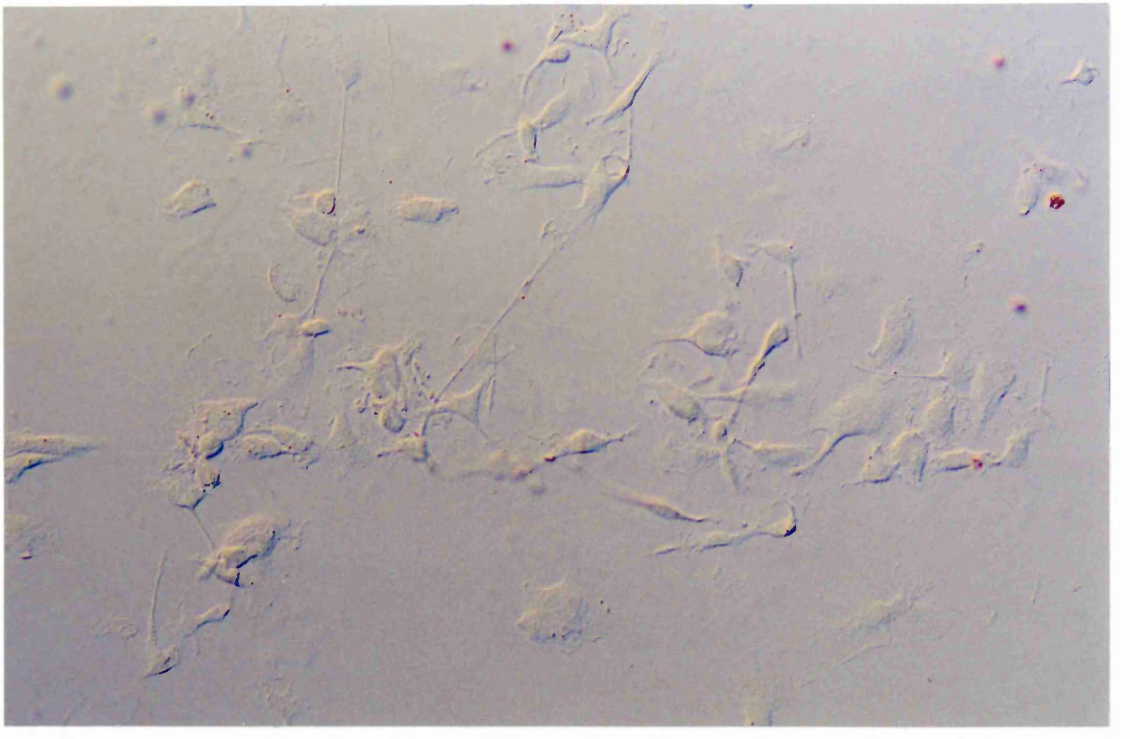
CHME3 cells stained positively for CD68/EBM-11 which is an antibody specific for human macrophages and negative for GFAP which demonstrates that these cells are not of astrocyte origin. (Figure 2.6a-c).

Astrocyte preparations isolated from 30 day old Wistar rats all stained positively for GFAP indicating a highly pure population of cells, demonstrated by immunofluorescence (Figure 2.7a and b)

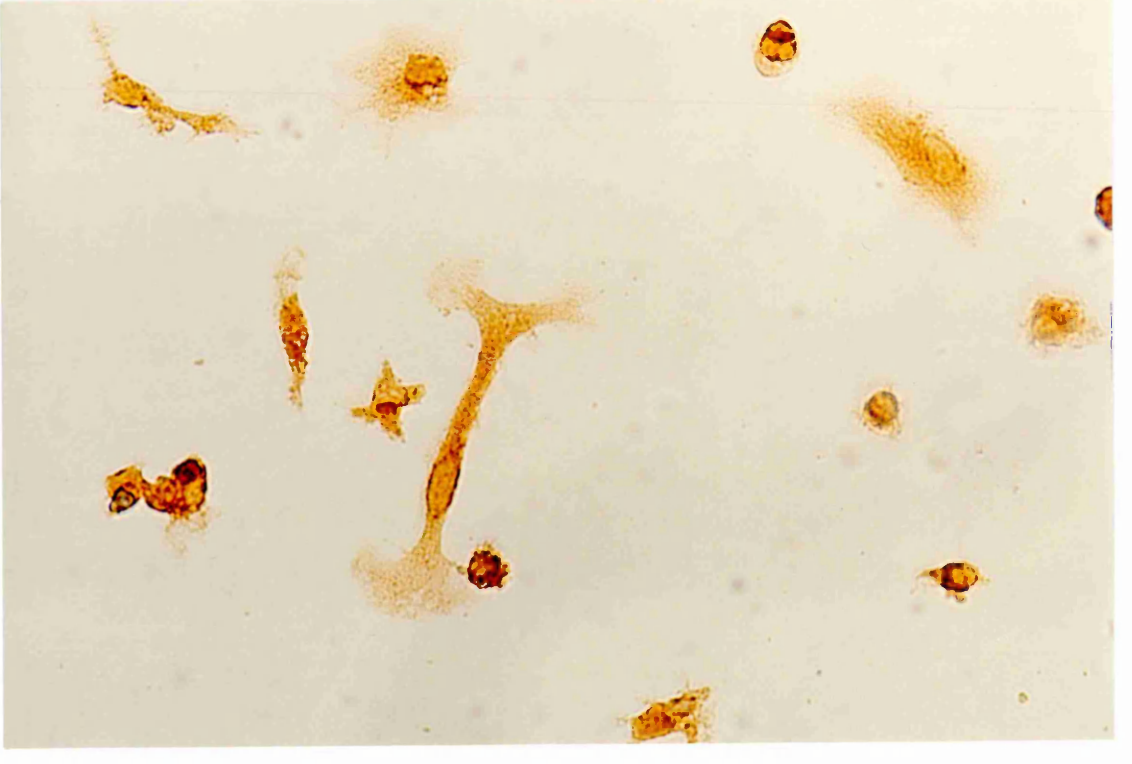
2.8.3 Proliferation assays on CHME3 cells

CHME3 cells when stimulated for 24 hours with the cytokines TNFα, IL-1β (0.1-10ng/ml) and IFNγ (1-100 IU/ml) or the chemokines MCP1, MIP1α, MIP1β,

Figure 2.4 Immunocytochemistry on 30 day old Wistar rat microglia

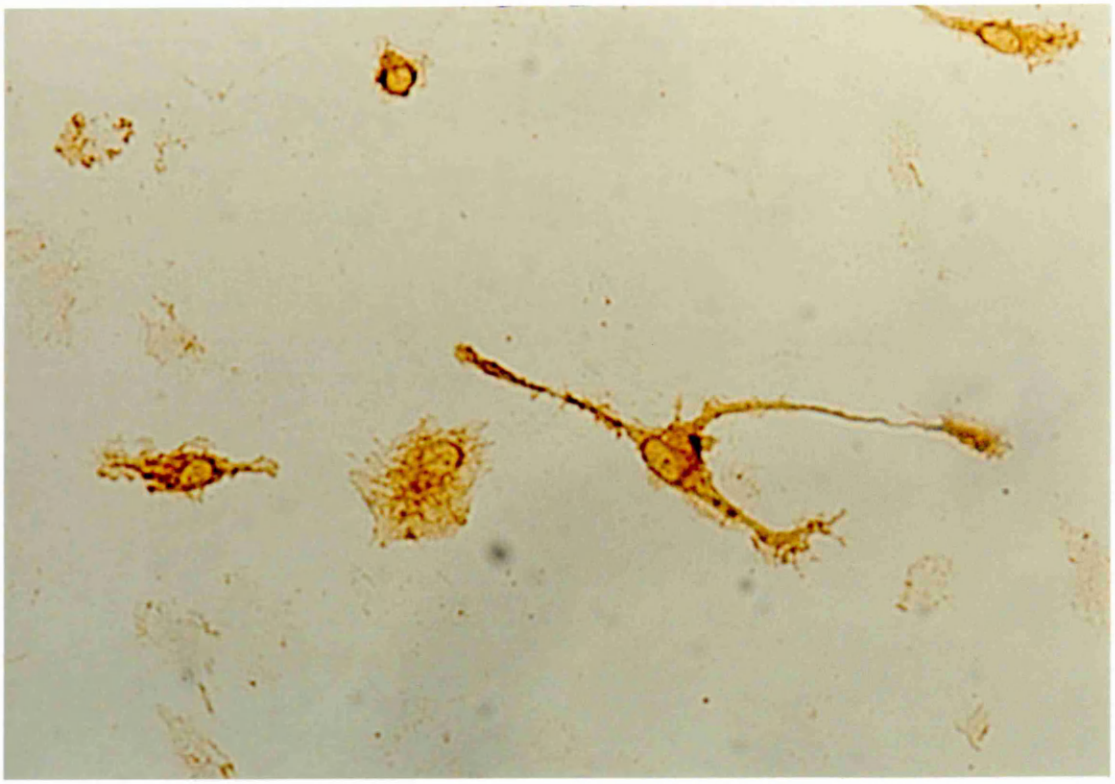


a) Control (X200) Cells show negative staining. Photographs of control cells were taken using phase contrast microscopy so that cells could be more easily observed.

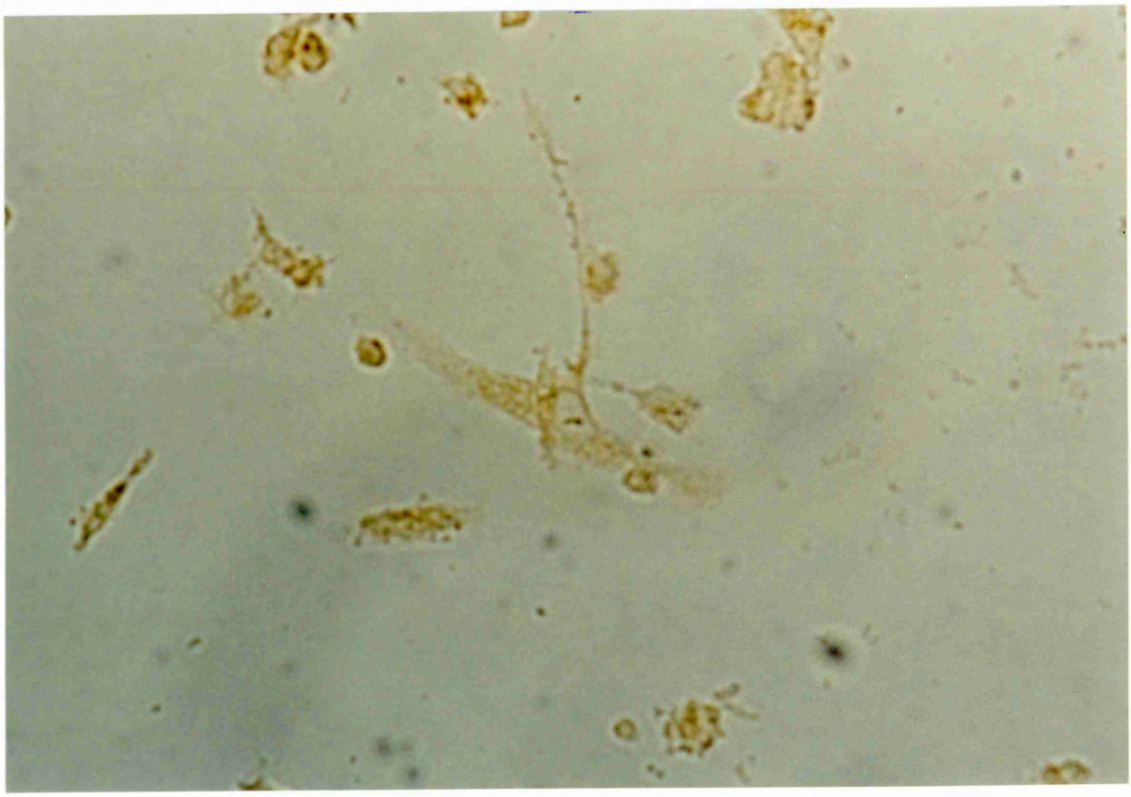


b) OX-42 antibody (X400) Cells are stained positively for OX-42.

Figure 2.4 continued. Immunocytochemistry on 30 day old Wistar rat microglia

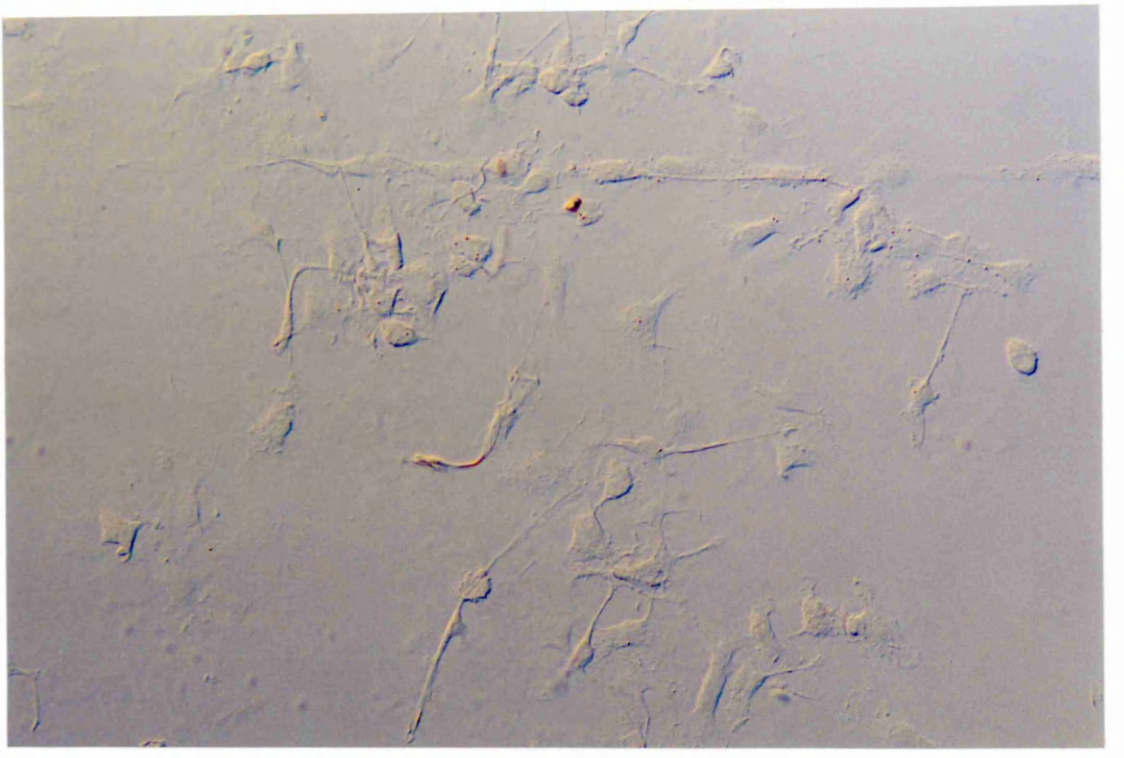


c) ED-1 positive microglia (X400)

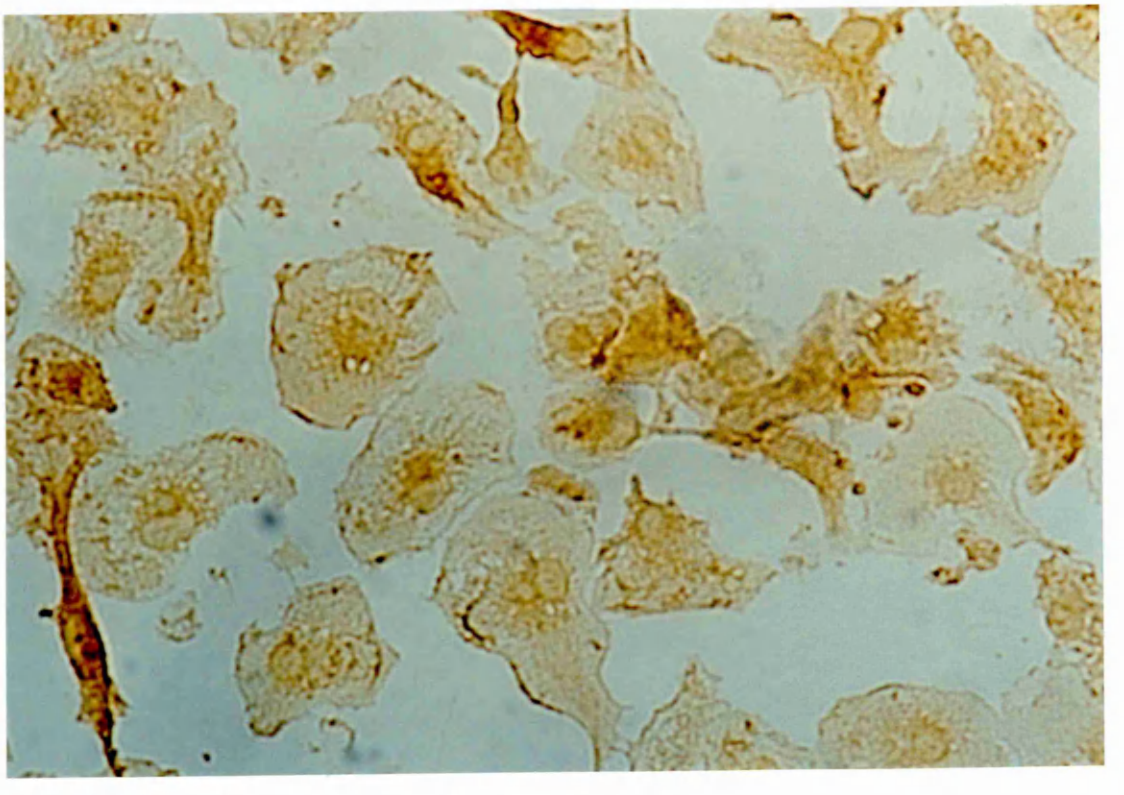


d) GFAP antibody to show the absence of contaminating astrocytes (X400)

Figure 2.5 Immunocytochemistry on 2-3 day old rat microglia

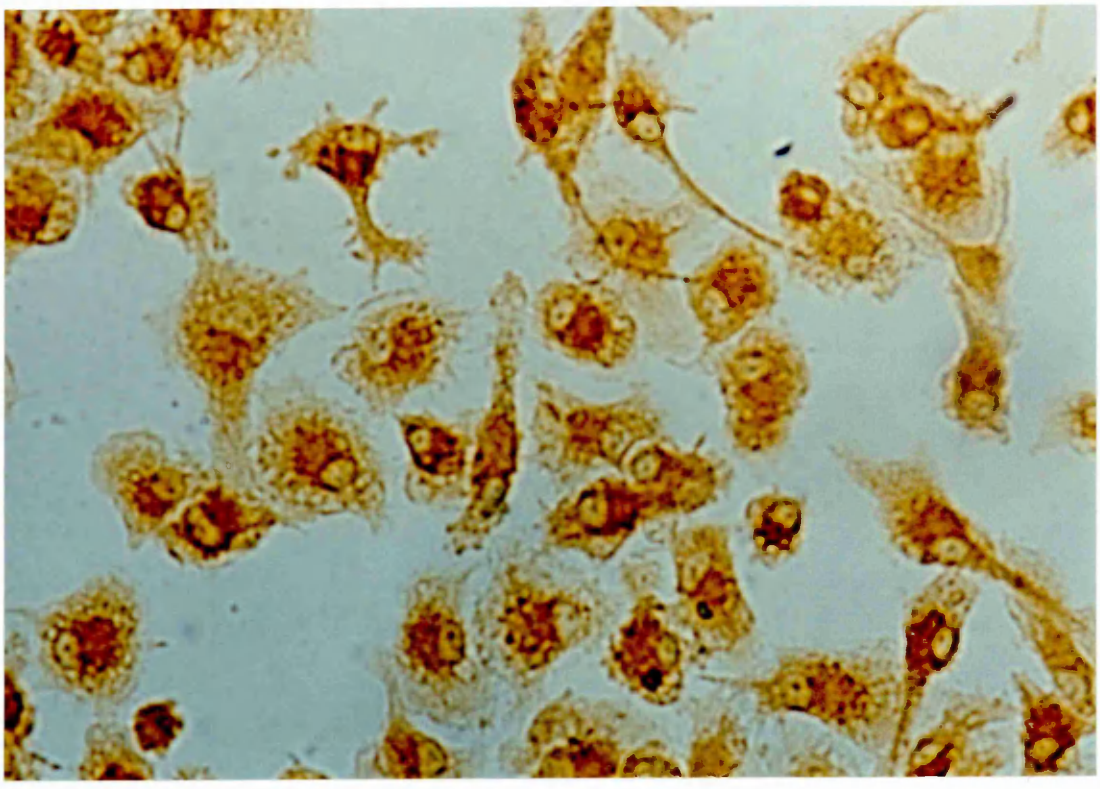


a) Control (X200). Cells show negative staining. Photographs of control cells were taken using phase contrast microscopy so that cells could be more easily observed.

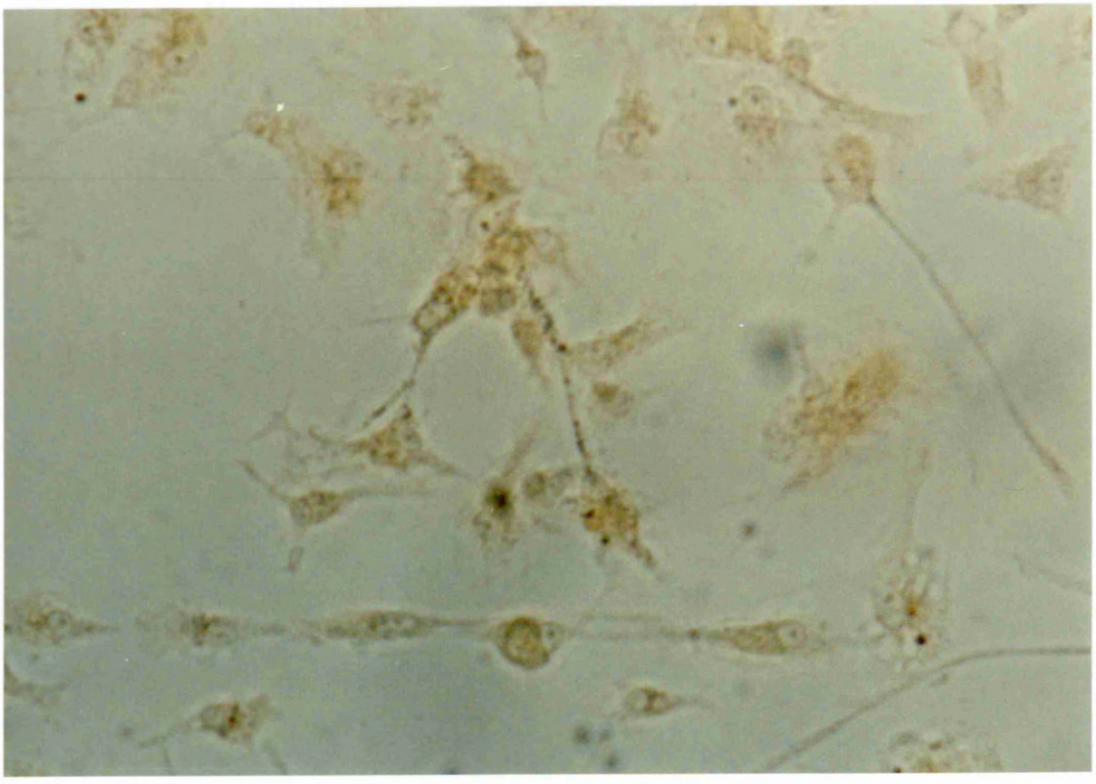


b) OX-42 antibody(X400). Cells are positive for OX-42.

Figure 2.5 continued. Immunocytochemistry on 2-3 day old rat microglia

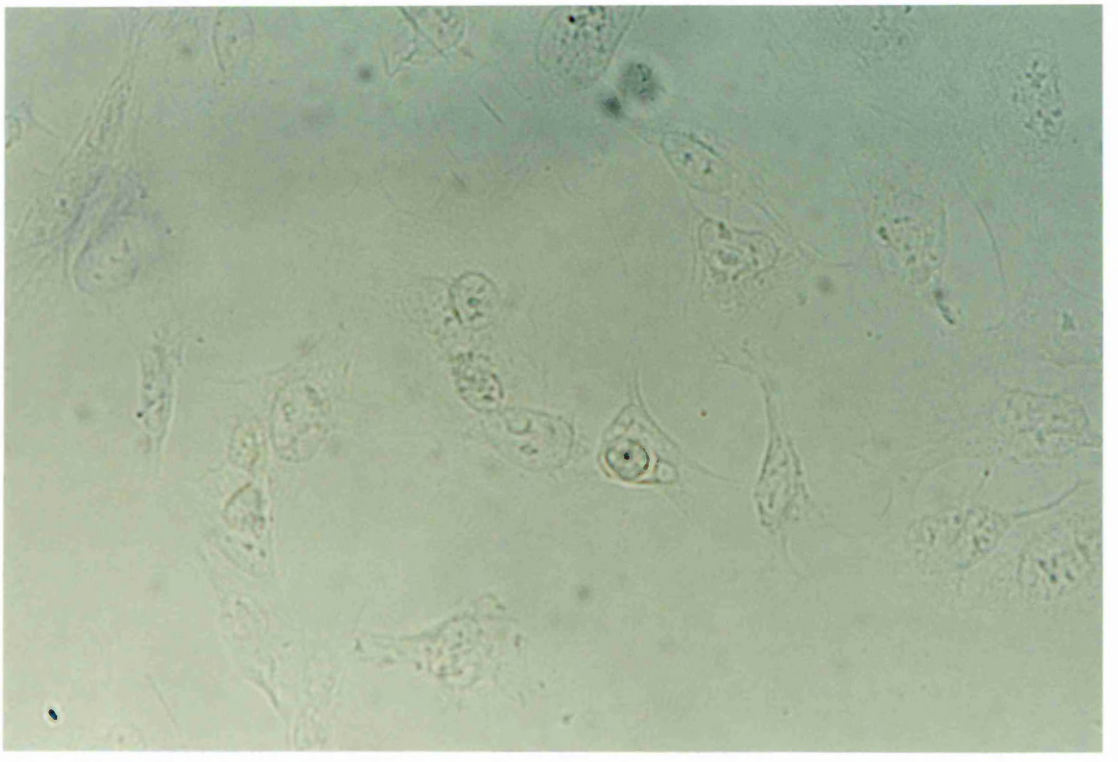


c) ED1 positive staining of 2-3 day old Wistar rat microglia (X400)

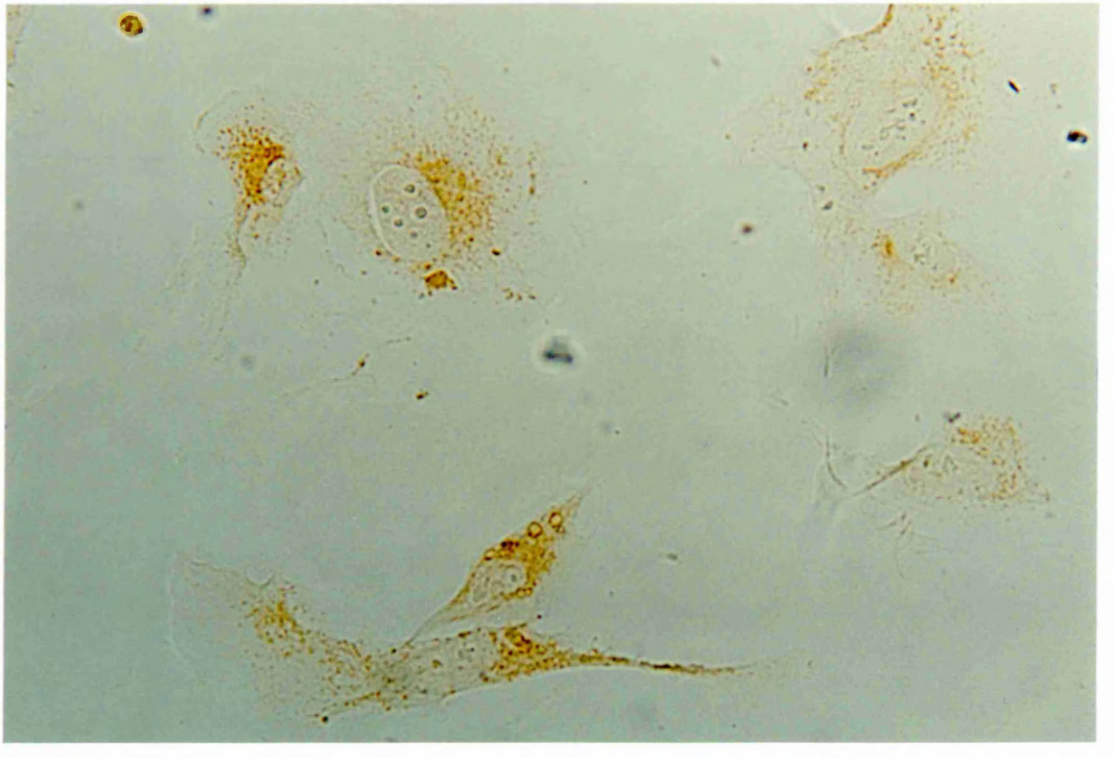


d) αGFAP (X400). Cells were negative for GFAP.

Figure 2.6 Immunocytochemistry on CHME3 cells

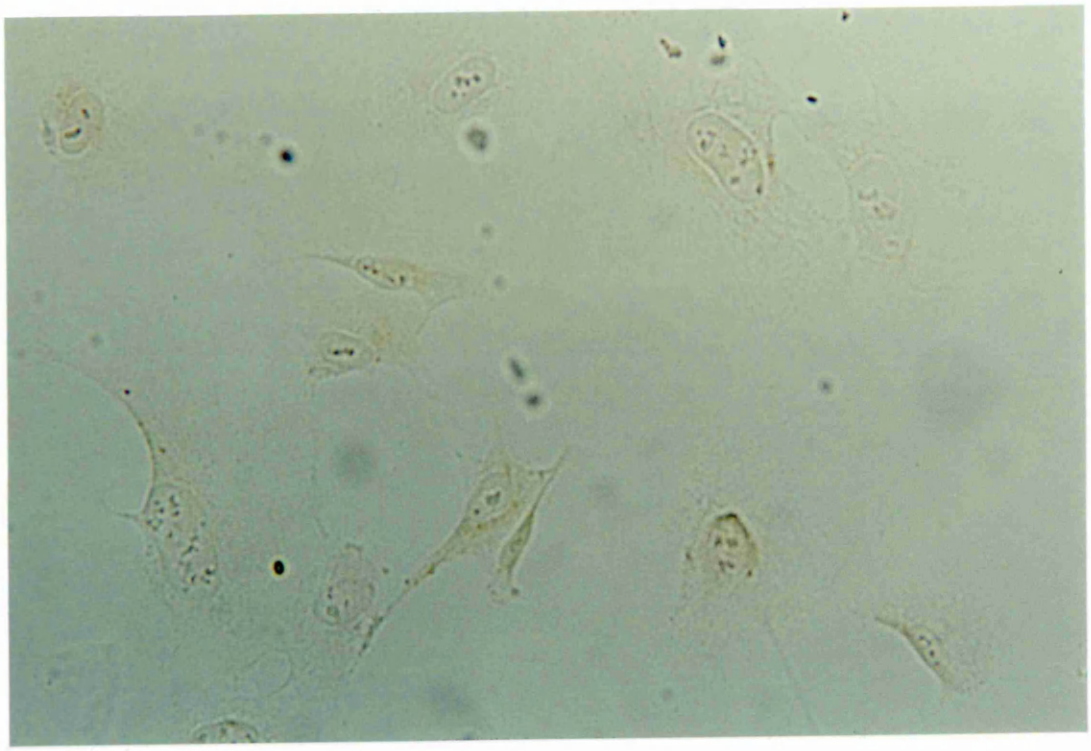


a) control (X400) Cells show negative staining.



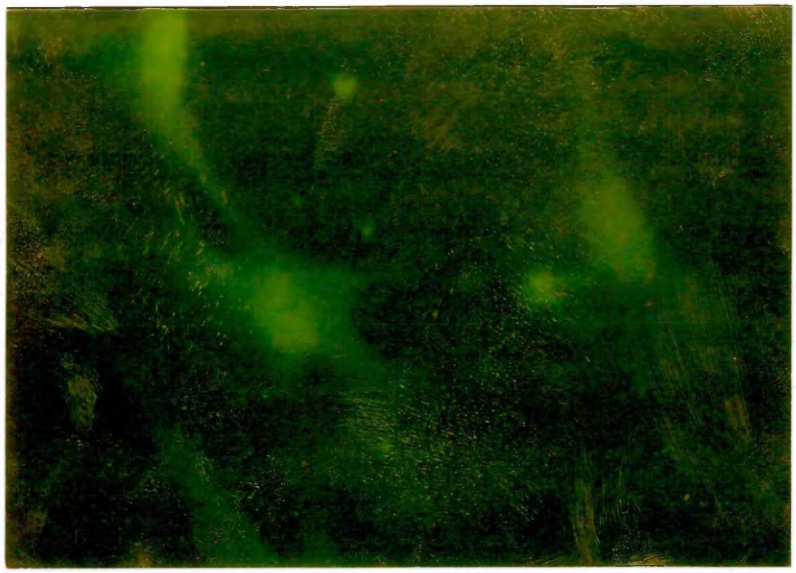
b) CD68/EBM-11 positive staining of CHME3 cells (X400)

Figure 2.6 continued. Immunocytochemistry on CHME3 cells

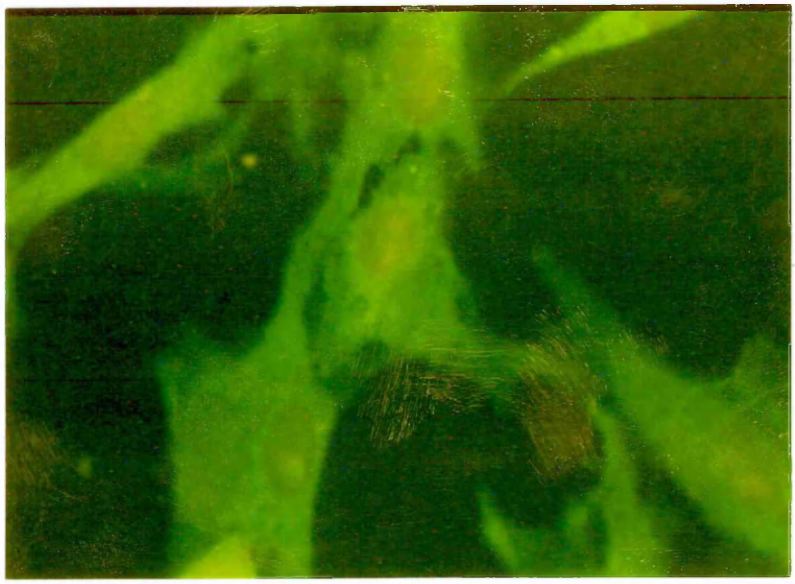


c) GFAP negative CHME3 cells.

Figure 2.7 Immunofluorescence on astrocytes isolated from 30 day old Wistar rats.



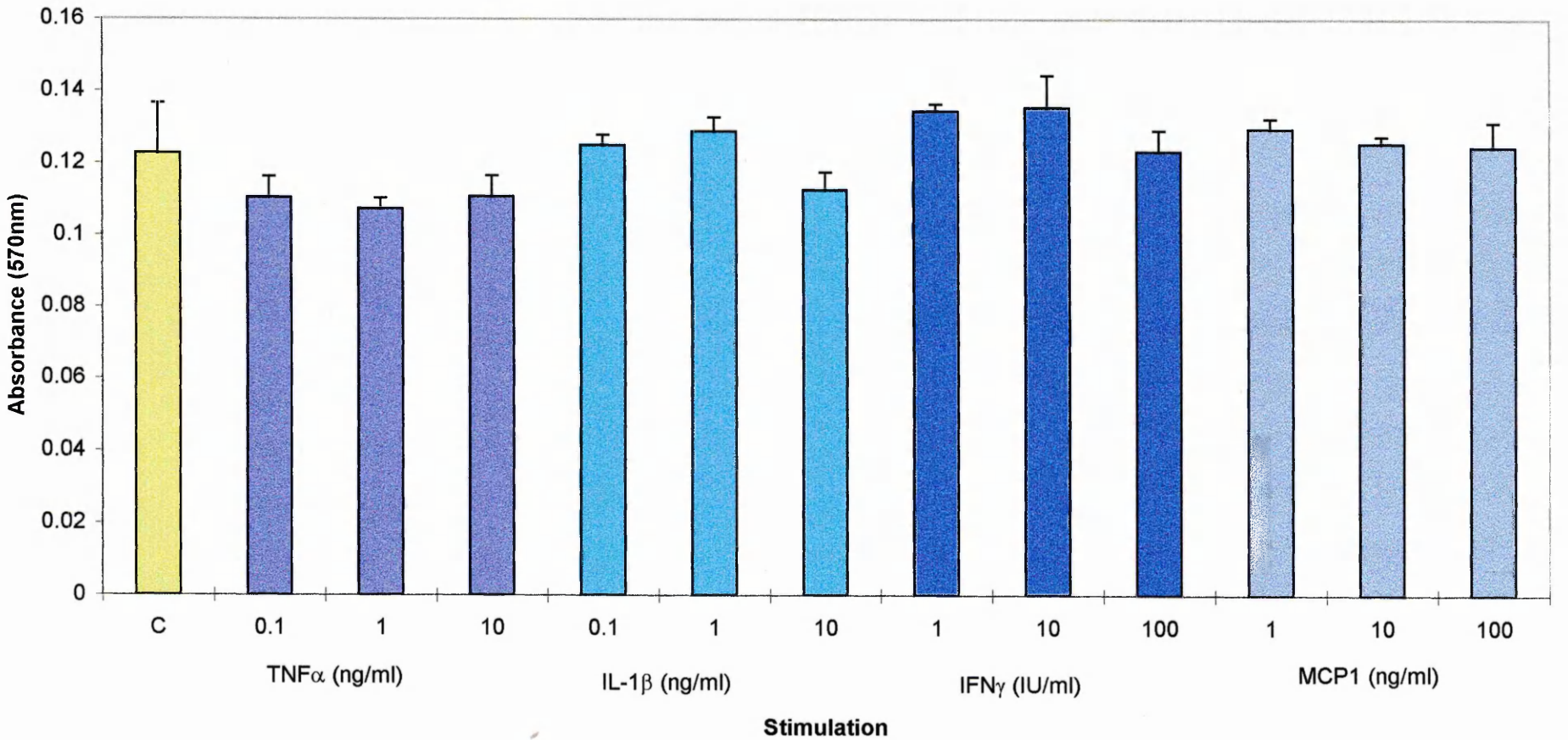
a) Control (no primary antibody) (X400)



b) Rat astrocytes were stained positively using αGFAP (X400)

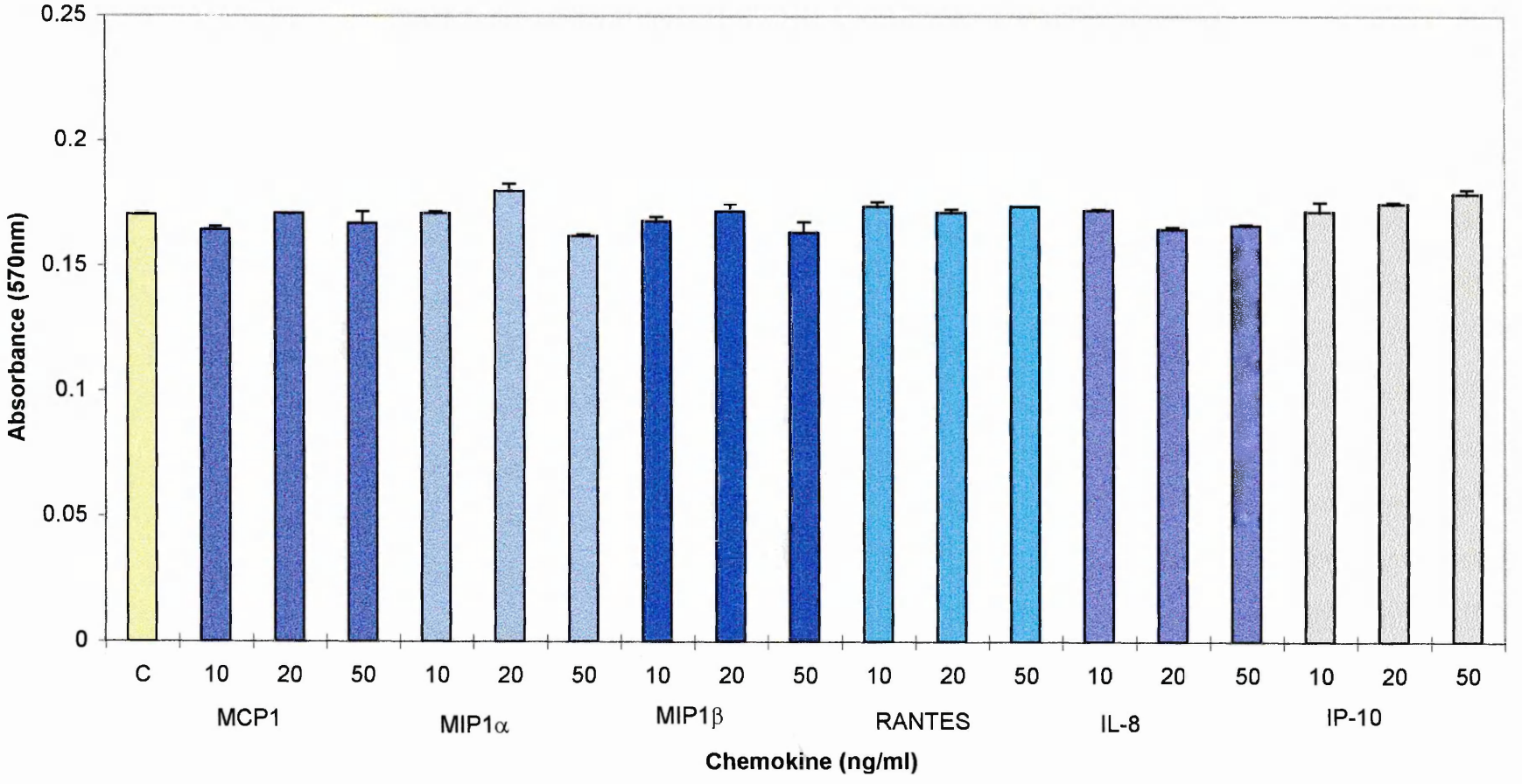
RANTES, IL-1β and IP-10 at 10-50ng/ml, did not significantly increase cell proliferation when compared to control unstimulated cells (using a paired Student's t test, $P<0.05$ was considered significant) thus demonstrating that at the concentrations used in the experiments in this thesis, these cytokines and chemokines had no significant effect on cell proliferation. This was shown using MTT and BCA assays (Figures 2.8 and 2.9).

Figure 2.8 MTT assay on CHME3 cells



Cells were stimulated in triplicate wells for 24 hours in culture before using the MTT proliferation assay. No significant increase in cell proliferation was observed ($P<0.05$) using a Students' t test.

Figure 2.9 BCA assay on CHME3 cells



Cells were stimulated with chemokine at 10-50 ng/ml for 24 hours in culture before using the BCA method to assess protein levels to determine the effect of chemokines on cell proliferation. No significant increase was observed ($P<0.05$) using a Students' t test.

2.9 Discussion

2.9.1 Cell yields of microglia isolated from Wistar rat CNS tissue

The numbers of microglia obtained from 30 day old rats were generally lower than the yields reported by Hayes *et al* (1988) who reported yields of $8.2x10^5$ cells/gram of original starting tissue, using the same isolation procedure. Typical yields obtained here were between $0.5\text{-}3x10^5$ cells/ gram of CNS tissue. One explanation for these lower yields may include the time between obtaining the CNS tissue and beginning the isolation process, which was up to an hour and involved storing the tissue on ice for transportation. Also, the large numbers of animals used each time may account for lower yields and this was also reported by Woodroofe *et al* (1989). It is estimated that there are around 3.5 million microglia in the rodent brain (Perry, 1994) which is far higher than the observed yields reported here. However, many cells are lost during the large number of steps in the isolation procedure, due to the adherent properties of microglia. Sedgwick *et al*, (1991) isolated cells by fluorescent activated cell sorting (FACS) and obtained yields of $CD45^{low}$ cells (reported as microglia) of $4.4x10^5$ cells per brain using 3-4 week old Lewis rats. Although this is higher than the mean yields obtained here it is similar to maximal yields of $3x10^5$ cells per animal using the rosetting method

The yields obtained from 2-3 day old rats were generally higher than those obtained from 30 day old rats which was also found by Hayes *et al* (1988). This could be accounted for by the remodelling process in the CNS in the pre-natal and early postnatal period, where microglia and macrophages are involved in phagocytosis of degenerating cells (Perry, 1994).

2.9.2 Cell purity

Microglia cells showed a typical bipolar morphology in culture. Both 30 day old and 2-3 day old rat microglia showed positive staining by immunocytochemistry with OX-42 and ED-1 and were negative for αGFAP and αGC demonstrating the high purity of the cell preparations and lack of contamination with astrocytes or oligodendrocytes. Cells sometimes contained small numbers of erythrocytes which have been phagocytosed during the isolation procedure.

CHME3 cells stained positively for the macrophage marker, CD68/EBM-11 and were negative for GFAP demonstrating the cells were of macrophage and not astroglial origin. This was also found by Janabi *et al* (1995) who originally immortalised the cell line by EBV transformation of human foetal microglia.

Astrocytes isolated from Wistar rats were shown to be pure cultures by strong positive staining for glial fibrillary acidic protein and absence of staining for other cell types.

2.9.3 Proliferation assays

Since cytokines and chemokines were used to stimulate cells in culture it was necessary to examine the effects of these stimulations on CHME3 proliferation. Using MTT and BCA assays it was observed that the cytokines, TNFα, IL-1β and IFNγ and the chemokines, MCP1, MIP1α, MIP1β, RANTES, IL-1 and IP-10 did not cause proliferation above control unstimulated cells. This indicates that any differences in results obtained in stimulation experiments with cytokines and chemokines, reported in the following chapters are not due to any effect on cell proliferation. Ganter *et al* (1992) reported that IL-1 and TNFα had no effect on proliferation of isolated microglia from Wistar rats although these cytokines did increase proliferation in mixed astrocyte/

microglia cultures suggesting a role for other astrocyte derived factors. Ganter *et al* (1992) did however demonstrate microglia proliferation stimulated by IL-13 and GM-CSF.

Chapter 3

EFFECTS OF CHEMOKINES ON MICROGLIAL MIGRATION

3.1 Introduction

3.1.1 Microglia accumulation at sites of inflammation/tissue damage.

An important function of macrophages is their ability to migrate to sites of tissue infection and injury. Microglia and macrophages are found closely associated with areas of inflammation and tissue damage in many CNS pathologies and are involved in tissue destruction, by release of free radicals and secretion of proteases, proinflammatory cytokines and complement components (Sriram and Rodriguez, 1997). In the CNS in MS, EBM-11^{+} macrophage type cells with microglial morphology are associated at increased densities in active plaques and at their borders (Esiri and Reading, 1987). Activated microglia are also associated with mature senile plaques in Alzheimer's disease and have been shown to accumulate at higher densities on cryostat sections of CNS senile plaques compared to the non-senile plaque area of the same tissue (Joshi and Crutcher, 1998). A model of experimentally induced glioma in the rat also demonstrated a massive infiltration of the tumour by activated microglial cells (Streit, 1996). The evidence from histopathological studies therefore suggest that microglia are able to migrate *in vivo* and a number of *in vitro* studies have been performed to reinforce this theory.

In vitro chemotaxis experiments, using microglia isolated from 2 day old rats, demonstrated their ability to migrate through polycarbonate filters in response to the chemotactic complement component, C5a (Yao *et al*, 1990) and this is thought to

account, in part, for the accumulation of microglia at tissue injury sites. Other *in vitro* chemotaxis experiments, using a method of chemotaxis in agarose gels containing a chemoattractant, have shown that microglia are able to migrate towards nerve growth factor (NGF) whereas astrocytes in the same experiment did not migrate (Giliad and Giliad, 1995). Time lapse video recording of microglia in culture have further proved that microglia are highly motile cells (Booth and Thomas, 1991, Haapaniemi *et al*, 1995). Foetal rat microglia in culture changed the size and shape of their cell body continuously and this was accompanied by constant extension and retraction of long processes. These cells showed random migration about a small local area measured in micrometers (Booth and Thomas, 1991). Haapaniemi *et al*, (1995) demonstrated the use of lamellipodia for microglial locomotion of up to 1.01 μm per second with the lamellipodia frequently observed as two horns, said to behave like 'blades of a pair of scissors' possibly to engulf opsonised particles.

3.1.2 Effect of chemokines on chemotaxis of leukocytes *in vitro*

The chemotactic family of cytokines known as chemokines have been studied intensively for their ability to chemoattract leukocyte sub populations. The α and β sub families of chemokines induce migration with distinct, and some overlapping, leukocyte specificities. Many of the ELR motif containing α chemokines attract neutrophils and T cells with little effect on monocytes, whereas the β chemokines attract monocytes, macrophages and T cells with little effect on neutrophils. Interestingly, the non ELR motif containing α chemokines, including IP-10 and PF4, fail to chemoattract neutrophils (Taub and Oppenheim, 1994). The β chemokines also show specificity for T cell subsets, such that MIP1α is more chemoattractive for $CD8^+$ T cells whereas MIP1β preferentially attracts $CD4^+$ T cells (Taub *et al*, 1993). The most extensively studied

monocyte/macrophage chemoattractant is MCP1 (Leonard and Yoshimura, 1990, Uguccioni *et al*, 1995, Rollins, 1996). MCP1 expression in the CNS has been detected following cerebral ischaemia and mechanical stab wound injury, consistent with macrophage chemoattraction (Ransohoff and Tani, 1998). Furthermore, intracerebral injection of recombinant MCP1 into mice leads to accumulation of large numbers of monocytes in a concentration dependant manner (Bell *et al*, 1996).

3.1.3 Chemokines expressed in diseases of the central nervous system

A number of chemokines have been detected in the CNS in MS. These include, MCP1, MCP2, MCP3, MIP1α/β, RANTES and IP-10 (Simpson *et al*, 1998, McManus *et al*, 1998). Chemokines present in the CNS of animals with EAE include MIP1α/β and RANTES (Eng *et al*, 1996, Miyagishi *et al*, 1997) as well as MCP1, IP-10 and GRO (Ransohoff and Tani, 1998). Although it appears that the β chemokines are the main sub group expressed in the CNS, fractalkine is also present and is produced predominantly by neurons in response to nerve damage (Harrison, 1998). IL-8 is produced by microglia in response to co-incubation with a cryptococcal fungus which infects the CNS (Lipovsky *et al*, 1998) or following stimulation with TNFα or IL-1β (Erlich *et al*, 1998). IL-8 was also found to be produced by a neuroblastoma cell line in response to IL-1α stimulation (Chuluyan *et al*, 1998).The α chemokine, MIG, has been found to be upregulated in acute MS lesions associated with perivascular leukocytes (Simpson *et al*, 1998b).

3.1.4 Migration of microglia to chemoattractants and chemokines *in vitro*

Human microglia have been previously shown to migrate *in vitro* to the chemokines MIP1α, MIP1β and MCP1 in a concentration dependant manner (Peterson *et al*, 1997) and recently, rat microglial migration has been demonstrated in response to

MCP1 and RANTES (Johnstone *et al*, 1999). Human microglia migrate in response to C5a and the classical chemoattractant peptide, f-met-leu-phe (fMLP) in higher numbers than to chemokines. In contrast, astrocytes did not respond to the chemokines tested (Peterson *et al*, 1997). Rat microglia were also shown to migrate to the complement component C5a but not fMLP (Yao *et al*, 1990).

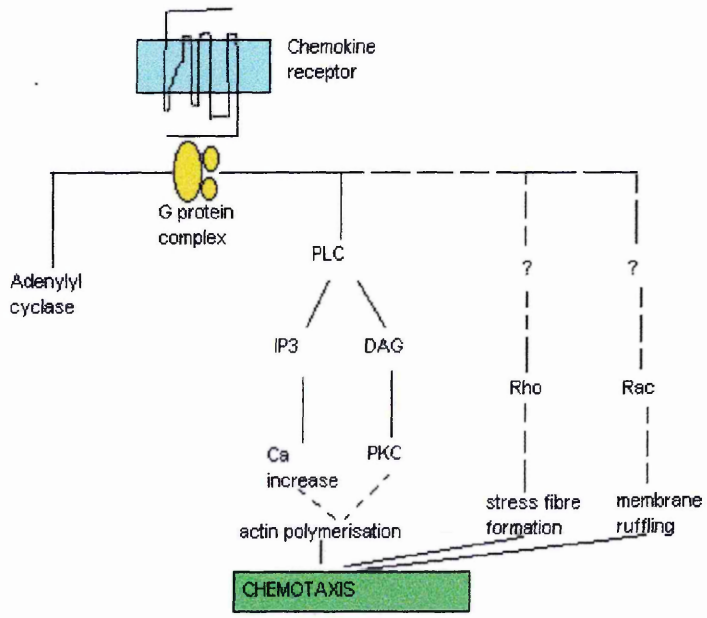
3.1.5 Effect of chemokines on reorganisation of actin through G protein/GTP binding proteins

Chemokine-induced migration involves binding of a chemokine to its receptor on the responding cell. The interaction of the chemokine with its receptor causes an increase in intracellular calcium and activation of second messenger enzymatic pathways such as adenylate cyclase, via the seven transmembrane domain, G protein coupled receptors (Petrek *et al*, 1995) and polarisation of the receptors to the leading edge of the cell (Nieto *et al*, 1997). This leads to polymerisation and breakdown of actin causing formation and retraction of lamellipodia enabling the responding cell to become motile (Baggiolini, 1998). Lymphocyte activation by chemokines including IL-8, RANTES and MCP1, induce cellular polarisation and development of a projection known as a uropod (del Pozo *et al*, 1996).

Chemokine signalling involves activation of GTP binding proteins including Rac and Rho which are involved in cell motility through regulation of actin dependant processes (Takai *et al*, 1995, Premack and Schall, 1996). Microinjection studies on Swiss 3T3 fibroblasts revealed that Rho controls the formation of stress fibres and focal adhesions, whereas Rac causes membrane ruffling. Both Rac and Rho cause clustering of integrins (Machesky and Hall, 1996) which is likely to be involved in cell locomotion. IL-8 has been shown to increase the amount of radiolabelled GDP bound to RhoA (the

predominant isoform in lymphocytes) in lymphoid cells in culture, with up to 12 times more bound than in control cells (Laudanna *et al*, 1996). The complex series of signalling events from chemokine receptor to cell migration is not fully elucidated, although these experiments indicate some of the pathways involved and are illustrated in Figure 3.1 which is modified from Premack and Schall (1996).

Figure 3.1 Illustration of pathways from chemokine receptor to chemotactic response.



Dashed lines indicate connections where intermediates are not yet known (Premack and Schall, 1996). Abbreviations: PLC = phospholipase C, IP3 = Inositol trisphosphate, DAG = diacyl glygerol, Ca = intracellular free calcium, PKC = protein kinase C. ? = not known.

3.1.6 Aims

Due to the reported observations on microglial migration to sites of tissue damage and inflammation and the localisation of chemokines within the CNS in pathological conditions, it is proposed that chemokines mediate the migration and recruitment of microglia through their actions on receptors on the cell surface and rearrangement of the cytoskeleton. The aims were to:

1. Examine microglial migration in response to a panel of chemokines.
2. Observe changes in the distribution of filamentous actin in response to chemokine stimulation.

3.2 Materials and methods

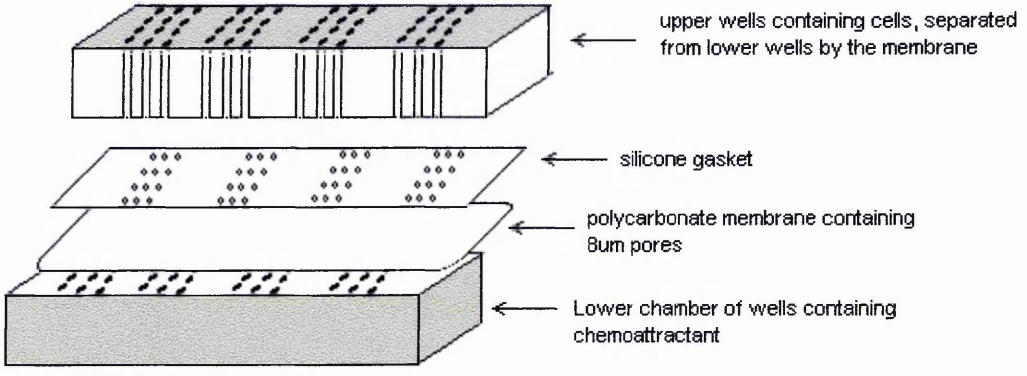
3.2.1 Chemotaxis assay.

Cell migration in response to chemokines was assessed using a 48 well microchemotaxis chamber method (Neuroprobe MD, USA) as previously described (Cross *et al.,* 1997). Nucleopore membranes (Costar, U.K) with 8μm pores were coated with fibronectin (6.5 μg/ml) (Sigma, U.K) in PBS for 20 minutes at 37°C. These were then air dried, rinsed in PBS and dried again. Stock chemokines (100ng/ml in PBS/0.1% BSA) were diluted in serum free DMEM and 28μl of each dilution pipetted in triplicate into the lower wells of the chemotaxis chamber. This was the volume required to fill the wells, with a slight positive meniscus. The human chemokines used included: MCP1, MIP1α, MIP1β, RANTES, IL-8 and IP-10 and were a kind gift from Prof. D Taub (NIH, Maryland, USA). 0.1% or 0.05% BSA in PBS was used as a negative control, as this was used in storage of the stock chemokines and present at these concentrations, in the highest chemokine concentration used, in experiments with rat microglia and CHME3 respectively. The response of rat microglia to rat MCP1 (Peprotech, UK) was also compared to the response with human MCP1, to examine the effects of species difference.

The filled lower chamber was then overlayed with the coated membrane and the top chamber assembled to form wells (Figure 3.2). 50μl of cell suspension ($5x10^5$/ml) in serum free DMEM was added to each of the wells and the chamber was incubated at 37°C, 95% air, 5% CO_2 in a humid environment for a fixed time period. The incubation period was determined by preliminary time course experiments assessing the response of microglia to MCP1 (20ng/ml) with incubation times of 2, 4 and 6 hours for CHME3 cells and 5, 10 and 17 hours for rat microglia. The time was extended for primary microglia to

allow recovery of the cell membranes, following the enzymatic isolation procedure. After incubation the chamber was dismantled and the cells on the membrane fixed in methanol for 5 minutes and stained. After fixing, the membrane was washed in distilled water and then placed in Harris' Haematoxylin (BDH) for 5 minutes. The membrane was rinsed in running tap water and then in Scots tap water (10g magnesium sulphate, 1.75g sodium bicarbonate, 500ml distilled water) for a further 2 minutes to define the nuclei. Following this, the membrane was inverted onto a microscope slide and a cover slip mounted with Immumount (Shandon, Life Sciences, UK). Migration was assessed by counting migrated cells in 5 microscopic fields per triplicate well (i.e. 15 fields per chemokine concentration) at x400 magnification.

Figure 3.2 Assembly of the 48 well chemotaxis chamber.



The assembled chamber is held together tightly by 6 screws.

3.2.2 Statistical Analysis

Significant results were calculated using a one way ANOVA followed by a multiple range analysis, to compare migration of cells to the control (0.05 or 0.1% BSA)

with chemokine induced migration and $P<0.025$ was considered significant. Results were expressed as the mean number of cells migrating per 3 wells in 5 microscopic fields +/- SEM.

3.2.3 Actin staining of the cytoskeleton using TRITC labelled phalloidin

Tetramethylrhodamine B Isothiocyanate (TRITC) labelled phalloidin (Sigma, UK) was used to visualise changes in actin fibre organisation in microglia, following incubation with chemokines. Phalloidin is highly toxic so the appropriate care must be employed when used i.e. wear protective clothing and use in a fume hood. Cells were seeded onto sterile fibronectin (6.5µg/ml) (Sigma, UK) coated coverslips (BDH, UK) in 6 well plates (Costar, Northumbria Biosciences, U.K) at a concentration of $5x10^4$/ml (300µl on each coverslip) for about one hour to allow cells to adhere. A further 2ml of cDMEM was added and cells cultured overnight. Cells were washed in serum free medium (SFM) and incubated with the appropriate chemokine diluted in SFM at 37°C for 15, 60 or 180 minutes. Shorter incubation times were chosen for the actin studies than for the migration studies as the chemokines should elicit an effect on cell shape immediately. Serum free medium (or SFM + 0.1%BSA) was used as the control. Cells were then washed in PBS and fixed for 20 minutes in freshly prepared 4% paraformaldehyde in PBS at room temperature. The paraformaldehyde was then removed, the cells were washed in PBS and then fixed in ice cold acetone for a further 5 minutes. Cells were then washed in PBS, and 200µl of TRITC labelled phalloidin (200ng/ml) in SFM, was added per well and incubated at room temperature for 45 minutes. The coverslips were washed twice in PBS and once in distilled water and then inverted onto microscope slides mounted in PBS/glycerol (1:1). Cells were visualised

under fluorescence microscopy (Olympus BX60) and photographed using Ilford HP5 film.

3.3 Results

3.3.1 Time course migration experiments

Time course experiments were carried out on CHME3 cells and rat microglia, to determine the optimal incubation time required to give a significant number of migrating cells. CHME3 cells were incubated in the chemotaxis chamber for 2, 4 or 6 hours and the number of cells migrating to MCP1 (20ng/ml) were counted in triplicate wells. Only small numbers of cells (mean of 10 per high power field), had migrated through the membrane after 2 hours and this was increased (mean of 30 cells per high power field) after a 4 hour incubation period. The number of cells migrating after 6 hours was substantially more than at 4 hours, therefore this was chosen as the appropriate incubation time for further experiments (Figure 3.3). Time course experiments for chemotaxis by rat microglia used incubation periods of 5, 10 or 17 hours, longer incubation times were used as cells were placed in the chemotaxis chamber directly after isolation. The number of primary rat microglia migrating after a 5 hour incubation time was comparable to the number of CHME3 cells that had migrated after the 4 hour incubation period (mean of 30 cells per high power field). When rat microglia were incubated for 10 hours in the chemotaxis chamber, the number of migrating cells more than doubled and after an overnight incubation of 17 hours the number of migrating cells increased further (Figure 3.4). The proportion of cells migrating after 17 hours was still relatively small compared to the number of cells added (approximately 0.6% in 5 microscopic fields). This could be due to the small surface area of the membrane in contact with the cells and although longer incubation times would probably produce higher numbers of migrating cells, the nutrients in the media would be consumed due to a high cell density in a small volume and also the number of cells in the top and bottom

Figure 3.3 Time course of CHME3 migration to MCP1 (20ng/ml)

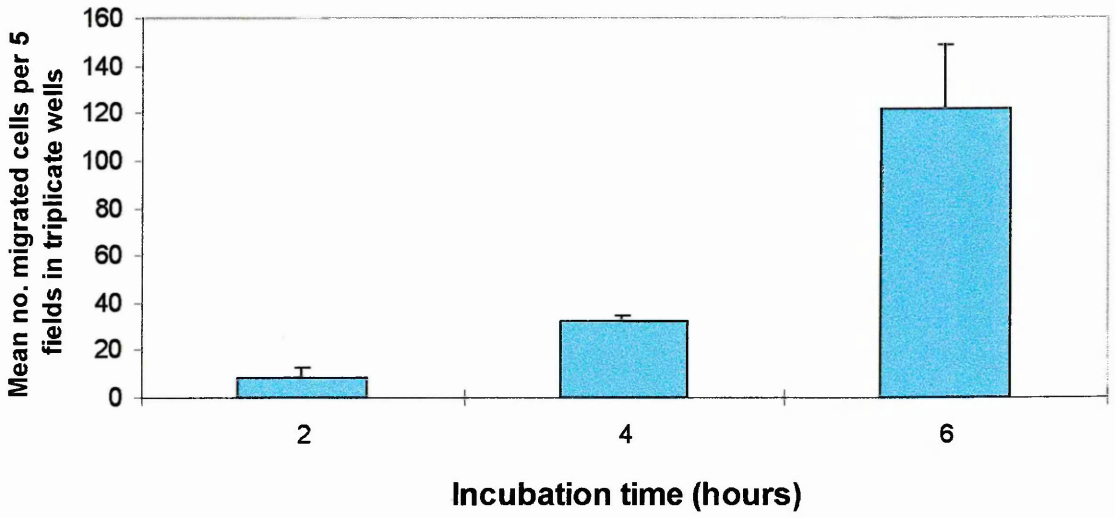
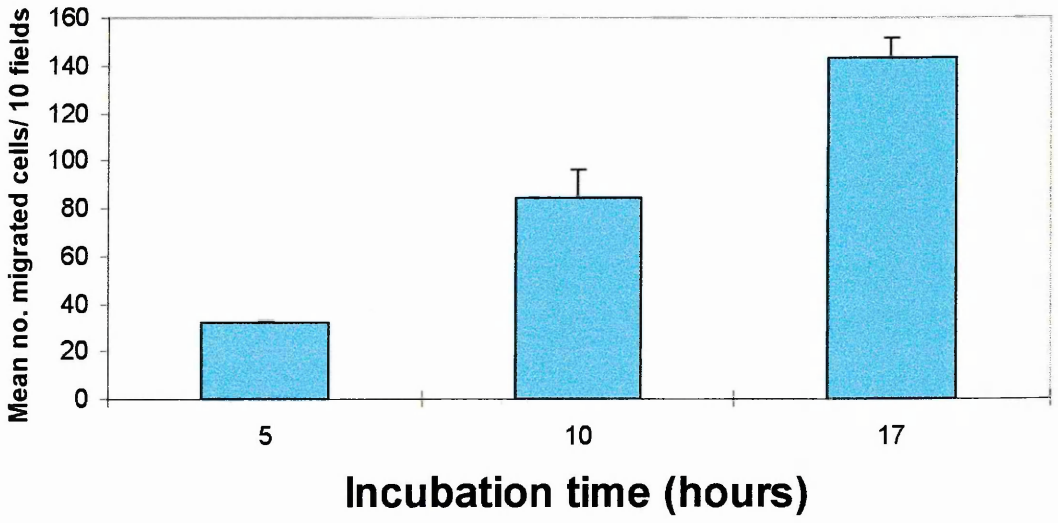


Figure 3.4 Time course of rat microglial migration to MCP1 (20ng/ml)



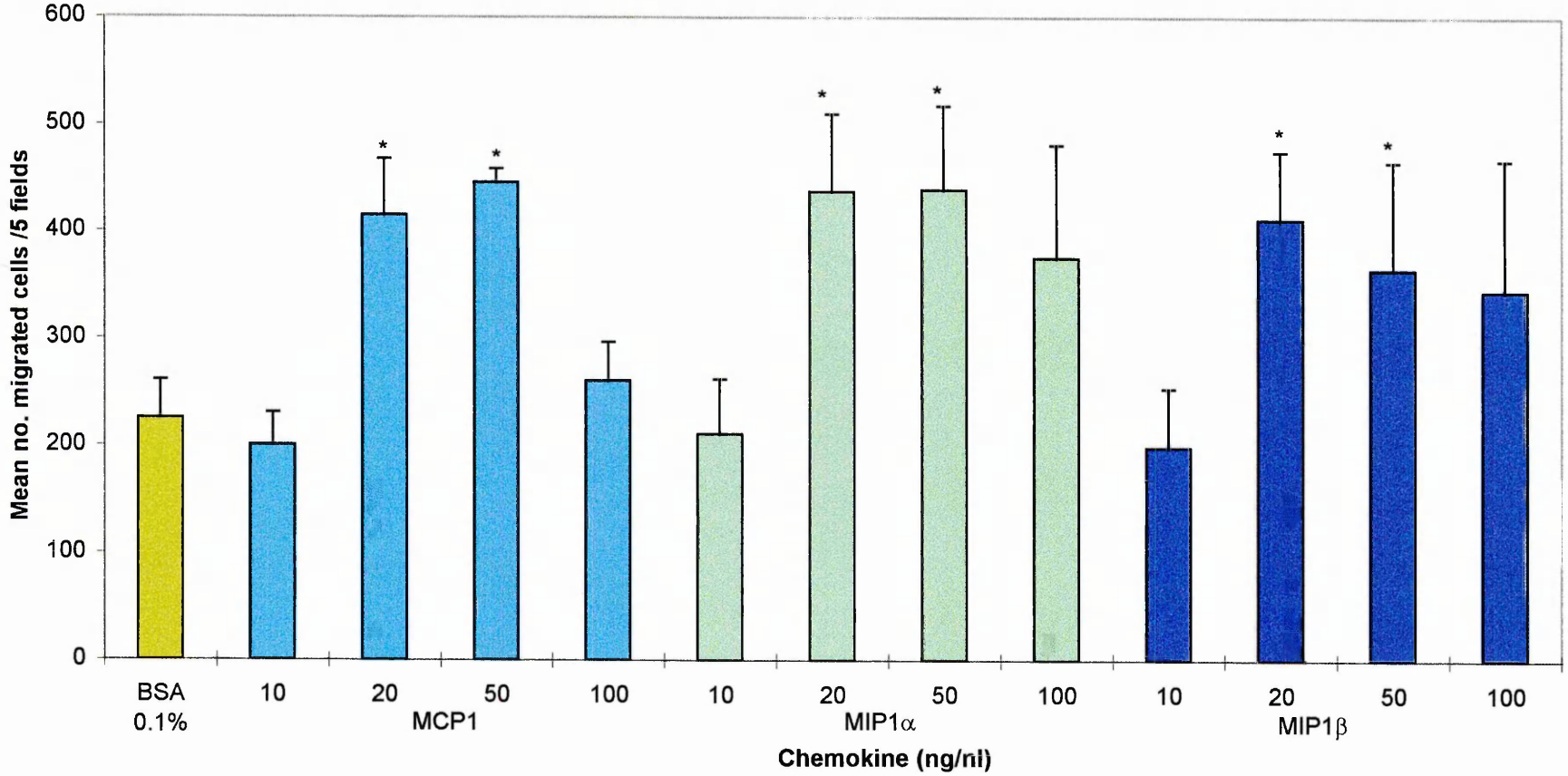
A time course migration experiment was performed using CHME3 cells (Figure 3.3) and rat microglia (Figure 3.4). Triplicate wells were used and expressed as the mean number of migrated cells per 5 microscope fields +/- SEM.

chambers would reach equilibrium as the chemokine gradient would equalise by diffusion across the membrane.

3.3.2 Migration of primary rat microglia in response to chemokines

Migration responses of 30 day old rat microglia were examined in response to the chemokines MCP1, MIP1α, MIP1β, RANTES, IL-8 and IP-10. Migration experiments using rat microglia showed typical bell shaped curves to all of the chemokines tested, where optimal migration reached a peak and then declined at higher chemokine concentrations. The migration of primary isolated rat microglia to MCP1, MIP1α and MIP1β, RANTES, IL-8 and IP-10 and data from all experiments is summarised in Tables 3.1 and 3.2. Figures 3.5 a and b show representative data from one of three individual experiments. A significant response was observed, $P<0.025$, for MCP1, MIP1α, MIP1β, RANTES, IL-8, and IP-10 in all three repeat experiments, although the actual numbers of migrating cells was quite variable between experiments. The concentration of chemokine required to give the highest migration response of rat microglia to MCP1, MIP1α and MIP1β was between 20 and 50ng/ml. For RANTES, IL-8 and IP-10, the concentration required to give the optimal migration response was variable between 20 and 100ng/ml. IL-8 and IP-10 gave the most variable responses in the actual numbers of cells responding between each experiment with counts between 164 and 617 migrating cells per 5 microscope fields, for IP-10 at 50ng/ml in one experiment. Rat microglia were also shown to migrate equally to rat and human MCP1 with similar numbers of cells responding (Figure 3.5c)

Figure 3.5a Migration response of rat microglia to human chemokines



The migration assay was incubated for 17 hours. The data represents one of three repeat experiments, each performed in triplicate wells. Bars represent mean number of migrated cells per 5 microscope fields +/-SEM * represents a significant increase in migration above control ($P<0.025$)

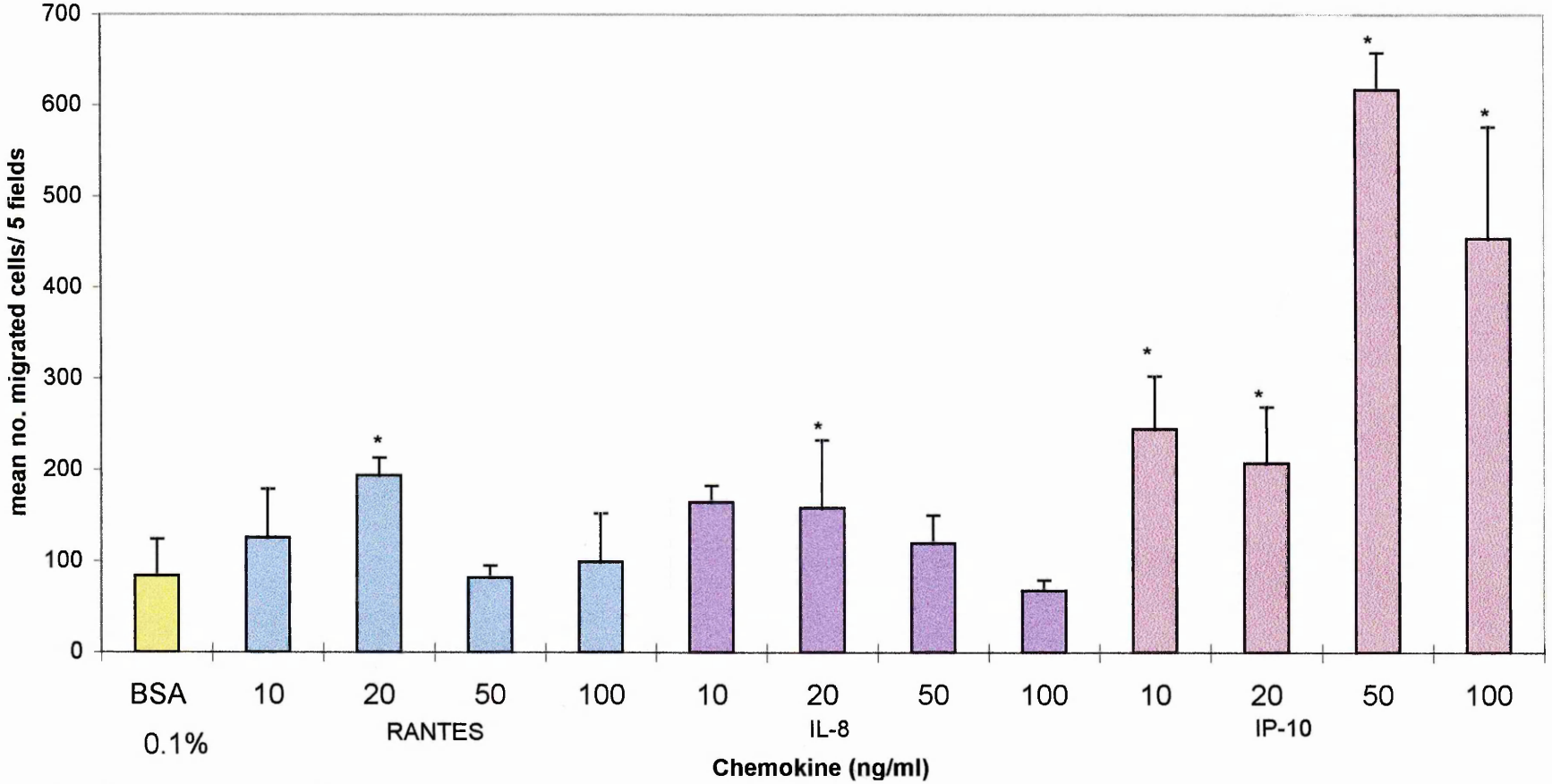
Table 3.1 Summary of migration responses of rat microglia to human MCP1, MIP1α and MIP1β in 3 individual experiments.

Chemokine (ng/ml)		mean number of migrated cells*.	SEM	Range	No. exp. showing significant migration above control#
0 (control)		118	71	40-225	-
MCP	10	214	105	69-372	1
	20	244	113	95-415	3
	50	249	137	42-445	2
	100	139	80	57-260	0
MIP1α	10	145	84	19-211	1
	20	188	166	31-437	1
	50	217	148	38-439	2
	100	185	126	43-375	1
MIP1β	10	126	48	74-198	0
	20	199	141	97-411	2
	50	170	129	52-364	1
	100	187	104	57-345	1

* represents mean of three individual experiments, each carried out in triplicate wells (i.e. 9 wells were counted for each chemokine concentration).

represents number of experiments showing significant migration above control migration ($P<0.025$) out of three individual experiments using a one way ANOVA followed by multiple range analysis.

Figure 3.5b Migration response of rat microglia to human chemokines



The migration assay was incubated for 17 hours. The data represents one of three repeat experiments, each performed in triplicate wells. Bars represent mean number of migrated cells per 5 microscope fields +/-SEM * represents a significant increase in migration above control ($P<0.025$)

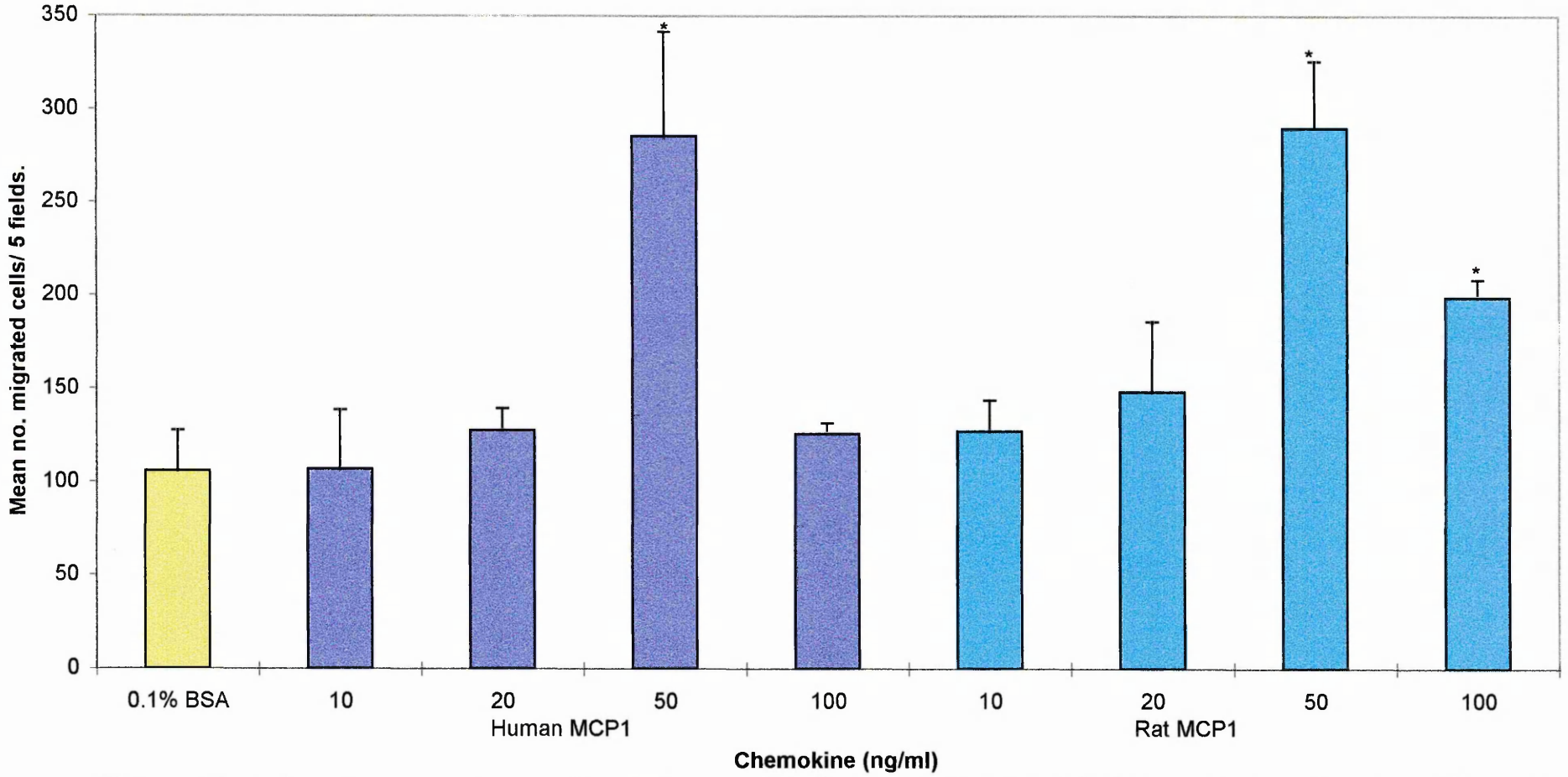
Table 3.2 Summary of migration responses of rat microglia to human RANTES, IL-8 and IP-10 in 3 individual experiments.

Chemokine (ng/ml)		mean number of migrated cells*.	SEM	Range	No. exp. showing significant migration above control#
0 (control)		100	27	75-142	-
RANTES	10	92	21	69-125	0
	20	163	38	105-193	2
	50	122	27	81-154	1
	100	160	71	99-267	1
IL-8	10	123	27	97-165	0
	20	153	4	147-158	2
	50	204	69	119-309	2
	100	251	206	68-561	1
IP-10	10	142	76	27-245	2
	20	164	42	100-208	2
	50	323	196	164-617	3
	100	239	143	91-454	2

* represents mean of three individual experiments, each carried out in triplicate wells (i.e. 9 wells were counted for each chemokine concentration).

represents number of experiments showing significant migration above control migration ($P<0.025$) out of three individual experiments using a one way ANOVA followed by multiple range analysis.

Figure 3.5c Comparison of migration response of rat microglia to human and rat MCP1



Human and rat microglia were used to examine whether rat microglia responded equally to both rat and human chemokine. *Represents significant migration response above control levels ($P<0.025$).

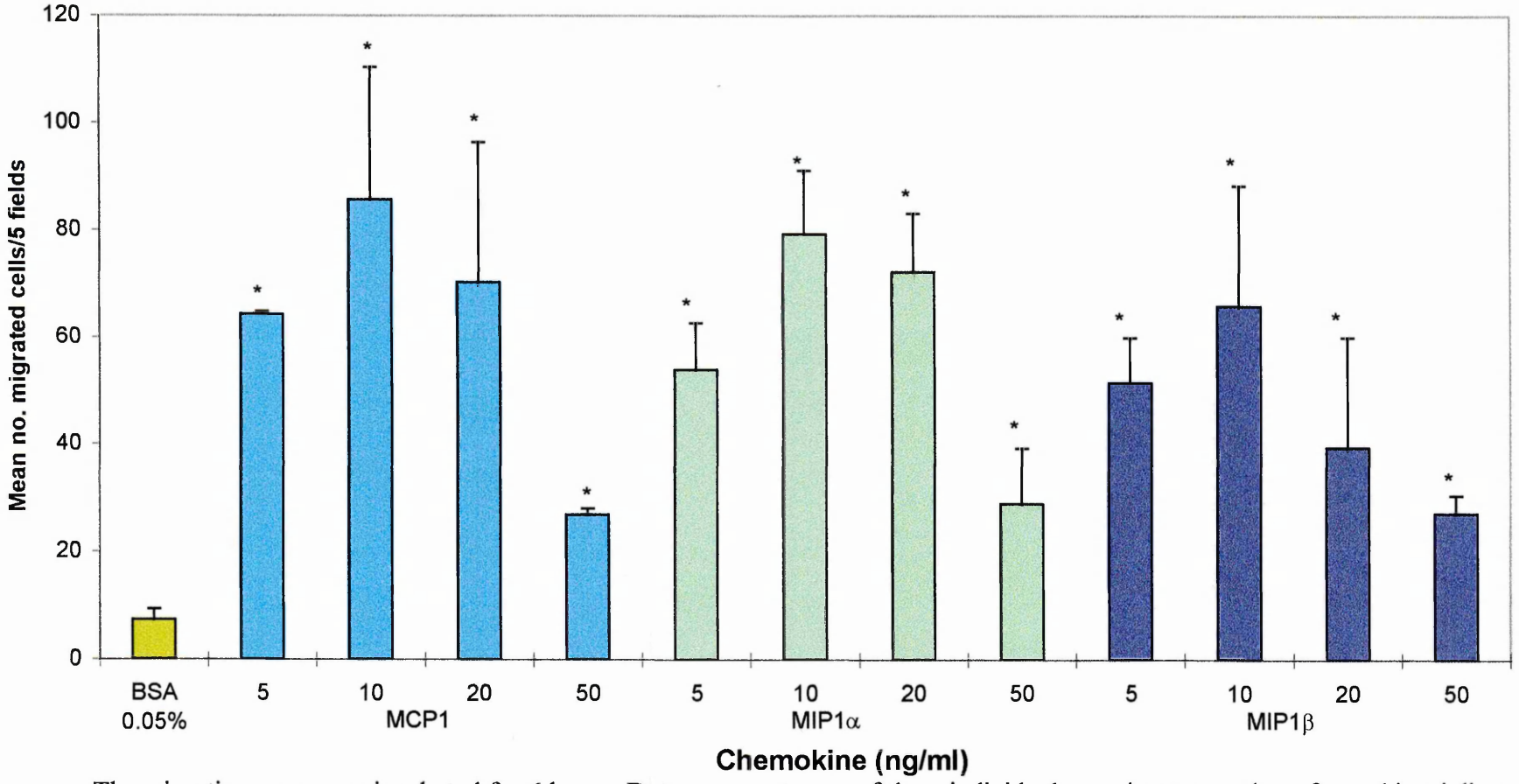
3.3.3 Migration of CHME3 cells in response to chemokines

Results in migration experiments using the human foetal cell line, CHME3 show that a significant response was observed ($P<0.025$) for all chemokines tested at, at least one concentration. Figures 3.6a and b show representative data from one of three individual migration experiments for MCP1, MIP1α, MIP1β and RANTES, IL-8 and IP-10 and results from all experiments are summarised in Tables 3.3 and 3.4. The concentration of chemokine required to produce optimal migration of CHME3 using MCP1 was between 10 and 20ng/ml, for MIP1α and MIP1β this was 10ng/ml, and using IP-10, a concentration range of 20 to 50ng/ml gave the highest response. The concentration of the chemokines RANTES and IL-8, required to produce the highest migration response was more variable. Numbers of migrated cells in experiments with CHME3 cells were generally lower than those seen using primary rat microglia with the highest response producing a count of just 113 migrated cells per 5 microscope fields in response to MIP1α compared to the 617 cells counted at the highest response for rat microglia in response to IP-10, although incubation times used for the two cell types were very different (5 hours and 17 hours respectively). The numbers of cells migrating to controls (BSA) was also much lower using CHME3 cells possibly suggesting they are less motile than primary microglial cells.

3.3.4 Chemokine induced changes in actin polymerisation

Chemokine induced changes in actin polymerisation and distribution in microglia were visualised by incubating cell cultures with chemokines for 15 to 180 minutes and staining with TRITC labelled phalloidin, a stain specific for f-actin. All of the chemokines tested demonstrated the ability to cause redistribution and polymerisation of f-actin in both rat microglia and CHME3 cells.

Figure 3.6a Migration of CHME3 cells to chemokines



The migration assay was incubated for 6 hours. Data represents one of three individual experiments, each performed in triplicate wells. Bars represent the mean number of migrated cells per 5 microscope fields +/- SEM. * represents significant migration above control levels.

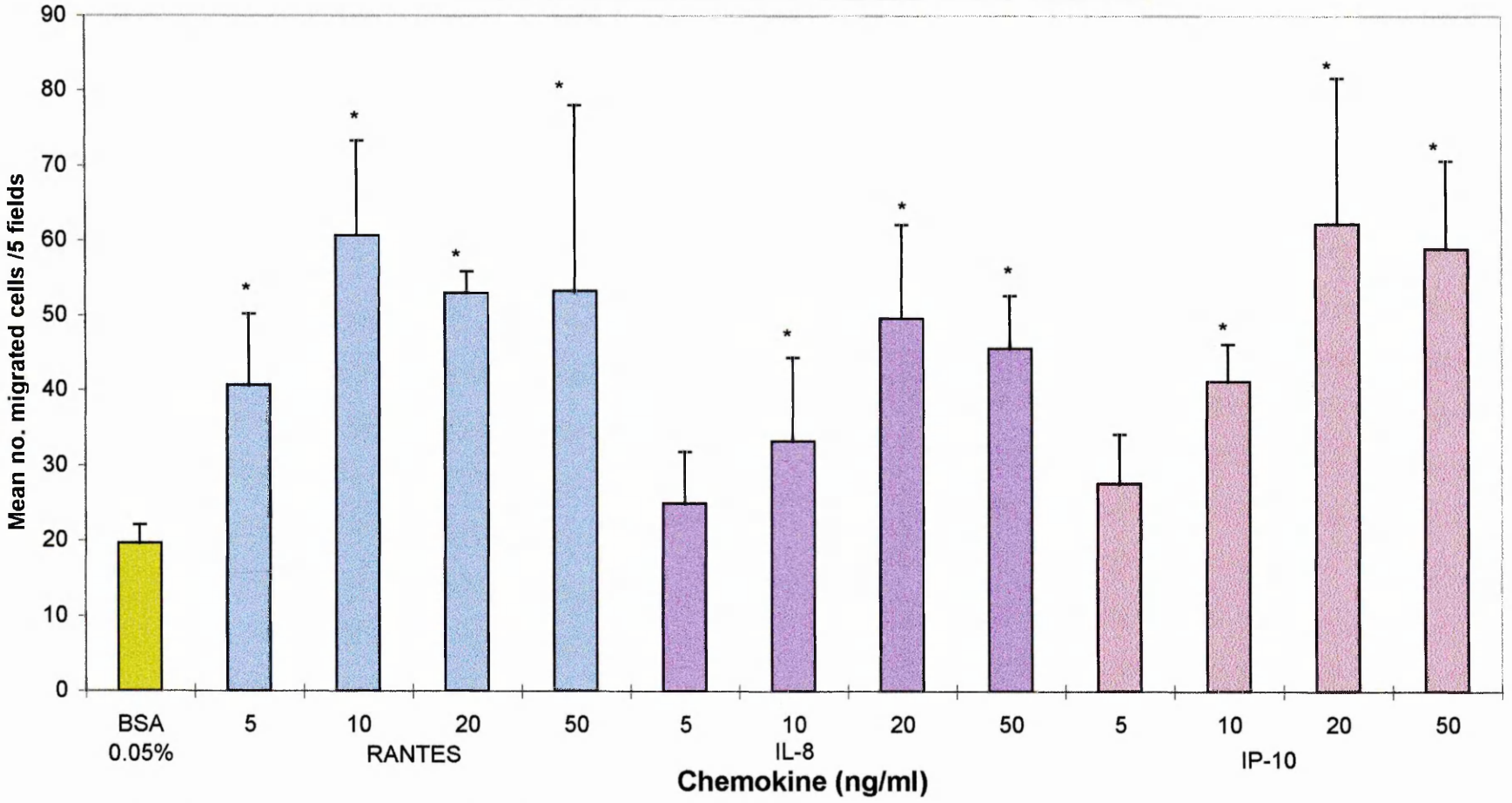
Table 3.3 Summary of migration responses of CHME3 cells to human MCP1, MIP1α and MIP1β in 3 individual experiments.

Chemokine (ng/ml)		mean number of migrated cells*.	SEM	Range	No. exp. showing significant migration above control#
0 (control)		12	3	7-17	-
MCP1	**5**	68	19	44-97	3
	10	69	11	51-70	3
	20	63	12	45-74	2
	50	24	7	14-33	1
MIP1α	**5**	71	27	47-113	3
	10	78	18	50-106	3
	20	57	15	34-72	2
	50	28	4	22-34	1
MIP1β	**5**	62	26	34-102	2
	10	62	3	57-66	3
	20	52	8	39-65	3
	50	39	10	27-54	2

* represents mean of three individual experiments, each carried out in triplicate wells (i.e. 9 wells were counted for each chemokine concentration).

represents number of experiments showing significant migration above control migration ($P<0.025$) out of three individual experiments using a one way ANOVA followed by multiple range analysis.

Figure 3.6b Migration of CHME3 cells to chemokines



The migration assay was incubated for 6 hours. Data represents one of three individual experiments, each performed in triplicate wells. Bars represent the mean number of migrated cells per 5 microscope fields +/- SEM. * represents significant migration above control levels.

Table 3.4 Summary of migration responses of CHME3 cells to human RANTES, IL-8 and IP-10 in 3 individual experiments.

Chemokine (ng/ml)		mean number of migrated cells*.	SEM	Range	No. exp. showing significant migration above control#
0 (control)		20	5	13-27	-
RANTES	5	45	4	40-51	2
	10	53	21	22-78	2
	20	56	19	30-86	3
	50	47	12	29-61	3
IL-8	5	48	20	25-79	2
	10	47	14	33-69	3
	20	55	19	31-84	3
	50	47	15	26-71	2
IP-10	5	38	8	27-51	1
	10	53	16	40-78	3
	20	53	6	46-62	2
	50	51	5	46-59	2

* represents mean of three individual experiments, each carried out in triplicate wells (i.e. 9 wells were counted for each chemokine concentration).

represents number of experiments showing significant migration above control migration ($P<0.025$) out of three individual experiments using a one way ANOVA followed by multiple range analysis.

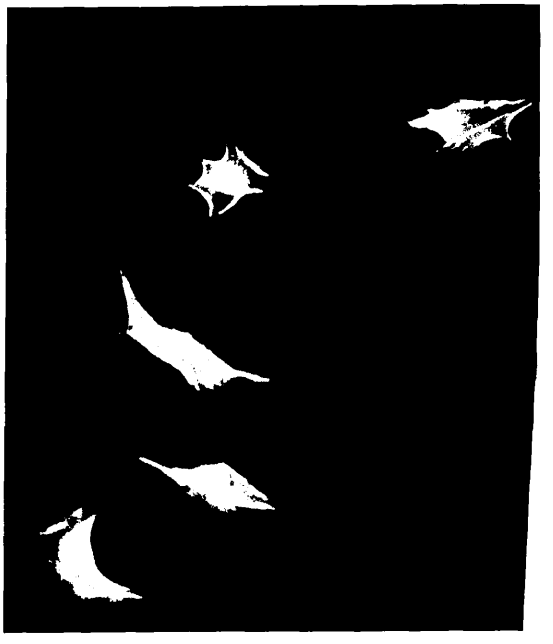
3.3.5 Chemokine induced changes in the actin cytoskeleton in CHME3 cells

Although morphological changes were evident following incubation with chemokines for only 15 minutes, the difference between treated and control cells was more pronounced after 60 minutes and after 180 minutes there was little further change in appearance of the cells. Figure 3.7 shows CHME3 cells treated with MCP1 (20ng/ml) for 15, 60 and 180 minutes compared to the control, untreated cells. Control cells show diffuse staining of actin with poorly defined edges (Figure 3.7a). After 15 minutes treatment with MCP1, the cells showed formation of stress fibres across and around the edges of cells (Figure 3.7b). After treatment for 60 minutes the stress fibre formation was much more pronounced with more f actin around the edges of cells (Figure 3.7c) and this was similar in the cells treated for 180 minutes (Figure 3.7d). Figure 3.8a and b show untreated and MCP1 treated CHME3 cells for 60 minutes at higher magnification to better illustrate the stress fibre formation after chemokine treatment. A comparison of untreated CHME3 cells compared with cells treated for 60 minutes with the chemokines MCP1, MIP1α, MIP1β, RANTES, IL-8, IP-10 and MIG is shown in Figure 3.8a-h. All the chemokines tested caused an increase in the amount of f actin and increased the stress fibre formation.

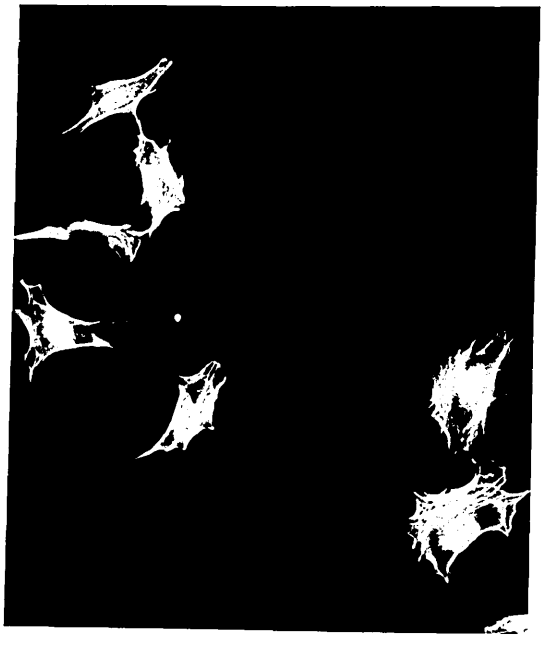
3.3.6 Chemokine induced changes in the actin cytoskeleton of primary rat microglia

Untreated rat microglia (Figure 3.9a) have the most intense stain of f-actin using TRITC phalloidin, around the nuclei and little staining was observed in the cytoplasmic regions as seen in similar experiments using CHME3 cells. Untreated cells had a bipolar branched morphology although only faint f actin staining appeared in these ramifications. The human chemokines MCP1, MIP1α, MIP1β, RANTES, IL-8 and MIG, when added to rat microglia for 60 minutes caused a change in morphology. Treated cells have a

Figure 3.7 Visualisation of F actin staining in CHME after treatment with MCP1 at 20ng/ml (X200 magnification).



a) Unstimulated cells

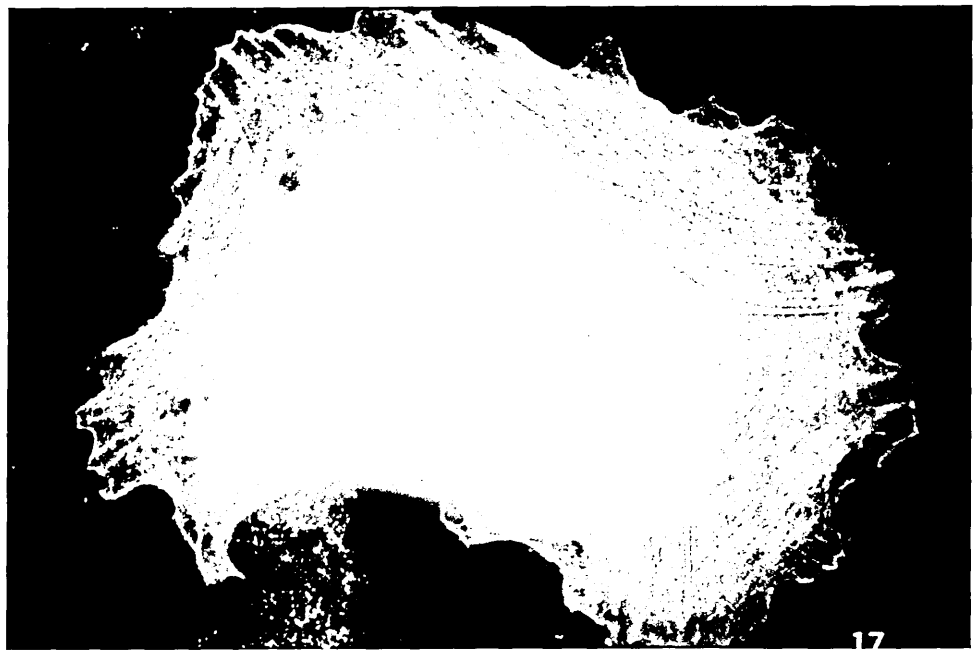


b) MCP1 stimulated (15minutes)

c) MCP1 stimulated (60 minutes)

d) MCP1 stimulated (180 minutes)

Figure 3.8 Visualisation of F actin staining in CHME3 cells using TRITC Phalloidin, after chemokine treatment or control media for 60 minutes. (X1000). Chemokine treated cells show more actin fibres and are stained more intensely.

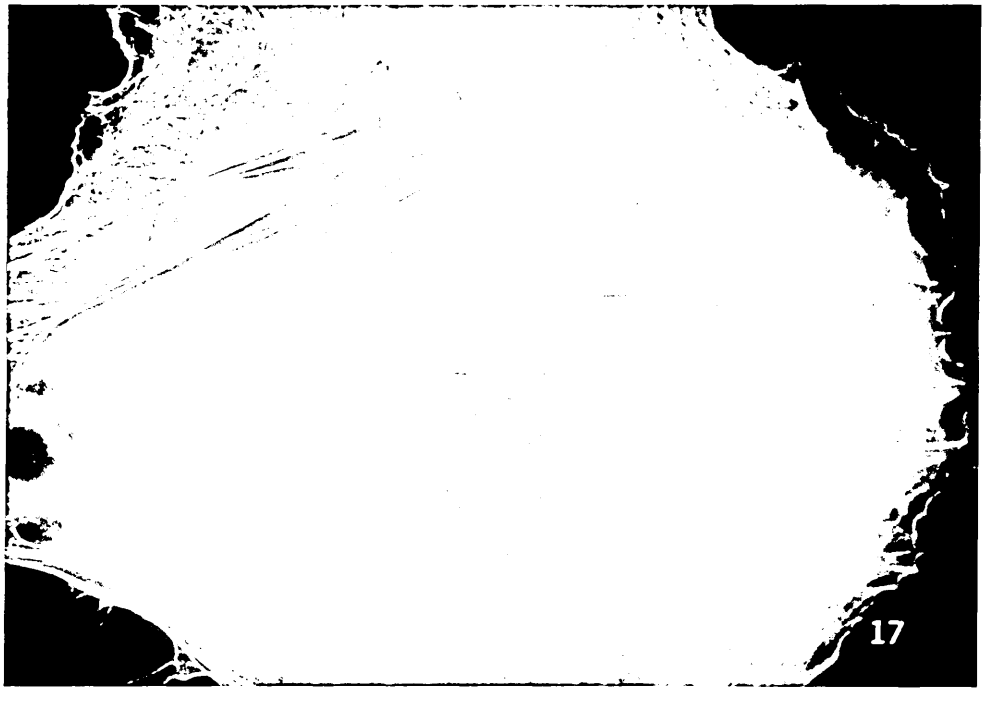


a) Control unstimulated cells

b) MCP1 treated (20ng/ml)

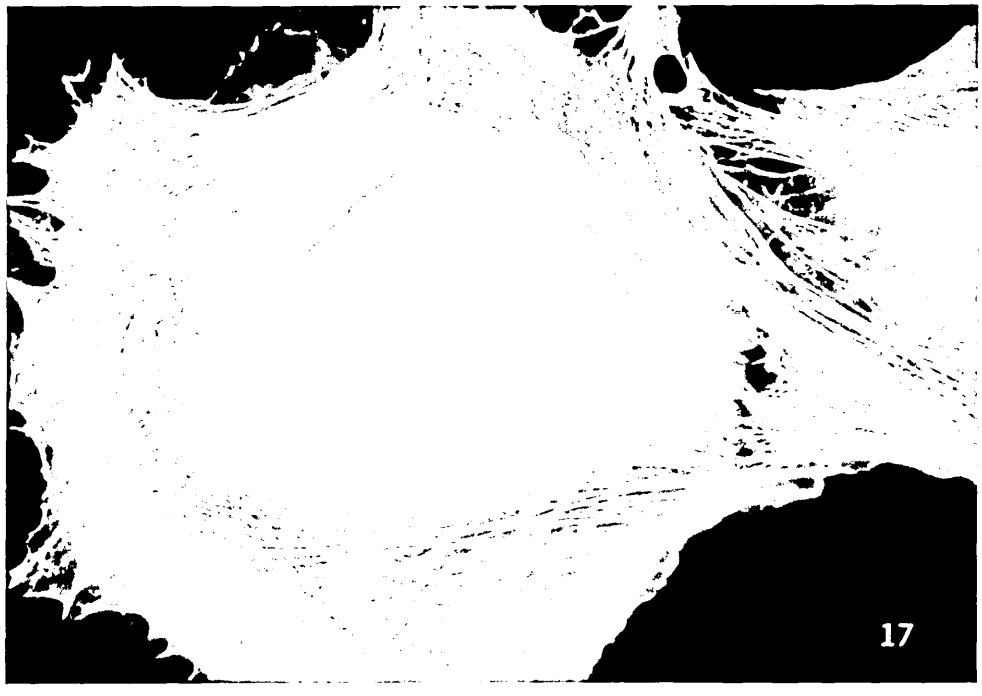
Figure 3.8 continued. Visualisation of F actin staining in CHME3 cells stained after chemokine treatment for 60 minutes. (X1000).

c) MIP1α (20ng/ml)



d) MIP1β (20ng/ml)

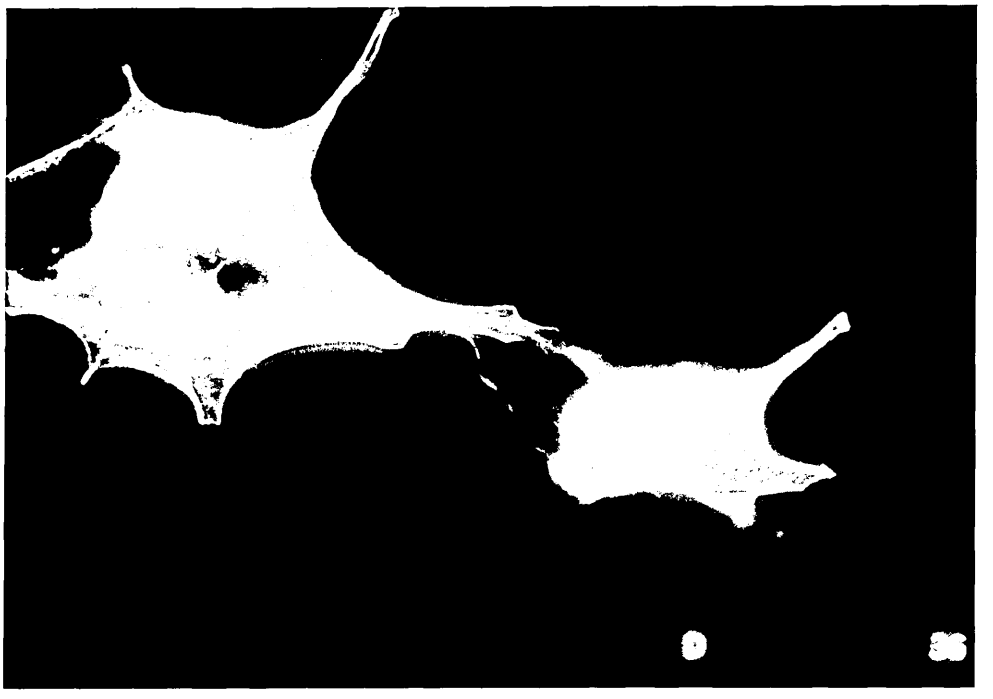
Figure 3.8 continued. Visualisation of F actin staining in CHME3 cells stained after chemokine treatment for 60 minutes. (X1000).



e) RANTES (20ng/ml)

f) IL-8 (20ng/ml)

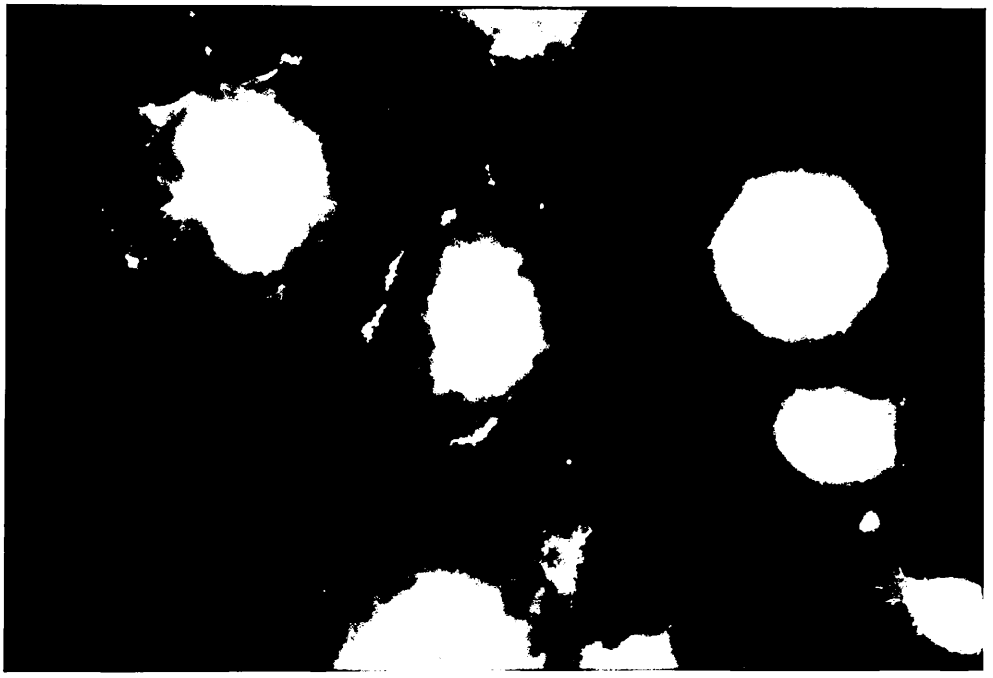
Figure 3.8 continued. Visualisation of F actin staining in CHME3 cells stained after chemokine treatment for 60 minutes. (X1000).



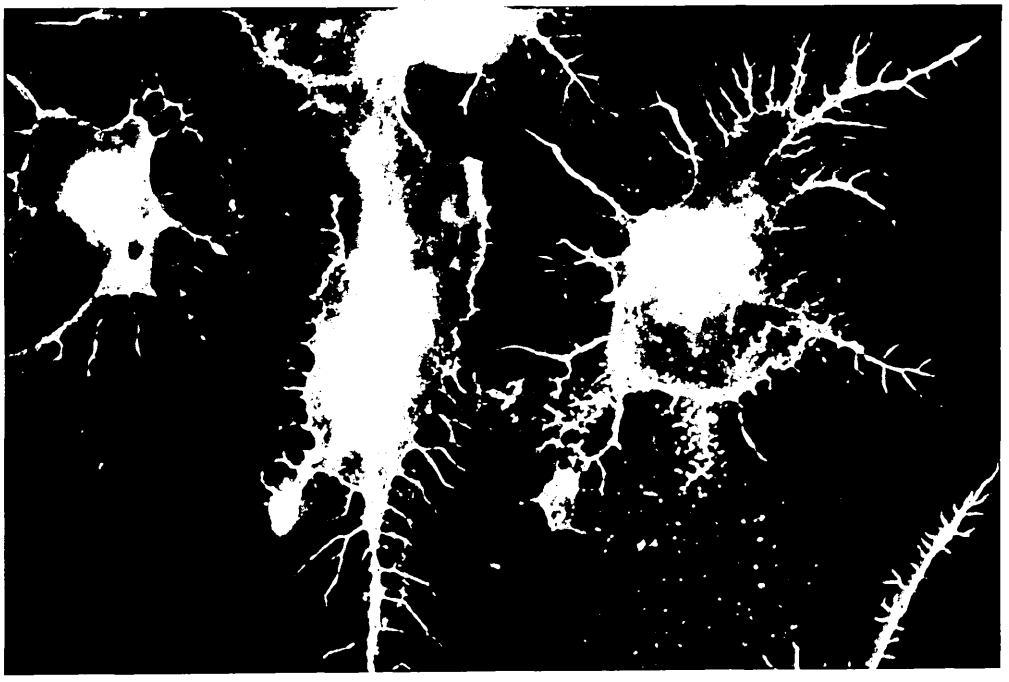
g) Mig (20ng/ml)

h) IP-10 (20ng/ml)

Figure 3.9 Visualisation of F actin in 30 day old primary rat microglia using TRITC phalloidin following incubation for 60 minutes with chemokine (all used at 20ng/ml) or control media (X1000). Chemokine treated cells show a more highly branched morphology with intense f actin staining in the pseudopodia.



a) Control unstimulated cells



b) rat MCP1 treated

much more highly branched structure with intense f-actin staining within the pseudopodia (Figure 3.9b-g and i). The α chemokine, IP-10 showed similar effects on the actin cytoskeleton however to a lesser extent than seen with the other chemokines in all experiments (Figure 3.9h). The effects of rat and human MCP1 on rat microglia were compared to assess if there was any species specific effect of the chemokine used, however the changes to the actin cytoskeleton using either chemokine were identical. Some of the chemokine treated rat microglia appeared to have a polarised morphology with 'horns' at one edge (shown in figure 3.9c) as described by Haapianiemi *et al* (1995). Rat microglia were treated with MCP1 that had been boiled for 5 minutes, prior to addition to cells, to determine if the chemokine effects were specifically due to functional activity of chemokine- chemokine receptor interactions. Cells treated with heat denatured chemokine appeared the same as control cells with only diffuse actin staining (Figure 3.9j).

The effects of chemokines on f actin formation were observed to be different in rat microglia compared to CHME3 cells, with the most obvious change being the stress fibre formation in CHME3 cells, which was not seen to the same extent in the primary rat microglia. The change in staining intensity in the rat microglia was mainly confined to the pseudopodia and although the staining intensity was increased around the edges of CHME3 cells, they showed a much less branched morphology (compare Figures 3.8b and 3.9b/c)

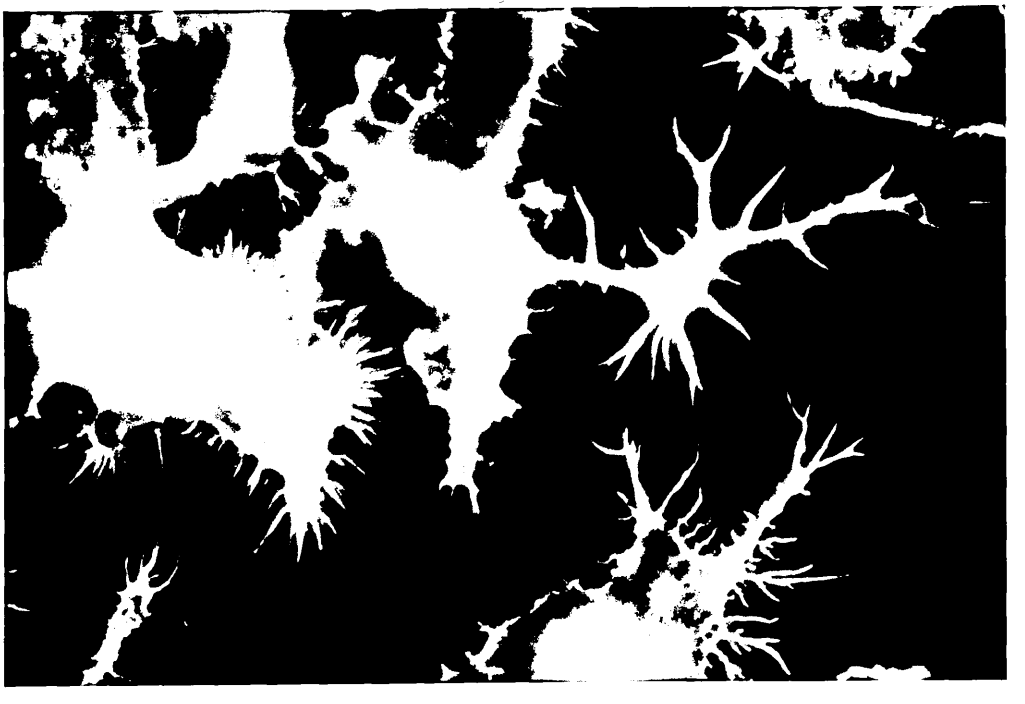
Figure 3.9 continued. Visualisation of F actin in 30 day old primary rat microglia after incubation for 60 minutes with chemokine (all used at 20ng/ml) (X1000)

c) human MCP1

d) human MIP1α

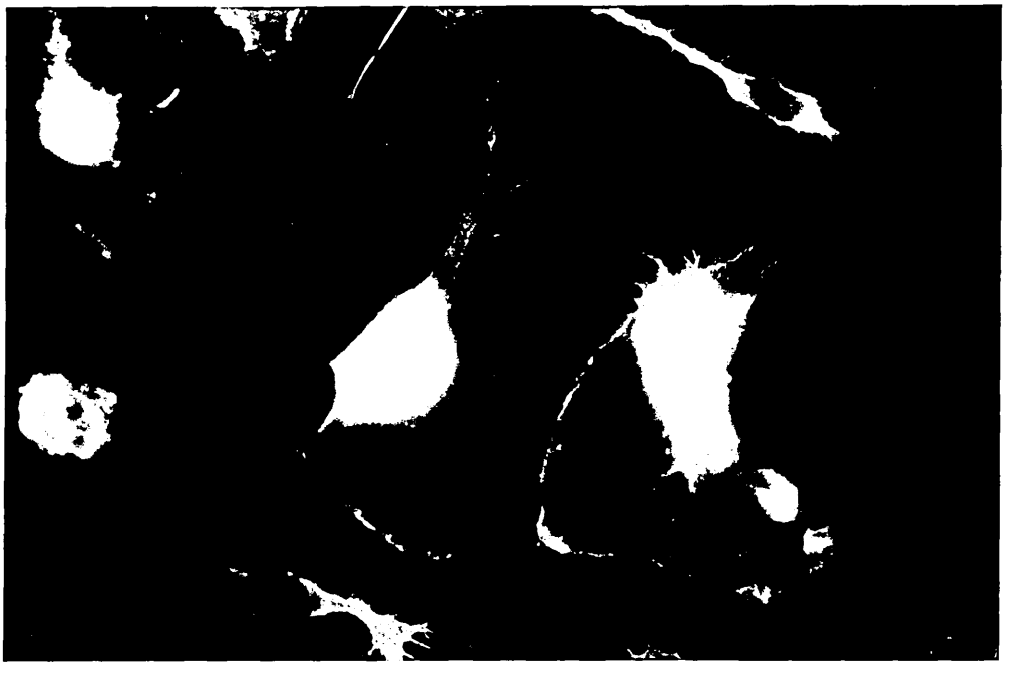
Figure 3.9 continued. Visualisation of F actin in 30 day old primary rat microglia after incubation for 60 minutes with chemokine (all used at 20ng/ml) (X1000)

e) human MIP1β

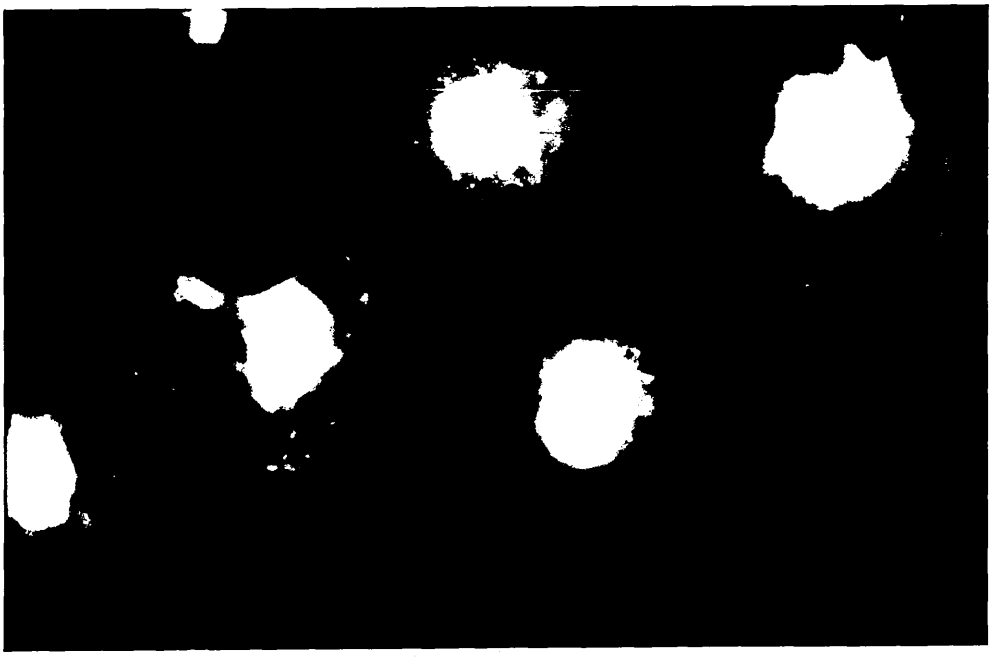


f) human RANTES

Figure 3.9 continued. Visualisation of F actin in 30 day old primary rat microglia after incubation for 60 minutes with chemokine (all used at 20ng/ml) (X1000)

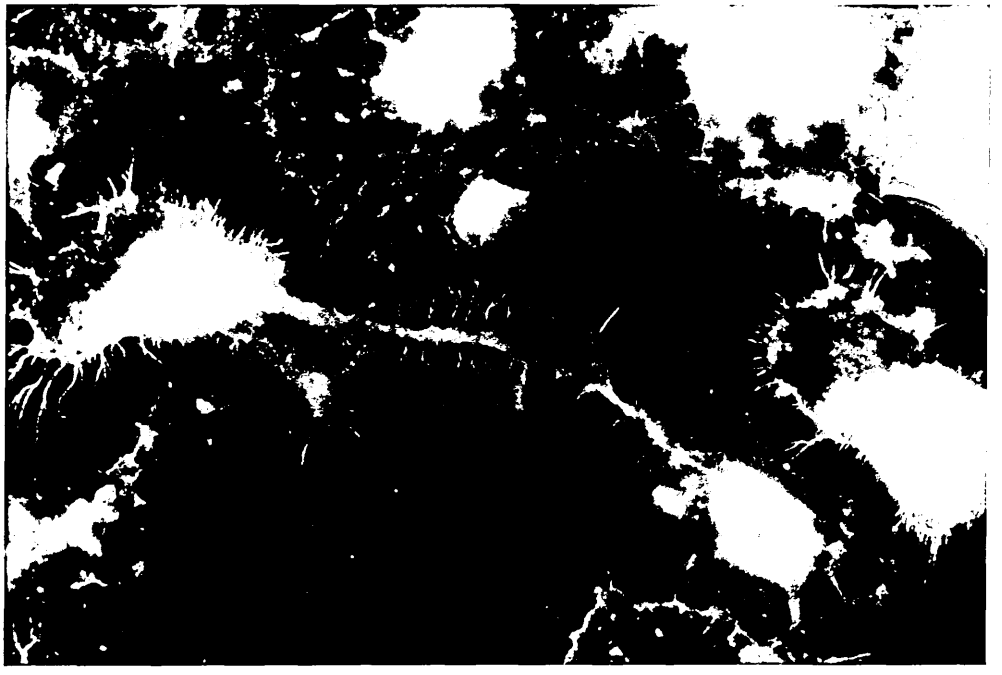


g) human IL-8



h) human IP-10

Figure 3.9 continued. Visualisation of F actin in 30 day old primary rat microglia after incubation for 60 minutes with chemokine (all used at 20ng/ml) (X1000)



i) human Mig

j) Denatured rat MCP1 The chemokine was boiled before adding to cells, to denature the protein.

3.4 Discussion

The results of this study clearly demonstrate that rat microglia and CHME3, the human foetal microglial cell line, migrate in response to chemokines. 30 day old rat microglia and not neonatal microglia were used in this study as they are more representative of neuroimmunological processes *in vivo* during inflammation. These primary microglia were compared to CHME3 in their migratory response to chemokines. Although CHME3 cells are of foetal origin, similar responses were observed to those of adult rat microglia. Both cell types migrated to a concentration range of chemokines giving a typical bell shaped curve, where the cells respond only weakly or not at all to low concentrations of chemokine and migration is attenuated at high concentrations. This presumably causes cells to migrate up a concentration gradient until they reach a critical point of receptor saturation.

To test whether the typically observed decrease in migration response at higher chemokine concentrations were significantly different from the peak response, further statistics were performed. The migration response at the highest chemokine concentration used with an apparent decrease in migration, was compared to the peak migration response, at a lower chemokine concentration. Paired T tests were used for all of the chemokines for both CHME3 cells and rat microglia, $P<0.05$ was considered significant.

The migration response of rat microglia to MCP1 was significantly less at the highest concentration used in two out of three experiments and only one out of three experiments using MIP1α, RANTES, IL-8 and IP-10. Experiments with MIP1β was not significantly reduced at the highest concentration used in any experiments suggesting that a higher concentration than 100ng/ml may be required to reach maximal migration.

The numbers of migrated cells were generally higher in experiments using rat microglia with numbers of migrated cells as high as 617 per 5 microscopic fields. However longer incubation times were used for the primary rat microglia since the harshness of the isolation procedure of the cells required time for the cell membranes to recover. The actual numbers of migrated cells cannot be directly compared between the two cell types studied, although their responses to the different chemokines does allow comparison. The rat microglia generally migrated optimally to higher chemokine concentrations than those required for optimal CHME3 migration. This could be attributed to the fact that the chemokines used were recombinant human proteins, although experiments using rat MCP1 with rat microglia demonstrated no differences in the numbers of cells migrating, or the concentration required to produce optimal migration to those for human chemokines. Also the rat and human MCP1 both induced changes in the actin cytoskeleton of rat microglia. Therefore species difference does not appear to contribute to this differing response in this particular chemokine. Studies on human and mouse microglial cell migration to MCP1 by Peterson *et al* (1997) and Hayashi *et al* (1995) respectively, reported optimal migration at a chemokine concentration of 10ng/ml which is comparable to the CHME3 migration to MCP1 reported here although a higher concentration of MCP1 (50ng/ml) was required to produce optimal migration using rat microglia. CHME3 cells responded equally well to all α and β chemokines tested whereas the migration response of rat microglia was greatest to the β chemokines tested.

It is now well documented that in areas of brain inflammation in MS lesions, there is perivascular cuffing consisting of inflammatory cell types including B cells, T cells and macrophages. (Woodroofe *et al,* 1986, Adams *et al,* 1989, Benveniste, 1997)

Both infiltrating monocytes and resident brain macrophages or microglia, contribute to the macrophage population in lesions. A recent study by Simpson *et al* (1998) has reported that β chemokines are expressed in MS brain sections, by astrocytes, microglia and endothelial cells in active lesions with inflammation and demyelination. This would provide the stimulus required to chemoattract other inflammatory cells such as T and B lymphocytes and monocytes from the circulation, as well as astrocytes and microglia from adjacent areas of the brain, where they are normally evenly distributed (Perry and Gordon, 1991), to areas of inflammation and demyelination or to sites of tissue injury. Macrophages recruited to these sites may then contribute to the inflammatory response by production of inflammatory mediators such as IL-1, IL-6, TNFα and reactive oxygen species which promote and intensify the immune response. Macrophages are also involved in the breakdown and destruction of the myelin sheath and phagocytosis of the breakdown products of myelin (Benveniste, 1997).

Accumulation of activated microglia at sites of tissue injury have been reported in a number of CNS pathologies including Alzheimer's disease (Dickson *et al.*, 1993, McGeer and McGeer, 1995) Creutzfelt-Jacob disease (Muhleisen *et al.*, 1995) and experimental glioma induced in rats (Streit, 1996). This present study would suggest that centrally produced chemokines may be involved in microglia recruitment in these CNS pathologies and is not restricted to the pathogenesis of MS.

The results of this study, both on isolated rat microglia and the human microglial cell line, demonstrate that the pro-inflammatory chemokines can induce microglial cell migration *in vitro* and this may be the mechanism of chemoattraction for microglia to areas of CNS inflammation or tissue damage *in vivo*. Chemokines are produced by activated cells already present in the lesion in MS (Simpson *et al.*, 1998) which may then chemoattract microglia, expressing chemokine receptors, to respond to these signals.

The chemokine gradient established, with the highest concentration being at the lesion centre, may elicit their response by initially altering actin polymerisation and therefore motility of the cells. Our results display a dramatic change, following chemokine stimulation of microglia, in their morphology and distribution of f-actin within one hour. Although cell migration cannot be attributed to f- actin changes alone, redistribution of actin fibres and extension of pseudopodia is an important event in cell locomotion.

Experiments by Hayashi *et al* (1995) also demonstrate that microglia migrate to MCP1 *in vitro* and propose that production of MCP1 by astrocytes, modulates recruitment and activation of microglial cells in the CNS. In comparison to the results reported by Hayashi *et al* (1995), the wider range of chemokines tested in this present study, demonstrate that microglia migrate in response to many other chemokines *in vitro* which are likely to be the products of both inflammatory cells invading the CNS as well as the resident astrocytes and microglia. Chemokine receptors 3 and 5 (CCR3, 5) have been shown to be expressed by microglia (He *et al.*, 1997) and CXCR4 has been shown to be expressed by the murine microglial cell line, N9 (Tanabe *et al.*, 1997) for which SDF-1 (stromal cell derived factor) is the only known ligand. MIP1α, MIP1β and RANTES are the ligands for chemokine receptor 5 (CCR5) and Eotaxin, RANTES, MCP3 and MCP4 are the ligands for CCR3. In the present study, microglia also responded to MCP1, IL-8 and IP-10 suggesting the presence of other chemokine receptors, possibly CCR2, CXCR1 and CXCR3, the receptors for MCP1, IL-8 and IP-10 respectively (Premack and Schall, 1996).

Table 3.5 Summary of migration experiments.

		CHME3	Rat Microglia
Chemokine	(ng/ml)	Migration score #	
MCP1	5	*****	NT
	10	*****	+++++
	20	*****	+++++
	50	*	+++++
	100	NT	+
MIP1α	5	*****	NT
	10	******	+
	20	****	++
	50	***	++++
	100	NT	++
MIP1β	5	*****	NT
	10	*****	+
	20	****	+++
	50	***	++
	100	NT	++
RANTES	5	**	NT
	10	**	+
	20	**	+++
	50	**	+
	100	NT	++
IL-8	5	**	NT
	10	**	+
	20	**	++
	50	**	+++++
	100	NT	+++++
IP-10	5	*	NT
	10	**	++
	20	**	+++
	50	**	++++++
	100	NT	+++++

Migration score is the percentage increase in the mean number of cells migrating to chemokine compared to the mean number of cells migrating to the control.

* 0-100%, ** 101-200%, ***201-300%, ****301-400%, ***** 401-500%, ****** greater than 500%

+ 0-40%, ++ 40-60%, +++ 61-80%, ++++ 81-100%, +++++ greater than 100%, ++++++ greater than 200%.

NT not tested.

This table summarises the means of three repeat migration experiments for both CHME3 and rat microglia. As mentioned previously (section 3.2.2), the rat microglia migrated in higher numbers than CHME3 cells, although the actual percentage increase in migration to chemokines above migration to control media is much greater by CHME3 cells giving a much higher migration score. From this table it can be seen that CHME3 cells migrate less well to α chemokines than rat microglia but also that the migration of both cell types to RANTES is less than to other β chemokines. It may be that RANTES is bound with a lower affinity to the shared receptor (CCR1) for MIP1α/β and RANTES or the downstream signalling may differ between chemokines. It may also be that microglia are signalling through other receptors, that bind MIP1α/β but not RANTES. A receptor has recently been discovered called CCR9 that could fit these criteria (Asensio and Campbell, 1999) although it is not yet known whether microglia express it. MCP1 was the best chemoattractant for both CHME3 and rat microglia although rat microglia showed a greater response to the α chemokines than CHME3 cells. This may be due to a species difference in the cell types or a difference between a cell line and primary cells which should be further investigated using primary human microglia.

The relevance of these results to the *in vivo* situation is that the chemokines, especially of the β sub-class, are the likely chemoattractant in CNS inflammation that recruit microglia. Chemokines are released by T cells, macrophages and microglia in the MS lesion probably under the control of cytokines. Cytokine release may be initiated by viral infection in the CNS or, by virally activated T cell populations in the periphery which may cross the BBB and react with CNS autoantigens. T cells infiltrating the CNS, may then be presented with self antigen with MHC class II and co-stimulatory molecules by

microglia, further activating them to release proinflammatory cytokines and chemokines. Chemokines may then form a concentration gradient, and microglia possessing the correct receptors undergo polymerisation of actin, ultimately allowing the cell to become motile. Once the microglia are attracted to the site of inflammation within the perivascular cuff, they may be activated themselves to release further cytokines and chemokines and also release enzymes and free radicals, potentiating the damage to the myelin sheath. The chemokines and their receptors are therefore possible targets for downregulating the immune response in MS.

Chapter 4

EFFECTS OF CHEMOKINES ON MATRIX METALLOPROTEINASE, UROKINASE TYPE PLASMINOGEN ACTIVATOR AND TISSUE INHIBITORS OF METALLOPROTEINASE PRODUCTION BY MICROGLIA.

4.1 Introduction

4.1.1 Effects of matrix metalloproteinases on myelin components

Demyelination is one of the central pathological features of MS and myelin proteins have been shown to be degraded by MMPs. MMP9 was found to cleave myelin basic protein *in vitro* and two cleavage sites were identified that are within conserved amino acid sequences in different species. The degraded fragments of MBP retained encephalitogenic epitopes for EAE (Gijbels *et al*, 1993, Proost *et al*, 1993).

4.1.2 Beneficial effects of tissue inhibitor of metalloproteinase production

The most obvious role of TIMP production is to control the activity of MMPs so it is not surprising that TIMPs can be detected in most body fluids and tissues (Gomez *et al*, 1997). TIMP1 and TIMP2 also demonstrate growth promoting activities on a variety of cultured cell lines (Gomez *et al* 1997). Both MMP and TIMP immunoreactivity have been detected in MS CNS tissue with TIMP1 found to be associated with macrophages and blood vessel matrix in MS plaques. MMP9 and TIMP1 was also detected in normal control white matter associated with glial cells (Cuzner *et al*, 1996) with microglia and astrocytes being the principal cells expressing the enzyme and inhibitor in control tissue.

4.1.3 Production of matrix metalloproteinases by microglia *in vitro*

MMP production by microglia has previously been studied *in vitro* and it has been found that rat brain microglia constitutively produce MMP9 which can be upregulated by treatment of the cells with bacterial lipopolysaccharide (Gottschall *et al*, 1995). Colton *et al* (1993) also found that rat microglia constitutively produced MMP9, as well as MMP2 by analysis of culture supernatants and proteinase activity was increased by IL-1β treatment of the cells. In addition, microglia have been shown to constitutively express MT-MMP (Yamada *et al*, 1995a), a membrane bound metalloproteinase involved in the local activation of MMP2.

4.1.4 Effect of Cytokines and chemokines on the regulation of MMP and TIMP expression

Cytokines and chemokines have previously been shown to regulate the secretion of MMPs and TIMPs by human lymphocytes *in vitro*. In a study by Johnatty *et al* (1997), pro-MMP9 and TIMP1 secretion was analysed in cultures of human $CD4^+$ and $CD8^+$ T cells in response to cytokine and chemokine stimulation. Pro MMP9 secretion by $CD4^+$ T cells was increased by the β chemokines MIP1α, MIP1β and RANTES and by the proinflammatory cytokines TNFα and IL-1. Secretion of pro MMP9 by $CD8^+$ T cells was also increased by the β chemokines whereas TNFα and IL-1 downregulated its secretion. The α chemokines IL-8 and IP-10 showed no effects on either cell subtype. In contrast, only a small increase in TIMP1 secretion was observed by the action of α and β chemokines on $CD4^+$ T cells with no increase at all on $CD8^+$ cells (Johnatty *et al*, 1997). This study indicated that there is a shift in the balance between MMP and inhibitor in favour of protease activity by T cells in response to proinflammatory stimuli.

Myelin proteolipid protein (PLP) specific T cell clones have also been shown to secrete pro MMP9 as well as MIP1α and MIP1β *in vitro* and the levels of chemokine secreted were increased in the presence of specific antigen stimulation (Biddison *et al*, 1997). The secretion of MMP9 by peripheral blood mononuclear cells (PBMCs) has been shown to be increased by MCP1 stimulation *in vitro* and this could be antagonised with IFNβ-1b treatment which also decreased migration of PBMCs through fibronectin coated membranes, probably due to downregulation of protease activity (Stuve *et al*, 1997). This illustrates the important role of metalloproteinases in enabling cells to migrate through extracellular matrices. Saren *et al*, (1996) found that MMP9 secretion by human macrophages was upregulated by the pro inflammatory cytokines, TNFα and IL-1 whereas IFNγ suppressed TNFα and IL-1induced production. TNFα and IL-1 also had no effect on TIMP1 secretion.

An increase in MMP activity by the effects of pro inflammatory cytokines and chemokines on cells in the inflammatory lesion in MS, is likely to lead to disruption of the blood brain barrier and demyelination as well as aiding the migration of further inflammatory cell types, including microglia, to the site of inflammation.

4.1.5 The plasminogen activator cascade

MMP activity is regulated at all levels, including activation of pro enzymes by the serine protease, plasmin (Cuzner and Opdenakker, 1999). Plasminogen, the inactive precursor of plasmin is cleaved by the plasminogen activators uPA and tPA. uPA localises the activation to the cell membrane as it binds to the cell surface receptor uPAR (Blasi, 1997). Increased levels of both uPA and tPA have been observed in the CSF of MS patients compared to healthy controls (Akenami *et al*, 1997) suggesting a role for these proteases in disease pathogenesis.

Neonatal rat microglia *in vitro* secrete both plasminogen (Nakajima *et al*, 1992a, Inoue *et al*, 1998) and uPA (Nakajima *et al*, 1992b). IL-1 stimulation of microglia increased uPA secretion into the culture media whereas stimulation with LPS markedly decreased the amount of uPA in the culture medium (Nakajima *et al*, 1992b). uPA itself can degrade the extracellular matrix (Quax *et al*, 1992) and uPAR plays a role in the migration of human monocytes *in vitro* and localises to the leading edge of migration (Gyetko *et al*, 1994).

4.1.6 Aims

The MMPs clearly play an important role in demyelination in which microglia are heavily implicated. The secretion of MMPs, TIMPs and plasminogen activator was therefore analysed in cultures of 30 day old rat microglia and the human foetal cell line, CHME3. With the increasing evidence of the importance of chemokines in the pathogenesis of MS, the aim of this study was to determine the effects of chemokines on MMP, TIMP and uPA secretion in microglia.

4.2 Materials and methods

4.2.1 Cell stimulation

CHME3 cells were seeded into 24 well plates at a concentration of $1x10^5$ cells/ml (1ml added per well) and cultured in serum containing medium (cDMEM) for 24 hours (see chapter 2.2.2). The cells were then washed with serum free DMEM and transferred to macrophage serum free medium (SFM) (Life Technologies) for 2 hours. Primary microglia were seeded at a density of $2x10^5$ cells/well in 1ml of cDMEM and maintained in culture for 3-7 days prior to transfer to SFM. Cells were then stimulated in duplicate with 300µl of chemokine (MCP1, MIP1α, MIP1β, RANTES, IL-8 or Fractalkine) TNFα, or IL-1β for 24 hours at a range of concentrations. Culture supernatants were harvested, centrifuged to remove cell debris at 10,000g on a bench top eppendorf centrifuge (Micro Centaur) at 4°C and either loaded immediately onto gelatin substrate SDS gels for electrophoresis or collected and stored at -20°C until required. In some experiments, cells were also used to isolate mRNA for RT-PCR experiments.

4.2.2 Substrate gel electrophoresis (Zymography)

Zymography is a sensitive qualitative technique that was developed to measure matrix metalloproteinases in cell culture supernatants and lysates (Kleiner and Stetler-Stevenson, 1994). Cell supernatants can be electrophoresed on SDS containing polyacrylamide gels to first separate the proteins by molecular weight. Following electrophoresis, the SDS is removed from the gel by exchange in a solution containing Triton X-100 to allow the proteolytic enzymes to renature. The gel can then be incubated in an appropriate buffer at 37°C to allow the enzyme to digest the substrate incorporated into the gel during polymerisation. The protein in the gel can then be

stained and the enzyme activity is revealed by an absence in protein staining in the region where the substrate has been digested. The addition of molecular weight markers and positive controls allows identification of the bands.

4.2.3 Preparation of solutions for zymography

All zymography solutions with the exception of ammonium persulphate and gelatin were prepared and stored for up to 2 months prior to use.

Resolving gel buffer:

Trizma base	18.16g
Sodium dodecyl sulphate	0.4g

The above chemicals were dissolved in 90ml of ultra pure water and the pH adjusted to 8.8 with concentrated HCl. The volume was then made up to 100ml with ultra pure water

Stacking gel buffer

Trizma base	6g
Sodium dodecyl sulphate	0.4g

The above chemicals were dissolved in 90ml of ultra pure water and the pH adjusted to 6.8 with concentrated HCl. The volume was then made up to 100ml with ultra pure water

Gelatin/

A 1% solution (w/v) of gelatin (Sigma) in ultra pure water dissolved at 54°C in a water bath. This was freshly prepared immediately prior to use

Ammonium persulphate

A 10% solution (w/v) of ammonium persulphate in ultra pure water was prepared immediately prior to use.

Casein solution

Casein Hammarstein (Sigma)	1g
Resolving gel buffer	25ml
Ultra pure water	75ml

Tank buffer (X10 concentrate)

Trizma base 30.27g

Glycine 150.14g

Sodium dodecyl sulphate 10g

The above chemicals were made up to 1L in ultra pure water and dissolved by continuous stirring. Tank buffer was made up as a 10X solution and diluted 1 in 10 in ultra pure water as required

Incubation Buffer for gelatin gels

Trizma base	30.27g
Sodium chloride	58.44g
Cacium chloride	2.78g

Made up to 1L in ultra pure water with pH adjustment to 7.4 with concentrated HCl Incubation buffer was made up as a 5X solution and diluted 1 in 5 in ultra pure water as

required. For some experiments, a 10mM solution of EDTA was made up in 1X incubation buffer to chelate divalent cations thus inhibiting metalloproteinase activity.

Incubation buffer for casein plasminogen gels (10X concentrate)

Trizma base 60.5g

This was dissolved in 500ml of ultra pure water. After dissolving, pH was adjusted to 8.1 with concentrated HCl. The solution was made up as a 10x liquid and diluted accordingly prior to use.

Non reducing sample buffer

Stacking gel buffer	10ml
Sodium dodecyl sulphate	2g
Glycerol	6.4ml

Sodium dodecyl sulphate was dissolved in stacking gel buffer and then mixed with the glycerol. A few grains of bromophenol blue were then added to allow easy visualisation of sample when loading onto gel.

2% Triton X-100

Triton X-100	20ml
Ultra pure water	980ml

Destaining solution

Glacial acetic acid	100ml
Methanol	300ml
Ultra pure water	600ml

Coomassie blue staining solution

Coomassie Blue (R-250) (Sigma) 0.5g dissolved in 150ml methanol

Ultra pure water	300ml
Glacial acetic acid	50ml

Stain was prepared and filtered through Whatman (Kent) filter paper to remove any un-dissolved particles.

Gel Recipes

Gelatin substrate gel (7.5%)

Component	Volume (µl)
Acrylamide/bis (29:1)	875
Resolving gel buffer	875
Ultra pure water	1400
Gelatin (warm)	350
Ammonium persulphate	30
TEMED	3

Approximately 3ml were loaded per gel.

Casein and Plasminogen substrate gel (12%)

Component	volume (µl)
Acrylamide/bis (29:1)	1060
Resolving gel buffer	780
Ultra pure water	1270
Casein solution	350
Plasminogen	20 (of 1mg/ml solution)
Ammonium persulphate	30
TEMED	3

Approximately 3ml were loaded per gel

Stacking gel (3.4%)

Component	Volume (µl)
Ultra pure water	1730
Acrylamide/bis (29:1)	330
Stacking gel buffer	690
TEMED	3

Approximately 2ml of stacking gel was loaded before inserting combs.

Gels were cast using a Biorad mini gel system with 0.5mm spacers between gel plates. Resolving gels were poured and then overlaid with n-butanol and allowed to polymerise for 30 minutes before the n-butanol was washed off with distilled water. The excess water was removed by blotting with filter paper and the stacking gels cast. Combs

were inserted to make 10 wells and the stacking gel allowed to polymerise for a further 30 minutes before combs were carefully removed. Gels were placed onto the electrodes and inserted into the buffer tank which was then filled with 1X tank buffer ensuring that the wells of the gel were filled and did not contain any air bubbles.

4.2.4 Sample preparation for zymography

Cell culture supernatants, including the positive control for MMP2 and 9 (DX3 cell supernatant, a human melanoma cell line), or human uPA (1:100) (Sigma) as a positive control for casein/plasminogen zymography, were thawed at room temperature as required and mixed 2:1 in non reducing sample buffer. Some cell supernatants were activated by the addition of P- aminophenylmercuric acetate (10mM APMA in 50mM sodium hydroxide) (Sigma). This was prepared freshly and added to the supernatants (1 :10) and incubated for 1 hour at 37°C, prior to loading onto the gel

After supernatants were removed from cell cultures, cell lysates were prepared. Cells were washed in PBS (Sigma) and lysed by the addition of cell lysis buffer (50mM Tris/HCl, pH 7.5, + 0.1% Triton X-100) for 5 minutes at room temperature. Lysates were removed by repeated pipetting into 1.5ml eppendorf tubes which were then centrifuged at 10,000g for 10 minutes at 4°C to remove cell debris.

4.2.5 Zymography

Cell supernatants or lysates were separated on 7.5% SDS- polyacrylamide gels containing gelatin substrate to analyse MMP2 and 9 activity. Plasminogen activator activity was determined on 12% gels containing casein and plasminogen. 10µl of marker or 20µl of sample was added per well. After electrophoresis for approximately 45 minutes at 200V, gels were removed from the apparatus into weighing boats and washed

with gentle shaking on an orbital shaker in 2% Triton X-100 for 1 hour. Gels were then incubated in incubation buffer for 18 hours at 37°C and stained with Coomassie blue for approximately 2 hours with gentle rotation. Gels were then destained until clear bands of protein degradation could be visualised against the blue background stain. Bands seen on gelatin gels were confirmed as metalloproteinases in replicate gels by addition of 10mM EDTA to the incubation buffer to inhibit enzyme activity. High molecular weight markers (Sigma) were also separated on the gels to determine the protein size by plotting $\log_{10}$ molecular weight against the distance travelled by the protein in millimeters.

4.2.6 Enzyme linked Immunosorbent assay (ELISA) for matrix metalloproteinases and tissue inhibitors of metalloproteinases

Chemokine modulation of MMP2, MMP9, TIMP1 and TIMP2 secretion by CHME3 cells was quantified using Biotrak ELISAs (Amersham International plc, UK) according to the manufacturer's instructions (see Table 4.1). All solutions were provided. Briefly, a range of standards were prepared in the same way as the samples, diluted in assay buffer provided in the kit. Samples and standards were added in duplicate to the antibody coated microtitre plate. Following incubation, plates were washed in PBS and peroxidase conjugate was added. Plates were washed again and 3,3',5,5'-tetramethylbenzidine (TMB) substrate added. 1M sulphuric acid was then added giving the resulting yellow colour which was read spectrophotometrically.

Samples were assayed in duplicate, diluted 1:1 for both MMPs and TIMPs, and standard curves were set up in duplicate on each plate according to the manufacturers instructions. Results were multiplied according to the dilution factor. Standard curves

Table 4.1 Specificities of Biotrak MMP/TIMP ELISAs

ELISA kit	Specificity	Detection range (ng/ml)
MMP2	pro-MMP2, pro-MMP2/TIMP complex but not active MMP2.	0.37-24
MMP9	pro-MMP9, pro-MMP9/TIMP complex but not active MMP9.	0.6-32
TIMP1	TIMP1, TIMP1 complexed with MMPs	3.13-50
TIMP2	TIMP2, TIMP2/active MMP complex but not TIMP2/proMMP2 complex.	3-128

were plotted on RIA calc (Wallac, USA) to calculate the MMP/TIMP concentration in samples from their absorbance readings at 450nm on a Titertek plate reader. The significant differences between controls and chemokine treated levels were calculated by a one way ANOVA followed by a multiple range analysis. Values of $P<0.05$ were considered significantly different from control.

4.2.7 ELISA for urokinase type plasminogen activator

Levels of uPA in cell supernatants from chemokine treated CHME3 cells were assayed using a uPA ELISA kit (Alpha laboratories, Eastleigh, Hants, UK.). The kit recognised: pro uPA, high molecular weight uPA and uPA bound to plasminogen activator inhibitors (PAI-1 and PAI-2). Samples were diluted 1:4 in the sample buffer provided and added to the microtitre plate in duplicate. Results were multiplied according to the dilution factor. A standard curve was provided in the kit between 0 and

1ng/ml. Briefly, standards and samples were pipetted in duplicate into the capture antibody coated wells and the plate was then incubated overnight at 4°C. Following this the plate was washed and a detection antibody was added and the plate incubated for one hour at room temperature. The plate was then washed again and an enzyme conjugate (horseradish peroxidase) added and incubated again for one hour at room temperature followed by a further wash step and the addition of the substrate solution. The substrate colour change was measured at 450nm on a Titertek plate reader and the concentrations of uPA in the samples calculated from the standard curve. The significance differences in uPA concentrations between controls and chemokine treated cell supernatants were calculated by a one way ANOVA followed by a multiple range analysis. Values of $P<0.05$ were considered significant.

4.3.1 RNA extraction from CHME3 cells for use in RT-PCR to detect MMP transcripts

RNA was extracted from CHME3 cells in 24 well plates using an extraction kit, 'Totally RNA' (Ambion, UK). All reagents were provided in the kit unless otherwise stated. After media was removed from the wells, 0.45ml denaturing solution per well was added and left for 2 minutes at room temperature. 45µl of 3M sodium acetate was then added and the solution mixed and transferred to 1.5ml autoclaved eppendorfs containing 0.45ml of phenol:chloroform:isoamyl-alcohol (25:24:1) (pH 6.2). Tubes were then shaken vigorously for 1 minute, left on ice for 5 minutes, followed by centrifugation at 10,000g for 10 minutes at 4°C in a Micro Centaur. The top phase was carefully removed into fresh eppendorf tubes containing 0.45ml of acid phenol:chloroform (1:1) (pH 4.5). This was shaken vigorously again for 1 minute, left to stand on ice for 5 minutes and the centrifugation step repeated (10,000g for 10 minutes at 4°C). The top phase was then removed and pipetted into fresh RNase free 0.5ml eppendorf tubes. An equal volume of

isopropanol (Sigma) was then added to each tube and mixed gently. The tubs were then placed in a -20°C freezer overnight to precipitate the RNA. Tubes were centrifuged in a benchtop centrifuge (MSE microfuge) at 10,000g for 20 minutes at 4°C to pellet the precipitated RNA. The isopropanol was then carefully removed, without dislodging the RNA pellet. 25µl of ultra pure water was added to each pellet, followed by vortexing to dissolve the pellets. The quality of the RNA was examined by electrophoresis on 1% agarose gels. Ribosomal RNA bands could be seen on agarose gels (Figure 4.13a).

4.3.2 Agarose gel electrophoresis

RT-PCR products and RNA were electrophoresed on 2 and 1% agarose gels respectively. Agarose gels were made up in 1X Tris Borate EDTA (TBE) buffer which was made as a 10X solution for storage and diluted in water as required. A 1 litre stock of 10X TBE in ultra pure water contained 108g Tris Base, 55g Boric acid and 9.3g Na_2EDTA (Sigma). 5µl of ethidium bromide (10mg/ml) (Sigma) was added to each 50ml of liquid agarose gel, immediately prior to casting. This allowed visualisation of nucleic acids under UV transillumination as ethidium bromide binds to DNA and fluoresces under UV light. The gel was allowed to polymerise with a comb inserted to make 12 sample wells. 1µl of extracted RNA samples were mixed with 2ml of loading dye (50% glycerol, 0.2% bromophenol blue, 0.1M EDTA) provided in the extraction kit and loaded into the wells. The tank buffer used was 1XTBE and samples were electrophoresed at 100V for approximately 45 minutes. PCR products were mixed 3:1 with loading dye (50% glycerol, 0.2% bromophenol blue, 0.1M EDTA) and 20µl loaded per well. A DNA marker was also electrophoresed on each gel (Phi X 174 DNA/ Hae III, 72-1353 base pairs (Promega, UK)) to allow calculation of the product size.

4.3.3 RT-PCR

RT-PCR was performed using Thermus Thermophillus (Tth) enzyme (Bioline U.K) which has both reverse transcriptase and DNA polymerase activity to allow both the cDNA synthesis and amplification steps to occur in one reaction mixture. Human primers for MMP 2, MMP3, MMP7 and MMP9 were a kind gift of Dr John Clements, (British Biotech Pharmaceuticals Ltd, Oxford, U.K) and were used at a final recommended concentration of 0.5μM with a recommended annealing temperature of 57°C.

Primer sequences

Human MMP2 product size: 202 base pairs

5' CCGCCTTTAACTGGAGCAAA 3'

3' GGACTTCGACCTCTTGGTTT 5'

Human MMP9 product size: 196 base pairs

5' GAAGATGCTGCTGTTCAGCG 3'

3' AACTTGGTCCACCTGGTTCA 5'

Human MMP7 product size: 219 base pairs

5' TTTGATGGGCCAGGAAACAG 3'

3' GATACCTTTACCTCTAGGGG 5'

Human MMP3 product size: 225 base pairs

5' GAGGAAAATCGATGCAGCCA 3'

3' GACCTAGAAGTGTCAACCTC 5'

Table 4.2 RT-PCR Mastermix

RT-PCR mastermixes were made using the following protocol:

Component	Volume (µl)
Water	22.5
5X reaction buffer (incl. 10mM $MnCl_2$)	10
100 mM DTT (Dithiothreitol)	2
Primers (40µM)	2.5
dNTP mixture (12.5mM) (Bioline)	1
100U RNase inhibitor RNAguard (Pharmacia U.K)	1
5U Thermus Thermophilus (Bioline)	1
RNA (or water for a negative control)	10

Each mastermix prepared as above (Table 4.2) was divided into two Eppendorf tubes (25µl in each) for the RT reaction and no RT reaction which serves as a negative control to ensure the amplified products are not from genomic DNA contamination in the RNA sample. The 'no RT' tubes were added to the PCR block at step 4, missing out the reverse transcriptase conditions. All tubes were overlaid with 25µl of mineral oil to prevent evaporation of samples during the reaction. RT-PCRs were performed on a Biometra trio-thermoblock (Biometra, UK) using the conditions given in Table 4.3.

Table 4.3 Thermal Cycler conditions for RT-PCR amplification of MMP transcripts

Step	Temperature (°C)	Time (minutes)
1	65	5
2	57	5
3	70	30
4	94	1
5	57	1
6	72	1
7	72	6
8	4	End of programme

Steps 4 - 6 were cycled 35 times. Products were stored at 4°C before being visualised on agarose gels (as described in section 4.3.2)

4.4 Results

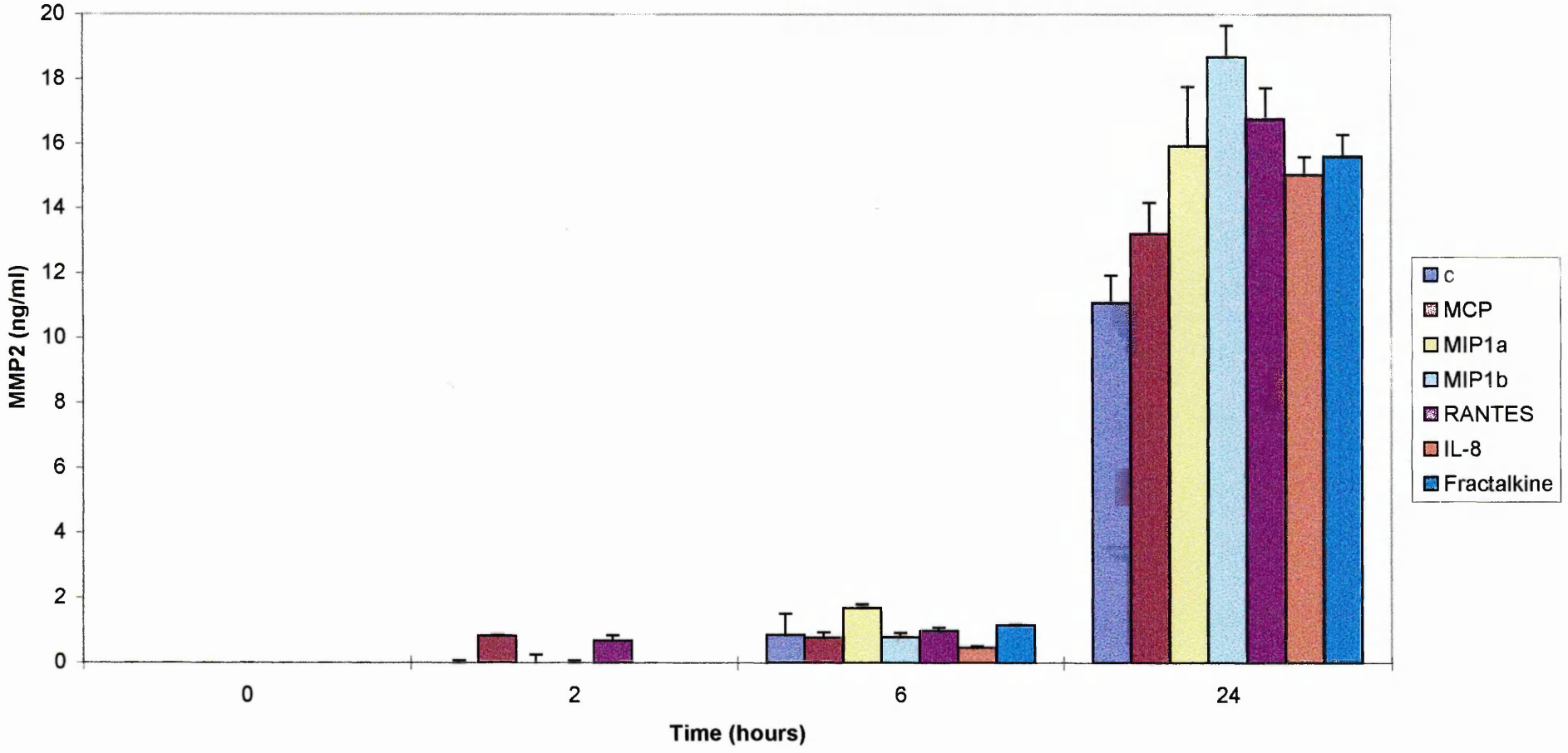
4.4.1 ELISA experiments on CHME3 cells following chemokine stimulation

ELISA experiments were only carried out on CHME3 cells to detect MMPs 2 and 9 and TIMPs 1 and 2. The human kits did not cross react with the rat proteins and only rat specific MMP and TIMP kits were unavailable. CHME3 cells were found to constitutively secrete MMP2 when assayed by ELISA (Figure 4.1). Using an ELISA kit which detected proMMP9 as well as MMP9 bound to TIMP1, gave negative results in two experiments (e.g. the zero control on the standard curve gave an absorbance reading of 0.045 and all samples were around this figure (+/-0.005) with none as high as the first standard of 4ng/ml) suggesting that only the active form of MMP9 was present 24 hours after stimulation, as shown by zymography experiments (Figure 4.7a). Time course experiments were carried out for MMP2 and TIMP1(Figures 4.1 and 4.2) TIMP1 and MMP2 levels were below the detection limit in supernatants after 2 hours in culture, however at 6 hours, slightly higher levels were detected although levels were much higher after 24 hours.

The production of MMP2 in response to the chemokines, MCP1, MIP1β, RANTES, IL-8 and Fractalkine at 50ng/ml, was significantly increased above control unstimulated values in 3 out of 3 experiments When stimulated with the chemokines at 20ng/ml, at least 2 out of 3 experiments for each chemokine produced a significant increase in MMP2 secretion (Table 4.4 and Figure 4.3). At the highest concentration of chemokine used (50ng/ml), there was an approximate 1.3-2 fold increase in MMP2 for all chemokines tested.

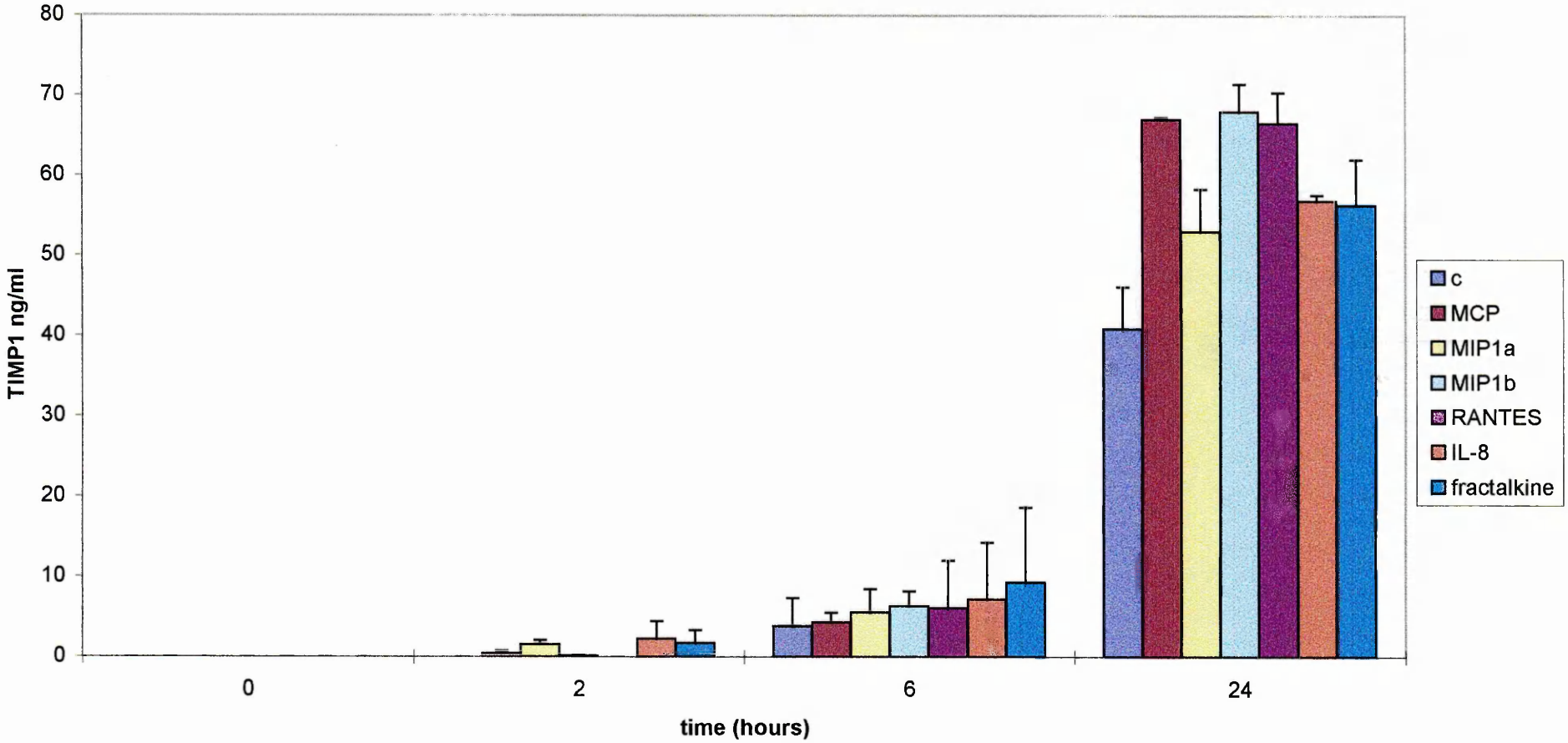
TIMP2 production was also significantly increased above control levels following treatment by all the chemokines tested with an approximate 2-5 fold increase at the

Figure 4.1 Time course of expression of MMP2 by CHME3 cells following chemokine stimulation



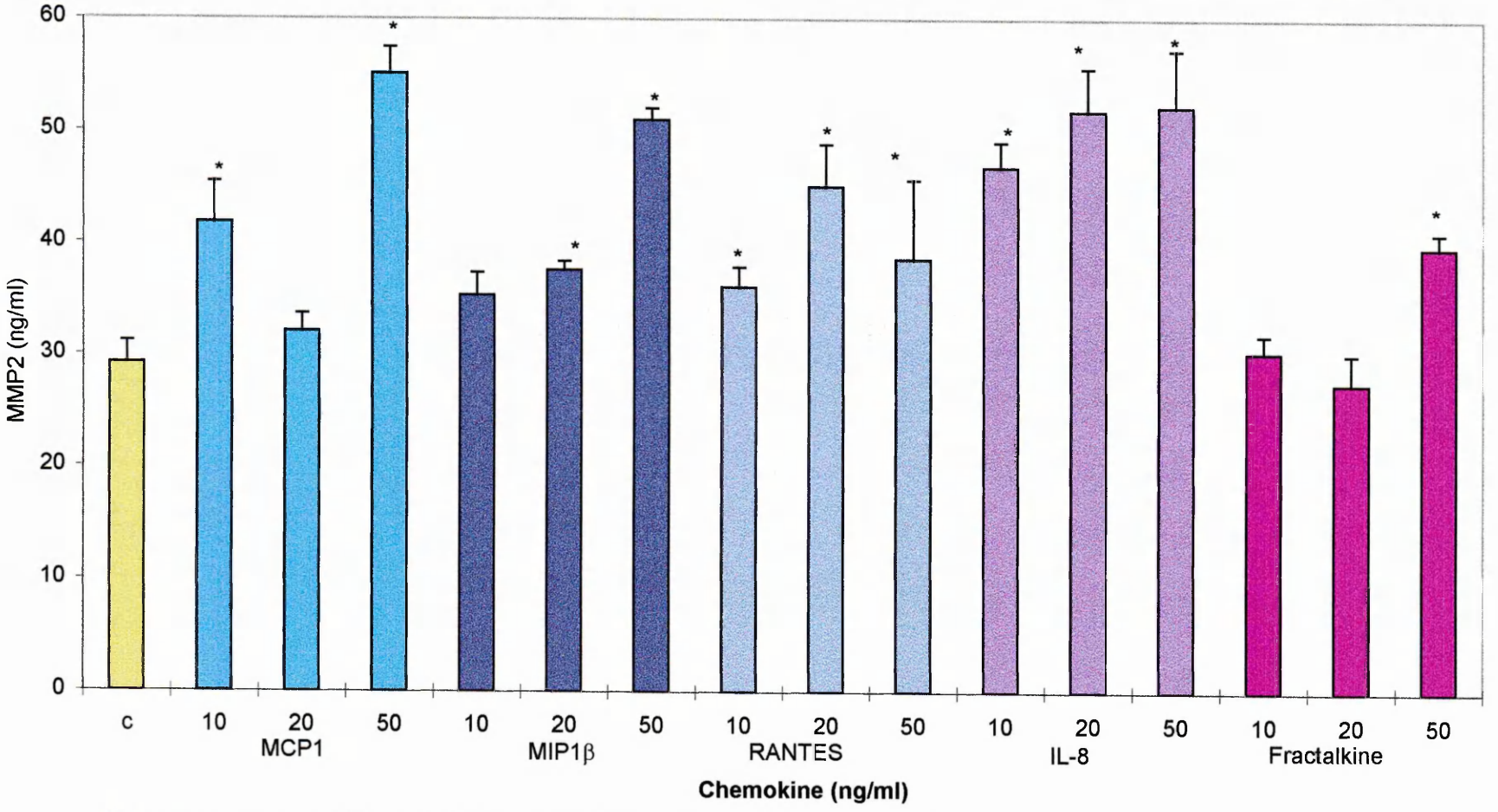
CHME3 cells were stimulated with chemokines (20ng/ml) for 2, 6 and 24 hours prior to use in the ELISA. Data represents the mean of 2 individual experiments +/- SEM.

Figure 4.2 Time course of expression of TIMP1 by CHME3 cells following chemokine stimulation



CHME3 cells were stimulated with chemokines (20ng/ml) for 2, 6 and 24 hours prior to use in the ELISA. Data represents the mean of 2 individual experiments +/- SEM.

Figure 4.3 MMP2 production by CHME3 cells following chemokine stimulation for 24 hours.

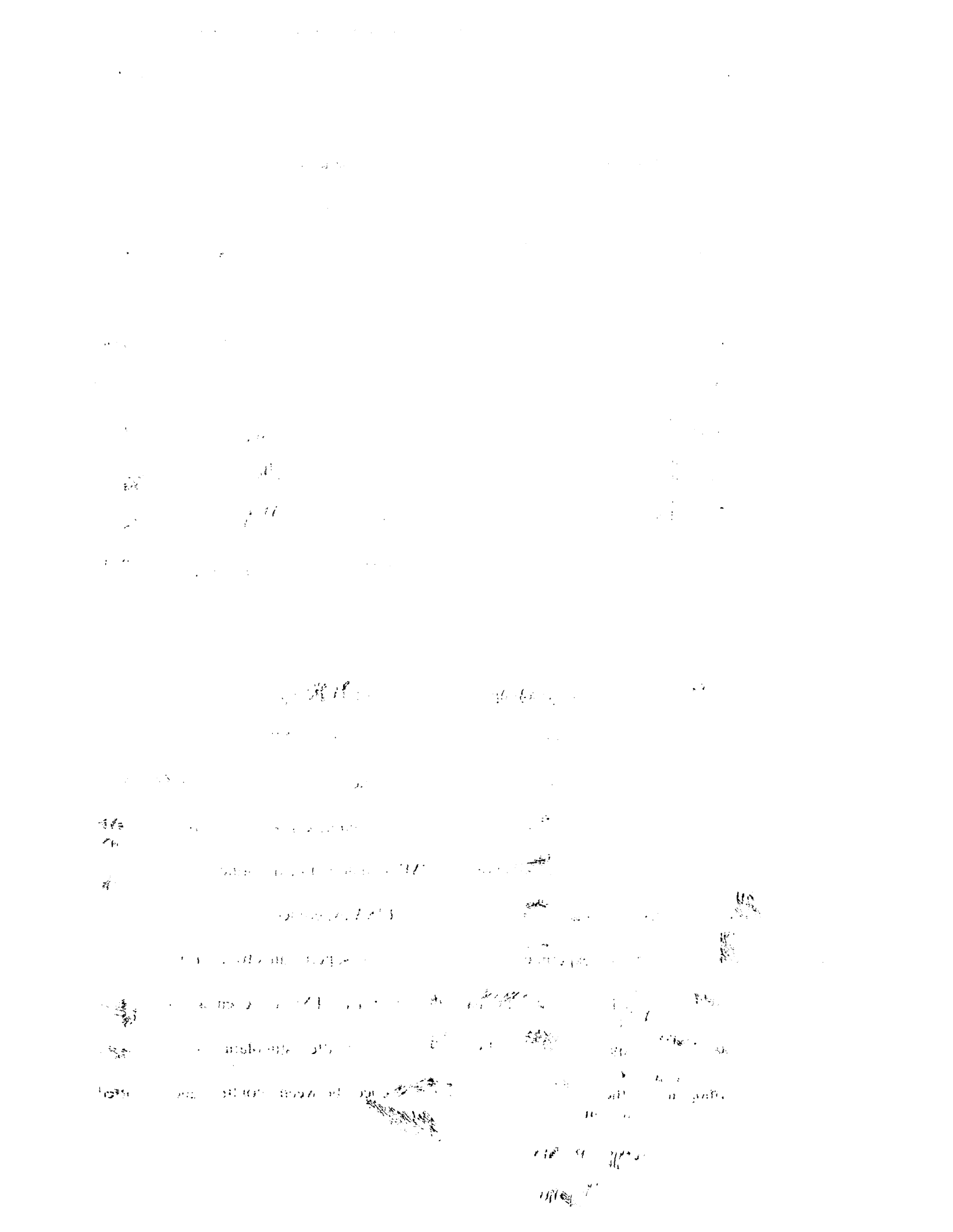


Data represents one of three individual experiments. Cells were stimulated for 24 hours prior to harvesting supernatants for use in the ELISA. * Represents significant increase in MMP2 above control levels ($P<0.05$) using a one way ANOVA.

Table 4.4 Chemokine stimulation of MMP2 production in CHME3 cells. Summary of 3 individual experiments.

Chemokine (ng/ml)		Mean MMP2 level (ng/ml)	SEM	Range	No. of experiments showing significant * increase above control
Control (unstimulated)		15.9	8.8	8-29.2	_
MCP 1	10	24.7	11.4	13.4-41.8	3/3
	20	25.6	5.4	17.5-32.1	2/3
	50	36.1	12.7	20.3-55.1	3/3
MIP1β	10	24.6	7.1	18.0-35.3	2/3
	20	29.0	5.7	20.6-37.6	3/3
	50	32.4	12.4	22.3-51.0	3/3
RANTES	10	20.3	10.5	10.1-36.1	1/3
	20	27.1	12.1	15.9-45.1	3/3
	50	27.9	7.1	19.2-38.6	3/3
IL-8	10	31.0	10.5	18.2-46.8	3/3
	20	37.7	17.1	12.1-51.8	2/3
	50	39.6	12.2	21.3-52.2	3/3
Fractalkine	10	24.6	4.6	17.7-30.3	2/3
	20	24.2	2.2	22.2-27.5	2/3
	50	29.3	6.9	21.1-39.7	3/3

*The significant increase above control levels was calculated by a one way ANOVA followed by multiple range analysis. $P<0.05$ was considered significant.



highest concentration of chemokine (50ng/ml) in at least 2 out of 3 experiments. Constitutive expression of TIMP2 was between 9 and 25ng/ml (Table 4.5 and Figure 4.4).

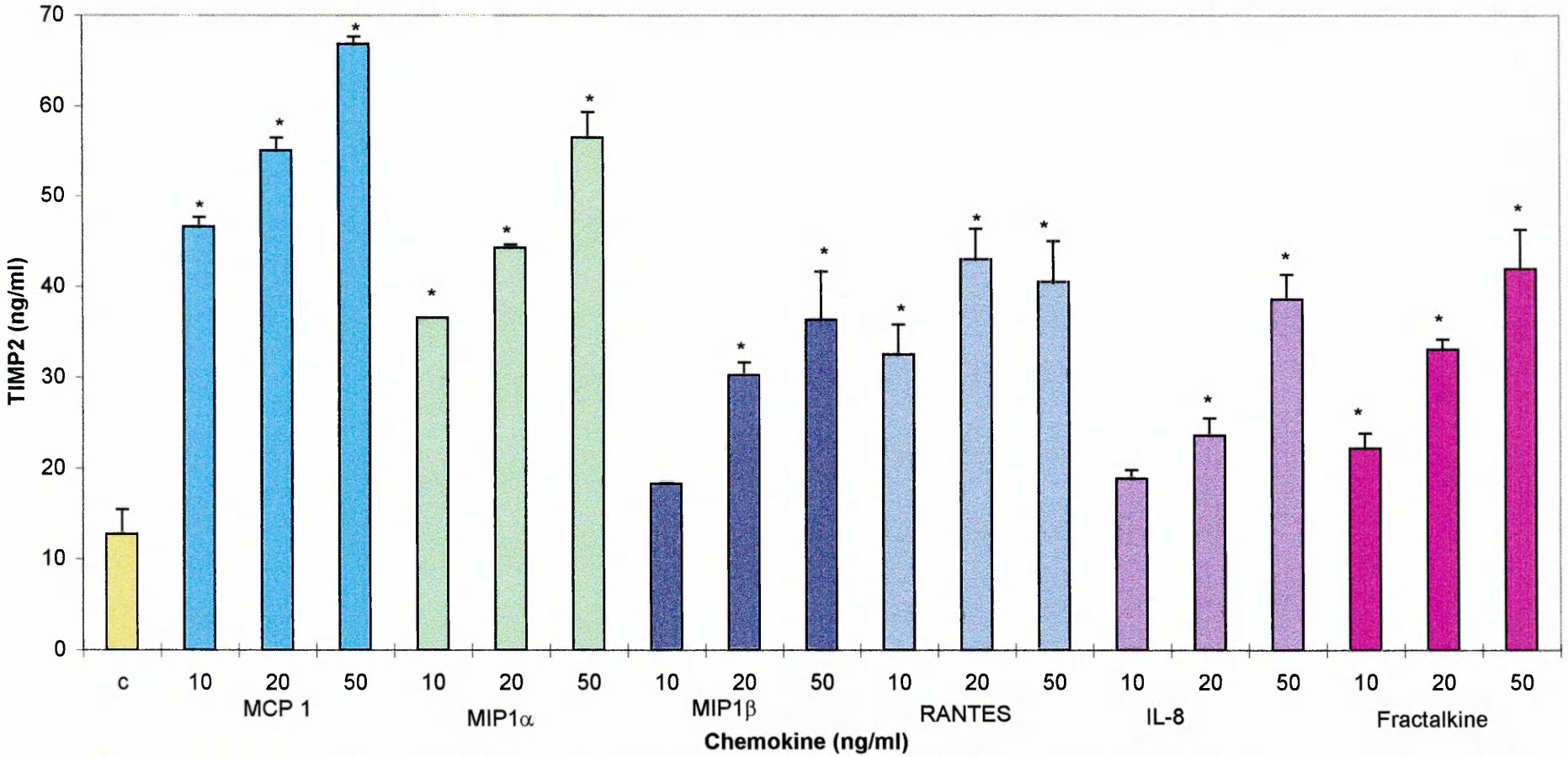
TIMP 1 production was significantly increased above unstimulated levels by the chemokines MCP1, MIP1α, MIP1β, and Fractalkine, in at least two out of three experiments with a maximum of a 2 fold increase at the highest concentration of chemokine (50ng/ml) for each individual experiment (Table 4.6 and Figure 4.5). Stimulation of CHME3 cells with IL-8 at 10ng/ml only gave a significant increase in TIMP1 secretion in 2 out of 3 experiments with a significant increase in only 1 out of 3 experiments at the higher concentrations of 20 and 50ng/ml. Inter-experimental variation was marked and constitutive levels of TIMP1 secretion ranged between 24.7 and 73.1 ng/ml (Table 4.6). The reasons for the variations are unknown but could be due to the passage number of the cells, although all experiments were performed between passages 3 and 12.

4.4.2 Gelatin substrate zymography for detection of MMPs 2 and 9

Gelatin zymography was used to detect changes in MMP2 and 9 production by CHME3 cells and primary rat microglia. Production of MMP2 and MMP9 by rat microglia could not be measured by ELISA, as commercial kits to measure rat MMPs are not available MMP9 production by CHME3 could not be quantified by ELISA as cell supernatants gave negative results in MMP9 ELISA experiments.

Preliminary experiments were carried out on supernatants from CHME3 cells at 6 hours and 24 hours after stimulation with PMA, IFNγ or chemokines. Although constitutive expression of MMPs were detectable after stimulation for 6 hours by zymography, there was no obvious difference between control and stimulated

Figure 4.4 TIMP2 production by CHME3 cells following chemokine stimulation for 24 hours.



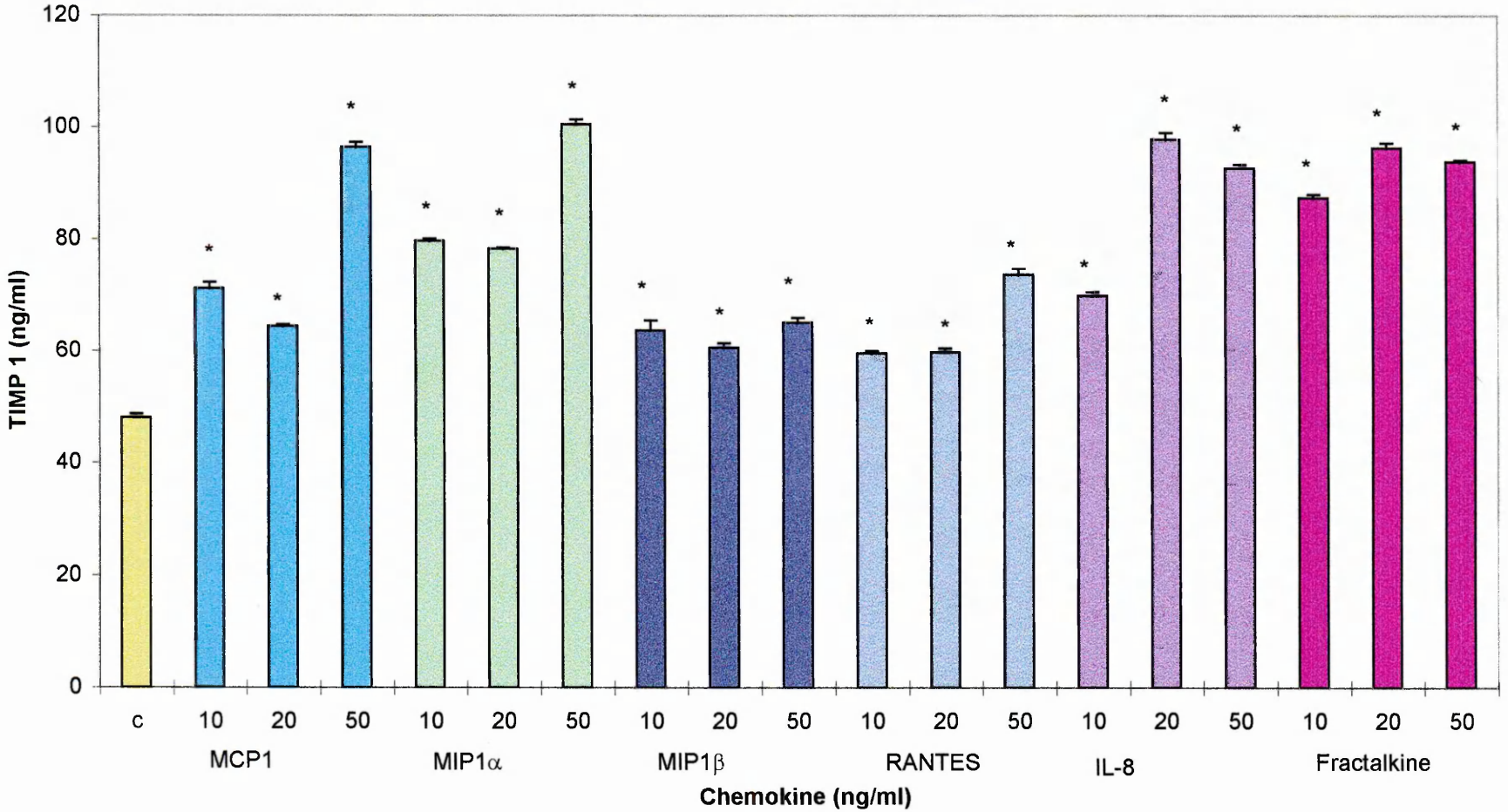
Data represents one of three individual experiments. Cells were stimulated for 24 hours prior to harvesting supernatants for use in the ELISA. * Represents significant increase in TIMP2 production above control levels ($P<0.05$) using a one way ANOVA.

Table 4.5 Chemokine stimulation of TIMP2 production in CHME3 cells. Summary of 3 individual experiments.

Chemokine (ng/ml)		Mean TIMP2 level (ng/ml)	SEM	Range	No. of experiments showing significant * increase above control
Control (unstimulated)		15.7	6.4	9.1-25.3	_
MCP 1	10	30.5	10.8	14.3-46.6	2/3
	20	37.6	14.4	15.6-55.1	3/3
	50	47.6	21.1	15.9-66.8	3/3
MIP1α	10	33.4	14.2	12.0-51.6	3/3
	20	35.2	13.5	15.3-46.3	3/3
	50	39.8	15.2	17.4-56.5	3/3
MIP1β	10	27.8	17.1	11.7-53.5	2/3
	20	31.2	11.2	15.2-48.0	3/3
	50	38.8	15.5	17.9-62.0	3/3
RANTES	10	23.2	6.9	12.9-32.6	1/3
	20	28.4	9.7	15.1-43.1	2/3
	50	30.2	7.0	19.7-40.6	2/3
IL-8	10	16.3	3.9	10.4-19.5	2/3
	20	17.9	4.9	10.5-23.7	1/3
	50	26.75	9.8	12.1-38.7	2/3
Fractalkine	10	16.5	3.9	12.9-22.3	2/3
	20	22.0	7.4	16.5-33.2	2/3
	50	32.4	7.7	20.9-42.1	3/3

*The significant increase above control levels was calculated by a one way ANOVA followed by multiple range analysis. $P<0.05$ was considered significant.

Figure 4.5 TIMP1 production by CHME3 cells following chemokine stimulation for 24 hours.



Data represents one of three individual experiments. Cells were stimulated for 24 hours prior to harvesting supernatants for use in the ELISA. * represents significant increase in TIMP1 above control levels ($P<0.05$) using a one way ANOVA.

Table 4.6 Chemokine stimulation of TIMP1 production in CHME3 cells. Summary of 3 individual experiments.

Chemokine (ng/ml)		Mean TIMP1 level (ng/ml)	SEM	Range	No. of experiments showing significant * increase above control
Control (unstimulated)		48.8	15.5	24.7-73.1	-
MCP 1	10	68.4	9.4	54.3-79.6	3/3
	20	72.0	14.4	57.8-93.5	3/3
	50	71.3	16.8	49.1-96.5	3/3
MIP1α	10	79.7	20.0	59.7-99.6	3/3
	20	78.5	22.0	56.5-100.5	3/3
	50	100.7	38.4	62.3-139.1	3/3
MIP1β	10	61.6	1.5	59.7-63.8	2/3
	20	67.0	4.2	60.7-72.1	2/3
	50	75.8	10.3	65.1-91.2	2/3
RANTES	10	47.8	18.3	20.4-63.1	1/3
	20	46.9	18.7	18.8-62.3	1/3
	50	56.7	19.3	27.7-73.7	1/3
IL-8	10	55.6	18.3	28.2-69.9	2/3
	20	66.0	28.7	23.0-97.9	1/3
	50	63.6	25.5	25.4-92.7	1/3
Fractalkine	10	64.7	18.2	37.3-87.5	2/3
	20	65.8	22.5	32.1-96.4	2/3
	50	66.3	18.4	42.2-93.9	2/3

*The significant increase above control levels was calculated by a one way ANOVA followed by multiple range analysis. $P<0.05$ was considered significant.

(See Figure 4.6) Cell culture supernatants from all experiments were subsequently harvested after 24 hours.

4.4.2.1 Detection of MMP2 and MMP9 secretion by Rat Microglia

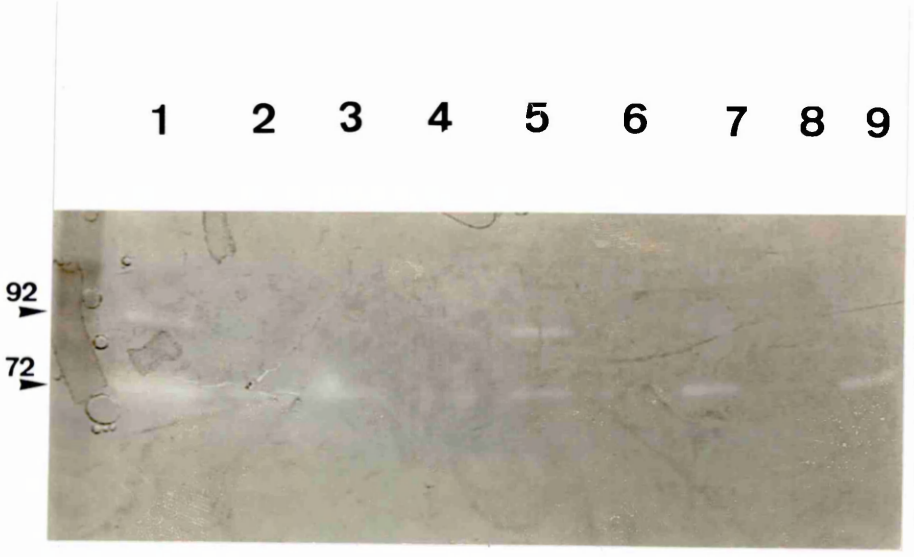
Primary rat microglia isolated from 30 day old Wistar rats, expressed both MMP2 and MMP9, constitutively and when stimulated with the cytokines TNFα (10ng/ml), IL-1β (10ng/ml) and IFNγ (10 IU/ml) there was an increase in both MMP2 and 9 production (Figure 4.7a). Following stimulation with PMA (100nM) there was a marked increase in MMP9 secretion (Figure 4.7b, lane 6). When stimulated with rat MCP1 (Figure 4.7c) or the human chemokines MIP1β (Figure 4.7d) or Fractalkine (Figure 4.7e) at concentrations of 10, 20, and 50ng/ml, MMP2 increased slightly or remained the same as control levels whereas MMP9 production was markedly increased. The increase in MMP9 secretion was most pronounced at chemokine concentrations of 20 and 50ng/ml.

4.4.2.2 Detection of MMP2 and MMP9 secretion by CHME3 cells

CHME3 cells constitutively expressed proMMP2 (72 Kda) (Figure 4.8a)and either lower or sometimes undetectable levels of proMMP9 (92 Kda) which can be easily observed when supernatants were electrophoresed along side DX3 supernatants (used as a positive control for MMP2 and 9) and/or a molecular weight marker. Treatment of supernatants with APMA confirmed these results. Activation of the pro enzymes occurred, resulting in both pro and active bands of MMP2 appearing on the gel (Figure 4.8a).

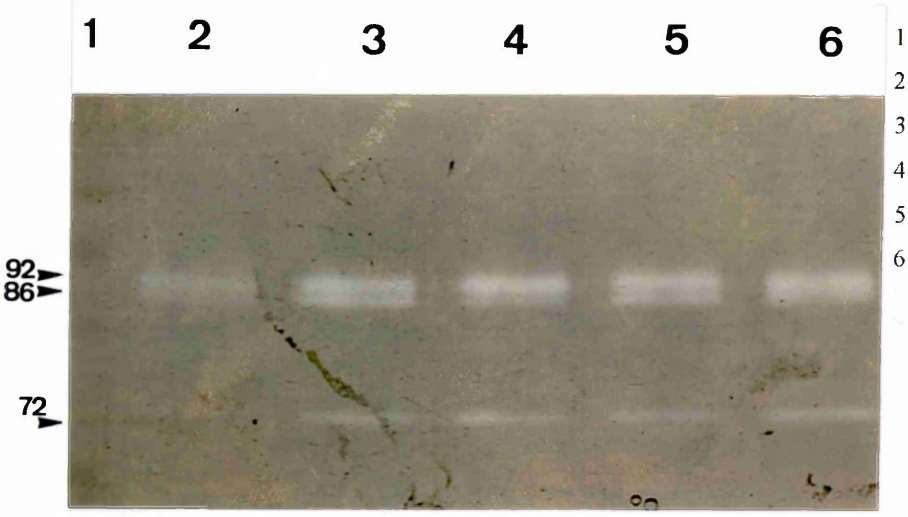
The chemokines MIP1α (Figure 4.8b), MIP1β and RANTES were used in preliminary experiments at 0.1, 1.0 and 10ng/ml to stimulate CHME3 cells, which

Figure 4.6 CHME3, Gelatin zymogram of supernatant following a 6 hour and a 24 hour stimulation period with PMA, IFNγ and MCP1. Arrows indicate molecular weights in kDa

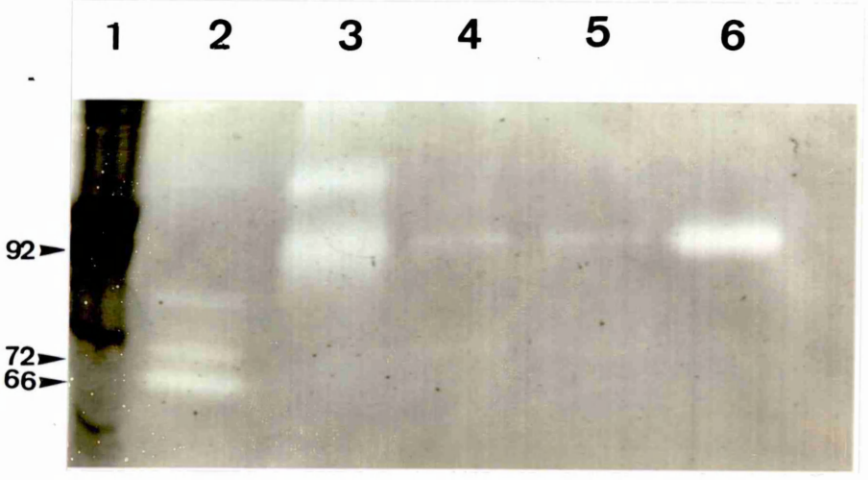


1 DX3,
2 control 6h,
3 control 24h,
4 PMA 100nM 6h,
5 PMA 100nM 24h,
6 MCP 10ng/ml 6h,
7 MCP 10ng/ml 24h,
8 IFNγ 10IU/ml 6h,
9 IFNγ 10IU/ml 24h.

Figure 4.7 Zymograms of rat microglia supernatants following chemokine and cytokine stimulation to observe MMPs 2 and 9.

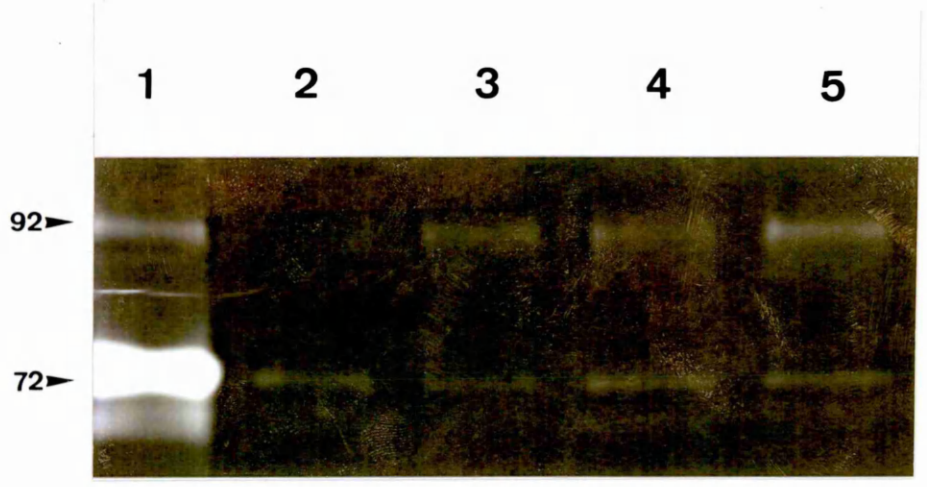


a) Rat microglia, gelatin zymogram.



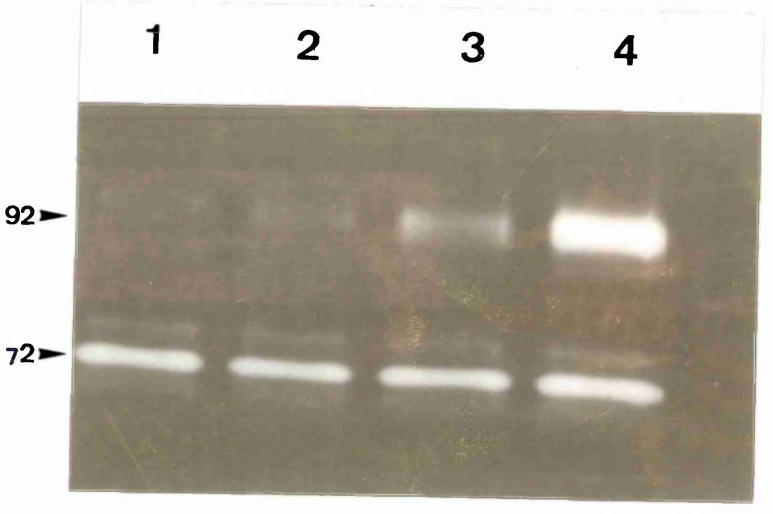
b) Rat microglia, gelatin zymogram.

Figure 4.7 continued. Zymograms of rat microglia supernatants following chemokine and cytokine stimulation to observe MMPs 2 and 9.



1 DX3,
2 control,
3 MCP1 10ng/ml
4 MCP1 20ng/ml
5 MCP1 50ng/ml

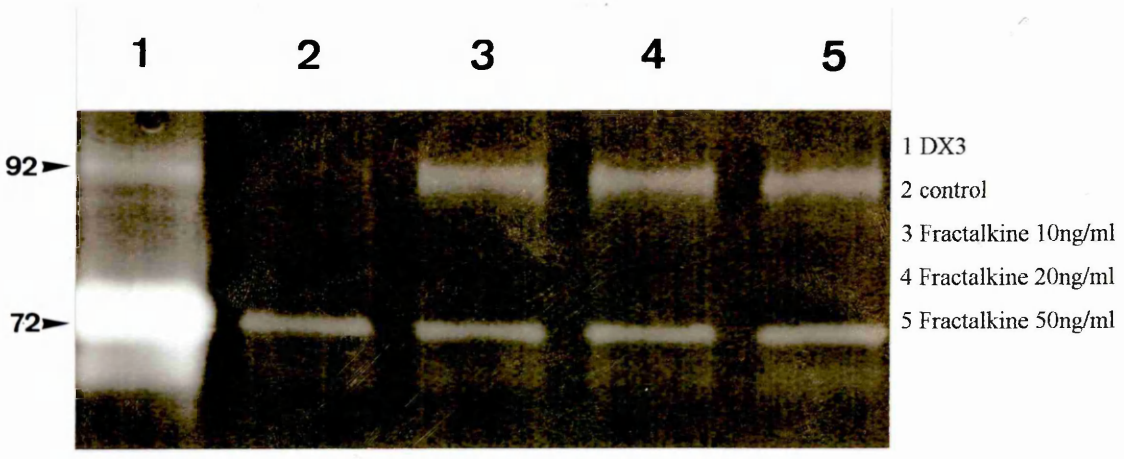
c) Rat microglia, gelatin zymogram.



1 control,
2 MIP1β 10ng/ml
3 MIP1β 20ng/ml
4 MIP1β 50ng/ml.

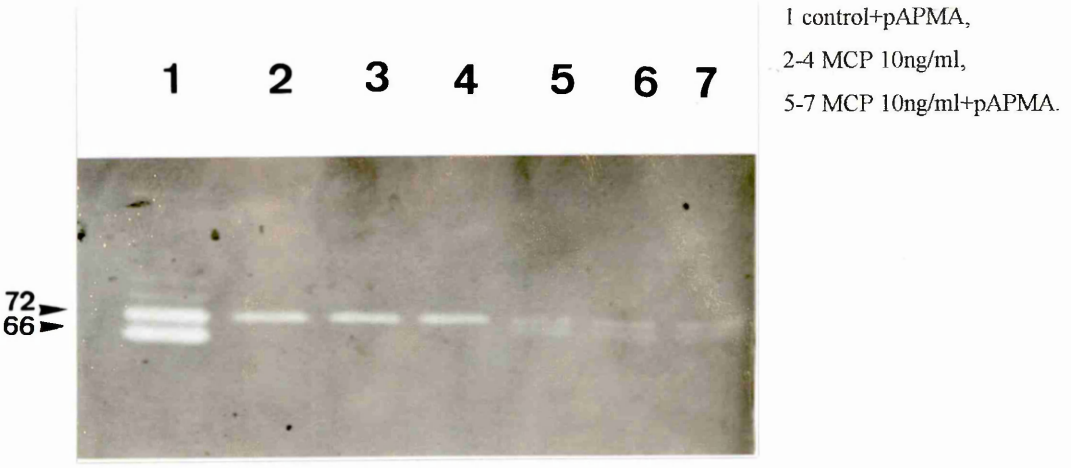
d) Rat microglia, gelatin zymogram.

Figure 4.7 continued. Zymograms of rat microglia supernatants following chemokine and cytokine stimulation to observe MMPs 2 and 9.

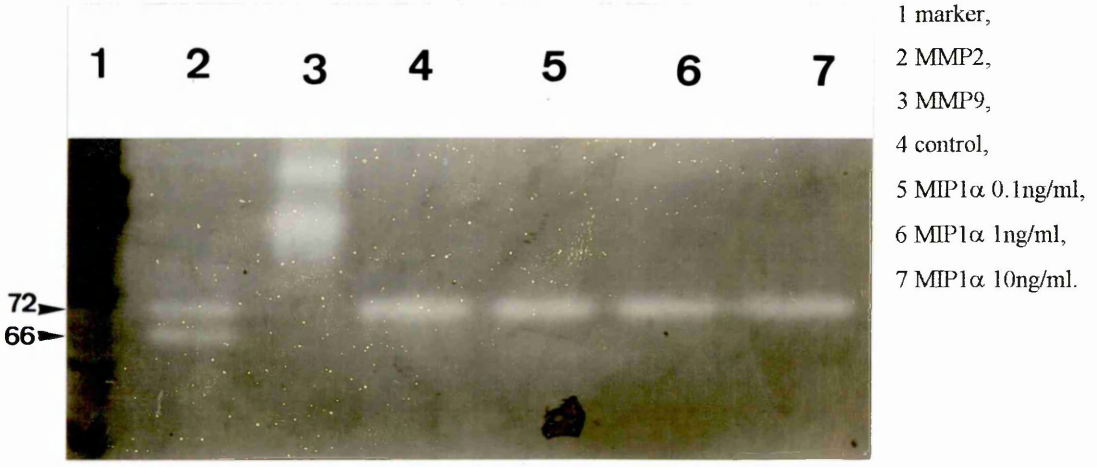


e) Rat microglia, gelatin zymogram.

Figure 4.8 Zymograms of CHME3 cell supernatants following chemokine stimulation for 24 hours to observe MMPs 2 and 9.



a) CHME3, gelatin zymogram.



b) CHME3 gelatin zymogram.

showed only a very slight increase in MMP2 secretion at the highest concentration used (10ng/ml). The chemokines tested, MCP1, MIP1α/β, RANTES, IL-8 and Fractalkine at 10, 20 and 50ng/ml, were all shown to cause an increase in both MMP2 and 9 in CHME3 cells (Figure 4.8c-g). TNFα at 1 and 10ng/ml also increased MMP2 and 9 above control levels (Figure 4.8g).

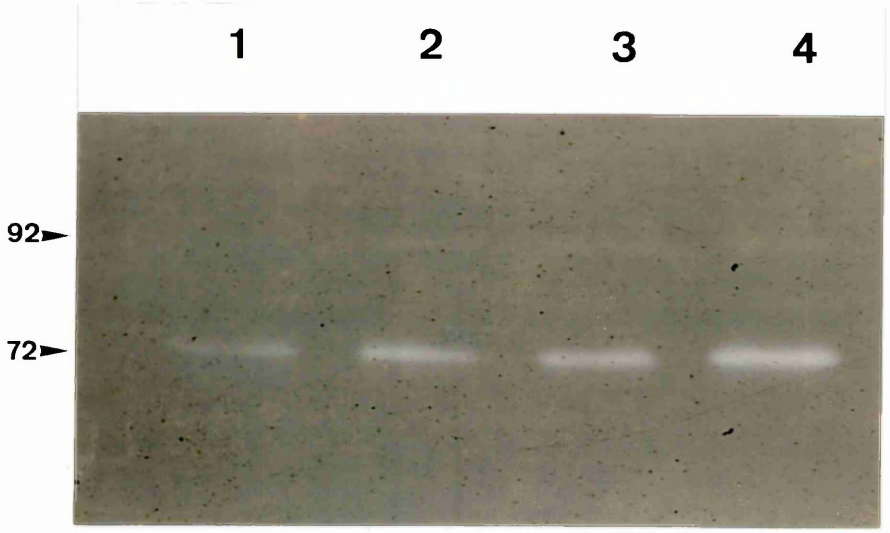
To assess levels of MMP2 and MMP9 within the cytoplasm of CHME3 cells, lysates as well as supernatants were collected following MCP1 and Fractalkine stimulation to compare the cytoplasmic content of MMPs to the secreted MMPs. MMP 2 and 9 could be detected in the cell supernatant as previously seen although only MMP2 was detectable in the lysate which did not appear to be altered by chemokine stimulation (Figure 4.8h and i).

In zymography experiments, bands were shown to be metalloproteinases by the addition of EDTA, which chelates metal ions, to the incubation buffer of replicate gels and the bands of degradation did not appear in either the samples or the DX3 positive control supernatants indicating that the activity was inhibited (Figure 4.8j).

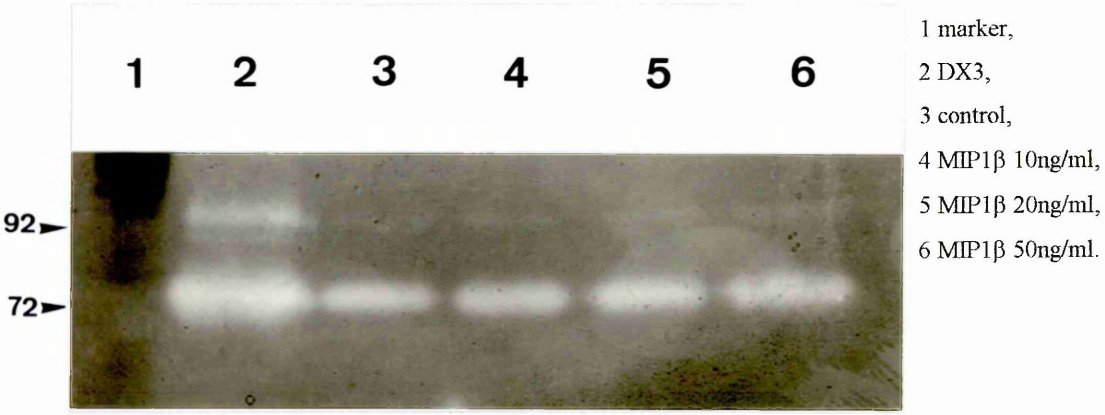
4.4.2.3 Rat Glioma cell line production of MMPs

The rat glioma cell line C6 was also used as a comparison for MMP secretion by microglia. Chemokines were used again at 20ng/ml and a panel of chemokines were chosen to compare the different effects of different chemokines (Figure 4.9). MMP 2 and 9 were constitutively expressed and secreted by C6 cells after 24 hours in culture. The β chemokines RANTES and MIP1β, the δ chemokine, Fractalkine and the α chemokine IL-8, increased MMP9 secretion with no effect on MMP2 whereas the β chemokine MCP1, and another α chemokine, MIG, increased MMP2 secretion with no effect on MMP9. This interesting observation implies that the chemokines do not always

Figure 4.8 continued Zymograms of CHME3 cell supernatants following chemokine stimulation for 24 hours to observe MMPs 2 and 9.

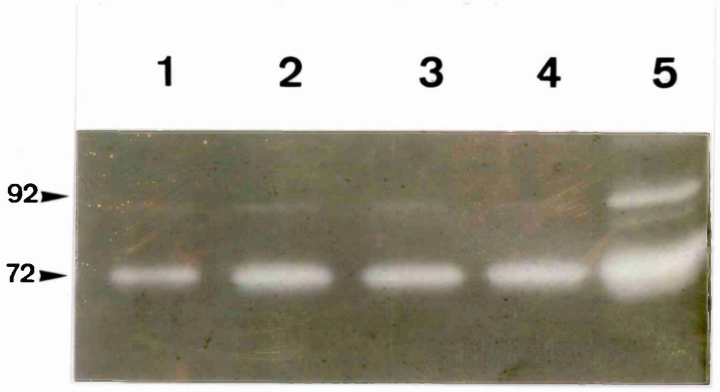


c) CHME3 gelatin zymogram.



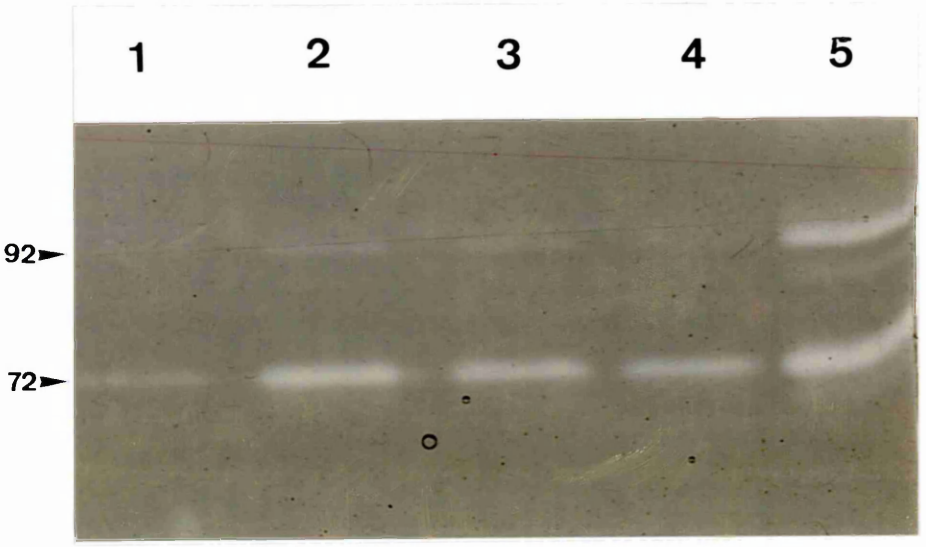
d) CHME3 gelatin zymogram.

Figure 4.8 continued. Zymograms of CHME3 cell supernatants following chemokine stimulation for 24 hours to observe MMPs 2 and 9.



1 control,
2 RANTES 10ng/ml,
3 RANTES 20ng/ml,
4 RANTES 50ng/ml,
5 DX3.

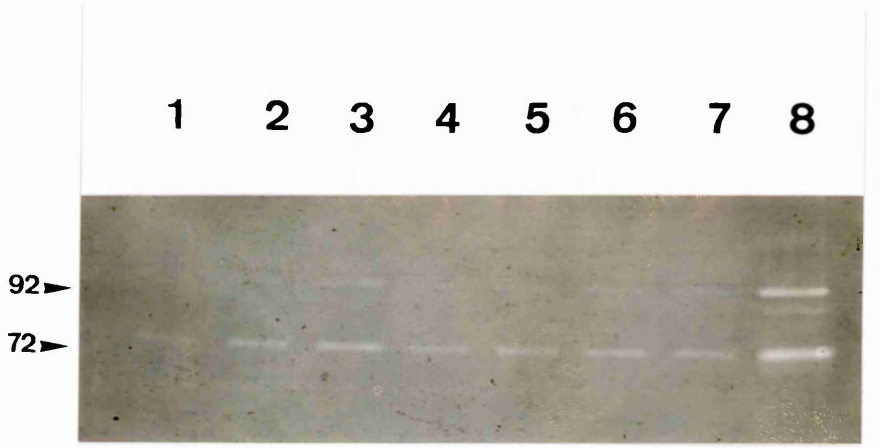
e) CHME3 gelatin zymogram.



1 control,
2 IL-8 10ng/ml,
3 IL-8 20ng/ml,
4 IL-8 50ng/ml,
5 DX3.

f) CHME3 gelatin zymogram.

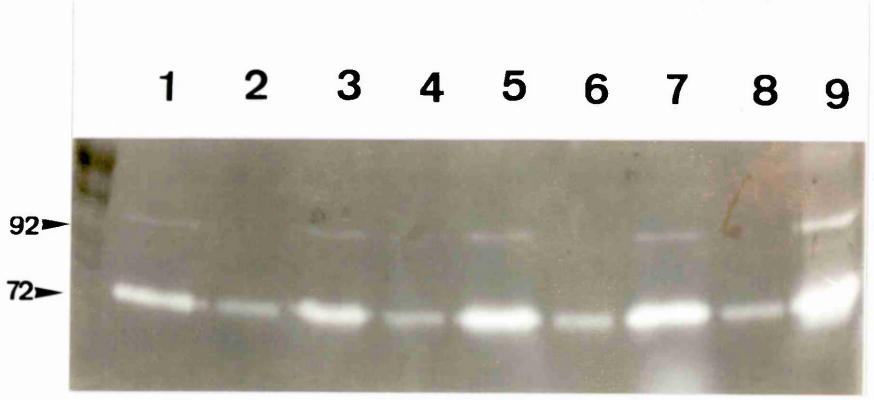
Figure 4.8 continued. Zymograms of CHME3 cell supernatants and cell lysates following chemokine stimulation for 24 hours to observe MMPs 2 and 9.



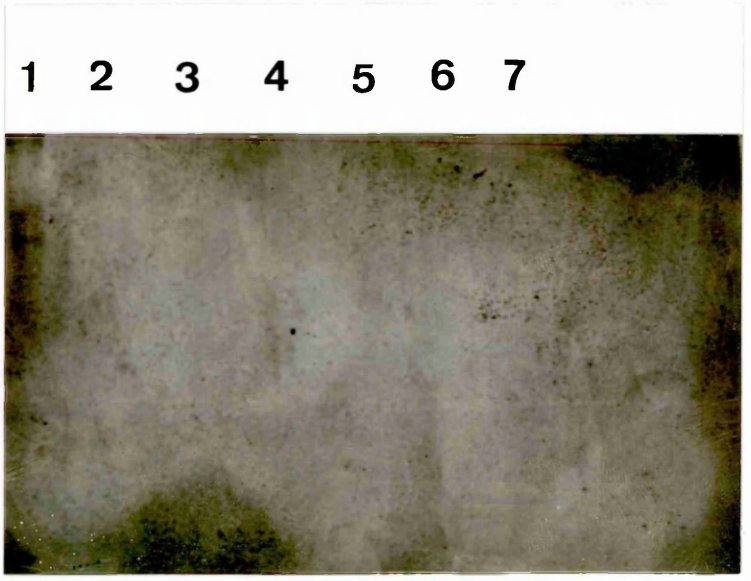
g) CHME3 gelatin zymogram.

h) CHME3 gelatin zymogram. Lanes 1, 3, 5 and 7 are cell supernatants, lanes 2, 4, 6 and 8 are cell lysates.

Figure 4.8 continued. Zymograms of CHME3 cell supernatants and cell lysates following chemokine stimulation for 24 hours to observe MMPs 2 and 9.

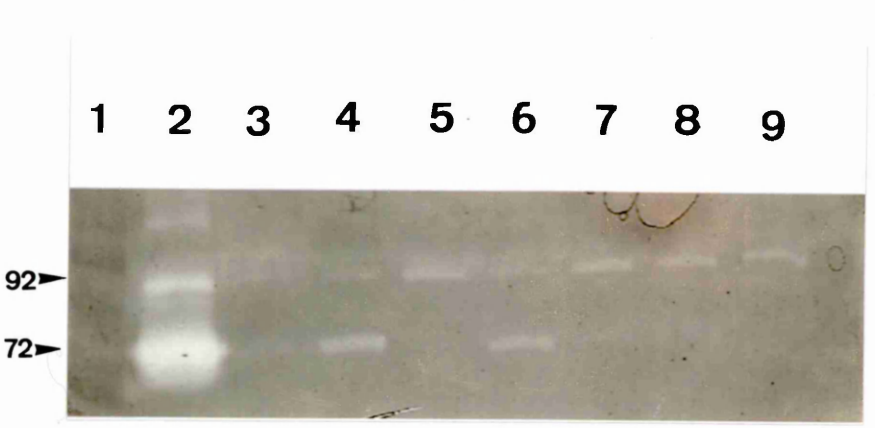


i)CHME3 gelatin zymogram. Lanes 1, 3, 5 and 7 are cell supernatants, lanes 2, 4, 6 and 8 are cell lysates.



j) CHME3 gelatin zymogram. No bands of gelatinase activity were observed as incubation buffer containing EDTA was used to show bands were metalloproteinases.

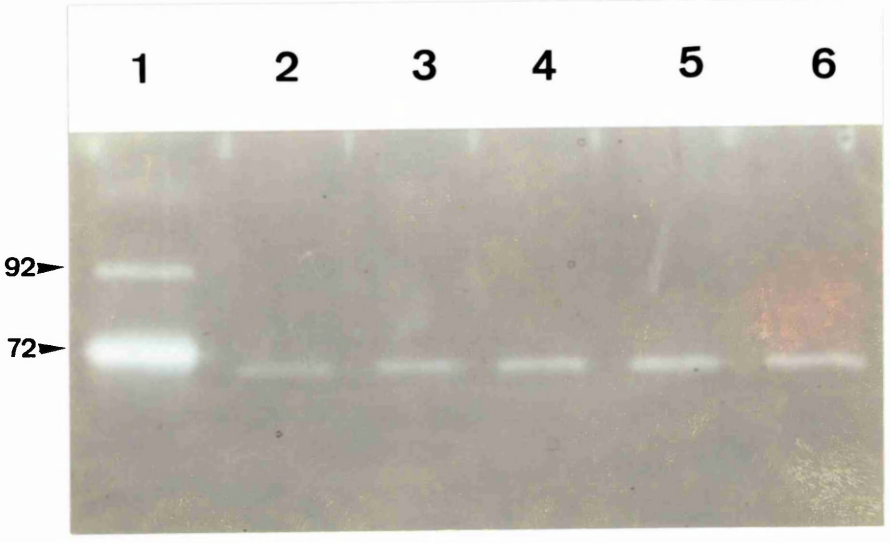
Figure 4.9. Gelatin zymogram of C6 rat glioma cell line supernatants following chemokine stimulation for 24 hours.



1 marker,
2 DX3,
3 Control,
4 MCP1 (20ng/ml),
5 MIP1β (20ng/ml),
6 MIG (20ng/ml),
7 RANTES 20ng/ml),
8 IL-8 (20ng/ml),
9 Fractalkine (20ng/ml).

C6 gelatin zymogram.

Figure 4.10 Gelatin zymogram of rat astrocyte cell supernatants following chemokine stimulation for 24 hours.



1 DX3,
2 control,
3 rMCP1 20ng/ml,
4 Fractalkine 20ng/ml,
5 MIP1β 20ng/ml,
6 RANTES 20ng/ml.

have the same effects on a particular cell type (as in microglia) and that these differences cannot be attributable to the different sub-classes of chemokine. As these results differ from those seen in primary rat astrocytes, the effects may be due to the heterogeneous and complex nature of tumour cell lines.

4.4.2.4 Primary rat astrocyte production of MMPs

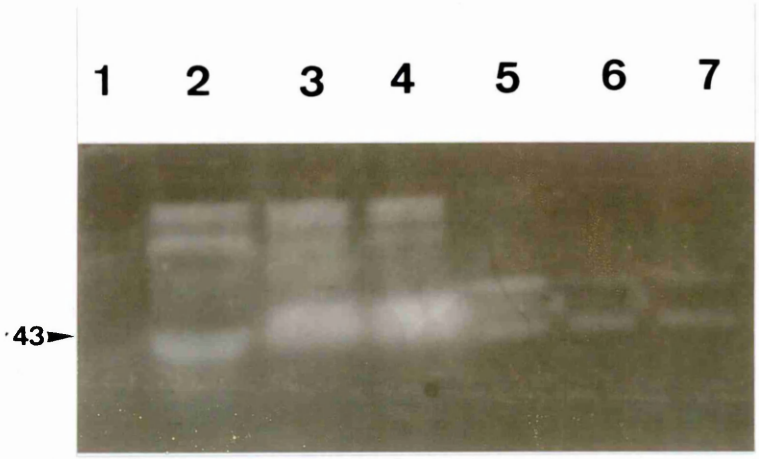
Astrocytes isolated from 30 day old Wistar rats were found to constitutively secrete MMP2 and there was no detectable constitutive MMP9 present after 24 hours in culture. Stimulation of astrocytes with the chemokines rat MCP1, or human Fractalkine, MIP1β and RANTES all used at a concentration of 20ng/ml (as this concentration was found to have a clear effect on microglia), showed an increase in the secretion of MMP2 with no detectable induction of MMP9 expression (Figure 4.10). This is in contrast with findings for both CHME3 cells and primary rat microglia which both demonstrated an increase in MMP9 secretion after stimulation with these chemokines.

4.4.3 Detection of urokinase type plasminogen activator using Casein/ plasminogen gels

Pure human urokinase type plasminogen activator (uPA) was electrophoresed on each gel as a positive control which had an observed molecular mass of approximately 43 Kda. Primary rat microglia were found to produce 2 bands close together at around the same size as the human uPA positive control. Rat microglia when stimulated with MCP1 appeared to show a slight decrease in both secreted plasminogen activator bands (Figure 4.11a).

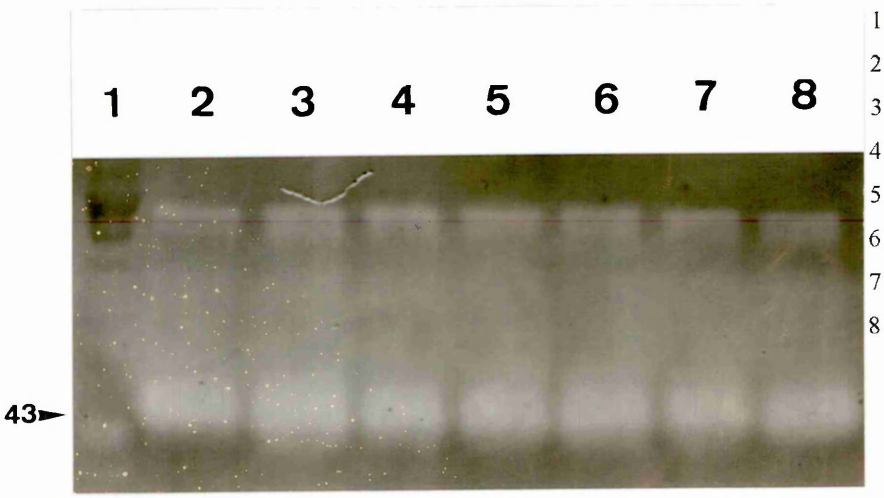
CHME3 supernatants constitutively secreted a plasminogen activator (Figure 4.11a), of the same RMM as the positive control and was later confirmed to be uPA secretion by ELISA experiments (See section 4.4.4). Unstimulated CHME3 cells as well

Figure 4.11 Casein/ plasminogen zymograms on rat microglia and CHME3 cell supernatants or cell lysates following chemokine stimulation for 24 hours to observe plasminogen activator activity.



1 marker,
2 uPA,
3 CHME3 control supernatant,
4 CHME3 control cell lysate,
5 rat microglia control,
6 rat microglia MCP1 50ng/ml,
7 rat microglia MCP1 50ng/ml.

a) CHME3 and rat microglia cell supernatants and lysates.



1 marker,
2 control,
3 IL-8 10ng/ml,
4 IL-8 20ng/ml,
5 IL-8 50ng/ml,
6 Fractalkine 10ng/ml,
7 Fractalkine 20ng/ml,
8 Fractalkine 50ng/ml.

b) CHME3 cell supernatants.

as MCP1, MIP1α, MIP1β, RANTES, IL-8 and Fractalkine (10-50ng/ml) stimulated cell supernatants and lysates were collected after 24 hours and separated on casein/ plasminogen gels. All appeared to have decreased uPA in the supernatants as the chemokine concentration increased to 50ng/ml (Figure 4.11). In the cell lysates the chemokines appeared to slightly increase the amount of uPA (Figure 4.11c and d). TNFα was found to increase uPA secretion (Figure 4.11e).

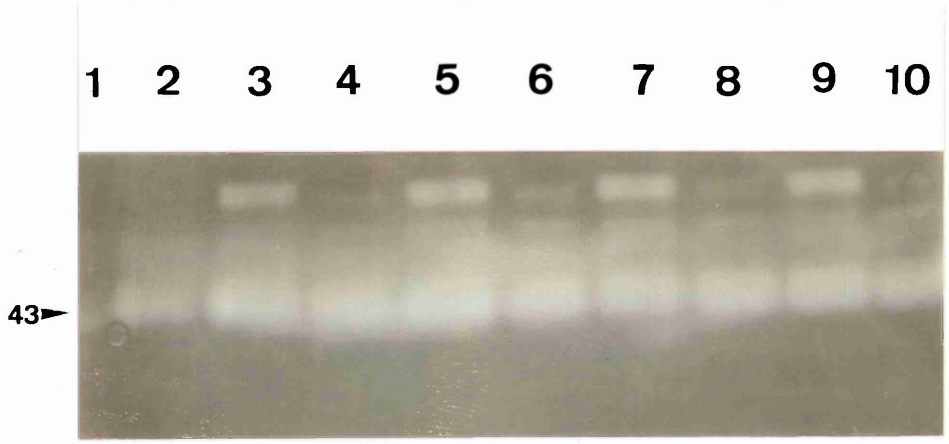
4.4.4 Urokinase plasminogen activator ELISA

Urokinase secretion in unstimulated and chemokine stimulated CHME3 cell supernatants were assessed by ELISA (Figure 4.12). The mean unstimulated levels of the three individual experiments was 1.1ng/ml and this was significantly reduced using a one way ANOVA followed by multiple range analysis ($P<0.05$), by all of the chemokines tested, MCP1, MIP1α, MIP1β and RANTES, at 50ng/ml in three out of three experiments. These levels were within the detection limit of the assay which was 10pg/ml. MCP1 also caused a significant decrease at 20ng/ml (Figure 4.12).

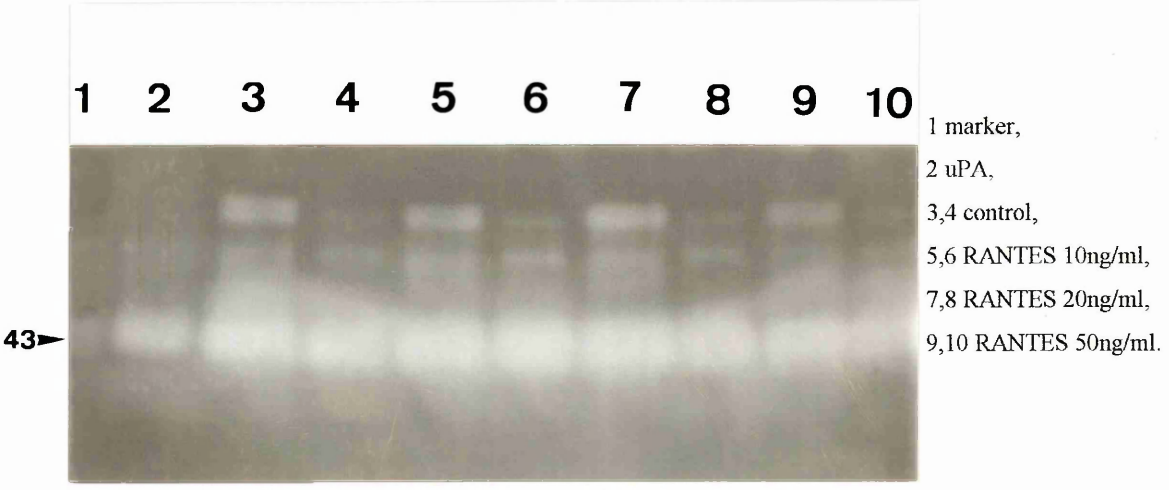
4.4.5 MMP RT-PCR

Expression of mRNA for MMPs 2, 3, 7 and 9 were assessed in CHME3 cells by RT-PCR. Although the experiments were repeated a number of times and care was taken with each of the steps, results were inconsistent, i.e. sometimes the mRNA was not detected in repeat experiments, probably due to experimental errors or lack of activity of reagents used in RT-PCR experiments. Bands corresponding to MMP 2, MMP9, MMP7 and MMP3 (Stromelysin1) mRNA were all detected in CHME3 cells. MMP2 and MMP3 were seen most consistently with MMP9 and faint MMP7 bands seen occasionally (Figure 4.13b).

Figure 4.11 continued. Casein/ plasminogen zymograms on CHME3 cell supernatants and cell lysates following chemokine stimulation for 24 hours to observe plasminogen activator activity.

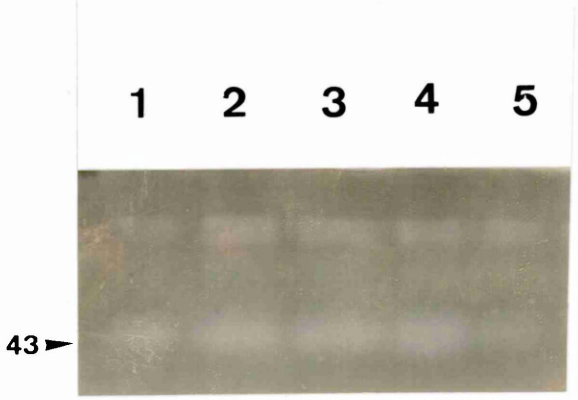


c) CHME3. Lanes 3,5,7,9 cell supernatants, lanes 4,6,8,10 cell lysates.



d) CHME3. Lanes 3,5,7,9 cell supernatants, lanes 4,6,8,10 cell lysates.

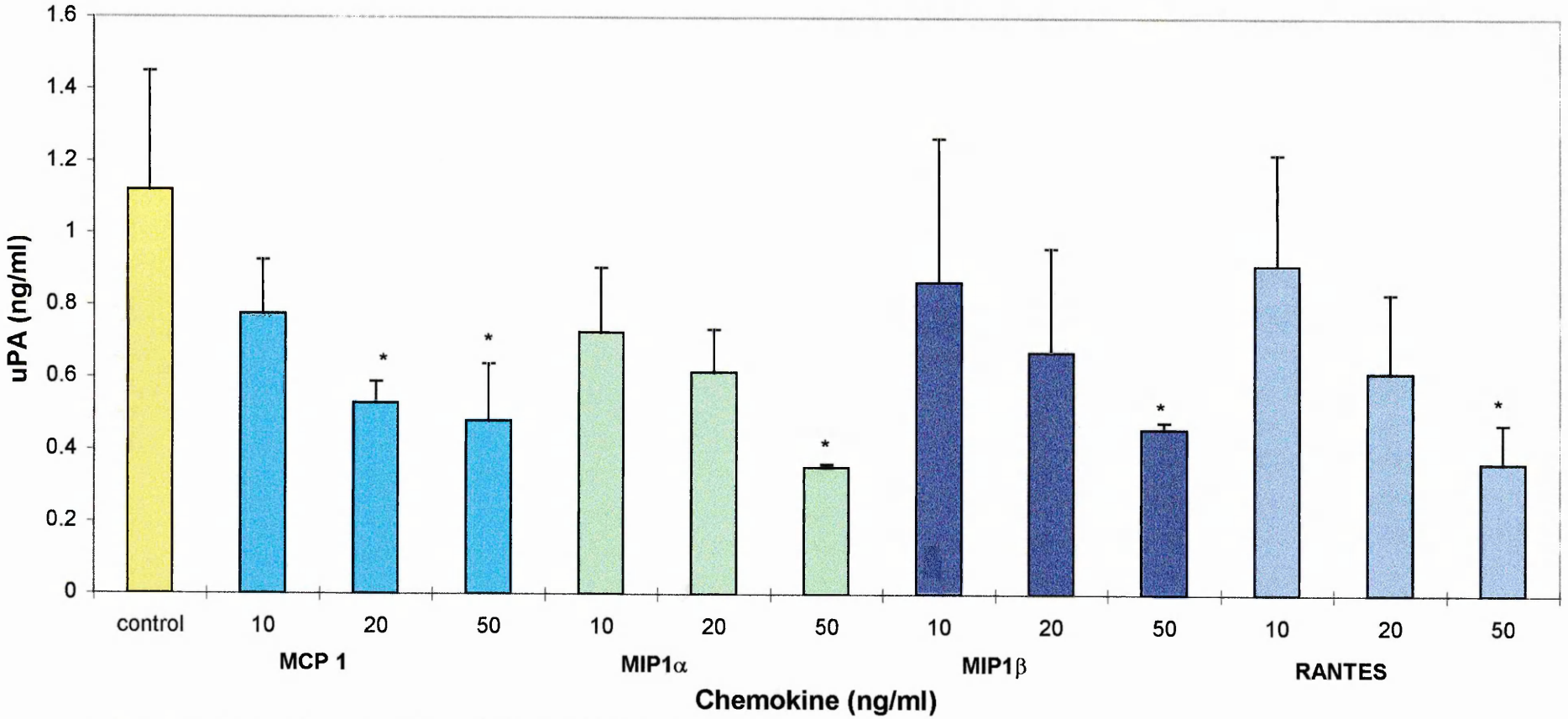
Figure 4.11 continued. Casein/ plasminogen zymograms on CHME3 cell supernatants following chemokine or cytokine stimulation for 24 hours to observe plasminogen activator activity.



1 control,
2 TNFα 1ng/ml,
3 TNFα 10ng/ml,
4 RANTES 20ng/ml,
5 RANTES 50ng/ml.

e) CHME3 Casein/ plasminogen zymogram.

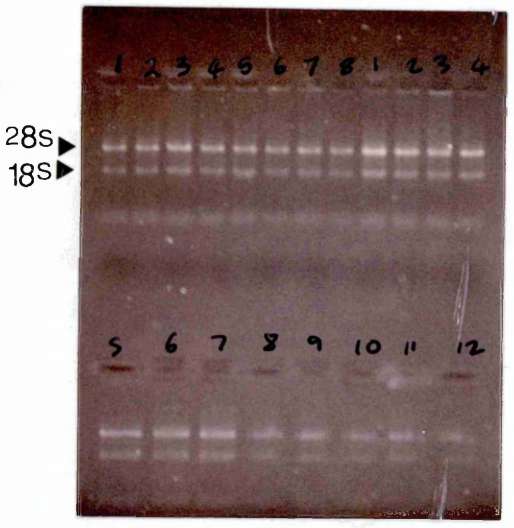
Figure 4.12 uPA secretion by CHME3 cells in response to chemokine stimulation for 24 hours



The graph shows uPA secreted by CHME3 cells into culture supernatant, detected by ELISA, following stimulation by chemokine for 24 hours. Data represents the mean of three individual experiments +/- SEM ($P<0.05$).

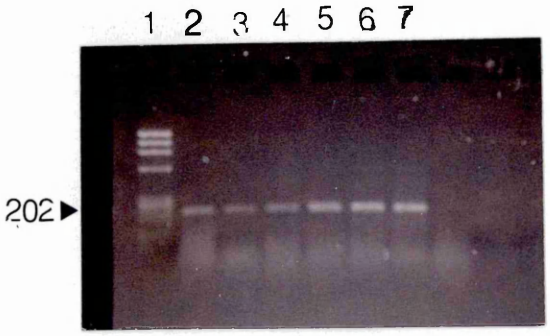
* Significant decrease in uPA secretion below that of control cells.

Figure 4.13a RNA extracted from CHME3 cells, separated on agarose gels containing ethidium bromide and visualised by UV transillumination.

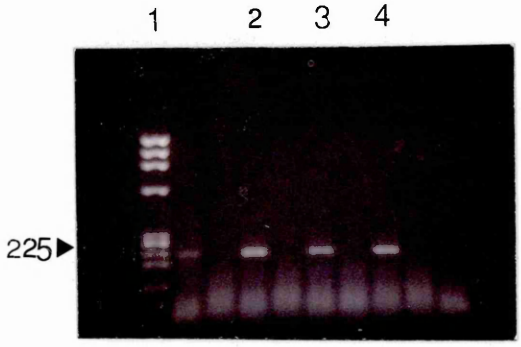


Each lane shows RNA extracted from one well of a 24 well plate using the Totally RNA extraction kit (Ambion, U.K) The bands are.18 and 28s ribosomal RNA.

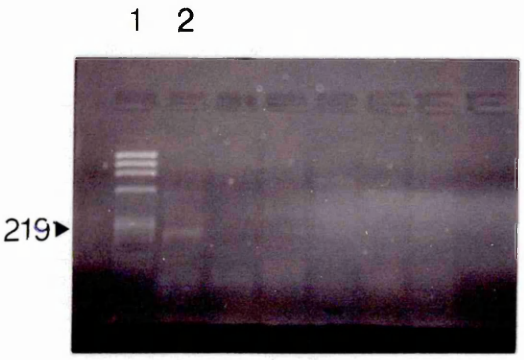
Figure 4.13b RT-PCR on CHME RNA using primers for MMPs 2, 3, 7 and 9. Arrows indicate numbers of base pairs. Lane 1 in all gels contains the molecular weight marker. All other lanes indicated contain PCR products from RT-PCR experiments on CHME3 cell RNA.



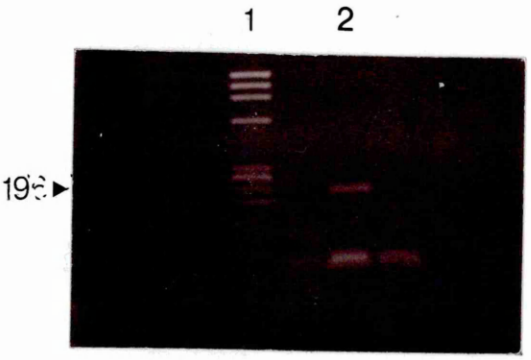
b) (i) MMP2



(ii) MMP3



(iii) MMP7



(iv) MMP9

4.5 Discussion

The presence of MMPs has been under much investigation in a number of CNS disease states including stroke (Todor *et al,* 1998), MS (Anthony *et al,* 1997), Alzheimer's disease (Yamada *et al,* 1995) and brain tumours (Freidberg *et al,* 1998). In the present study, the secretion of MMPs, TIMPs and urokinase type plasminogen activator by microglia *in vitro* was investigated and the influence of chemokines and other inflammatory cytokines on their secretion was assessed. The results demonstrated that all the chemokines tested were able to upregulate CHME3 cell secretion of MMP 2 and TIMPs 1 and 2 assayed in ELISA experiments and upregulation of MMP2 and 9 in cultured rat microglia was observed assessed by zymography. Since MMPs have been implicated in causing BBB breakdown and myelin degradation and are involved in cell migration, these results further support a role for chemokines in (1) the regulation of breakdown of the blood brain barrier (2) demyelination in MS as well as (3) chemotaxis of inflammatory cells, including microglia, *in vitro* (Cross and Woodroofe, 1999). This evidence would support the postulated role of chemokines in the CNS in the recruitment of T cells and resident microglia to areas of inflammation and tissue damage. MMPs produced by microglia could contribute to the formation of the plaque in MS by proteolysis of myelin and also by aiding migration of microglia through the extracellular matrix in response to chemoattractants produced at the site of inflammation. Baseline levels of TIMP1 were higher than TIMP2 in CHME3 cells although both were constitutively expressed by resting cells in culture after 24 hours. Pagenstecher *et al* (1998) found MMP gene products and TIMP gene products coincided at sites of inflammation and tissue damage in EAE and suggested a 'dynamic state' in which the

levels of each of these proteins and their interaction may determine the extent of the inflammatory lesion.

The levels of MMPs and TIMPs cannot be compared directly in these experiments due to the nature of the ELISA specificities, i.e. the ELISA for MMP2 measures both free pro enzyme and that bound to the inhibitor but not active enzyme, however it may be that there is a time lag in the secretion of the inhibitor. This would allow initial MMP activity which is then regulated by TIMP secretion as well as other factors involved in the activation of pro enzymes. This time lag in TIMP secretion was found in human T lymphocyte cells by Johnatty *et al* (1997). The upregulation of microglial secretion of both gelatinases and TIMPs by chemokines may be a regulatory mechanism to limit and tightly control extracellular matrix breakdown. It has also been shown that TIMP 1 has cell growth promoting activity and therefore could be beneficial to remyelination of axons (Hayakawa *et al* 1992).

It is also likely that the plasminogen activator cascade will fit into this model, by exerting an effect on the extent of pro-MMP activation. Plasminogen activators are involved in the tightly regulated cascade as they cleave plasminogen to plasmin which activates proMMPs and also degrades several components of the extracellular matrix alone (Vassalli *et al*, 1994). CHME3 cells were shown to constitutively secrete a urokinase-type plasminogen activator and all the chemokines tested significantly reduced the amount of uPA in the supernatant, when cells were stimulated for 24 hours with chemokine at a concentration of 50ng/ml assessed by ELISA experiments. This may be due to an increase in the number of uPA receptors expressed on the cell surface or an increase in the binding affinity of uPA for its receptor. Microglial uPAR expression is shown to be increased in the MS lesion (Washington *et al*, 1996) and this increase in expression could be due to the increase in chemokines present in the lesion. If this is the

case, in the cell culture system, this would suggest more binding sites are then available to bind uPA, decreasing the amount detectable in the supernatant. The decrease in uPA in culture supernatants also appeared to be evident in zymography experiments, using primary rat microglia, when stimulated with chemokine. The decrease in uPA in stimulated microglia cultures has also been shown by Nakajima *et al,* (1992b) when cells were stimulated with LPS.

Several protease inhibitors have been shown to have a beneficial effect in EAE. An inhibitor, D-penicillamine, was found to decrease gelatinase A and B activity in CSF from patients with MS or optic neuritis and also reduced mortality and morbidity in mice developing acute demyelination in EAE (Norga *et al* 1995). The metalloproteinase inhibitor, Ro31-9790 was found to prevent disease onset when given to rats at the same time as induction of EAE (Hewson *et al* 1995). The inhibitors of MMPs are important considerations in the treatment of MS, but it is becoming clear that the molecules that upregulate the MMPs, such as the chemokines, are also important targets for potential therapies to restore the balance of MMP and TIMP activities. This study provides a base for further research which should include inhibition of chemokine stimulated gelatinase production by microglia, using chemokine and/or MMP inhibitors.

Chemokines indicated in figure 4.14 were used to stimulate the cells for 24 hours in culture. The summary of the effects produced and measured by ELISA and/or zymography are shown.

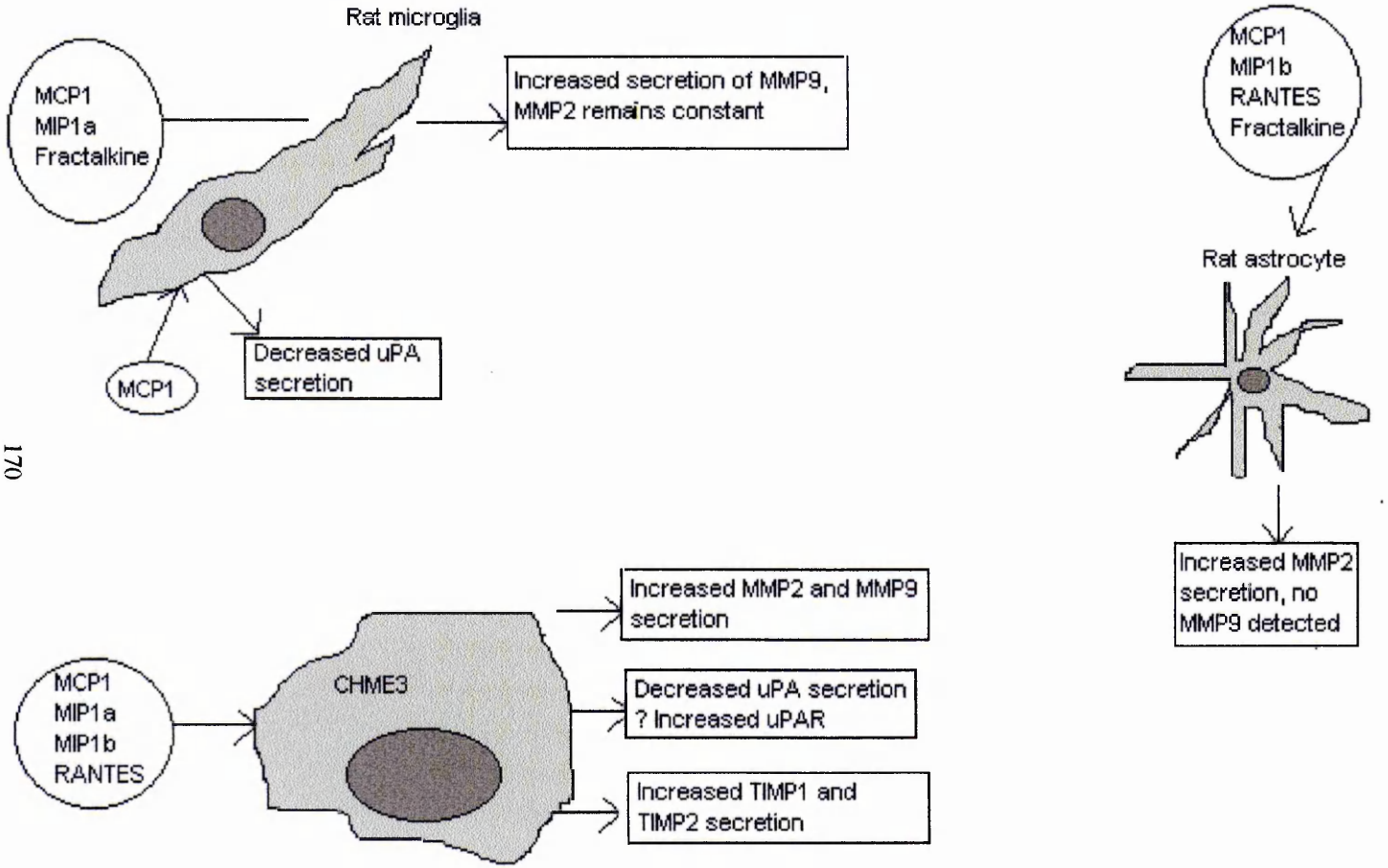


Figure 4.14 Schematic representation of chemokine effects on MMP2 and 9, TIMPs and uPA secretion by rat microglia, CHME3 cells and rat astrocytes *in vitro*.

The results discussed here, have important implications in the study of MS, in that once chemokines, released by infiltrating T cells under the control of pro-inflammatory cytokines, have recruited microglia to the inflamed site, the microglia (and also astrocytes) are able to further act in response to β chemokines and Fractalkine, by release of gelatinases, uPA and their natural inhibitors, TIMPs. The fact that microglial secretion of both MMPs and TIMPs were increased by chemokines, may suggest that the overall lytic activity in MS is due to a time lag in inhibitor secretion or incomplete inhibition. MMP2 requires TIMP2 for its own activation from the pro-enzyme form, increasing the complexity of the fine balance. The release of uPA adds to this complexity as it can activate plasmin which cleaves pro-MMPs and is also involved in chemotaxis when bound to its receptor, uPAR. Chemokine upregulation of MMP secretion may therefore be of critical importance in damage to the blood brain barrier allowing increased leukocyte migration as well as in myelin damage, leading to demyelination making them potential targets for MS therapy.

Chapter 5

PRODUCTION OF NITRIC OXIDE, SUPEROXIDE AND FC RECEPTOR EXPRESSION.

5.1 Introduction

5.1.1 Microglia are the effector cells of the CNS

Microglia are believed to play a central role in both inflammatory and degenerative processes in the CNS and are able to respond to a wide range of stimuli. They are often the first cell type to be activated in CNS injury and respond by secretion of cytokines, chemokines, proteases, nitric oxide and superoxide radicals (Peterson *et al*, 1997, Minghetti and Levi, 1998).

5.1.2 Nitric oxide production and its effects in the CNS

Nitric oxide (NO) is an inorganic free radical generated through an enzymatic reaction involving the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS) with consequent release of NO radicals (See section 1.6). The reaction requires molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates (Minghetti and Levi, 1998). Inflammation in the CNS can increase the levels of nitric oxide produced by induction of NOS in microglia and astrocytes (Smith *et al*, 1999). iNOS activity is regulated mainly at the level of transcription and several upstream transcription factors have been identified including sites for NF-κB, IFNγ response element, TNFα response element and AP-1 (Minghetti and Levi, 1998). iNOS mRNA is elevated in MS plaques and is located mainly in monocytes and microglia (Bagasra *et al*, 1995, Smith *et al*, 1999). Nitric oxide synthase

activity can be induced by the cytokines IL-1β, TNFα and IFNγ in rat macrophages, microglia and astrocytes, however expression by human monocytes/macrophages is controversial (Brosnan *et al*, 1995). iNOS activity could be induced after just 2 hours, following stimulation with these cytokines, in human foetal mixed glial cultures with translation of iNOS protein expressed after 24 hours (Ding *et al*, 1997). NO radicals can affect lipid peroxidation which in turn affects membrane fluidity and permeability and can change the functions of proteins embedded in the lipids (Smith *et al*, 1999). Nitric oxide also causes cell damage particularly by its effects on mitochondrial enzymes and *in vitro* experiments have shown that oligodendrocytes are more susceptible to damage by NO, with a 40% loss of succinate dehydrogenase activity, than are the producers of NO, the microglia (Mitrovic *et al*, 1994). Oligodendrocytes were also found to be more susceptible to single stranded DNA breaks caused by NO than either microglia or astrocytes (Mitrovic *et al*, 1994). IFNγ activated microglia were shown to be cytotoxic to oligodendrocytes *in vitro* and this effect was shown to be reduced by inhibitors of nitric oxide synthase (Merrill *et al*, 1993). This provides further evidence to suggest that NO plays a direct role in demyelination in the CNS.

Although the effects of chemokines on NO production have not been studied until now, chemokines themselves have been shown to be regulated by NO. A competitive inhibitor of L-arginine dependant NO synthase, N^G-methyl-L-arginine (NMA), was shown to increase IL-8 and ENA-78 production from peripheral blood monocytes, during a mixed lymphocyte reaction *in vitro*, whereas levels of MIP1α and MCP1 were unaffected (Orens *et al*, 1994). This suggests that NO production may have downregulatory effects on some chemokines in certain cell types, which could be a feedback mechanism to prevent further chemokine release and could also infer selectivity

on the cell types recruited by the downregulation of some chemokines in favour of others.

5.1.3 Superoxide production by microglia and its effects on the CNS

Cultured microglia, activated by phorbol myristate acetate (PMA) can respond by production of superoxide radicals produced as part of a 'respiratory burst' mediated by NADPH oxidase (Colton and Gilbert, 1987, Smith *et al*, 1999). Superoxide radicals are implicated in the pathogenesis of MS (Smith *et al*, 1999), as they are involved in lipid peroxidation and can affect the lipid and protein content of myelin (Bongarzone *et al*, 1995). PMA triggered production of superoxide by mouse or rat microglia can be increased by pre-treatment of cultures with TNFα or IFNγ (Hu *et al*, 1995, Woodroofe *et al*, 1989). Stimulated blood monocytes, isolated from MS patients have been found to produce significantly more superoxide than monocytes from healthy controls, which may be due to the monocytes from MS patients being 'primed' to trigger superoxide production (Fisher *et al*, 1988). Reactive oxygen species may contribute to demyelination by killing oligodendrocytes. This has been demonstrated *in vitro* by culture of dog oligodendrocytes in the presence of a reaction to generate superoxide anions, which killed the oligodendrocytes at concentrations that did not appear to affect astrocytes or brain macrophages (Griot *et al*, 1990).

Further effects of superoxide can be attributed to the formation of peroxynitrite in the presence of nitric oxide free radicals. Peroxynitrite, although not a highly reactive free radical itself (Beckman and Koppenol, 1996), can lead to cell death by mechanisms including nitration of tyrosine residues affecting cell signalling or by direct interaction with DNA causing DNA strand breakages. Peroxynitrite also affects lipid peroxidation (Smith *et al*, 1999). Peroxynitrite has been shown to induce the release of active MMPs

from their precursors by interaction with the cysteine switch mechanism in the pro-peptide autoinhibitory domain (Maeda *et al*, 1998).

Peritoneal macrophages have been shown to produce superoxide in response to phagocytosis of myelin in *in vitro* experiments and treatment with NADPH oxidase inhibitors, which prevent reactive oxygen species generation, resulted in inhibition of myelin phagocytosis (Van der Goes *et al*, 1998). This evidence suggests that reactive oxygen species can activate macrophages to phagocytose myelin as well as its role in the direct damage of myelin.

5.1.4 Phagocytosis by microglia and its relevance to the study of MS

Rat microglia and peritoneal macrophages have been demonstrated to phagocytose myelin *in vitro* and antibody opsonisation of myelin resulted in increased phagocytosis by peritoneal macrophages, which could be further increased by stimulation with IFNγ (Mosley and Cuzner, 1996). There appears to be some controversy in the regulation of respiratory burst following phagocytosis. Myelin and opsonised myelin uptake were shown to increase nitrite production, although opsonised myelin uptake caused a decrease in superoxide release (Mosley and Cuzner, 1996) in contrast with findings by Van der Goes, (1998). Schwacha *et al* (1993), found that PMA-triggered release of hydrogen peroxide, by rat peritoneal macrophages, was decreased following phagocytosis of opsonised erythrocytes but only when PMA was used at maximum stimulating concentrations of 50ng/ml.

Myelin phagocytosis has been shown to be increased *in vivo* when anti-myelin antibody is present. This was demonstrated by injection of antibodies against myelin oligodendrocyte glycoprotein, after induction of EAE in Lewis rats which lead to enhanced demyelination (Smith, 1999). Furthermore, cytokines have been shown to

modulate the expression of Fc receptors on microglia *in vitro*. Fc receptor expression can be increased on rat microglia by treatment with IFNγ (Woodroofe *et al*, 1989), TNFα, LPS, IL-1β (Loughlin *et al*, 1992) and IL-4 (Loughlin *et al*, 1993), demonstrated by binding of antibody opsonised erythrocytes to microglia. TGF-β reduces Fc receptor expression by microglia and also antagonises the induction by IFNγ (Loughlin *et al*, 1993). Increased Fc receptor expression has also been observed on reactive microglia in MS tissue (Ulvestad *et al*, 1994).

5.1.5 Aims

The aim of the following investigations were to:

a) Examine the effects of chemokines on primary rat microglial nitrite and PMA triggered superoxide production *in vitro*.

b) Observe the effects of chemokines on Fc receptor expression and phagocytosis of opsonised erythrocytes by microglia

c) Compare the findings for (a) and (b) with the human neonatal microglial cell line, CHME3 and rat peritoneal macrophages

5.2 Materials and methods

5.2.1 Cell culture

Rat microglia were isolated as in chapter 2 (Section 2.2.8) and plated into 96 well plates at a concentration of $2.5x10^5$/ml in cDMEM (200µl/well). Plates were incubated overnight in a humid atmosphere at 37°C, 5%CO_2/95% air and then washed in warm cDMEM to remove any non adherent cells or remaining erythrocytes.

Rat peritoneal macrophages were isolated from adult Wistar rats by peritoneal flushings with ice cold $EBSS^-$ (Chapter 2, section 2.3). Briefly, these cells were pelleted by centrifugation at 180g for 10 minutes at 4°C, washed in $EBSS^-$, counted and plated into 96 well plates, at a concentration of $5x10^5$/ml in cDMEM (200µl/well).

CHME3 cells were trypsinised and plated into 96 well plates at a concentration of $1x10^5$/ml (200µl/well) in cDMEM and cultured for 24 hours. Cells were then washed in cDMEM before use in the assays.

5.2.2 Cell stimulation

Cells were stimulated in triplicate wells (unless otherwise stated) with the human chemokines MCP1, MIP1β, RANTES and IP-10 between 0 and 50ng/ml in cDMEM or the cytokine IFNγ, at 1 and 10 IU ml for 24 hours. cDMEM alone was used in control wells.

5.2.3 Nitrite assay

Nitric oxide release from cells was estimated by measuring nitrite levels in cell supernatants, as nitric oxide has a half life of only milliseconds before it is converted to nitrite. Following the 24 hour stimulation period, cell supernatants were harvested and used to determine the amount of nitrite released compared to control, unstimulated levels. Nitrite levels were measured colourimetrically using Greiss reagent (consisting of 1% sulphanilamide (Sigma), 0.1% napthylethylene diamine dihydrochloride (Sigma) and 2.5% phosphoric acid (Vincent *et al*, 1997). The sulpanilamide in H_2O and napthylethylene diamine dihydrochloride in 5% phosphoric acid were made up separately and stored at 4°C. The two components were mixed 1:1 immediately prior to use. 100µl of cell supernatants were mixed 1:1 with freshly mixed Greiss reagent in clean 96 well plates and incubated at room temperature for 10 minutes and the absorbance read at 550nm on a Wallac spectrophotometer. Standard curves were prepared by dissolving sodium nitrite in cDMEM (0-25µM) and pipetted in duplicate into the 96 well plates alongside the test samples. A standard curve was plotted of nitrite concentration against absorbance at 550nm. Nitrite levels in test supernatants were calculated from the curve using Microsoft Excel. Nitrite levels (µM) in the supernatants are expressed as a mean of three experiments, unless stated otherwise.

5.2.4 Fc receptor assay

Fc receptor expression was estimated on microglia and CHME3 cells using the method of Woodroofe *et al* (1989). Following stimulation of cells in 96 well plates with chemokines or cDMEM alone (as above), plates were washed in warm PBS, and 50µl of 1% EA (see chapter 2, section 2.2.6) in $EBSS^+$ /0.5%BSA was added to each well and the plates incubated at 37°C for 90 minutes. The plates were washed four times in warm

PBS by immersion and flicking out. The cells were then solubilised by the addition of 100µl of 0.3% SDS (Sigma) in distilled water for 10 minutes at room temperature followed by 100µl of substrate solution made according to the manufacturers' instructions (Sigma fast OPD (O-phenylene diamine) tablets, Sigma) using one tablet dissolved per 20ml of PBS, to measure peroxidase activity of bound and ingested erythrocytes. The plates were further incubated for 30 minutes at 37°C and the absorbance read at 450nm on a Wallac spectrophotometer. The mean absorbance values of control unstimulated cells was subtracted from the mean absorbance values of chemokine stimulated cells and then expressed as a mean of 3 separate experiments performed in triplicate wells.

5.2.5 Phagocytosis assay

To visualise the extent of phagocytosis by neonatal rat microglia, cells were plated into 8 well chamber slides (Nunc, Life Technologies, UK) following isolation, at a density of $2x10^5$ cells/well in 400µl of cDMEM. After 24 hours in culture, cells were washed in cDMEM and 4 wells of the chamber slide stimulated with MIP1β (10ng/ml) as this chemokine gave a good response in the Fc receptor assay. After a further 24 hours, cells were washed in warm PBS and 100µl of 0.5% EA in $EBSS^+$ /0.5% BSA and the cells were incubated at 37°C for 90 minutes. The chamber slides were then washed 4 times in PBS and the cells fixed in cold acetone for 5 minutes followed by washing again in PBS and addition of the peroxidase substrate, diaminobenzidine hydrochloride (DAB) (Sigma), 0.2mg/ml in PBS containing 0.4µl/ml hydrogen peroxide (30%w/v) for 5 minutes at room temperature. Slides were then washed in tap water, mounted in Immumount (Shandon, UK) and observed by light microscopy (Olympus BX60) to

compare the phagocytosis of unstimulated and MIP1β stimulated microglia. Cells were photographed on Kodak colour film (200ASA).

5.2.6 Assay for superoxide production

Superoxide production by microglia, CHME3 cells and splenic macrophages was assessed colourimetrically using a modified method of Pick and Mizel (1981) utilising the reduction of ferricytochrome c by superoxide anions in the absence or presence of superoxide dismutase. Following stimulation, for 24 hours, cells were washed in DMEM without phenol red (Sigma) and incubated at 37°C with ferricytochrome c (from horse heart), 160μM (Sigma) and phorbol-12-myristate-13-acetate (PMA) (20nM) to trigger superoxide production, in phenol red free DMEM (Life Technologies, U.K) for 60 minutes. To calculate the reduction of ferricytochrome c by superoxide only, superoxide dismutase (from bovine erythrocytes) (Sigma) at a final concentration of 375 IU/ml was included in replicate experiments. Triplicate wells were averaged and superoxide expression per 10^5 cells was calculated using the formula by Pick and Mizel (1981):

$$\frac{A_{550nm} \text{ without SOD} - A_{550nm} \text{ with SOD}}{6.3} \times 100.$$

This gives the amount of superoxide produced in nmoles per 10^5 cells per hour. CHME3 results were calculated based on the number of cells plated out at the start of the experiment.

5.2.7 Statistical analysis

Significant differences between control and stimulated cells were calculated using one way ANOVAs followed by multiple range analyses for all experiments. P values of <0.05 were considered significant.

5.3 Results

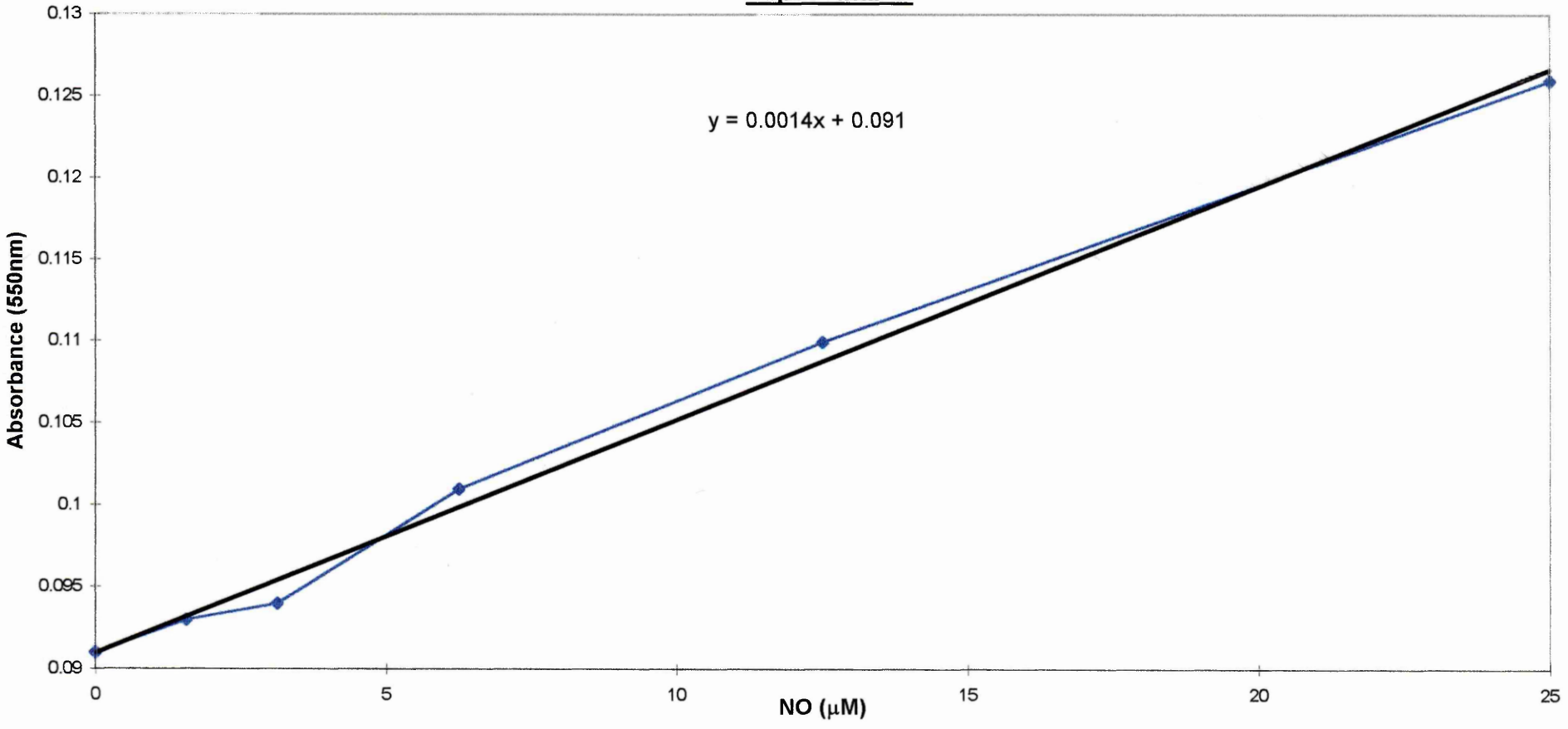
5.3.1 Nitrite Results

A standard curve was produced for each experiment, to determine the amount of nitrite in cell culture supernatants. All standard curves were highly reproducible and a typical example is shown in Figure 5.1. The plot is linear and absorbance values obtained from test supernatants were converted into nitrite levels in μM, directly from the curve using Microsoft Excel spreadsheets.

5.3.2.1 Nitrite production by rat microglia and peritoneal macrophages following IFNγ stimulation

To assess the assay protocol, IFNγ , which has previously been demonstrated to upregulate nitrite production in microglia (Chao *et al,* 1992), was used to stimulate rat microglia and rat peritoneal macrophages. Nitrite levels produced by 30 day old rat microglia (2 experiments) and peritoneal macrophages (3 experiments) were compared in response to stimulation with IFNγ at 1 and 10IU/ml (concentrations reported by Woodroofe *et al* (1989) to stimulate microglia). Primary microglia showed a significant increase in nitrite levels above unstimulated levels ($P<0.05$) (Table 5.1) when stimulated for 24 hours with IFNγ at 1IU/ml (1/2 experiments) and 10IU (2/2 experiments) using a one way ANOVA followed by multiple range analysis. Similarly, rat peritoneal macrophages also showed a significant increase in nitrite levels when stimulated with IFNγ at 1 and 10IU/ml (3/3 experiments). Nitrite levels produced by peritoneal macrophages were generally higher in response to IFNγ compared to microglia (Table 5.1), although the levels produced in response to the chemokines tested were very similar.

Figure 5.1 A typical sodium nitrite standard curve for determination of nitrite concentration in cell supernatants



Standard curves were set up using duplicate dilutions of sodium nitrite in DMEM.

Table 5.1 Nitrite production by 30 day old rat microglia and peritoneal macrophages in response to IFNγ.

	Mean nitrite production (μM) +/- SEM	
IFNγ (IU/ml)	microglia	peritoneal macrophages
0	2.52+/-1.96	4.07+/-2.94
1	6.47+/-0.026 (1/2)*	37.0+/-11.8 (3/3)
10	7.26+/-0.52 (2/2)	42.7+/-15.9 (3/3)

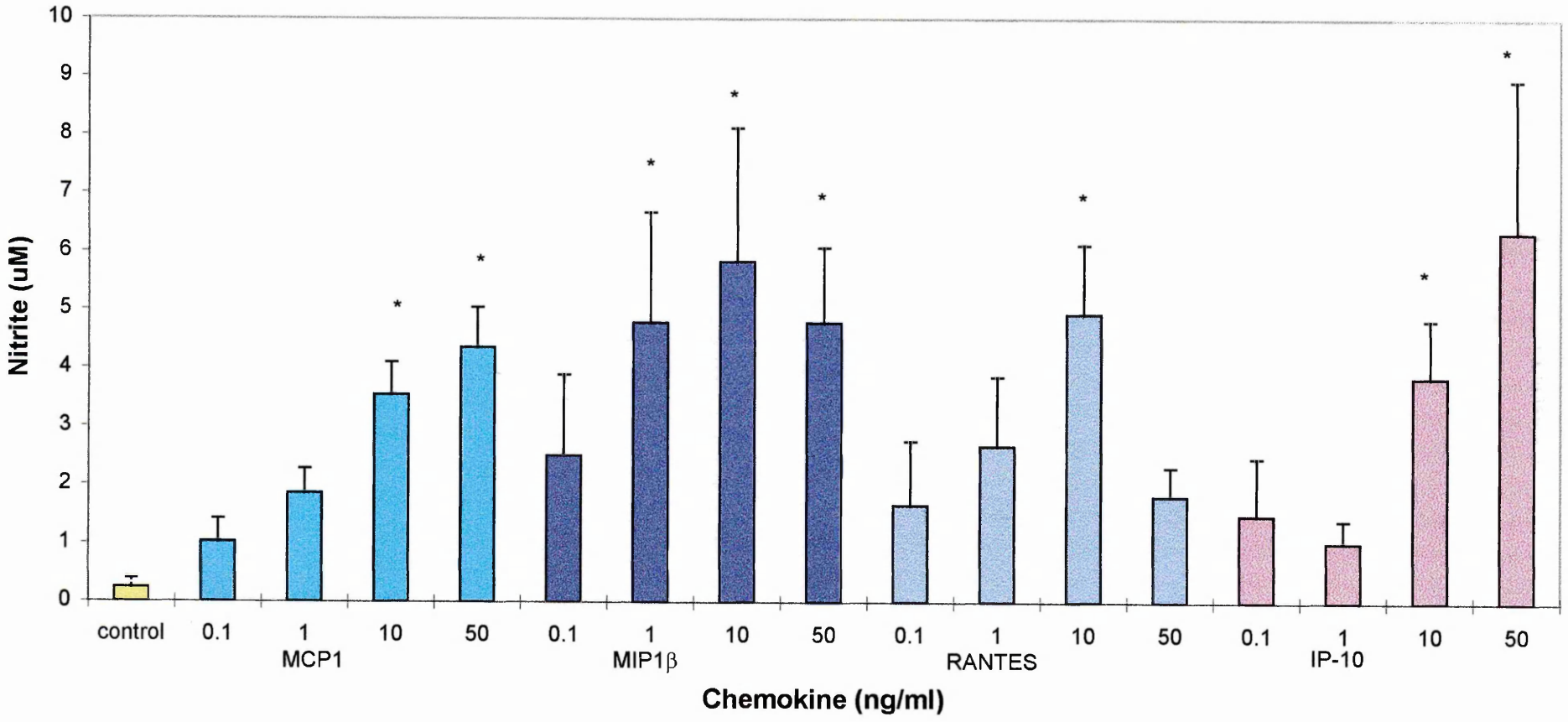
Results are expressed as the mean +/- SEM.

*Brackets show number of experiments giving a significant ($P<0.05$) response above control levels. Experiments were performed twice using microglia and three times using peritoneal macrophages.

5.3.2.2 Nitrite production by neonatal rat microglia following chemokine stimulation

Nitrite production by neonatal microglia was assessed *in vitro* following stimulation for 24 hours with the β chemokines MCP1, MIP1β and RANTES and the α chemokine IP-10, used at concentrations between 0.1 and 50ng/ml. Experiments were performed three times and the mean nitrite levels +/- SEM determined. Control levels were very low in 2/3 experiments (0.23μM and 0.49μM) and were undetectable in the third experiment. Nitrite levels were significantly increased above control levels ($P<0.05$) by all of the chemokines tested at at least one concentration of chemokine (Figure 5.2a). The response to stimulation with MIP1β was consistently the highest above control levels, when compared to the other chemokines with a peak increase in nitrite production

Figure 5.2a Nitrite production by neonatal microglia following chemokine stimulation for 24 hours



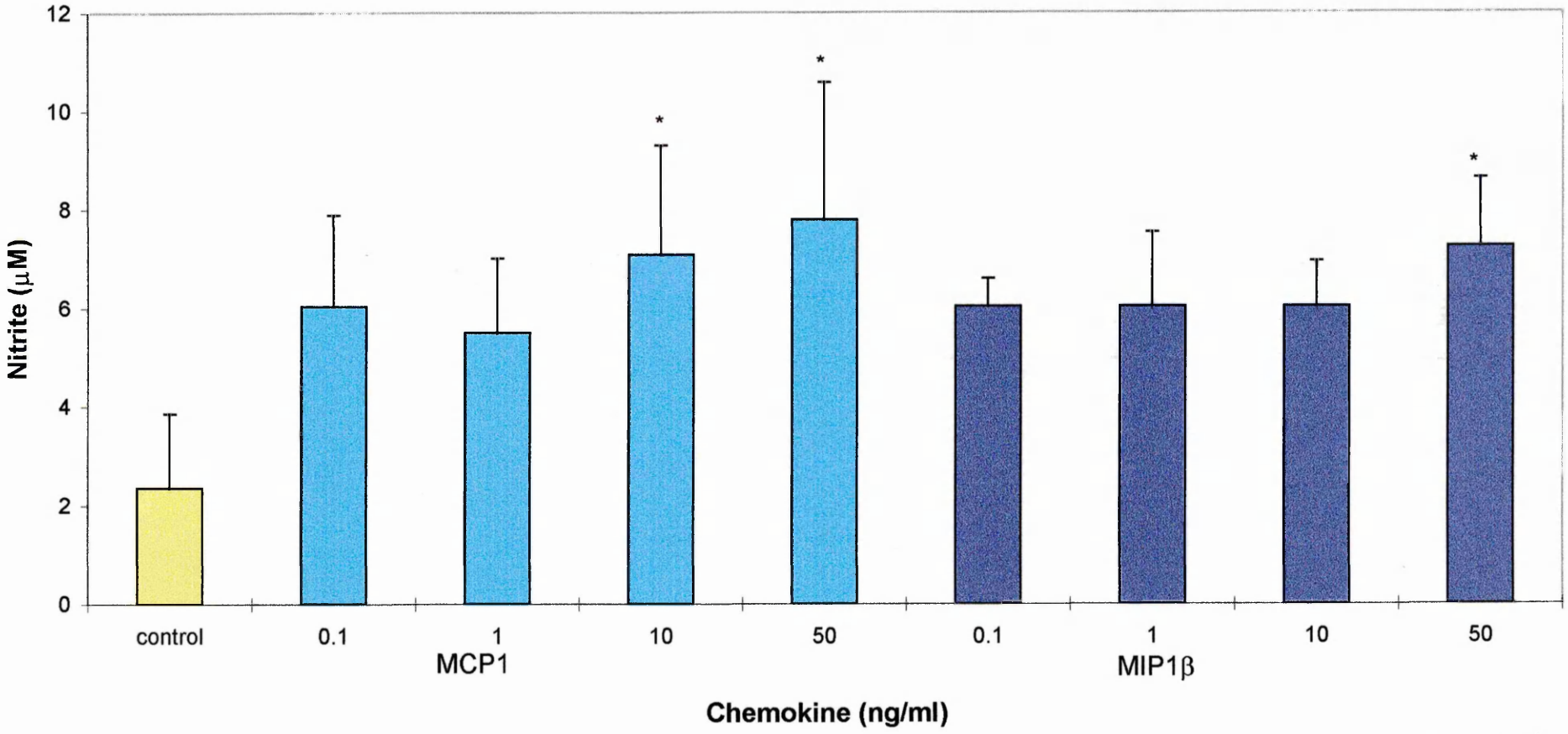
The graph shows a mean of three individual experiments +/- SEM. * Represents a significant increase in nitrite production above control levels using a one way ANOVA followed by multiple range analysis.

at 10ng/ml. IP-10 showed a comparable increase at 50ng/ml (Figure 5.2a) in 2/3 experiments. Stimulation with MIP1β also appeared to have an effect over a wider range of concentrations when compared to the other chemokines tested. Nitrite production was decreased by MIP1β and RANTES at the higher concentration of 50ng/ml when compared to results at 10ng/ml. RANTES only caused a significant increase in nitrite production by neonatal rat microglia above control levels at 10ng/ml, and was not significantly different from control when used at 50ng/ml, whereas stimulation with MCP1 and IP-10 gave the highest response above control levels at 50ng/ml (Figure 5.2a).

5.3.2.3 Nitrite production by 30 day old rat microglia

Microglia isolated from 30 day old rat brains were stimulated with the β chemokines, MCP1 and MIP1β, to compare their response with that of neonatal microglia. These experiments also showed a significant increase in nitrite production above control levels when stimulated for 24 hours, under the same conditions (Figure 5.2b). Control unstimulated production of nitrite was generally higher than in the neonatal microglia with a range of between 0.4 and 4.6 μM in three experiments. Both chemokines showed the maximal increase above control levels at the higher end of the concentration range (50ng/ml). The increased nitrite production seen with increasing chemokine concentrations, was not as marked with the 30 day old rat microglia as seen with the neonatal microglia, although the levels obtained were higher e.g. with MIP1β at 10ng/ml the mean nitrite levels for neonatal microglia were 10μM and was 8μM using 30 day old rat microglia.

Figure 5.2b Nitrite production by 30 day old rat microglia following chemokine stimulation for 24 hours



The graph shows a mean of three individual experiments +/- SEM. * Represents a significant increase in nitrite production above control levels using a one way ANOVA followed by multiple range analysis.

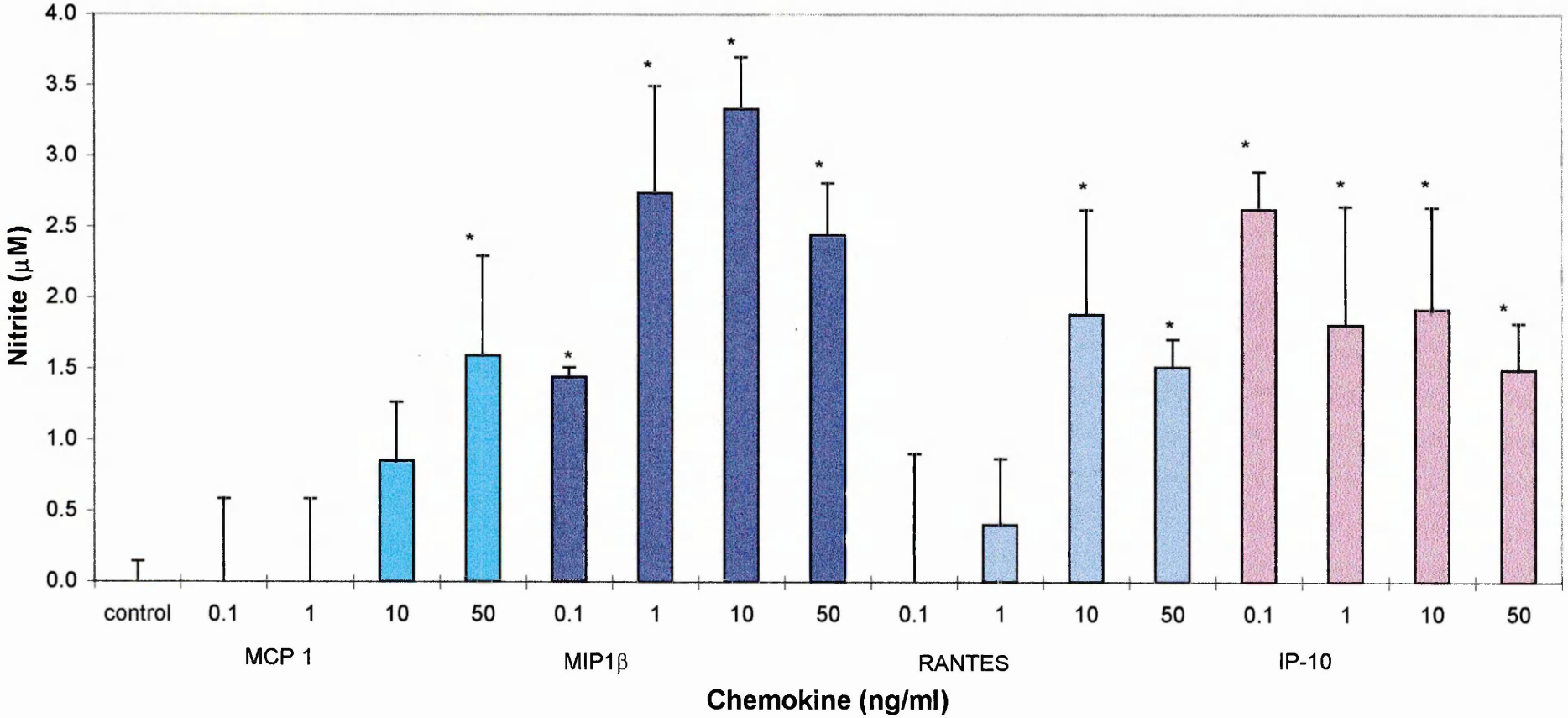
5.3.2.4 Nitrite production by CHME3 cells

CHME3 cells were used in the same experiments, to compare their nitrite production to that of primary rat microglia. Unstimulated levels of nitrite produced were comparable to the neonatal microglia and lower than the 30 day old microglia. The response to the four chemokines, MCP1, MIP1β, RANTES and IP-10, showed a very similar pattern to that of the primary microglia, although the actual amount of nitrite produced was lower (Figure 5.2c). The highest increase above control levels was after stimulation with MIP1β which also gave a significant increase above controls over the full range of concentrations tested. MCP1 gave the highest response at the higher concentrations. When stimulated with RANTES, there was a peak response at 10ng/ml, whereas IP-10 gave a significant increase in nitrite over the whole range of concentrations tested.

5.3.2.5 Nitrite production by rat peritoneal macrophages

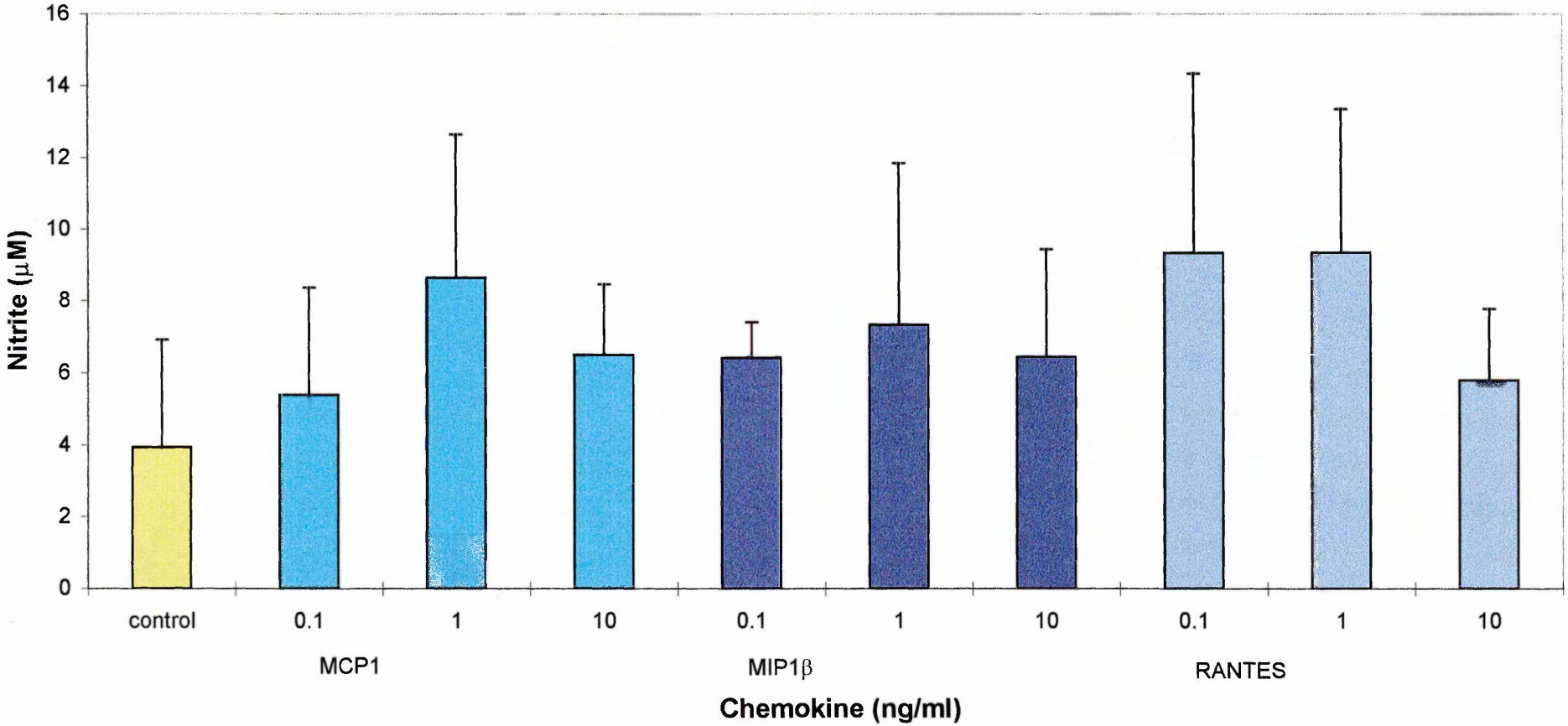
Nitrite levels in rat peritoneal macrophages were also tested following chemokine stimulation, for comparison. Nitrite levels in unstimulated peritoneal macrophages in these experiments were generally higher than with microglia, although the percentage increase above the control levels, when stimulated with α chemokines was comparable to the microglia. The intra and inter experimental variation was greater and although there is an apparent increase in nitrite production, this was not a significant increase above control levels, at the 5% level when stimulated with MCP1, MIP1β or RANTES at concentrations of between 0.1 and 10ng/ml (Figure 5.2d). There appears to be a slight increase in nitrite production at 1ng/ml for all three chemokines but these were not significant at the $P<0.05$ level of significance due to the variability of the results. Nitrite levels in rat peritoneal macrophages were also tested in response to the a chemokines IP-

Figure 5.2c Nitrite production by CHME3 cells following chemokine stimulation for 24 hours



The graph shows the mean of three individual experiments +/- SEM. + Represents a significant increase in nitrite production above control levels using a one way ANOVA followed by multiple range analysis.

Figure 5.2d Nitrite production by rat peritoneal macrophages following chemokine stimulation for 24 hours



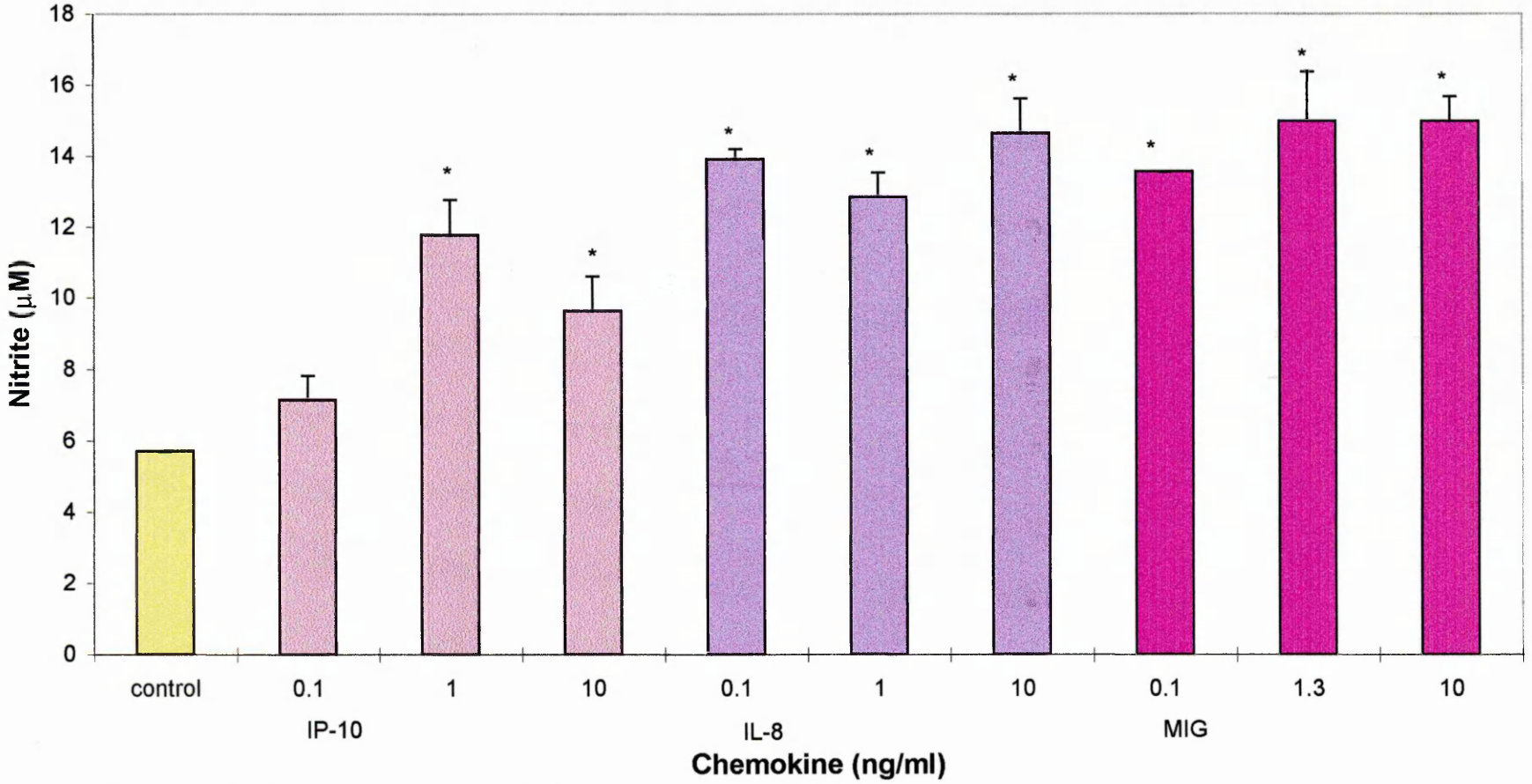
The graph shows the mean of three individual experiments +/-SEM. The values obtained were not significantly greater than control levels as individual experiments or as the mean of three, using a one way ANOVA followed by multiple range analysis.

10, IL-8 and MIG in two experiments (Figure 5.2e). In contrast to the response using the β chemokines, these all gave a significant increase in nitrite produced above the control levels. The chemokines IL-8 and MIG had a similar effect on nitrite production at all three concentrations used and IP-10 had a significant effect at only the higher concentrations used (1 and 10ng/ml).

5.4.1 Superoxide production by rat peritoneal macrophages, microglia and CHME3 cells

Superoxide anion production by rat microglia and peritoneal macrophages was assessed *in vitro* in response to stimulation with IFNγ for 24 hours as a positive control. Peritoneal macrophages were stimulated with IFNγ at 1, 10 and 100 IU/ml and compared to unstimulated controls in medium alone. The experiments were performed in quadruplicate wells and were repeated 3 times (Table 5.2) Unstimulated levels of superoxide release were 0.012 nmoles/10^5 cells/hour. The superoxide release was markedly and significantly ($P<0.05$) increased by IFNγ at 1 IU/ml in all three experiments. At the higher concentrations of 10 and 100 IU/ml, the levels of superoxide release were not significantly different from control levels ($P<0.05$). With this prior knowledge, the response of neonatal and 30 day old rat microglia was assessed when stimulated with IFNγ at 1 IU/ml and compared to unstimulated levels (Table 5.3). Cells were stimulated in quadruplicate and the experiments were performed twice. Both neonatal and 30 day old rat microglia did not release a measurable amount of superoxide anions under control, unstimulated conditions although there was a marked induction by IFNγ stimulation. 30 day old rat microglia produced slightly higher levels of superoxide when stimulated with IFNγ than the neonatal microglia, e.g.0.253 nmoles/10^5 cells/hour

Figure 5.2e Nitrite production by rat peritoneal macrophages following chemokine stimulation for 24 hours



The graph shows the mean of two individual experiments +/- SEM. * Represents a significant increase in nitrite production above control levels using a one way ANOVA followed by multiple range analysis.

compared with 0.206 nmoles/10^5 cells/hour. Conversely, CHME3 cells did not release any detectable amount of superoxide when stimulated with IFNγ at 1 or 10 IU/ml.

Table 5.2 Induction of superoxide production by IFNγ stimulation of rat peritoneal macrophages for 24 hours.

IFNγ (IU/ml)	mean superoxide levels (+/- SEM) (nmoles/10^5cells/ hour)
0	0.063 (0.063)
1	0.243 (0.036) *
10	0.032 (0.042)
100	0.032 (0.042)

Mean superoxide production is shown from three individual experiments. Significance was calculated using a one way ANOVA followed by multiple range analysis. * indicates significant increase above control levels ($P<0.05$)

Table 5.3 Induction of superoxide production by IFNγ stimulation of neonatal and 30 day old rat microglia and peritoneal macrophages for 24 hours.

	Superoxide production (nmoles/10^5 cells/hour)		
IFNγ (IU/ml)	neonatal microglia	adult microglia	peritoneal macrophages
0	0	0	0.012+/-0.012
1	0.206+/- 0.016 *	0.253+/-0.001 *	0.243+/-0.036 *

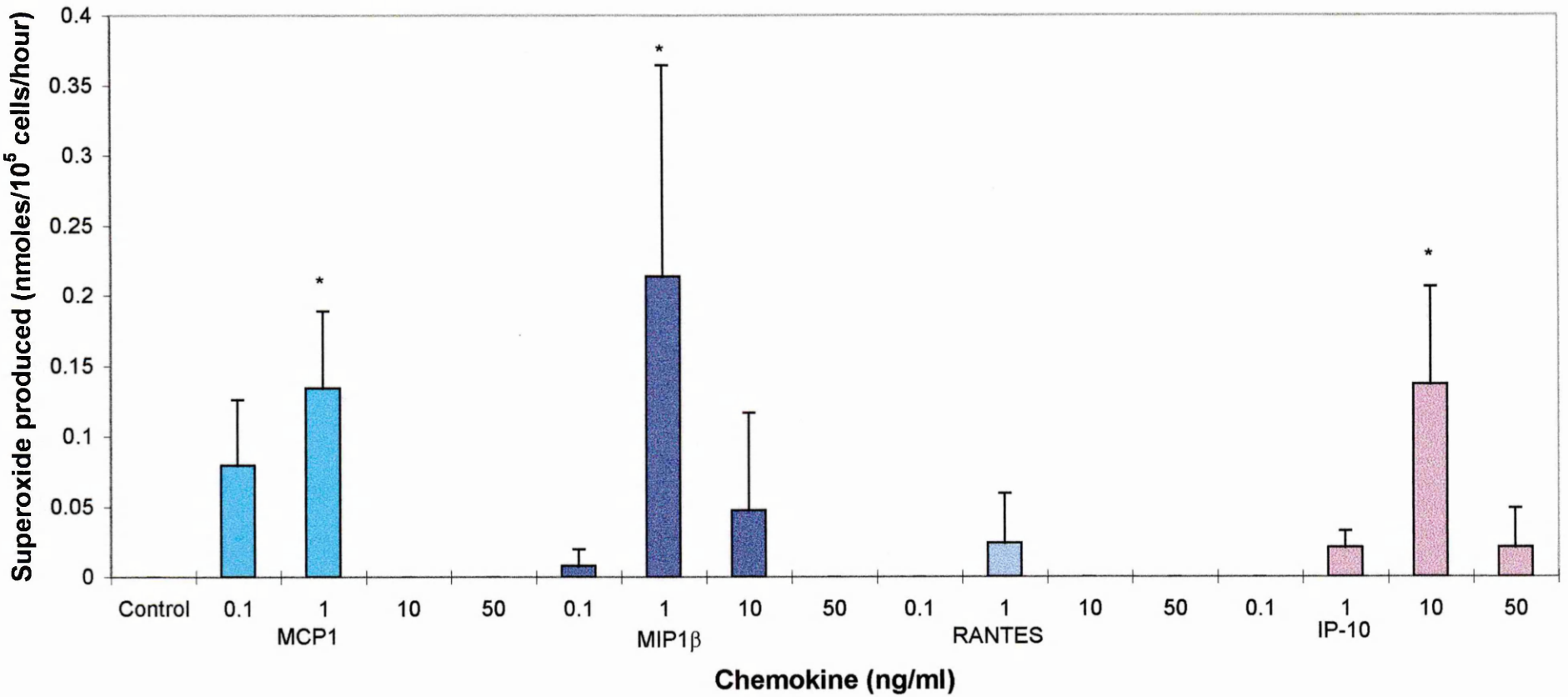
Results are expressed as mean superoxide production +/- SEM for 2 experiments for neonatal and adult microglia, and 3 experiments for peritoneal macrophages. Each stimulation was performed in quadruplicate wells. Significance was calculated by a one way ANOVA followed by multiple range analysis. * indicates significant increase above control unstimulated cells ($P<0.05$).

Superoxide anion production by rat microglia, CHME3 cells and rat peritoneal macrophages was then assessed *in vitro* in response to stimulation with chemokines for 24 hours. The effects of the chemokines, MCP1, MIP1β, RANTES and IP-10 were examined on neonatal rat microglia. Stimulations were performed in triplicate wells and each experiment was repeated three times.

5.4.2.1 Superoxide production by neonatal rat microglia in response to chemokines

Neonatal microglial cells did not produce any measurable amount of superoxide anions under resting, unstimulated conditions in any experiments (Figure 5.3a). There was a significant induction of superoxide anion production ($P<0.05$) in response to

Figure 5.3a Effects of chemokine stimulation on superoxide production by neonatal microglia



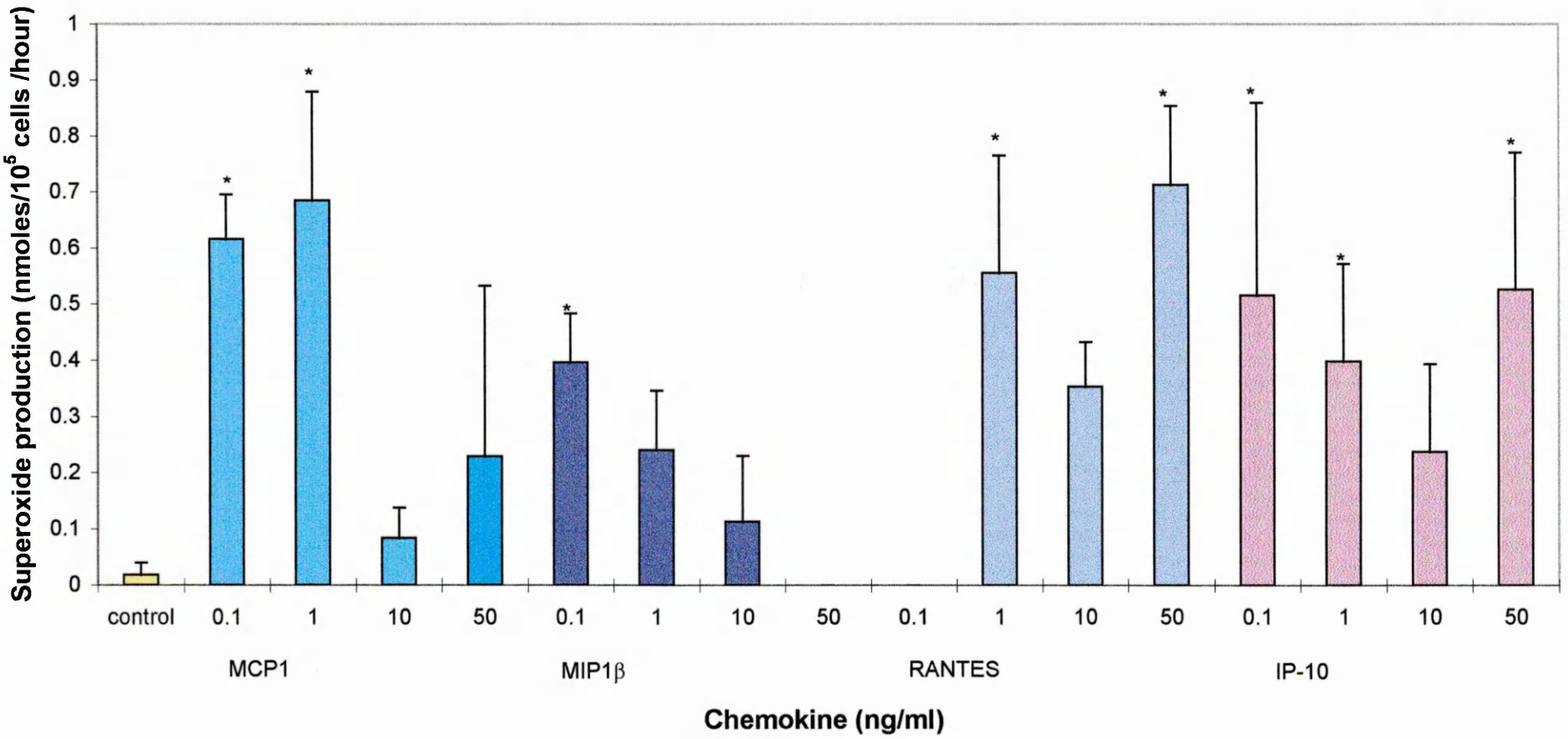
The graph shows a mean of three individual experiments +/- SEM. * Represents a significant increase in superoxide production above control levels.

MCP1 and MIP1β at 1ng/ml, and to IP-10 at 10ng/ml, although actual levels were quite variable between experiments. MIP1β at 1ng/ml consistently induced the greatest amount of superoxide release, when compared to the other chemokines tested. RANTES did not induce a significant release of superoxide anions in response at any of the concentrations tested and only one experiment out of three showed a small positive response above control, when cells were stimulated using RANTES at 1ng/ml.

5.4.2.2 Superoxide production by CHME3 cells in response to chemokines

As a comparison, the superoxide anion released from CHME3 cells in response to the same chemokines was examined. Stimulations were carried out again in triplicate wells and experiments repeated 3 times. Control, unstimulated levels of superoxide release were undetectable in two out of three experiments and were very low (0.01nmoles/10^5cells/hour) in the third experiment. Superoxide release was significantly increased above control levels ($P<0.05$) by all of the chemokines tested, in at least one concentration used, although similarly with the neonatal microglia, the levels of superoxide produced were variable between experiments (Figure 5.3b). A significant increase in superoxide production was observed when CHME3 cells were stimulated with MCP1, MIP1β and IP-10 at 0.1ng/ml, with no detectable superoxide production following stimulation by RANTES at this concentration under the same conditions. MCP1 stimulation also produced a significant increase at 1ng/ml although 10 and 50ng/ml MCP1, produced a much smaller and non significant, increase above control levels with superoxide levels being undetectable in two out of three stimulation experiments with MCP1 at 50ng/ml. Stimulations with MIP1β at the highest concentration tested, 50ng/ml, did not produce detectable levels of superoxide by CHME3 cells in any of the three experiments. CHME3 cells showed a significant

Figure 5.3b Effects of chemokine stimulation on superoxide production by CHME3 cells



The graph shows a mean of three individual experiments +/- SEM. * Represents a significant increase in superoxide production above control levels using a one way ANOVA followed by multiple range analysis.

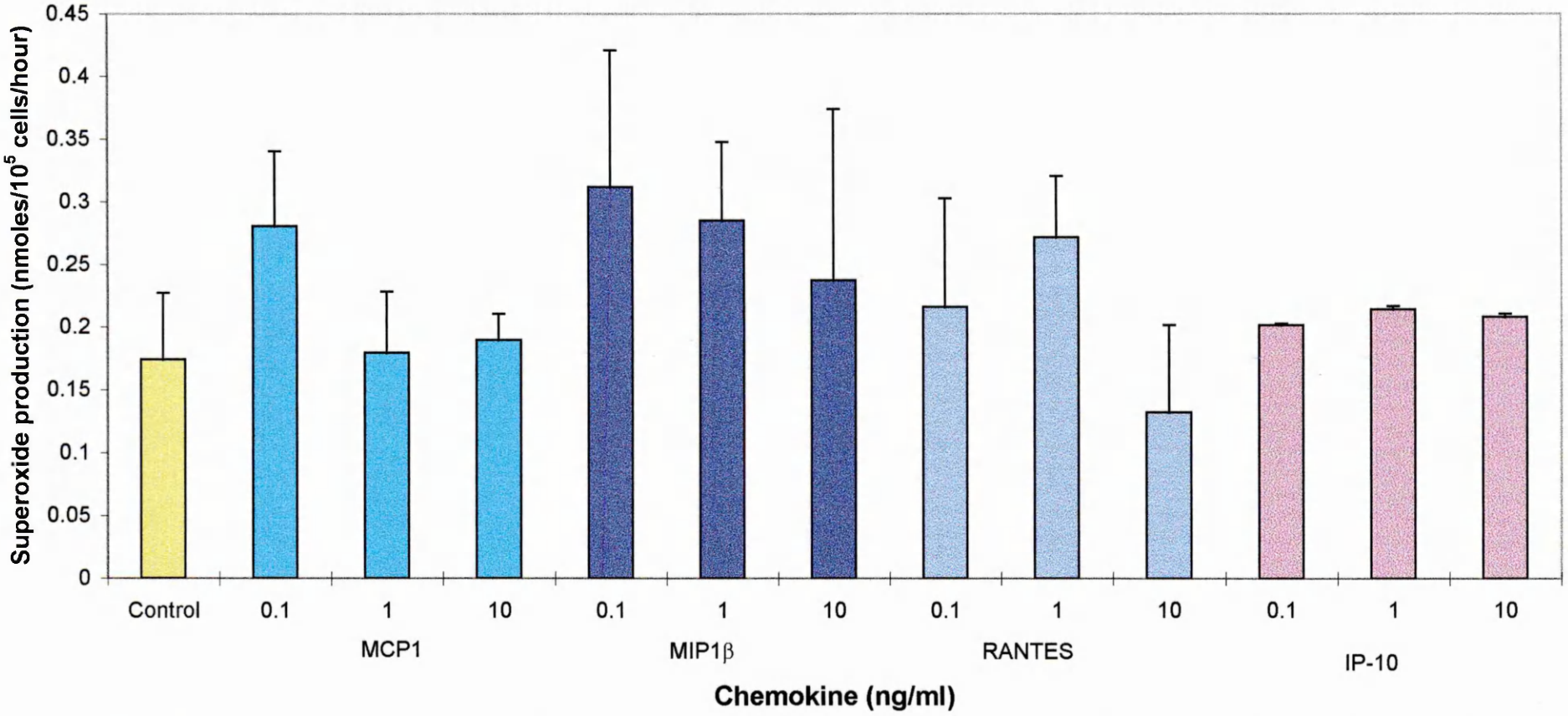
response to RANTES and IP-10 at 1 and 50ng/ml however an apparently lower and non significant increase above control was observed at 10ng/ml probably due to the inter-experimental variability.

Levels of superoxide production by CHME3 cells were generally higher than from neonatal rat microglia with maximal levels of up to 0.9nmoles/10^5cells/hour, when stimulated with MCP1 whereas the highest level of superoxide production by neonatal microglia following chemokine stimulation with MIP1β was just 0.44nmoles/10^5 cells/hour. The major difference between the response of CHME3 and primary neonatal rat microglia was in their response to RANTES, whereas all the other chemokines appeared to significantly increase superoxide release in both types of cells, RANTES only produced this effect in CHME3 cells (Figures 5.3a and b.)

5.4.2.3 Superoxide production by rat peritoneal macrophages in response to chemokines

Rat peritoneal macrophages were used as a comparison for microglia and CHME3 cells to assess the difference in the levels of superoxide produced when stimulated with chemokines (Figure 5.3c). Cells were stimulated with MCP1, MIP1β, RANTES and IP-10 between 0.1 and 10ng/ml for 24 hours. Unstimulated levels of superoxide production were between 0.1 and 0.25 nmoles/10^5cells/hour which was higher than that observed for CHME3 cells and neonatal rat microglia, which did not produce any superoxide under resting conditions. When stimulated with the chemokines MCP1 and MIP1β at 0.1ng/ml, there was an apparent increase in superoxide production by peritoneal macrophages although this was not statistically significant at the $P<0.05$ level. RANTES appeared to increase superoxide production at 1ng/ml in all three experiments but similarly, this was not statistically significant. IP-10 stimulation had no

Figure 5.3c Effects of chemokine stimulation on superoxide production by rat peritoneal macrophages



The graph shows a mean of three individual experiments (two for IP-10) +/- SEM. None of the apparent increases in superoxide production were statistically significant using a one way ANOVA followed by multiple range analysis.

effect on superoxide production by peritoneal macrophages in any experiments (Figure 5.3c).

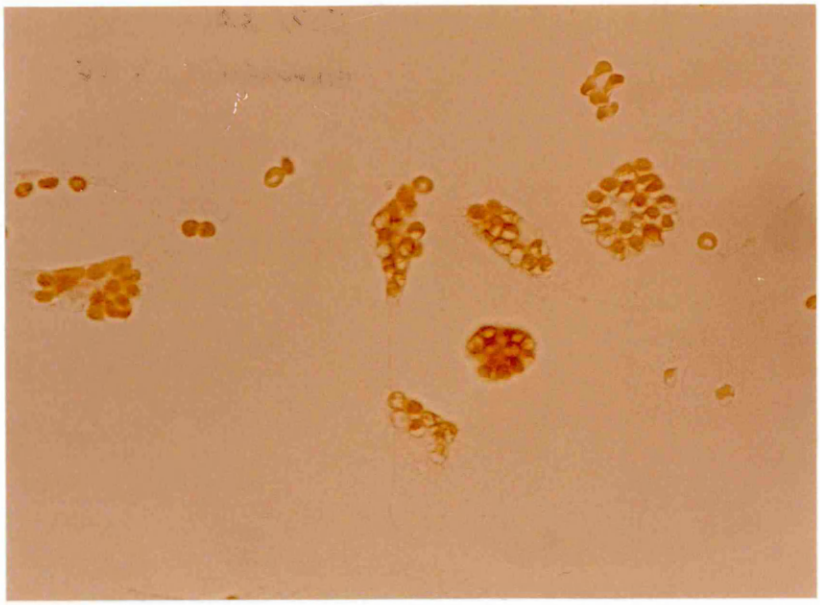
5.5.1 Fc receptor expression by rat peritoneal macrophages, microglia and CHME3 cells, assayed by measurement of phagocytosis of opsonised erythrocytes

Fc receptor expression was assessed by the ability of macrophages and microglia to bind and/or ingest antibody coated erythrocytes via the Fc portion of the opsonising antibody. Phagocytosis of antibody coated erythrocytes after 90 minutes was first observed using light microscopy, comparing chemokine stimulated and unstimulated neonatal and 30 day old rat microglia. (Figure 5.4). The mean number of phagocytosed erythrocytes per microglial cell was increased above that of unstimulated cells by stimulation with MIP1β at 10ng/ml for 24 hours. To quantitate the amount of erythrocytes bound to or ingested by cells, the pseudoperoxidase activity of the bound/ingested erythrocytes was examined colourimetrically in 96 well plates. Fc receptor expression was examined by neonatal and adult rat microglia, peritoneal macrophages and CHME3 cells to allow comparison between the cell types.

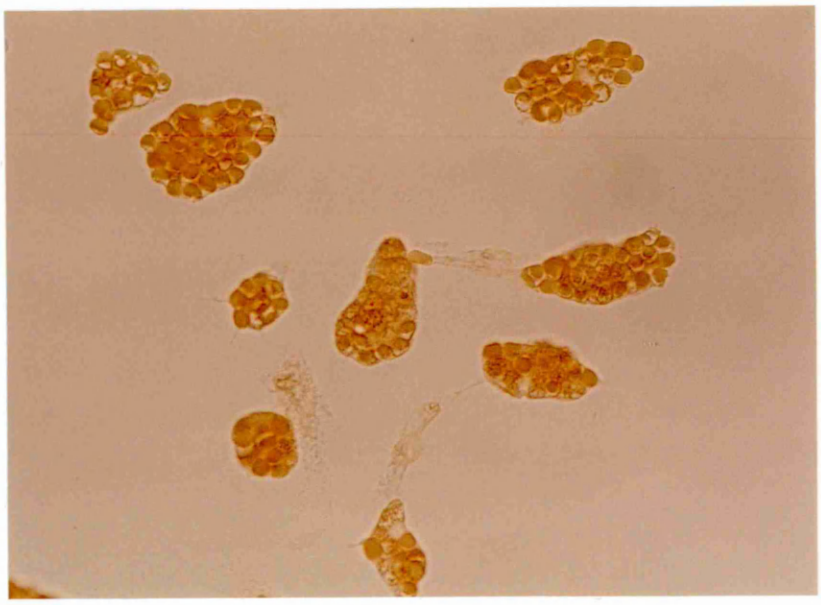
5.5.2.1 Fc receptor expression by neonatal rat microglia in response to chemokines

Fc receptor expression by neonatal microglia was assessed, with or without stimulation by the chemokines, MCP1, MIP1β, IP-10 and RANTES at 0.1, 1.0, 10, and 50ng/ml. Experiments were carried out three times (twice with RANTES) and the mean absorbance +/- SEM calculated. No statistically significant increase ($P<0.05$) in Fc receptor expression above control was observed due to inter-experimental variation, therefore individual experiments were plotted and the significant increase above control levels calculated (Figure 5.5a and b). MCP1 appeared to increase Fc receptor expression

Figure 5.4 Phagocytosis of antibody coated erythrocytes by neonatal microglia after 90 minutes.

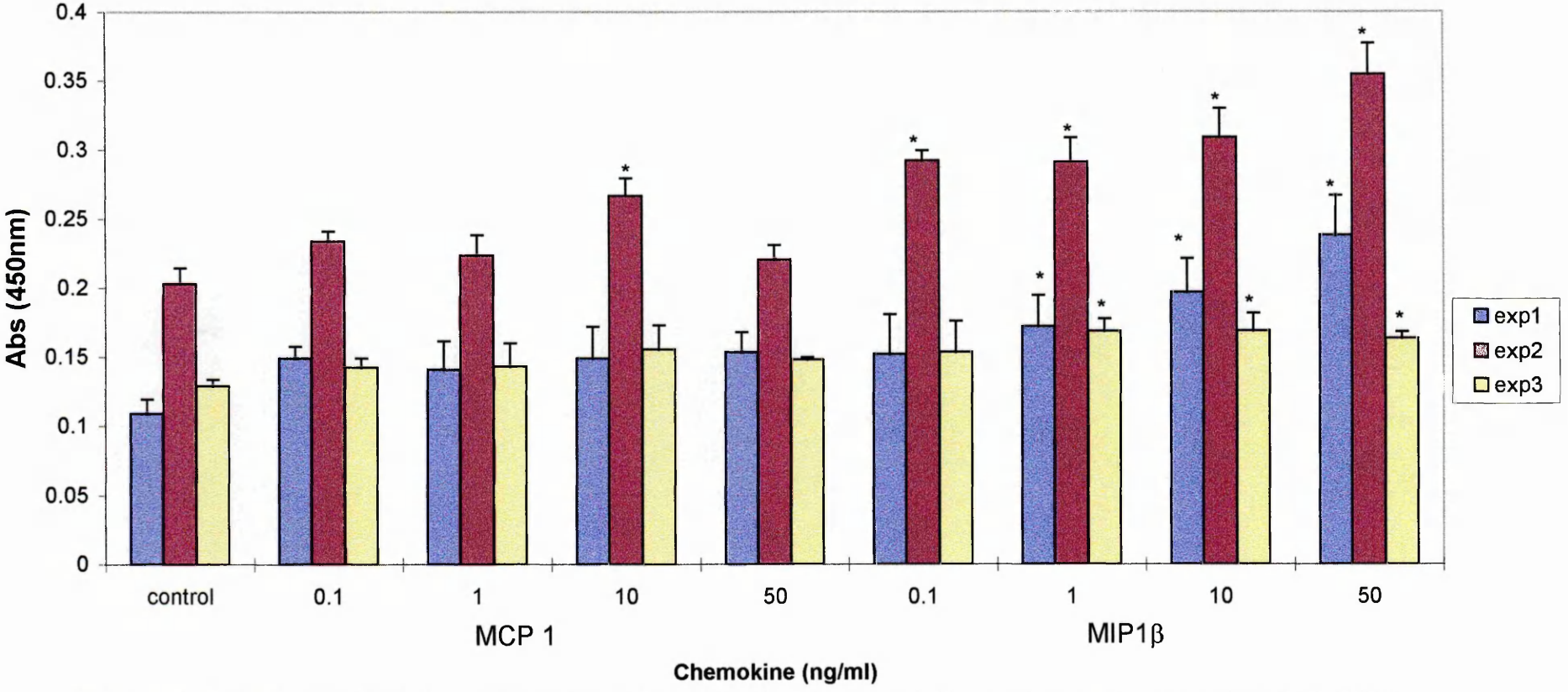


a) Control, microglia incubated with opsonised erythrocytes without pre-treatment with chemokine (X400)



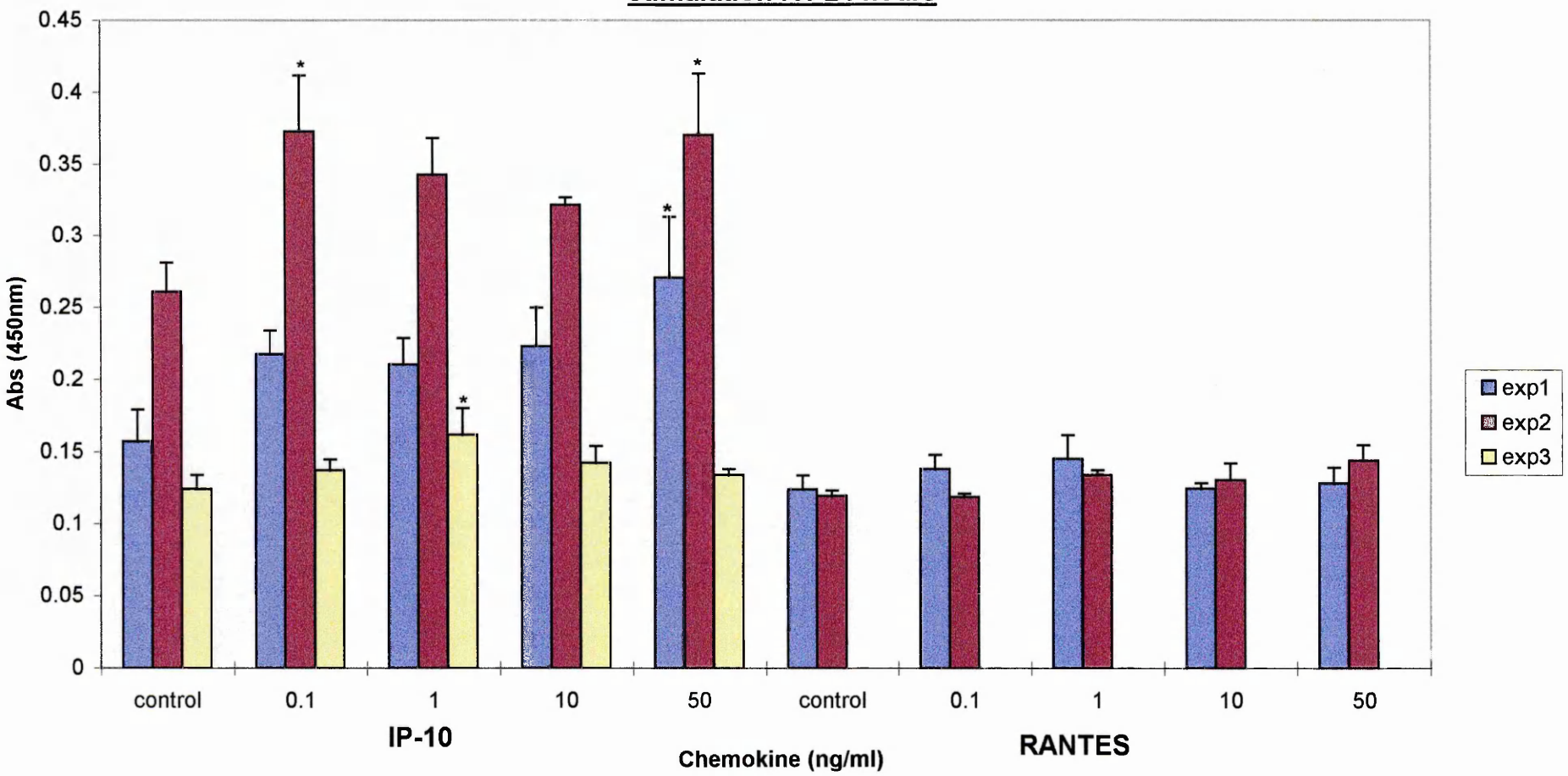
b) Microglia incubated with opsonised erythrocytes. Microglia were incubated with MIP1β (10ng/ml) for 24 hours before addition of erythrocytes (X400).

Figure 5.5a Fc receptor expression by neonatal microglia after chemokine stimulation for 24 hours



The graph shows the results of three experiments. The means of triplicate wells from each experiment are plotted +/- SEM.
* Represents a significant increase in Fc receptor expression above centrol levels using a one way ANOVA followed by multiple range analysis.

Figure 5.5b Fc receptor expression by neonatal rat microglia in response to chemokine stimulation for 24 hours



The graph shows the results of three experiments (two for RANTES). The means of triplicate wells from each experiment are plotted +/- SEM. * Represents a significant increase in Fc receptor expression above centrol levels.

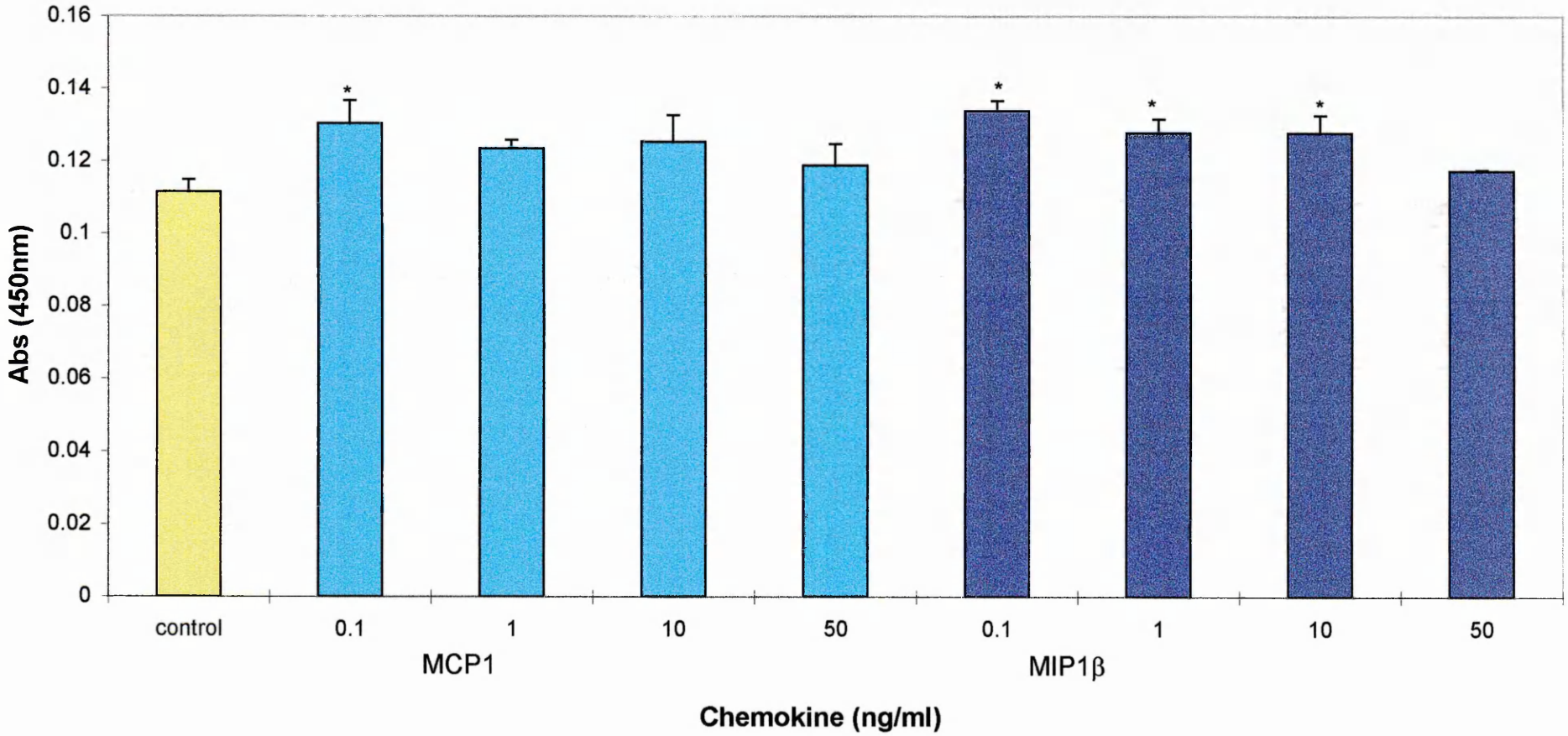
above control levels in all three experiments although this was only statistically significant ($P<0.05$) in one out of three experiments, at a concentration of 10ng/ml. MIP1β, however produced a much greater, statistically significant increase in Fc receptor expression, up to 1.75 fold, when compared to control levels, at all concentrations tested. MIP1β at concentrations of 1, 10, and 50ng/ml, in all three experiments a significant increase above control was observed.

When neonatal rat microglia were stimulated with IP-10 at 0.1, 1, 10 and 50ng/ml (Figure 5.5b) there was a significant increase in Fc receptor expression in 3/3 experiments although the concentration giving the most significant response was variable between experiments. Neonatal rat microglia did not show any significant increase in Fc receptor expression above control levels in response to RANTES stimulation at any of the concentrations tested.

5.5.2.2 Fc receptor expression by 30 day old rat microglia in response to chemokines

30 day old rat microglia were also used in this assay to assess the effect of chemokine stimulation on Fc receptor expression (Figure 5.5c). The chemokines used were MCP1 and MIP1β, at concentrations between 0.1 and 50ng/ml. A similar effect was observed as with neonatal microglia in response to MCP1 where there appeared to be an increase in absorbance above control levels but this was only statistically significant ($P<0.05$) at one concentration (0.1ng/ml). MIP1β, however, again showed a more significant increase than MCP1 stimulated cells with a statistically significant increase ($P<0.05$) at concentrations between 0.1 and 10ng/ml.

Figure 5.5c Fc receptor expression by 30 day old rat microglia following chemokine stimulation for 24 hours



The graph shows the mean of two individual experiments +/- SEM. * Represents a significant increase ($P<0.05$) in Fc receptor expression above control levels using a one way ANOVA followed by multiple range analysis.

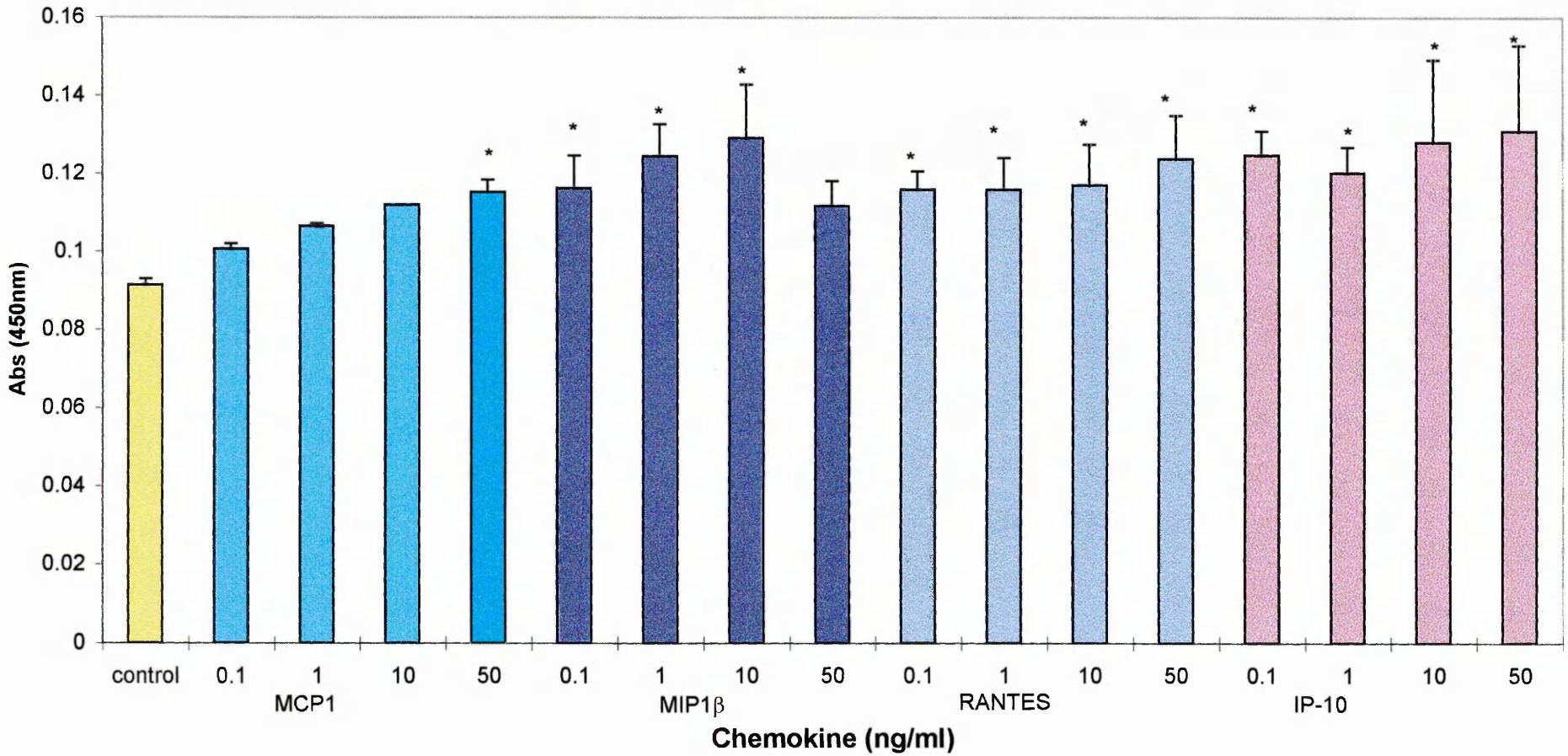
5.5.2.3 Fc receptor expression by CHME3 cells in response to chemokines

CHME3 cells were compared to neonatal rat microglia in their Fc receptor expression in response to the same chemokines as above (Figure 5.5d). There was less inter-experimental variation, therefore a mean of results from three individual experiments could be taken. Stimulation with MCP1 gave a similar response to that of the rat microglia in that there appears to be an increase in Fc receptor expression above control levels, although the response was only significant ($P<0.05$) at a concentration of 50ng/ml. MIP1β induced a greater response above control, which was statistically significant at concentrations of 0.1, 1 and 10ng/ml. RANTES and IP-10 caused a significant increase above control levels at all concentrations tested on CHME3 cells in contrast to rat microglia which did not show any response to RANTES stimulation. Absorbance values were generally lower in CHME3 cells compared to rat microglia suggesting a lower level of expression of Fc receptors.

5.5.2.4 Fc receptor expression by rat peritoneal macrophages in response to chemokines

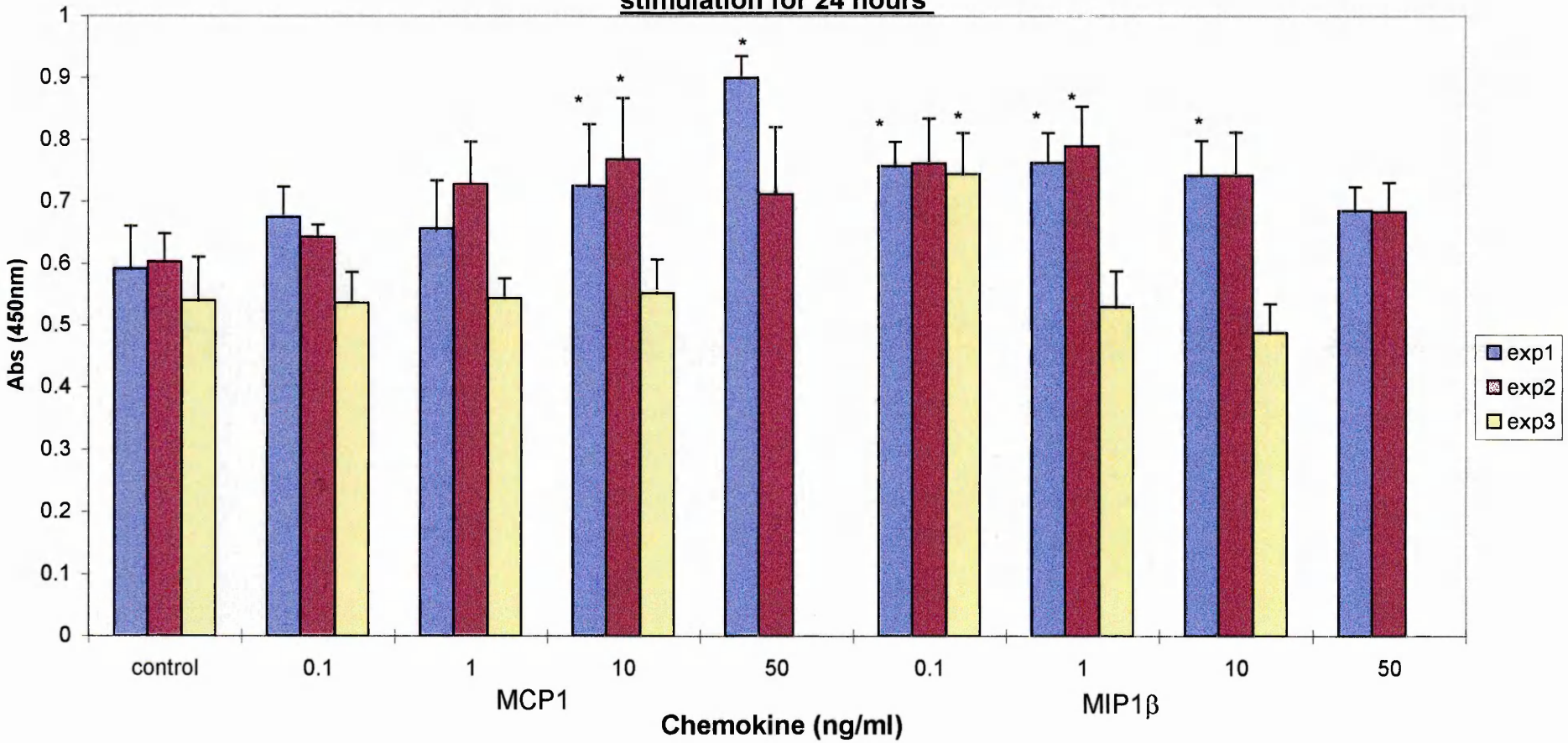
Rat peritoneal macrophages were also used in the Fc assay and tested in their response to MCP1 and MIP1β. Due to inter-experimental variation, all three repeat experiments (two for MCP1 and MIP1β at 50ng/ml) were plotted together on one graph (Figure 5.5e). A significant increase in absorbance was observed when cells were stimulated with MCP1 at 10ng/ml (2/3 experiments) and at 50ng/ml (1/2 experiments). MIP1β, showed a significant increase above controls over a wider range of concentrations which was consistent with results seen with neonatal and 30 day old rat microglia, as well as with CHME3 cells. The mean level of Fc receptor expression by peritoneal macrophages was similar to those seen in experiments with CHME3 cells, but this was lower than found using primary rat microglia.

Figure 5.5d Fc receptor expression by CHME3 cells after chemokine stimulation for 24 hours



The graph shows the mean of three individual experiments +/- SEM. * Represents a significant increase ($P<0.05$) in Fc receptor expression above control levels using a one way ANOVA followed by multiple range analysis.

Figure 5.5e Fc receptor expression by rat peritoneal macrophages following chemokine stimulation for 24 hours



The graph shows the results of three experiments (two for MCP1 and MIP1β 50ng/ml). The means of triplicate wells from each experiment are plotted +/- SEM. * Represents a significant increase ($P<0.05$) in Fc receptor expression above control levels using a one way ANOVA followed by multiple range analysis.

5.5.2.5 Summary of results

Table 5.4 summarises the results of chemokine stimulation experiments on nitrite and superoxide production and Fc receptor expression by microglia, CHME3 cells and peritoneal macrophages.

Table 5.4 Summary of the effects of chemokines on nitrite and superoxide production and Fc receptor expression by microglia, CHME3 cells and peritoneal macrophages.

		Chemokine			
Cell type	**Assay**	**MCP1**	**MIP1β**	**RANTES**	**IP-10**
2-3 day old rat microglia	Nitrite	+	+	+	+
	Superoxide	+	+	-	+
	Fc receptors	+/-	+	-	+
30 day old rat microglia	Nitrite	+	+	NT	NT
	Superoxide	NT	NT	NT	NT
	Fc receptors	+/-	+	NT	NT
CHME3	Nitrite	+	+	+	+
	Superoxide	+	+	+	+
	Fc receptors	+/-	+	+	+
Rat peritoneal macrophages	Nitrite	-	-	NT	+
	Superoxide	-	-	-	-
	Fc receptors	+	+	NT	NT

+ represents significant increase above control levels ($P<0.05$)

- represents no significant increase above control levels ($P<0.05$) using a one way ANOVA. NT represents not tested

5.6 Discussion

Microglia are thought to cause cellular damage *in vitro* by the production of reactive oxygen and nitrogen intermediates and are capable of phagocytosing products of demyelination. NO, measured as nitrite production, has been previously shown to be upregulated by IFNγ in rat microglial cell cultures *in vitro* (Chao *et al*, 1992) and these effects were confirmed in the present study. Nitrite levels reported by Chao *et al* (1992) were much higher than those found in this study although this could be accounted for by both the longer stimulation period (72 hours) used, allowing greater accumulation of nitrite in the culture medium and a much higher concentration of IFNγ (500IU/ml). In this present study, an increase in nitrite production by microglia was observed using only 1 and 10 IU/ml IFNγ.

IFNγ was also found to increase superoxide production by rat microglia and peritoneal macrophages, in agreement with experiments by Woodroofe *et al*, (1989). Treatment with IFNγ induced the greatest amount of superoxide production by peritoneal macrophages followed by 30 day old rat microglia and slightly less by neonatal microglia. This also mirrored findings by Woodroofe *et al*, (1989). The human foetal microglial cell line, CHME3 did not release any detectable amount of superoxide in response to IFNγ, indicating a difference between the primary rat microglial cultures and the cell line, although this effect may be accounted for by species differences. The generation of reactive oxygen intermediates by microglia from different species has been noted by Colton *et al*, (1996).

Since the generation of superoxide and nitric oxide and the subsequent formation of peroxynitrite in the CNS has clearly important implications in the pathogenesis of MS, and since a plethora of cytokines as well as the more recently documented chemokines,

are present in the MS lesion, it was hypothesised that chemokines may regulate the production of reactive oxygen and nitrogen intermediates by microglia. This study clearly indicates that the chemokines are able to increase their release by microglia at least *in vitro* and this may indicate a detrimental role for chemokines in causing damage to myelin *in vivo,* in MS.

MCP1, MIP1β, RANTES and IP-10, all increased nitrite production in both microglia and CHME3 cells above control levels, although nitrite levels were generally found to be higher in rat microglia than in CHME3 cell supernatants. This may also be accounted for by the species difference. It has often been debated whether or not human microglia produce nitric oxide, although it may be that stimuli capable of inducing iNOS in human microglia may be different from those effective in rat microglia. Species differences have previously been described along the signal transduction cascade required for the induction of the iNOS gene which may be responsible for the hypo-responsiveness of human iNOS to IFNγ stimulation (Zhang *et al*, 1996) and this could also be the case for the differences found here following chemokine stimulation.

The chemokines MCP1, MIP1β and IP-10, all increased superoxide production by both rat microglia and CHME3 cells. A difference was observed in the responsiveness to RANTES whereby only CHME3 cells produced a significant increase in superoxide production above unstimulated levels. In contrast, rat peritoneal macrophages did not show a significant increase in superoxide production following stimulation with any of the chemokines. This is an interesting difference because as mentioned previously, the peritoneal macrophages produced the highest response to IFNγ stimulation, yet seem unresponsive in generating superoxide after chemokine stimulation.

Important biological links between chemokines and reactive oxygen species/nitric oxide have been observed in previous studies. NO has been shown to inhibit the

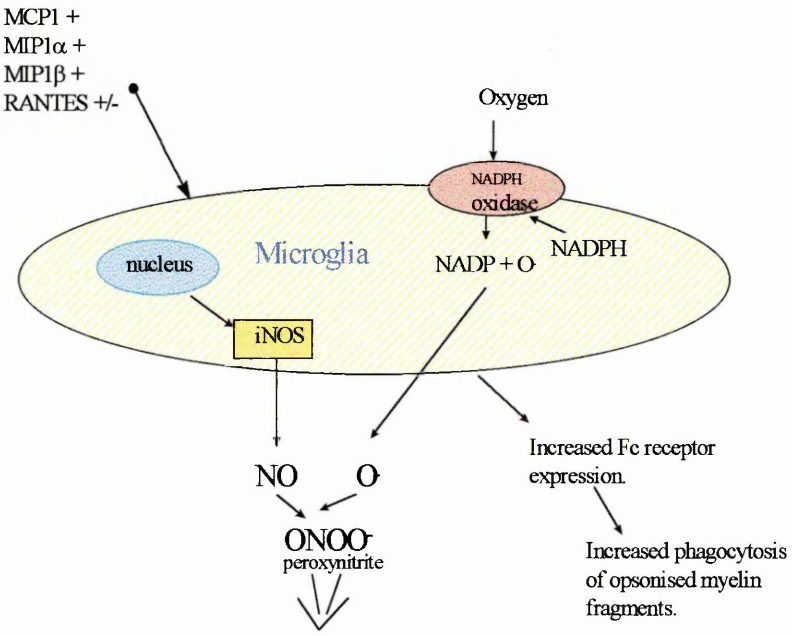
expression of IL-8 and MCP1 in endothelial cells and upregulates IL-8 transcription in leukocytes and melanoma cells (Merrill and Murphy, 1997). Reactive oxygen species including H_2O_2 and OH^-, have also been shown to upregulate IL-8 (DeForge *et al*, 1993). This present study is the only one to datethat has examined the effects of chemokines on nitrite production and superoxide production by microglia.

The present study also indicates that chemokines play a role in the regulation of Fc receptor expression by microglia, which may in turn increase the cells' phagocytic abilities. The effects of the chemokines tested on Fc receptor expression by microglia were quite variable between experiments and whereas MCP1, MIP1β and IP-10 all increased Fc receptor expression in rat microglia, RANTES had no effects at all on these cells, which was similar to the response by these cells on the production of superoxide. This cannot be explained by a lack of receptor for RANTES as a positive response was seen in microglia in NO production, migration experiments and MMP production in response to RANTES stimulation. CHME3 cells however did respond to RANTES by an increase in Fc receptors as well as to all the other chemokines tested and peritoneal macrophages also showed an increase in Fc receptor expression following chemokine stimulation. Experiments on rat microglia in culture showed that MIP1β stimulation did in fact increase phagocytosis of opsonised erythrocytes.

Many studies have focused on downregulating the effects of reactive oxygen intermediates in experimental models of MS. The use of peroxynitrite scavengers including uric acid, was shown to decrease the severity of disease symptoms in EAE, whether treatment was initiated before or after clinical symptoms of EAE had appeared (Hooper *et al*, 1997). This group found that the incidence of MS and gout, where

patients are hyperuricaemic, are virtually mutually exclusive and suggest that hyperuricaemia may protect against MS due to its effects on peroxynitrite mediated CNS damage (Hooper *et al*, 1998). The present study has demonstated that chemokines play an important role in the production of reactive oxygen and nitrogen species and it may be that chemokines could be a potential therapeutic target in downregulating CNS damage.

Figure 5.6 Summary of chemokine effects on microglial nitric oxide and superoxide production and Fc receptor expression with possible pathological consequences in MS.



Damage to oligodendrocyte cell membrane by fatty acid oxidation, release of membrane components, lipid/protein crosslinking.

The chemokines MCP1, IP-10, MIP1β and RANTES were all found to increase nitrite and superoxide production by CHME3 cells as well as increasing Fc receptor expression. MCP1, IP-10 and MIP1β had similar effects on rat microglia, although RANTES did not increase superoxide production and Fc receptor expression in these cells. This may be due to different signalling pathways in different cell types through the receptors CCR1/CCR5 or a lower affinity binding of RANTES to the receptor. Chemokines released by activated T cells within MS plaques could therefore be responsible for the recruitment and activation of microglia which then release free radicals causing damage

to the myelin sheath. The increase in Fc receptor expression would increase antibody mediated phagocytosis of opsonised myelin fragments which could be processed and presented to T cells, exacerbating the inflammatory response. Targeting the chemokines responsible for microglia activation, or their receptors may be an important consideration in the development of MS treatments

Chapter 6

DISCUSSION

Accumulation of microglia at sites of CNS injury is common to many neurological diseases, including MS (Esiri and Reading, 1987, Streit, 1987, Joshi and Crutcher, 1998). Immunohistochemical analysis of MS lesions has clearly shown that macrophages are a major cell type in perivascular cuffs and throughout the lesion and that they are present in an activated state. Activated microglia are capable of expressing MHC class II as well as a cascade of proinflammatory molecules from cytokines to proteases (Sriram and Rodriguez, 1997, Minghetti and Levi, 1998). Recent investigations have demonstrated the presence of chemokines in MS lesions (Simpson *et al,* 1998, McManus *et al* 1998a, Van der Voorn *et al,* 1999) and the use of animal model systems has shown the importance of chemokine expression for disease progression and correlate with disease severity (Miyagishi *et al,* 1995).

The purpose of this series of experiments was firstly to assess the migration response of microglia to a panel of chemokines, as these are obvious candidates for the recruitment of microglia *in vivo* to the inflammatory lesion in MS. Chemokines have been shown to be expressed by infiltrating T cells, astrocytes, endothelial cells and macrophages/ microglia in the lesion which could be the signal required for the further recruitment of microglia (Simpson *et al,* 1998). Microglia have previously been shown to migrate *in vitro* towards C5a and fMLP, the classical chemoattractants. Also a number of β chemokines have been shown to chemoattract human foetal microglia *in vitro* in migration studies (Peterson *et al,* 1997).

The present study has shown that primary rat microglia from 30 day old rats, representative of an adult phenotype, migrate to the chemokines MCP1, MIP1α, MP1β, RANTES, IL-8 and IP-10. The rat microglia were able to respond to both rat and human chemokines in chemotaxis experiments, probably due to the high degree of amino acid homology. The human foetal microglial cell line, CHME3 also showed a consistent migration response to the same panel of chemokines and the migration of both cell types was dose dependant, showing a typical bell shaped curve, where migration reached an optimal level and was then attenuated at higher concentrations of chemokine. This may cause the microglia to respond to a concentration gradient of a chemokine produced *in vivo* by cells present in the MS lesion by migrating up the concentration gradient until a point of receptor saturation where the microglia may become arrested and perform their functions within the lesion. Obviously in the *in vivo* situation, it is not as simple as in the migration studies reported here with one chemokine attracting the cells at one time, there seems to be an overwhelming presence of chemotactic signals produced *in vivo*. For the microglia to respond, there has to be the presence of the particular chemokine receptor on the responding cell as well as the chemokine gradient. It may be that there is a hierarchy of chemoattractants *in vivo* whereby some are preferential to others, or that binding of one particular chemokine to its receptor may downregulate the expression of another receptor. Chemoattractant receptor crosstalk in neutrophils has been demonstrated *in vitro* by Campbell *et al* (1997) and Foxman *et al* (1997), who showed 'multistep navigation' of neutrophils in a more complex environment of two different chemoattractants. Cells were able to migrate up a concentration gradient to a chemoattractant and then down that concentration gradient, towards a more distant, different chemoattractant. This may explain how microglia can negotiate the complex array of chemotactic stimuli *in vivo*.

Migration of cells involves changes in the actin cytoskeleton, allowing the formation and retraction of pseudopodia with focal adhesions for the cell to 'pull' itself along a substratum. Chemokine signalling through G protein coupled receptors involves the activation of small GTP binding proteins of the Ras superfamily. Rac and Rho lead to membrane ruffling, where cells form lamellipodia which extend outwards and then fold back over the cell as the cell moves forward. Rho induces the formation of stress fibres and focal adhesions (Machesky and Hall, 1996). The present study has demonstrated that the chemokines MCP1, MIP1α, MIP1β, RANTES, IL-8, IP-10 and MIG, cause morphological changes and actin reorganisation of the cytoskeleton in rat microglia and CHME3 cells. Stress fibre induction was the most obvious change in CHME3 cells, suggesting a typical Rho activated morphology, whereas an increase in F actin in pseudopodia was a more common feature in chemokine treated rat microglia, suggesting Rac activation. Some rat microglia also demonstrated a polarised morphology, indicating a response causing directed migration possibly due to formation of a local gradient in the microenvironment. The study clearly demonstrates that chemokines are likely candidates in the recruitment and accumulation of microglia to areas of inflammation or tissue damage in the CNS.

Migration of microglia through CNS tissue probably involves breakdown of the extracellular matrix. Microglia have been previously demonstrated to produce a whole range of proteases including MMPs in response to IL-1 and LPS (Colton *et al* 1993, Gottschall *et al,* 1995). The second part of this present study was undertaken to examine whether chemokines were able to regulate MMP and plasminogen activator production by microglia, as well as the inhibitors of MMPs, the TIMPs. The chemokines MCP1, MIP1β, RANTES, IL-8 and Fractalkine were all found to increase the secretion of MMP2 (gelatinase A) as well as TIMP1 and TIMP2 above control unstimulated levels.

Gelatin substrate zymography also showed an increase or induction of MMP9 (gelatinase B) by CHME3 cells. The increase in MMP secretion occurred slightly earlier than the TIMP secretion in preliminary experiments suggesting an initial release of proteolytic activity, followed by a regulation of this activity by the inhibitors. The chemokines tested including MCP1, MIP1β and Fractalkine were also shown to produce a similar effect on MMP expression by primary rat microglia. The effect of chemokines on MMP9 secretion by rat microglia was much more pronounced than the response seen in CHME3 cells. This may be explained by species differences or the use of a transfected immortalised cell line rather than primary cells. These results suggest that the chemokines exert an effect on the production of MMPs by microglia to allow them to break down the extracellular matrix, allowing them to migrate through the tissues' ECM along a chemokine gradient. This again may depend on the receptors expressed at any particular time. MMPs also play an important role in the breakdown of the blood brain barrier which in turn allows leakage of substances into the CNS which are normally excluded as well as further recruitment of circulating cells into the CNS. Myelin degradation in MS is also thought to be due partly to the actions of proteases and MMPs are known to cleave myelin basic protein *in vitro* (Gijbels *et al,* 1993, Proost *et al,* 1993). Chemokines may therefore, not only recruit microglia, but contribute to the progression of MS by increasing protease secretion by microglia. Even though the chemokines also increased TIMP secretion, there may be a time lag or a local imbalance to favour lytic rather than anti-lytic activity.

Plasminogen activators have also been previously detected in MS lesions (Cuzner *et al,* 1996) therefore the present study examined the effect of chemokine stimulation on the secretion of urokinase-type plasminogen activator. Interestingly the chemokines appeared to downregulate uPA secretion into culture supernatants in a dose dependant manner. uPA is active when bound to a cell surface receptor which localises the

activation of plasmin to the cell surface. The effect of the chemokines apparently downregulating the secretion of uPA may in fact be an effect of upregulating the expression of the cell surface receptor with an increase in binding of uPA. The effect of this *in vivo* may be localised breakdown of the ECM at the microglia cell surface to aid migrationin response to chemokines at the lesion site.

Microglia produce many different responses following cytokine stimulation which are thought to promote a pro-inflammatory response resulting in tissue damage. These cells are also involved in late 'cleaning up' events following inflammation. The third aim of this study was to examine the effects of chemokine stimulation on microglia effector functions. Reactive oxygen and nitrogen species are heavily implicated in inflammatory CNS lesions and are associated with microglia/macrophages (Bagasra *et al,* 1995, Hooper *et al,* 1997, Smith *et al,* 1999). Microglia have also been shown to produce nitric oxide and superoxide *in vitro* (Chao *et al,* 1992, Woodroofe *et al,* 1989), which together produce highly reactive peroxynitrite which would damage the myelin sheath by lipid peroxidation. The present study demonstrated that chemokines could in fact increase nitrite and superoxide in rat primary microglia and CHME3 cells. The relevance of this may be that the chemokines not only recruit microglia but also activate them, with resultant oligodendrocyte cytotoxicity *in vivo*. Reactive nitrogen species have also been shown to activate pro-MMPs *in vitro* (Maeda *et al,* 1998) which may suggest an important biological link between chemokines, nitric oxide and MMPs. Nitric oxide has also been shown to regulate chemokine expression (Orens *et al,* 1994) thus the findings in the present study that chemokines regulate nitrite production by microglia suggests an important feedback mechanism.

This present study has further demonstrated that chemokines increase Fc receptor expression on rat microglia and CHME3 cells and were able to increase the phagocytic

ability of rat microglia. The effects of this, if they also occur *in vivo,* may be the promotion of phagocytosis of myelin degradation products, following the proteolysis and lipid degradation by peroxynitrite. The chemokines may firstly attract the microglia at the onset of the inflammatory event and also promote the removal of debris by phagocytosis.

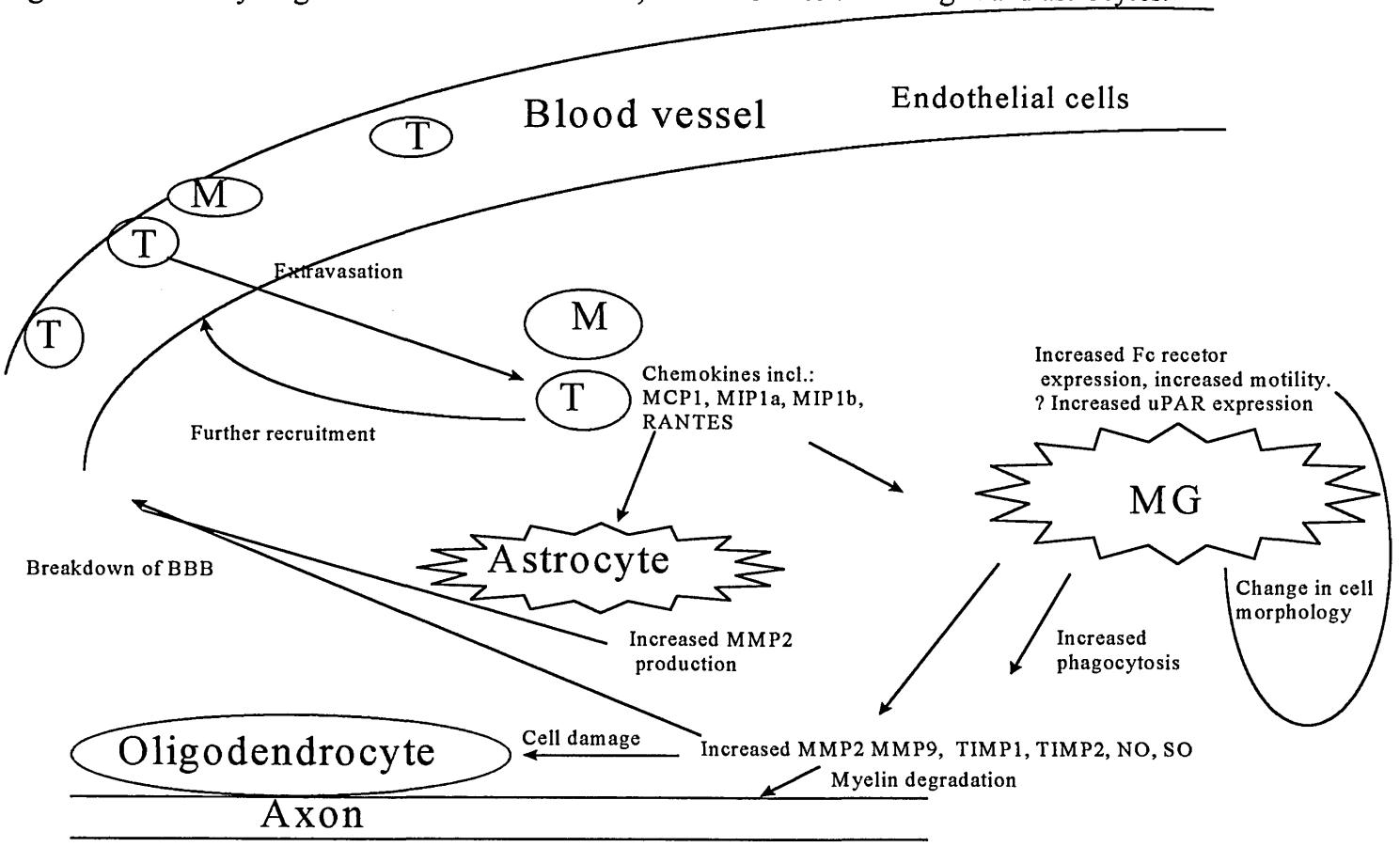
Activated T cells and monocytes/macrophages recruited from the circulation into inflamed CNS tissue, as well as astrocytes and microglia themselves, are sources of chemokine secretion. These chemokines exert multiple effects on microglia/ CHME3 cells *in vitro,* as demonstrated in this study. Chemokines act on microglia causing an increase in filamentous actin, allowing the cells to become motile and may allow recruitment of microglia to areas of inflammation *in vivo.* Chemokines induce an increase in the amount of MMP2 and MMP9 secretion as well as the inhibitors of the MMPs, TIMPs 1 and 2. This suggests either an imbalance in the proteinase/inhibitor ratio to favour lytic activity or an initial time lag in the secretion of the inhibitor to allow some lytic activity followed by a tighter control. Chemokine upregulated MMP activity could aid in microglia motility by breakdown of the extracellular matrix but may also cause demyelination of axons and disruption of the blood brain barrier. This allows further recruitment of inflammatory cells into the CNS as well as proteins such as antibodies, that are normally excluded.

Chemokines were found to increase free radical production by microglia, which could cause oligodendrocyte damage *in vivo* by lipid peroxidation and release of membrane components. An increase in their Fc receptor expression was observed following chemokine stimulation which would allow increased phagocytosis of antibody opsonised myelin fragments.

Figure 6.1 represents a tiny portion of the whole CNS inflammatory picture and shows only the chemokine effects observed in this thesis.

Many studies to date have observed the chemotactic effects of chemokines on various cells types but this study represents one of the few to examine other effects of chemokines on cell functions and probably the only study to date to show these effects on microglia. This study has clearly shown the importance of the chemokines present in MS lesions in the CNS and has underlined the importance of the actions of microglia. It may be that depletion of these chemokines using antibody therapy or the use of chemokine receptor agonists could limit the amount of damage to the CNS and improve the clinical outcome for patients.

Figure 6.1 Summary diagram of the identified effects, of chemokines on microglia and astrocytes.



MG- microglia/CHME3, M=macrophage, T=activated T cells, NO=nitric oxide, SO-superoxide radicals.

7 FURTHER WORK

The present study has highlighted the importance of chemokines in the recruitment and activation of microglia *in vitro*. Further work should focus on the roles of chemokines in the recruitment of microglia by utilising different methods to assess chemotaxis in sequential migration experiments to more than one chemokine. Also, pre-treating the cells with antibodies to the chemokines receptors may determine if the migration response could be blocked.

The chemokines clearly have an effect on the production of MMPs by microglia *in vitro* and it would be interesting to see if the chemokines together work in synergy or in opposition. This study has also shown a link between chemokines and uPA, which itself is involved in the activation of MMPs. Future work should include examining uPA receptor expression by microglia in response to chemokines as this may indicate why the uPA secretion by microglia was lower than control levels after chemokine stimulation. The chemokine control of uPA receptor expression by microglia may also regulate chemotaxis.

8 PUBLICATIONS RELEVANT TO THIS THESIS

Woodroofe MN, Cross AK, Harkness K and Simpson JE. (1998) The expression of chemokines in the central nervous system in multiple sclerosis. Chapter in The function of glial cells in health and disease: Dialogue between glia and neurons. Eds. Matsas and Tsacoupoulos, Plenum Press.

Cross AK, Woodroofe MN. (1999) Chemokines induce migration and changes in actin polymerization in adult rat brain microglia and a human foetal microglial cell line in vitro. *Journal of Neuroscience Research.* 55: 17-23.

Cross AK, Woodroofe MN. (1999) Chemokine modulation of Matrix Metalloproteinase and TIMP production in adult rat brain microglia and a human microglial cell line in vitro. *Glia* 28: 183-189.

ABSTRACTS AND MEETINGS

Cross AK, Simpson JE, Monk P, Murdoch C and Woodroofe MN (1997) Comparison of chemotaxis by isolated rat brain microglia and a human microglial cell line in response to chemokines. MS Society Grantholders meeting, Birmingham, March 26-27th.

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Cross AK and Woodroofe MN (1998) Matrix metalloproteinase expression by rat microglia in vitro and a human foetal microglial cell line, CHME3. Poster presentation, International Society of Neuroimmunology, 5th International Congress, Montreal. J.Neuroimmunol. 90(1)47.

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Chemokines Induce Migration and Changes in Actin Polymerization in Adult Rat Brain Microglia and a Human Fetal Microglial Cell Line In Vitro

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Microglia, the resident macrophages of the central nervous system, are the primary cells to respond to injury in the brain, both in inflammation, e.g., in multiple sclerosis, and trauma. Chemokines are potential mediators of microglial cell recruitment to sites of injury; thus, the ability of microglia to migrate in response to a number of chemokines was assessed. The chemokines monocyte chemoattractant protein 1, macrophage inflammatory protein 1α, macrophage inflammatory protein 1β, RANTES (regulated upon activation normal T cell expressed and secreted), interleukin 8, and IP-10 (interferon gamma inducible protein-10), induce migration and changes in the distribution of f-actin in adult rat microglia and a human microglial cell line, CHME3, in vitro. Both cell types show a significant migration response, above control levels, to all the chemokines tested in a typical dose-dependent manner. These chemokines also induced a reorganization of the actin cytoskeleton of the cells. This study indicates that chemokines play an important role in the recruitment of microglia to areas of central nervous system inflammation. J. Neurosci. Res. 55: 17–23, 1999. © 1999 Wiley-Liss, Inc.

Key words: chemokines; microglia; inflammation; injury; cytoskeleton

INTRODUCTION

Microglia are the resident macrophages of the brain and are able to produce and respond to a wide range of inflammatory stimuli, including tumor necrosis factor α, interferon gamma, interleukin (IL) 1, and IL-6 (Benveniste, 1997; Gehrmann et al., 1995). Microglia have been studied extensively for their role in the pathogenesis of the demyelinating disease multiple sclerosis (MS). It has been shown by immunocytochemistry that post mortem central nervous system (CNS) white matter from patients with MS often shows perivascular cuffing with inflammatory T lymphocytes, macrophages, and plasma cells (Woodroofe et al., 1986; Adams et al., 1989; Benveniste, 1997). It has been proposed that cytokines in the brain are localized to inflammatory cuffs (Woodroofe and Cuzner, 1993; Brosnan et al., 1995), and their production by activated immune cells may directly damage myelin and make it a target for phagocytosis by activated microglia (Sriram and Rodriguez, 1997). Microglia have been shown to be present in active MS lesions, expressing increased levels of major histocompatibility complex class II (Woodroofe et al., 1986). An important factor to consider in the functional role of microglia in CNS disease is their ability to respond to chemoattractants to reach areas of CNS injury or inflammation. Although few studies have been performed, it has been shown that rat microglia are able to migrate towards the classical chemoattractant C5a (Yao et al., 1990) and that human fetal microglia migrate to the β chemokines, macrophage inflammatory proteins (MIPs) 1α and 1β, and monocyte chemoattractant protein (MCP) 1 in vitro (Peterson et al., 1997). For any cell to migrate by chemotaxis or haptotaxis to areas of inflammation, there must be a soluble chemoattractant gradient in which cells move from a low concentration to a high concentration. The process of cell migration also involves polymerization and depolymerization of filamentous actin, which in turn leads to spatial asymmetry of the cytoskeleton and elongation of lamellipodia and filopodia (Lauffenburger and Horwitz, 1996). Binding of a chemokine ligand to its seven transmembrane domain receptor on a cell causes

Abbreviations: CNS, central nervous system; DMEM, Dulbecco's modified Eagle medium; IL, interleukin; IP, interferon gamma inducible protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MS, multiple sclerosis; PBS, phosphate-buffered saline; RANTES, regulated upon activation normal T cell expressed and secreted.

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the release of intracellular second messengers via G-protein complexes. This in turn causes effects, such as the reorganization of the cytoskeleton, formation of focal adhesions, and pseudopod extension, that ultimately lead to movement of the cell. Chemokine signaling also involves activation of the guanosine triphosphate–binding proteins of the Ras, Rac, and Rho families. Rac and Rho are involved in actin reorganization events and motility, including membrane ruffling (Premack and Schall, 1996).

Chemokines are a rapidly expanding family of chemotactic cytokines of which there are more than 30 recognized members to date. These are subdivided into four groups depending on the positions of conserved cysteine residues in their amino acid sequences. These are termed the CXC, CC, and C chemokines (Taub, 1996) and also a more recent CX_3C chemokine motif of which fractalkine, also known as neurotactin (Pan et al., 1997), is the only member to date (Bazan et al., 1997; Schall, 1997). The chemokine subfamilies were also originally distinguished on the basis of their ability to cause migration of the different leukocyte populations. The CXC or α chemokines that contain the ELR peptide motif, including IL-8 and granulocyte chemotactic peptide-2 (GCP-2), are potent mediators of neutrophil chemotaxis, whereas the α chemokines that lack the ELR peptide motif, including interferon gamma inducible protein (IP-10) and platelet factor-4 (PF-4) fail to chemoattract neutrophils. The CC or β chemokines were thought to act mainly on monocytes. This subdivision of the chemokines based on the cells they chemoattract in vitro is becoming increasingly invalid as more cell types, such as natural killer cells and B and T lymphocytes, were examined to find that there was an overlap of activities (Taub, 1996).

Several of these chemokines have been identified in CNS disease and detected in cultures of cells from the CNS. Miyagashi et al. (1995) detected elevated levels of the β chemokine MIP-1α in the cerebrospinal fluid of patients with MS during relapse. This increase was also observed in the cerebrospinal fluid of patients with other neurological disorders including human T-cell lymphotrophic virus type 1–associated myelopathy and meningitis. In this laboratory, a study of β chemokine expression in MS tissue showed that MIP-1β and MCP-1 were the predominant chemokines expressed in CNS inflammatory lesions in MS (Simpson et al., 1998). MCP-1 has been shown to be present in CNS sections of Lewis rats after stab wound injury, which was not found in the normal rat brain. This correlated with the earliest detection of inflammatory cells in the area (Berman et al., 1996). After this study, it was found that two other β chemokines, regulated upon activation normal T cell expressed and secreted (RANTES) and MIP-1β, were also expressed in rat stab wound brain injury and that MIP-1β was localized to reactive astrocytes and macrophage-like cells at the sites of injury (Ghirnikar et al., 1996).

This present study was undertaken to assess the potential of microglia to respond to a range of α and β chemokines. The results indicate that a number of human chemokines, but especially the chemokines belonging to the β family, are able to induce migration of rat microglia and a human fetal microglial cell line (CHME3) (Janabi et al., 1995). The β chemokines MCP-1, MIP-1α, MIP-1β, and RANTES are also shown to elicit a change in the organization of the f-actin cytoskeleton within these cells.

MATERIALS AND METHODS

Cell Culture

Rat microglia and a human fetal microglial cell line, CHME3 (a gift from Prof. M. Tardieu, Université Paris Sud, France) were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Paisley, England) supplemented with 10% heat-inactivated fetal calf serum (GIBCO BRL), 100 IU/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL).

Isolation of Microglia

Microglia were isolated using a rosetting method as described by Hayes et al. (1988). Briefly, a mixed glial suspension was prepared by enzyme digestion of chopped CNS tissue obtained after cervical dislocation of 30-day-old Wistar rats of both sexes. Cerebrae were dissected out, and the meninges were removed. The enzymes collagenase (1,000 U/ml), deoxyribonuclease I (200 μg/ml), and trypsin (1.5%, w/v) (Sigma Ltd., Dorset, England) at 1 ml/g tissue along with 7 ml Earle's balanced salt solution (EBSS)/g, were incubated at 37°C for 15 min with the chopped tissue. The enzyme digestion was repeated, and the resultant suspension was forced through 132- and 80-μm pore nylon mesh. Microglia were then isolated from the single mixed cell population by incubation of 1:1 with a 2% suspension of opsonized erythrocytes. Rosetted cells were then separated by density gradient centrifugation on Percoll (Sigma Ltd.), and erythrocytes were flash lysed. Cells were then counted and resuspended in DMEM alone or DMEM + 10% fetal calf serum and used directly in the chemotaxis chamber or placed into culture.

Chemotaxis Assay

Cell migration in response to chemokines was assessed using a 48-well microchemotaxis chamber method (Neuroprobe, Cabin John, MD) as described previously (Cross et al., 1997). Nucleopore membranes (Costar, High Wycombe, England) with 8-μm pores were coated with fibronectin (6.5 μg/ml) (Sigma Ltd.) in

phosphate-buffered saline (PBS) for 20 min at 37°C. These were then air dried, rinsed in PBS, and dried again. Stock chemokines (100 ng/ml in PBS containing 0.1% bovine serum albumin) were diluted in serum-free DMEM, and 28 µl of each dilution was pipetted in triplicate into the lower wells of the chemotaxis chamber. The human chemokines used included MCP-1, MIP-1α, MIP-1β, RANTES, IL-8, and IP-10 and were a kind gift from Prof. D. Taub (National Institutes of Health, Bethesda, MD). Bovine serum albumin (0.1% or 0.05%) in PBS was used as a negative control because this was used in storage of the stock chemokines and present at these concentrations in the highest chemokine concentration used in experiments with rat microglia and CHME3, respectively.

The filled lower chamber was then overlaid with the coated membrane, and the top chamber was assembled to form wells. Fifty microliters of cell suspension (5×10^5/ml) in serum-free medium was added to each of the wells, and the chamber was incubated at 37°C, 95% air, and 5% CO_2 in a humid environment for a period of 17 hr for microglia and 6 hr for CHME3 as previously determined by time course experiments (data not shown). After incubation, the chamber was dismantled, and the cells on the membrane were fixed in methanol and stained. Briefly, after fixing, the membrane was washed in distilled water and then placed in Harris' hematoxylin (Sigma Ltd.) for 5 min. The membrane was rinsed in running tap water and then in scots tap water (10 g magnesium sulphate, 1.75 g sodium bicarbonate, and 500 ml distilled water) for a further 2 min to define the nuclei. After this, the membrane was inverted onto a microscope slide, and a coverslip was mounted with Immumount (Shandon, Life Sciences, UK). Migration was assessed by counting migrated cells in five microscopic fields per triplicate well (i.e., 15 fields per chemokine concentration) at ×400 magnification.

Statistical Analysis

Significant results were calculated using a one-way analysis of variance comparing migration of cells to the control (0.05% or 0.1% bovine serum albumin) with chemokine-induced migration. Results were expressed as mean number of cells migrating per three wells in five microscopic fields ± SD ($P < 0.025$; significant).

Actin Stain (Using Tetramethylrhodamine Isothiocyanate–Labeled Phalloidin)

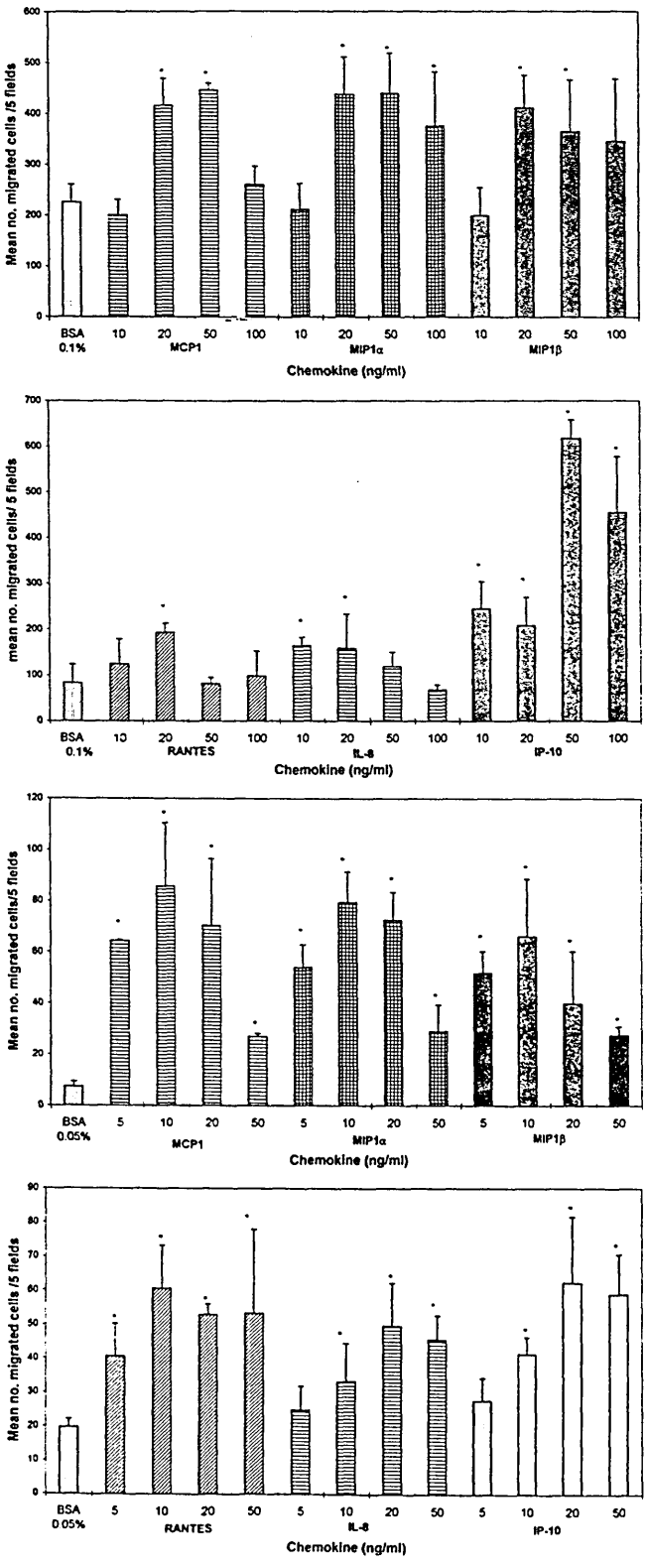
Tetramethylrhodamine isothiocyanate–labeled phalloidin (Sigma Ltd.) was used to visualize changes in actin fiber organization in microglia after incubation with chemokines. Cells were seeded onto sterile fibronectin-coated (Sigma Ltd.) coverslips (6.5 mg/ml) in six-well plates at a concentration of 5×10^4/ml (300 µl on each coverslip) for about 1 hr to allow cells to adhere. A further 2 ml DMEM (10% fetal calf serum) was added. and cells were cultured overnight. Cells were washed and incubated with the appropriate chemokine in serum-free media under study at 37°C for 15, 60, or 180 min. Shorter times were chosen for the actin studies than for the migration because the chemokines should elicit an effect on cell shape immediately. To examine whether the effect of chemokine on cells was specific, boiled MCP-1 was added to some cells. Serum-free medium (or serum-free media with 0.1% bovine serum albumin) was used as the control. Cells were then washed in PBS and fixed for 20 min in 4% paraformaldehyde at room temperature. The paraformaldehyde was then removed, cells were washed in PBS, and acetone was added for a further 5 min at room temperature. Cells were then washed in PBS, and 200 µl of tetramethylrhodamine isothiocyanate–labeled phalloidin (200 ng/ml) (Sigma Ltd.) was added per well and incubated at room temperature for 45 min. The coverslips were washed twice in PBS and once in distilled water and then inverted onto microscope slides mounted in PBS/glycerol ratio of 1:1. Cells were visualized under fluorescence microscopy (Olympus, Tokyo, Japan; BX60) and photographed using Ilford (Mobberley, England) HP5 film.

RESULTS

Adult primary rat microglia isolated by the rosetting method were shown to be >95% OX42 positive by immunocytochemistry and have been previously shown to express Fc receptors (Hayes et al, 1988), produce cytokines (Loughlin and Woodroofe, 1996), and release superoxide (Woodroofe et al., 1989) on stimulation with interferon gamma. Immunocytochemistry on cultures and cytospins of CHME3 cells showed them to be EBM/11 positive, and they did not stain positively for glial fibrillary acidic protein (GFAP) (not shown).

Migration responses of microglia in response to the chemokines MCP-1, MIP-1α, MIP-1β, RANTES, IL-8, and IP-10 were determined using an in vitro chemotaxis assay method (Fig. 1A and B). Preliminary time course experiments for rat microglia at 5, 10, and 17 hr showed an increase in cell migration with time. The 17-hr migration time was used in all further experiments. Similarly for CHME3. 2-, 4-, and 6-hr times were used in preliminary migration experiments, and the 6-hr time point was selected for further experiments. All migration experiments using rat microglia and CHME3 showed typical bell-shaped curves to all of the chemokines tested. Results using primary isolated rat microglia show representative data from one of three individual experiments for MCP-1, MIP-1α and MIP-1β, RANTES, IL-8, and IP-10. A significant response was observed: $P < 0.025$ for MCP-1, MIP-1α, MIP-1β, RANTES, IL-8, and IP-10 in all three repeat experiments. The concentration of chemo-

kine required to give the highest migration response of rat microglia to MCP-1, MIP-1α, MIP-1β, and IP-10 was between 20 and 50 ng/ml. For RANTES and IL-8, the concentration required to give the optimal migration response was variable between 20 and 100 ng/ml.



Results using the human fetal cell line CHME3 show that, for all chemokines tested, a significant response was observed ($P < 0.025$) at least at one concentration. Figure 1C and D show representative data from one of three individual experiments for MCP-1, MIP-1α, MIP-1β, RANTES, IL-8, and IP-10. The concentration of chemokine required to produce optimal migration of CHME3 using MCP-1 was between 10 and 20 ng/ml; for MIP-1α and MIP-1β, this was 10 ng/ml, and using IP-10, a concentration range of 20 to 50 ng/ml gave the highest response. The concentration of the chemokines RANTES and IL-8 required to produce the highest migration response was variable. Numbers of migrated cells in experiments using rat microglia were higher than those using CHME3. Migration studies gave inter-experiment variation such that exact numbers of migrating cells were different, although all showed similar pattern of migration to the different concentrations of chemokine used.

Chemokine-induced changes in actin polymerization and distribution in microglia were visualized by incubating cell cultures with chemokines and staining with tetramethylrhodamine isothiocyanate–labeled phalloidin, a stain specific for f-actin. All of the chemokines tested showed the ability to cause redistribution and polymerization of f-actin in both rat microglia and CHME3. Although both cell types showed morphological changes after incubation with chemokines for only 15 min (not shown), the difference between treated and control cells was more pronounced after 60 min. Rat microglia treated with boiled MCP-1 looked the same as control cells (not shown). Results from cells treated for 3 hr were similar to those at 60 min. Figure 2A and C shows untreated CHME3 cells at low and high magnification, respectively, with f-actin concentrated around the nuclei, whereas CHME3 cells treated for 60 min with MCP-1 or RANTES (Fig. 2B and D) show more cytoplasmic f-actin staining. Untreated rat microglia (Fig. 2E) again have the most intense stain around the nuclei, and little staining

Fig. 1. Chemokine-induced migration was assessed using a 48-well chemotaxis chamber. Human chemokines were diluted in serum-free DMEM and were added to the lower wells of the chamber in triplicate. CHME3 cells and primary isolated rat microglia were placed into the upper chamber of the chemotaxis chamber at a concentration of 5×10^5 cells/ml. **A:** Migration response of rat microglia to β chemokines. **B:** Migration response of rat microglia to the β chemokine RANTES and the α chemokines IL-8 and IP-10. **C:** Migration response of CHME3 to β chemokines. **D:** Migration response of CHME3 to RANTES and the α chemokines IL-8 and IP-10. Representative data from one experiment is expressed as the mean migration per three wells ± SD. Significant migration above control (0.05%–0.1% bovine serum albumin [BSA]) was calculated using a one-way analysis of variance. *Significant migration above control levels ($P < 0.025$).

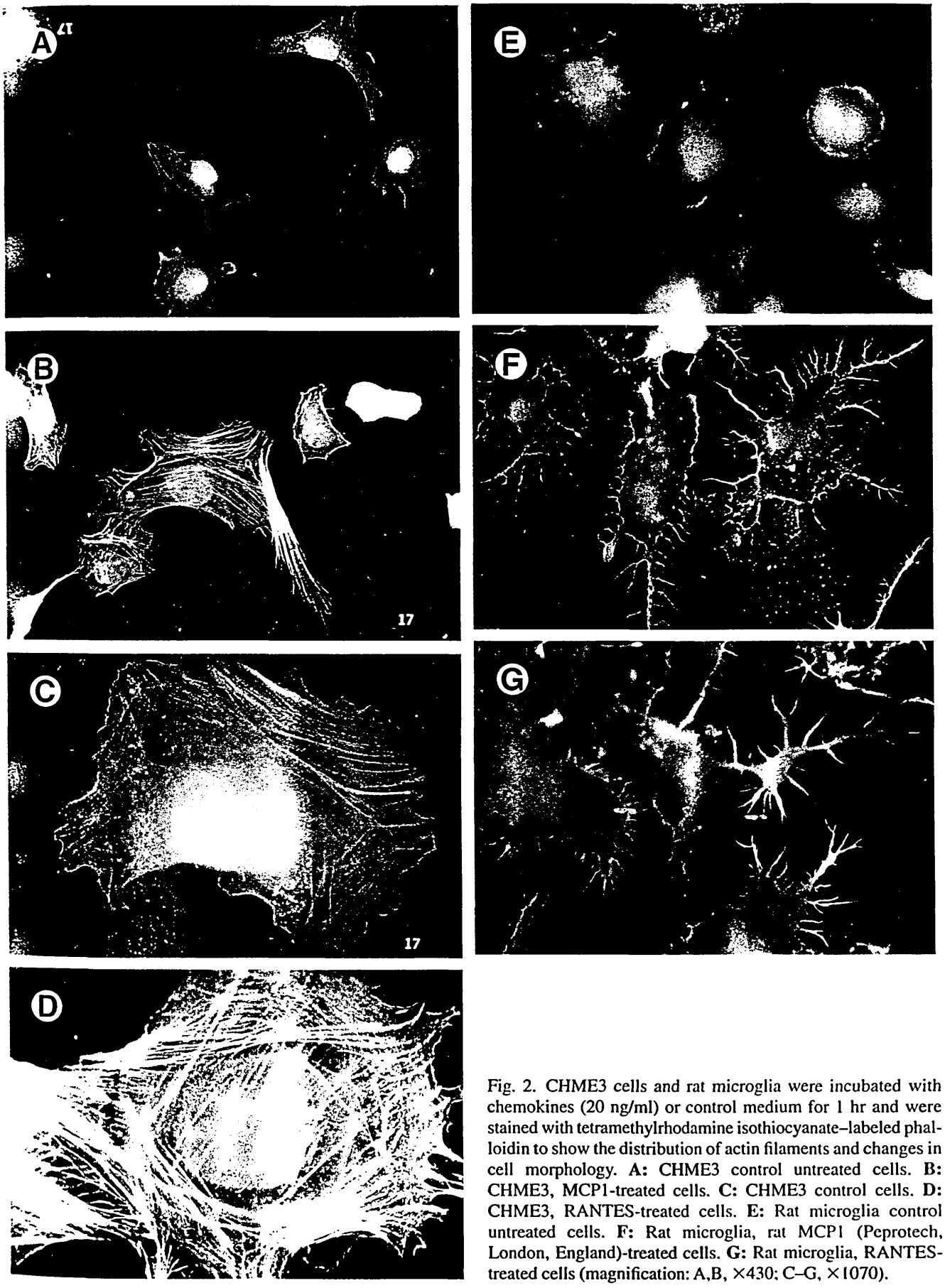


Fig. 2. CHME3 cells and rat microglia were incubated with chemokines (20 ng/ml) or control medium for 1 hr and were stained with tetramethylrhodamine isothiocyanate–labeled phalloidin to show the distribution of actin filaments and changes in cell morphology. **A:** CHME3 control untreated cells. **B:** CHME3, MCP1-treated cells. **C:** CHME3 control cells. **D:** CHME3, RANTES-treated cells. **E:** Rat microglia control untreated cells. **F:** Rat microglia, rat MCP1 (Peprotech, London, England)-treated cells. **G:** Rat microglia, RANTES-treated cells (magnification: A,B, ×430; C–G, ×1070).

was observed in the cytoplasmic regions. Rat MCP-1– and RANTES-treated rat microglia show a more highly branched morphology with intense f-actin staining within the pseudopodia (Fig. 2F and G). The effects of rat and human MCP-1 on rat microglia were compared to examine if there was any species specific effect; however, the changes to the actin cytoskeleton using either chemokine were identical. All the chemokines used in the migration studies caused similar effects on the cytoskeleton of both cell types.

DISCUSSION

The results of this study clearly show that rat microglia and CHME3, the human fetal microglial cell line, migrate in response to chemokines. Thirty-day-old rat microglia, not neonatal rats, were used in this study because they are more representative of neuroimmunologic processes in vivo. These cells were compared with CHME3 in their response to chemokines. Although CHME3 are of fetal origin, similar responses were observed to those of adult rat microglia. Both cell types migrated to a concentration range of chemokines giving a typical bell-shaped curve where the cells respond only weakly or not at all to low concentrations of chemokine and where migration is attenuated at high concentrations. This presumably causes cells to migrate up a concentration gradient until they reach a critical point of receptor saturation. The numbers of migrated cells were generally higher in experiments using rat microglia with numbers of migrated cells as high as 900 per five microscopic fields. However, longer incubation times were used for the primary rat microglia because the harshness of the isolation procedure of the cells required time for the cell membranes to recover. The actual numbers of migrated cells cannot be directly compared between the two cell types studied, although their responses to the different chemokines does allow comparison. The rat microglia migrated optimally to chemokine concentrations higher than those required for optimal CHME3 migration. This could be attributed to the fact that the chemokines used were of human origin, although recent experiments in this laboratory using rat MCP-1 with rat microglia showed no difference to those reported here for human chemokines in the numbers of cells migrating or the concentration required to produce optimal migration (data not shown). The rat and human MCP-1 both induced changes in the actin cytoskeleton of rat microglia. Therefore, species difference does not appear to contribute to this differing response in this particular chemokine.

Activated microglia are implicated in a number of CNS pathologies and are present in an activated state. The use of immunostaining techniques on post mortem tissue has shown dense staining of microglia associated with amyloid deposits in Alzheimer's disease (Dickson et al., 1993; McGeer and McGeer, 1995) and microglial nodules in human immunodeficiency virus encephalitis (Dickson et al., 1993). Activated microglia have also been observed in high densities in association with prion protein deposits in Creutzfelt-Jacob disease (Muhleisen et al., 1995). A review by Streit (1996) documented massive microglia infiltration into malignant rat glioma produced by intracerebral injection of glioma cells. This present study would suggest that centrally produced chemokines would be involved in microglia recruitment in these CNS pathologies and is not restricted to the pathogenesis of MS.

Our results show a dramatic change after chemokine stimulation of microglia in their morphology and distribution of f-actin within 1 hr. Although cell migration cannot be attributed to f-actin changes alone, redistribution of actin fibers and extension of pseudopodia are important events in cell locomotion. Chemokine signaling through G protein–coupled receptors includes activation of the guanosine triphosphate–binding proteins Rac and Rho that are involved in regulation of actin-dependent processes, i.e., formation of pseudopodia, ultimately leading to cell motility (Premack et al., 1996). Rho protein is well documented as a connector between membrane receptor interactions and regulation of the cytoskeleton (Takai et al., 1995; Machesky and Hall, 1996).

Because there is strong evidence of a role for chemokines in the pathogenesis of MS, we have shown here that microglia can respond to many of these signals in vitro, giving further support to the conclusion that chemokines produced at sites of inflammation, demyelination, and tissue damage are able to promote the recruitment of microglia. Further research in this laboratory in this area will concentrate on the effects chemokines have on the activation state of these cells that would complement the well-documented effects of other inflammatory cytokines on microglia.

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Chemokine Modulation of Matrix Metalloproteinase and TIMP Production in Adult Rat Brain Microglia and a Human Microglial Cell Line In Vitro

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KEY WORDS urokinase; microglia; MMP; TIMP; chemokines; ELISA; zymography

ABSTRACT Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes, capable of degrading proteins found in the extracellular matrix. MMPs 2 and 9 are known to be produced by microglia, the resident macrophages of the central nervous system. The control of the secretion of these proteases and the activation of proenzymes by other proteases such as plasmin, as well as the balance between MMP secretion and the secretion of their natural inhibitors (TIMPs), have an important relevance in the pathogenesis of multiple sclerosis (MS). The in vitro control of MMPs 2 and 9, TIMPs 1 and 2, and urokinase-type plasminogen activator by microglia was examined in response to a panel of chemokines (chemotactic cytokines), using ELISA and zymography techniques. The chemokines MCP1, MIP1β, RANTES, IL-8, and Fractalkine were all found significantly to increase the secretion of MMPs and TIMPs by a human foetal microglial cell line, CHME3, after 24 h stimulation. The chemokines tested, MCP1, MIP1β, and Fractalkine, were also shown to increase MMP9 secretion by primary isolated rat brain microglia in vitro. MCP1, MIP1α/β, and RANTES significantly decreased the secretion of uPA into culture supernatants in ELISA experiments. These findings suggest an important potential role for the involvement of chemokines in the breakdown of the blood–brain barrier and also the destruction of myelin basic protein in MS. *GLIA 28:183–189, 1999.*

INTRODUCTION

Microglia represent the primary immune effector cells of the adult central nervous system (CNS) and are often the first cell type to respond to CNS injury, displaying a wide range of immunologic activities. They are thought to contribute directly to a number of CNS pathologies, including Alzheimer's disease and multiple sclerosis (MS), by production of proinflammatory cytokines, chemokines, free radicals, and proteases.

Matrix metalloproteinases (MMPs) are proteolytic enzymes involved in remodelling of the extracellular matrix and are implicated in the pathogenesis of MS because of their detection in MS brain tissue (Cuzner et al., 1996) and in the CSF of patients with MS (Miyagishi et al., 1995). MMPs are suggested to play a role in the influx of inflammatory cells into the CNS, disrupt the blood–brain barrier, and have been shown to degrade myelin in vitro (Chandler et al., 1995; Gijbels et al., 1993), suggesting that these enzymes may contribute to myelin destruction in vitro.

MMPs, at least 14 of which have been characterised to date, are divided into three subgroups, gelatinases, stromelysins, and collagenases (Chandler et al., 1997),

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and are expressed as inactive zymogens, which are activated by autocatalysis or cleavage by membrane-type MMPs or plasmin. Following activation, their activity is tightly controlled by complexes formed with specific tissue inhibitors of metalloproteinases or TIMPs and also by α_2-macroglobulin (Woessner, 1991). There are at least four TIMPs to date, and they bind to MMPs in a 1:1 ratio.

Urokinase-type plasminogen activator (uPA) cleaves plasminogen to its active form, plasmin, which in turn is able to cleave pro-MMPs. This forms another level of control of proteolysis along with the expression of MMP inhibitors and regulation at the gene level. uPA has been previously detected in the culture medium of rat microglia (Nakajima et al., 1992), and modulation of its secretion was observed by treatment with interleukin-1 and lipopolysaccharide.

MMPs 2, 9 (gelatinases), 3 (stromelysin), and 7 (collagenase) have been previously detected in plaques or cerebrospinal fluid (CSF) of patients with MS, and activated T cells (Biddison et al., 1997), astrocytes (Chandler et al., 1997), brain endothelial cells (Harkness et al., 1998), and microglia have been shown to express a wide range of MMPs both in vitro and ex vivo (Colton et al., 1993; Gottschall et al., 1995; Yamada et al., 1995). uPA is also associated with many neuropathologies, including MS, as well as an involvement in normal physiological functions (Cuzner and Opdenakker, 1999).

MMP secretion by activated microglia may facilitate their ability to move toward and accumulate at sites of inflammation or tissue damage in response to chemoattractants such as chemokines, as well contributing to the demyelination. Chemokines are a superfamily of chemotactic cytokines, which can be divided into four subgroups depending on the presence and position of conserved cysteine residues and are known as the CC, CXC, C, and CX_3C chemokines. The CC chemokines include monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory proteins 1α and β (MIP1α and -β), and RANTES. The most well-characterised CXC chemokine is IL-8, and the only C chemokine discovered to date is lymphotactin. Chemokines play an important role in the attraction of leukocytes to areas of inflammation and infection and also are important in trafficking and homing of lymphocytes (Baggiolini, 1998) The human chemokines, MCP1, MIP1α, MIP1β, RANTES, IL-8, and IP-10, have previously been shown to cause the in vitro migration of rat brain microglia (Cross and Woodroofe, 1999). A recently discovered CX_3C chemokine called *Fractalkine* (also called *neurotactin*) was found to be expressed on the surface of microglia and is up-regulated in brain inflammation (Pan et al., 1997). Fractalkine has more recently been suggested to be most dominantly expressed by neurons with receptors for Fractalkine being expressed by microglia, suggesting this chemokine to be a source of communication between neurons and microglia (Harrison et al., 1998; Nishiyiori et al., 1998) It is now well-documented that chemokines have a role to play in autoimmune disease of the CNS from studies on CNS tissue (Simpson et al., 1998; McManus et al., 1998) and CSF from MS patients (Sørensen et al., 1999), and from work on the animal model of MS, experimental allergic encephalomyelitis (EAE) (Berman et al., 1996; Ransohoff et al., 1996; Miyagishi et al., 1997). The specific chemokines identified in MS and/or EAE lesions include MCP1, MIP1α/β, RANTES, and IP-10. Chemokines have been shown to modulate the production of MMP9 expression by peripheral blood lymphocytes in vitro (Johnatty et al., 1997). Thus, understanding of the regulation of these proteases by chemokines in CNS cell types may lead to a further understanding of the pathogenesis of MS.

In this study we report that the expression of MMPs by rat microglia and the human foetal microglial cell line CHME3 (Janabi et al., 1995) is modulated by chemokines in vitro and also that uPA and the inhibitors of metalloproteinases, TIMPs, are modulated by chemokines in the CHME3 microglial cell line.

MATERIALS AND METHODS

Isolation of Microglia

Microglia were isolated using a "rosetting" method as described by Hayes et al. (1988) and placed into culture in 24-well plates.

Cell Culture

Rat microglia and a human foetal microglial cell line, CHME3 (Janabi et al., 1995; a gift from Prof. M. Tardieu, Université Paris Sud, France), were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, U.K.) supplemented with 10% heat-inactivated foetal calf serum (Life Technologies, U.K.), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Life Technologies). DX3 cells (a human cutaneous melanoma cell line) were cultured in the same medium as CHME3 cells under the same conditions, and the supernatant was collected and used as a positive control for MMP2 and -9 secretion in zymography experiments.

Cell Stimulation

CHME cells were seeded into 24-well plates at a concentration of 1×10^5/well and cultured in serum containing medium for 24 h. The cells were then washed and transferred to macrophage serum-free medium (SFM; Life Technologies) for 2 h. Primary microglia were seeded at a density of 2×10^5/well and maintained in culture for 3–7 days prior to transfer to SFM. CHME3 cells were then stimulated in duplicate with 300 µl of human chemokine (MCP1, MIP1α, MIP1β, RANTES, IL-8, or Fractalkine) hTNFα, or hIL-1β for 24 h. Rat microglia were stimulated with rat

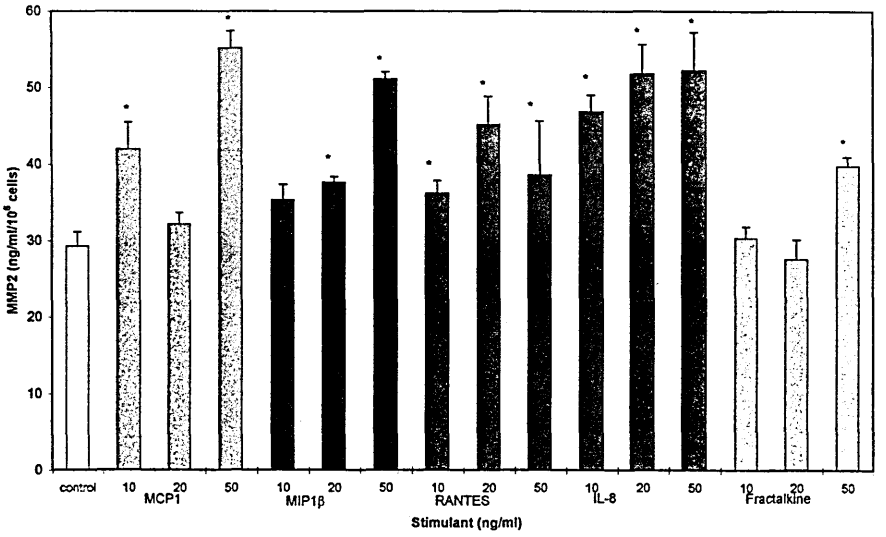

Fig. 1. MMP2 modulation in CHME3 cells by chemokines. ELISA results show one of three representative experiments. *Significant increase above control levels ($P < 0.05$).

MCP1; the rat cytokines TNFα, IL-1β, and IFNγ; or the human chemokines MIP1β and fractalkine under the same conditions. Chemokines (except rat MCP1 and Fractalkine, purchased from Peprotech, U.K.) were a kind gift from Prof. D Taub (National Institutes of Health, Bethesda, MD). Culture supernatants were then harvested, centrifuged to remove cell debris, and either loaded onto gelatin substrate SDS gels for electrophoresis or collected and stored at −20°C until required.

Zymography

Cell supernatants were separated on SDS-polyacrylamide (7.5%) gels containing gelatin substrate to analyse MMP2 and -9 production. After electrophoresis, gels were washed in distilled water containing 2% Triton X-100 for 1 h, then incubated in incubation buffer (50 mM Trizma base, 0.2 M NaCl, 5 mM$CaCl_2$, pH 7.4) for 18 h and stained with Coomassie blue. Gels were then destained in 30% methanol containing 10% glacial acetic acid, and clear bands of protein degradation were visualised. Bands seen on gelatin gels were confirmed as metalloproteinases by addition of 10 mM EDTA to the incubation buffer to inhibit enzyme activity.

TIMP/MMP/uPA ELISA

Chemokine modulation of MMP2, MMP9, TIMP1, TIMP2, and uPA secretion by CHME3 cells following stimulation with chemokines was quantified using Biotrak ELISAs for MMPs and TIMPs (Amersham International, U.K.) and a uPA ELISA kit (Alpha Laboratories, Eastleigh, Hants, U.K.) according to the manufacturer's instructions. Samples were assayed in duplicate, and the significant differences from control values were calculated by a one-way ANOVA on each individual experiment followed by a multiple range test for each chemokine concentration. P values of < 0.05 were considered significant.

RESULTS

ELISA

ELISA experiments were only carried out on CHME3 cells, because only human kits were available and there was no cross-reactivity between rat MMPs or TIMPs and the human antibodies supplied in the kits. CHME3 cells were found to secrete MMP2 constitutively by ELISA (Fig. 1). Use of an ELISA kit that detects pro-MMP9 as well as MMP9 bound to TIMP1 gave negative results (not shown) in two experiments, suggesting that only the active form of MMP9 was present 24 h after stimulation, as shown by zymography (see Fig. 3c,d). The chemokines tested, MCP1, MIP1β, RANTES, IL-8, and Fractalkine, all significantly increased MMP2 production above control, unstimulated values in ELISA experiments at all concentrations tested, in at least two of three repeat experiments. Constitutive secretion of MMP2 ranged between 9 and 29 ng/ml/10^5 cells At the highest concentration of chemokine used (50 ng/ml), there was an approximate 1.2–6 fold increase for all chemokines tested in all three experiments. TIMP2 production was also significantly increased above control levels by all the chemokines tested in at least two of three experiments (with the exception of IL-8 at 20 ng/ml and RANTES at 10 ng/ml giving significant increases above control levels in just one of three experiments) with an approximate two- to fivefold increase at the highest concentration of chemokine (50 ng/ml) in three of three experiments. Figure 2a shows one of three representative experiments. Consti-

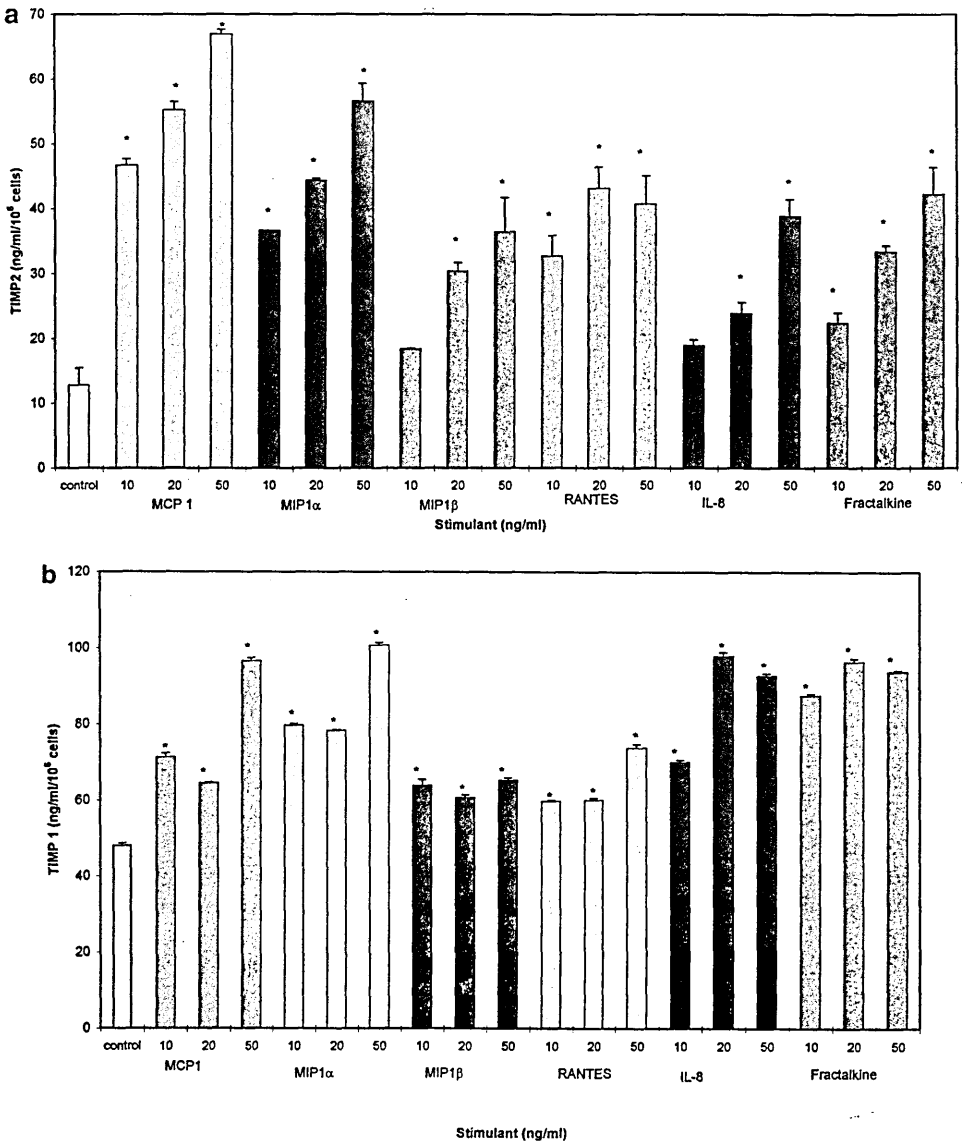

Fig. 2. **a:** TIMP2 modulation in CHME3 cells by chemokines. ELISA results show one of three representative experiments. *Significant increase above control levels ($P < 0.05$). **b:** TIMP1 modulation in CHME3 cells by chemokines. ELISA results show one of three experiments. *Significant increase above control levels ($P < 0.05$).

tutive expression of TIMP2 was between 10 and 25 ng/ml/10^5 cells. TIMP1 production was significantly increased above unstimulated levels by the chemokines MCP1, MIP1β, and Fractalkine at all concentrations tested in at least two of three experiments. IL-8 gave a significant increase in TIMP1 secretion in only one of three experiments at 20 and 50 ng/ml, and RANTES caused a significant increase in TIMP1 secretion in only one of three experiments for each of the cencentrations tested. Interexperimental variation was marked, and constitutive levels of TIMP1 secretion ranged between 24.7 and 71 ng/ml. Figure 2b shows the results of one of three experiments.

Gelatin Substrate Zymography

Gelatin zymography was used to detect changes in MMP2 and -9 production in CHME3 cells and primary rat microglia; MMP9 could not be measured by ELISA, because there were no commercial kits available to measure rat MMPs. Primary rat microglia stimulated with rTNFα (10 ng/ml), rIL-1β (10 ng/ml), and rIFNγ (10 IU/ml; not shown) caused an increase in both MMP2 and MMP9 production. When stimulated with rat MCP1 or the human chemokines MIP1β (Fig. 3a) or Fractalkine (Fig. 3b) at concentrations of 10, 20, and 50 ng/ml, MMP2 increased slightly or remained the

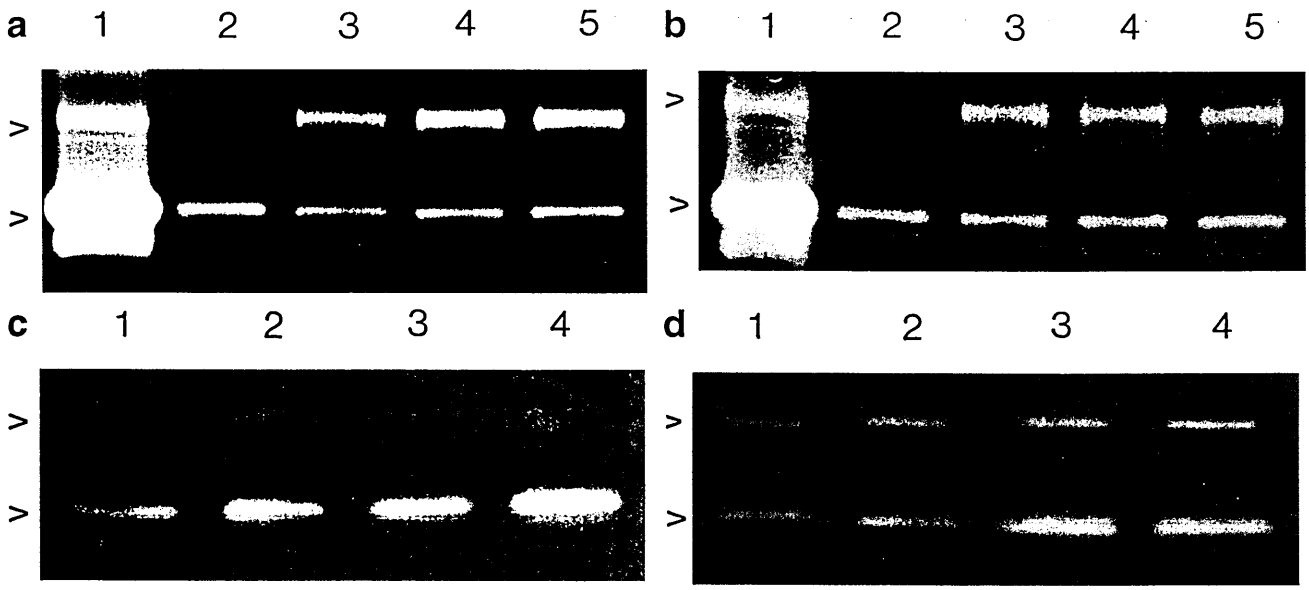

Fig. 3. Gelatin zymography on supernatants from rat microglia (a,b) and CHME3 cells (c,d) stimulated with chemokines. The upper arrowhead indicates MMP9 and the lower arrowhead MMP2. Each zymogram represents one of three repeat experiments. **a: Lane 1:** Supernatant from cell line DX3 (positive control to show MMP2 and -9). **Lane 2:** Control supernatant (unstimulated microglia). **Lanes 3–5:** Supernatants from cells stimulated with MIP1β at 10, 20, and 50 ng/ml, respectively. **b: Lane 1:** DX3 supernatant. **Lane 2:** Control supernatant. **Lanes 3–5:** Supernatants from cells stimulated with Fractalkine at 10, 20, and 50 ng/ml, respectively. **c: Lane 1:** Control (unstimulated) CHME3 supernatant. **Lanes 2–4:** Supernatants from cells stimulated with MCP1 at 10, 20, and 50 ng/ml, respectively. **d: Lane 1:** Control CHME3 supernatant. **Lanes 2–4:** Supernatants from cells stimulated with IL-8 at 10, 20, and 50 ng/ml, respectively.

same as control levels, and MMP9 production was markedly increased. Stimulations with cytokines and chemokines all caused a comparable increase in gelatinase band intensity. Rat microglia have been previously shown to respond to human chemokines in chemotaxis experiments in vitro (Cross and Woodroofe, 1999).

The chemokines tested, MCP1 (Fig. 3c), MIP1α/β, RANTES, IL-8 (Fig. 3d), and Fractalkine, were all shown to cause an increase in both MMP2 and MMP9 in CHME3 cells, and these were increased by comparable amounts by the different chemokines. TNFα at 1 and 10 ng/ml also increased MMP2 and -9 above control levels, although the effects were not seen using IL-1β at the same concentrations (not shown). Bands were demonstrated to be metalloproteinases by the addition of EDTA to the incubation buffer in some of the gels. Gelatin gels were stained blue throughout, and the bands of degradation normally observed did not appear (not shown).

uPA ELISA

Urokinase secretion in unstimulated and MCP1-, MIP1α-, MIP1β-, and RANTES-stimulated CHME3 supernatants was assessed by ELISA. The mean, unstimulated level of uPA from three individual experiments was 1.1 ng/ml, and this was significantly reduced in three of three experiments by all of the chemokines tested at 50 ng/ml (Fig. 4). uPA secretion was also significantly reduced by MCP1 stimulation at 20 ng/ml.

DISCUSSION

The presence of MMPs has been under much investigation in a number of CNS disease states, including stroke (Todor et al., 1998), MS (Anthony et al., 1997), Alzheimer's disease (Yamada et al., 1995), and brain tumours (Freidberg et al., 1998). We have investigated the secretion of MMPs, TIMPs, and uPA by microglia and the influence of chemokines and other inflammatory cytokines on their secretion. Our results show that all the chemokines tested were able to up-regulate CHME3 cell secretion of MMP2 and TIMPs 1 and 2 in ELISA experiments and up-regulated MMP2 and -9 in cultured rat microglia. This may suggest a potential role for chemokines in the regulation of breakdown of the blood–brain barrier and demyelination in MS as well as their role in chemotaxis of inflammatory cells, including microglia, in vitro (Cross and Woodroofe, 1999) and their postulated role in the recruitment of T cells and resident microglia to areas of inflammation and tissue damage. MMPs produced by microglia could contribute to the formation of the plaque in MS by proteolysis of myelin and also by aiding migration of microglia through the extracellular matrix in response to chemoattractants produced at the site of inflammation. Baseline levels of TIMP1 were higher than TIMP2 in CHME3 cells, although both were constitutively expressed by resting cells in culture after 24 h. Pagenstecher et al. (1998) found that MMP gene products and TIMP gene products coincided with sites of inflammation and tissue damage in EAE and suggested a "dy-

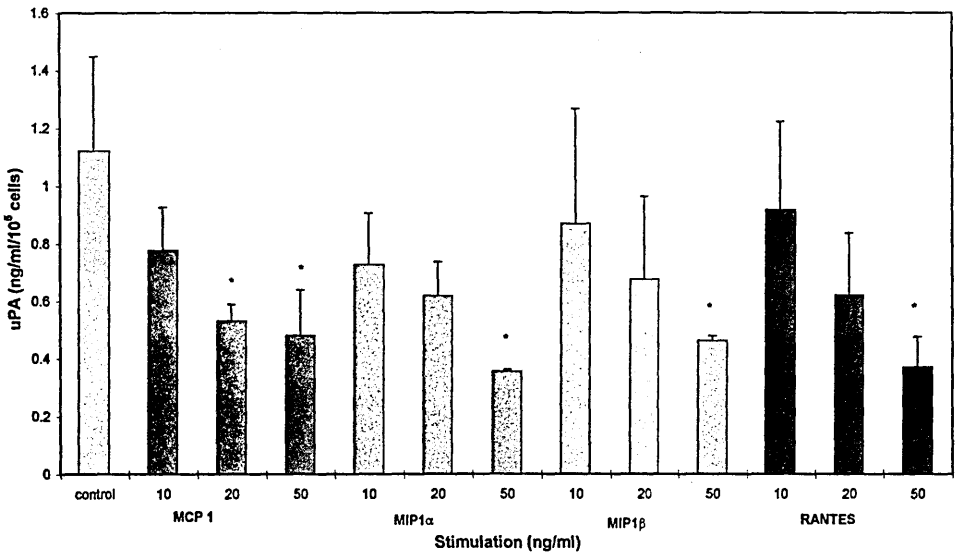

Fig. 4. Secretion of urokinase-type plasminogen activator by CHME3 cells, showing down-regulation of uPA in the supernatant of cells stimulated with chemokines. ELISA results show the mean of three individual experiments. *Significant decrease below control levels ($P < 0.05$).

namic state" in which the levels of each of these proteins and their interaction may determine the extent of the inflammatory lesion. Although the levels of MMPs and TMPs cannot be compared directly in these experiments owing to the nature of the ELISA specificities (i.e., the ELISA for MMP2 measures both free proenzyme and that bound to the inhibitor but not active enzyme), preliminary experiments in our laboratory show the presence of MMP2 in culture supernatants after just 2 h in culture, whereas TIMP1 was not detectable at this time, suggesting that there may be a time lag in the secretion of the inhibitor. This would allow initial MMP activity, which is then regulated by TIMP secretion as well as other factors involved in activation of proenzymes. Further experiments are in progress to investigate this. The up-regulation of both gelatinases and TIMPs by chemokines may be a regulatory mechanism to limit and tightly control extracellular matrix breakdown. It has also been shown (Hayakawa et al., 1992) that TIMP1 has cell growth-promoting activity and therefore could be beneficial to remyelination of axons. It is also likely that the plasminogen activator cascade will fit into this model, by exerting an effect on the extent of pro-MMP activation. Plasminogen activators are involved in the tightly regulated cascade as they cleave plasminogen to plasmin, which activates pro-MMPs and also degrades several components of the extracellular matrix alone (Vassalli and Pepper, 1994). CHME3 cells were shown to secrete constitutively a urokinase-type plasminogen activator, and all the chemokines tested significantly reduced the amount of uPA in the supernatant when cells were stimulated for 24 h with chemokine at a concentration of 50 ng/ml. This may be due to an increase in the number of uPA receptors on the cell surface or an increase in the binding affinity of uPA for its receptor. Ongoing research in this laboratory will examine this further and should determine the effects, if any, of chemokines on plasminogen activator receptors.

Several protease inhibitors have been shown to have an effect on EAE. An inhibitor, D-penicillamine, was found to decrease gelatinase A and B activity in CSF from patients with multiple sclerosis or optic neuritis and also reduced mortality and morbidity in mice developing acute demyelination in EAE (Norga et al., 1995). The metalloproteinase inhibitor Ro31–9790 was found to prevent disease onset when given to rats at the same time as induction of EAE (Hewson et al., 1995). The inhibitors of MMPs are important considerations in the treatment of MS, but it is becoming clear that the molecules that up-regulate the MMPs, such as the chemokines, are also important targets for potential therapies to restore the balance of MMP and TIMP activities. This study provides a base for further research, which should include inhibition of chemokine-stimulated gelatinase production by microglia, using chemokine and/or MMP inhibitors.

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