Functional properties of microglia relevant to inflammation and
demyelination and their regulation by cytokines.

A thesis submitted for the degree of
Doctor of Philosophy in the Faculty of Science,
University of London.

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ABSTRACT

Immunocytochemical and electron microscopic techniques have implicated microglia in the processes of inflammation and demyelination in the central nervous system (CNS). The activity of microglia and other cellular participants in these events is subject to modulation by the local action of cytokines. Interleukin-1 (IL-1), IL-2, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNFα) have all been identified in MS brain tissue.

To facilitate the study of microglial functional properties in vitro an existing procedure for rapid isolation of microglia was further optimised to improve yields and shorten the isolation time. Isolated rat microglia produce IL-1 and IL-6 on stimulation in vitro with lipopolysaccharide (LPS) and this effect is down-regulated by IFN-γ. Using in vivo microdialysis techniques, IL-1 and IL-6, secreted in response to injury within the CNS, have been monitored and, on the basis of immunocytochemical analysis, microglia have been identified as the likely source of these cytokines.

Functional assays were used to assess the regulatory influence (inhibitory / stimulatory) of individual or combinations of cytokines on indicators of microglial antigen presenting and phagocytic capacities in vitro, these being judged particularly relevant to the processes of inflammation and demyelination in vivo. Whilst microglial phagocytic capacity was sensitive to LPS and a broad range of individual cytokines (IFN-γ, TNFα, IL-1, IL-3, IL-4, GM-CSF, TGFβ1), of the cytokines tested only IFN-γ and TGFβ1 affected antigen presenting capacity (MHC class II expression). However the stimulatory effect of IFN-γ on microglial antigen presenting capacity was modulated by TNFα, IL-4, TGFβ1 and LPS. The combined effect of two cytokines
appears to depend on the sequence in which the cell is exposed to them and their relative concentrations. Particularly striking was the shift between an additive stimulatory and a mutually antagonistic effect of IL-4 and IFN-γ on phagocytic capacity when the IL-4 concentration was increased. Differences in the responses of microglia and peritoneal macrophages to cytokine treatment indicate that different regulatory mechanisms operate in these two cell types.

Use has been made of a foetal rat CNS aggregate culture system with the capacity to myelinate, demyelinate and remyelinate, to investigate the role of macrophages in and the effect of cytokines on these processes. This culture system simulates the in vivo situation in terms of cell-cell interactions. Enrichment of aggregate cultures with macrophages enhances the degree of myelination which occurs, while demyelination is induced in this system by treatment with IFN-γ, TNFα, IL-1α and LPS. Interestingly, whilst the extent of demyelination induced by cytokines is not affected by the presence of greater numbers of macrophages, antibody-mediated demyelination is more extensive in these than in normal aggregate cultures. Cytokines may be acting directly on myelin or oligodendrocytes as well as modulating effector cell behaviour. The effect of macrophage enrichment on antibody-mediated demyelination may be indicative of an Fc and/or complement receptor-mediated mechanism. These results suggest that different mechanisms operate in cytokine and antibody-mediated demyelination.
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
<td></td>
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<tr>
<td>BSA/PBS</td>
<td>solution of BSA in PBS (eg. 1% BSA/PBS)</td>
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<tr>
<td>C</td>
<td>complement</td>
<td></td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
<td></td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
<td></td>
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<tr>
<td>CNPase</td>
<td>cyclic nucleotide phosphodiesterase</td>
<td></td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>corticosterone</td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
<td></td>
</tr>
<tr>
<td>DAB</td>
<td>3’3-diaminobenzidine</td>
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<tr>
<td>DEX</td>
<td>dexamethasone</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagles medium</td>
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<tr>
<td>E</td>
<td>erythrocytes</td>
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<tr>
<td>EA</td>
<td>antibody-coated erythrocytes</td>
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<tr>
<td>EAE</td>
<td>experimental allergic encephalomyelitis</td>
<td></td>
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<tr>
<td>EBSS</td>
<td>Earles balanced salt solution</td>
<td></td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Fc</td>
<td>constant fragment of the immunoglobulin molecule</td>
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<td>FcR</td>
<td>receptor for the Fc portion of immunoglobulin</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>g</td>
<td>gram</td>
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<td>GC</td>
<td>galactocerebroside</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
<td></td>
</tr>
<tr>
<td>GS</td>
<td>glutamine synthetase</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
<td></td>
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<tr>
<td>IL</td>
<td>interleukin eg. IL-1, IL-2, IL-3, IL-4, IL-6</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MBP</td>
<td>myelin basic protein (also referred to as BP)</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>μg</td>
<td>microgram</td>
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</table>
ml millilitre
μl microlitre
mM millimolar
μM micromolar
MS multiple sclerosis
Mφ macrophage
MOG myelin-oligodendroglial protein
n number of experiments
NA noradrenaline
NBS newborn bovine serum
ng nanogram
nM nanomolar
OD optical density
PBS phosphate buffered saline
pM picomolar
RBC red blood cell
RT room temperature
s second
SEM standard error of the mean
TNF tumour necrosis factor
TGFβ transforming growth factor-β
U unit
v/v volume for volume
w/v weight for volume

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ACKNOWLEDGEMENTS

I would like to thank Dr Louise Cuzner, my supervisor, and Dr Nicola Woodroofe for their guidance and encouragement throughout this study. I am grateful to Meenu Wadwha at the National Institute of Biological Standards and Controls for performing the cytokine bioassays. I am also grateful to Dr Jean-Marie Matthieu at the Centre Hospitalier Universitaire Vaudois, Lausanne and Dr Paul Honegger at the University of Lausanne, Switzerland for their hospitality and for sharing their expertise in aggregate culture in the course of a collaboration which has contributed to this study.

This work was supported by the Multiple Sclerosis Society of Great Britain.

Dedicated to my parents.
CHAPTER 1

INTRODUCTION

Microglia, the resident macrophages of the brain, have been implicated in inflammation and demyelination, the two pathological features which characterise multiple sclerosis (MS). Cytokines detected in MS tissue are the probable regulators of cellular events during inflammation and demyelination and may, in the future, provide a means of influencing the disease course. Questions arise as to the properties and behaviour of these cells in normal and disease tissue and the manner in which these properties are regulated by cytokines and other mediators. To put this study in context, the following are discussed: inflammation and demyelination in the central nervous system (CNS), the functions and properties of microglia and their role in these events and the regulatory influences of cytokines.

1.1 THE CNS AS AN IMMUNOLOGICALLY PRIVILEGED SITE

In healthy animals under normal conditions the CNS contains few lymphocytes and the major histocompatibility complex (MHC) antigen is expressed at a very low level (Lampson and Hickey, 1986). The immunologically privileged status of the CNS (Barker and Billingam, 1977; Griffin et al, 1984), has been attributed to the blood-brain barrier (BBB) which restricts the free passage of large molecules such as immunoglobulins as well as lymphocytes and other cells from the circulation into the CNS whilst allowing active transport of certain molecules. The BBB is formed by tight junctions between the endothelium of the blood vessels (Reese and Karnovsky, 1967). The
BBB is however 'leaky' at the circumventricular organs allowing entry of plasma proteins into the extracellular space. There is also a low level of lymphocyte trafficking across the BBB and a small number of these cells are found throughout the normal CNS (Calder et al, 1989). Clearly the potential for initiation of an immune response within the CNS does exist.

1.2 MULTIPLE SCLEROSIS: AN AUTOIMMUNE DEMYELINATING DISEASE

Multiple sclerosis (MS) is a relapsing-remitting disease affecting the mature human CNS. In terms of pathology, MS is characterised by regions of myelin loss within the white matter. Demyelination at the site of a lesion is preceded by inflammation. Conduction of signals via the nerve axons is impaired by myelin loss and it is this irreversible breakdown of myelin which causes the functional deficit exhibited by MS patients. Whilst the usual pattern is one of progressive disability over a matter of years, the disease can follow a benign course in which there is minimal demyelination. During isolated and shortlived episodes of acute inflammation there is virtually no demyelination (Miller et al, 1988). However with persistent or recurrent inflammation considerable myelin loss can occur. Though the disability experienced by MS patients is linked to demyelination of CNS axons, it is possible for extensive demyelination to occur without evidence of neurological deficit (Vuia, 1987).

The animal model, experimental allergic encephalomyelitis (EAE) is induced in susceptible animals following active sensitisation with suitable adjuvants and spinal cord or CNS antigen such as myelin basic protein (MBP). In contrast, no auto-antigen has as yet been identified in MS. Depending on the animal and mode of
sensitisation, an acute monophasic or a chronic relapsing-remitting form of EAE can be induced. In the former case there is evidence of inflammation but not of demyelination whereas the latter form exhibits both pathological characteristics (Glynn and Linington, 1989; Lassmann, 1983). Evidence (reviewed in Calder et al, 1989) supports the view of EAE as a T cell mediated disease. The initial T cell response in EAE occurs in the peripheral lymphoid tissue and activated T cells cross the BBB, the permeability of which is enhanced by encephalitogenic T cells.

In studies of MS tissue, activated lymphocytes are commonly identified by the presence of interleukin-2 (IL-2) receptors, detected by the anti-Tac monoclonal antibody (MAb) (Uchiyama et al, 1981), and the presence of cytoplasmic or membrane-bound IL-2, detected by the 3.9C2 MAb. Activated lymphocytes have been identified in the perivascular areas of the brain of acute MS cases (Woodroose et al, 1986; Booss et al, 1983). Roughly half of the T helper cells found in the CSF of such patients are activated and in a comparison of activated T cells in the CSF and blood, their numbers are greater in the former (Bellamy et al, 1985). This data suggests that, unlike in EAE, cell activation in MS occurs in the CNS rather than in the periphery.

1.3 INFLAMMATION AND DEMYELINATION IN THE CNS

1.3.1 Evidence that microglia participate in inflammation and demyelination in vivo

T cells recognise antigen in association with the major histocompatibility complex (MHC). Immunocytochemical analysis of
MS and EAE tissue reveals that both astrocytes and microglia express MHC class II antigen (Traugott et al, 1985; Hofman et al, 1986; Hayes et al, 1987). Whereas astrocytes are negative for class II in normal tissue, Hayes et al (1987) report constitutive expression of MHC class II on microglia in normal white matter. Furthermore, in MS tissue, microglia express increased levels of MHC class II and comprise the major class II positive population (Hayes et al, 1987). Similarly the dominant class II positive cells in EAE lesions are of monocyte/macrophage lineage (Lassmann et al, 1986). The capacity of perivascular microglial cells to present antigen has been successfully demonstrated in vivo (Hickey and Kimura, 1988). As the major antigen presenting cells (APCs) in the CNS, microglia may be critical, if not to the initial T cell activation step, then to presentation of antigen to T cells at the edge of the plaque (Woodroffe et al, 1986). Perivascular infiltrates in MS lesions include lymphocytes, plasma cells and macrophages (Booss et al, 1983; Woodroffe et al, 1986; Esiri and Reading, 1987). Evidence that the immune response is initiated in the CNS (Bellamy et al, 1985) does not tell us whether the cellular participants are in fact infiltrating cells, gaining access to the CNS via a compromised BBB, or resident cells.

The case for involvement of microglia with the process of demyelination rests primarily on results of immunocytochemistry and electron microscopy studies. Prineas and Connell (1978) noted active demyelination of fibres in contact with reactive microglia. Microglia packed with myelin debris are observed at the edge of demyelinating lesions. However these brain macrophages are not only involved in clearing-up and degrading myelin membrane fragments but also, it appears, in the initial stages of myelin damage. Processes of mononuclear cells appear to be peeling off and phagocytosing the
outer lamella of the myelin sheath. The phagocytosed myelin is contained within coated pits which suggests that uptake is receptor-mediated (Epstein et al, 1983). Various ways in which microglia / brain macrophages might mediate myelin or oligodendrocyte damage, are proposed on the basis of evidence from in vitro experiments using tissue explants or mixed glial cell cultures as well as in vivo studies.

1.3.2 Demyelination in EAE

EAE can be induced in rats and guinea-pigs by injection of a crude brain/spinal cord homogenate with complete Freund’s adjuvant (CFA) (ie. CNS/CFA). In this form of the disease there is little demyelination in the CNS. In contrast in the disease induced by purified myelin basic protein (MBP) and CFA injection (MBP/CFA), there is evidence of perivascular demyelination, as well as oedema and leukocyte infiltration (Lampert and Kies, 1967). Much of the work concerned with the processes and mechanisms of demyelination has made use of this model (reviewed by Glynn and Linington, 1989).

Two principal events in demyelination in EAE have been described. Firstly, macrophages appear to be stripping the myelin sheath. Their processes, infiltrating underneath the outer lamella appear to peel it away and phagocytose it (Raine, 1984). The second commonly observed feature is vesiculation and swelling of sheaths (Dal Canto et al, 1975). With splitting of the sheath, lamellae form vesicles which are broken down into extracellular fragments and phagocytosed by macrophages. The periodicity of the normally compacted lamellae of the sheath increases and the interlamellar space is continuous with the extracellular space (Glynn and Linington, 1989). Mononuclear cells are always present at sites of vesiculation and
swelling of the myelin sheath. The marked absence of cellular organelles in the processes of these cells suggests that lysis of myelin may be caused by macrophage secretions. Similar effects are observed in vitro on treatment of myelinated cultures from neonatal rat CNS with sera from CNS/CFA-immunised animals (Bornstein and Appel, 1961). In another in vitro study, using CNS explants, rapid migration of macrophages and their association with the myelin sheath was observed on treatment with this sera (Yonezawa et al, 1976).

1.3.3 Macrophage function in myelin damage / demyelination

Clearly, on the basis of in vivo and in vitro evidence, macrophages play an important part in the process of demyelination, being involved in the extracellular destruction of the sheath as well as the clearing-up of debris. Brain macrophages, including those recruited from the circulation and activated microglial cells, express receptors for the Fc portion of IgG (FcR) (Perry et al, 1985) and binding via this receptor to the antibody-coated myelin sheath will activate phagocytic responses. Brain macrophages also possess type 3 complement receptors (CR3) (Perry et al, 1985) and may interact with C3b-opsonised myelin membrane. Binding of C3b to the CR3 receptor leads to activation of the macrophages. Other potential macrophage activators are locally produced cytokines including interferon-γ (IFN-γ) produced by the activated T cells and tumour necrosis factor (TNF) and interleukin-1 (IL-1) produced by macrophages / microglia and astrocytes. Fc receptor binding also activates the macrophages, and apart from inducing phagocytosis, it induces the respiratory burst with release of reactive intermediates (Wright and Silverstein, 1986). When anti-CNS sera is decomplemented before addition to explant
cultures, instead of the usual demyelination there is merely myelin swelling though the myelin is coated with antibody (Johnson et al, 1979), suggesting that the major signal for macrophage activation and demyelination is complement rather than Fc receptor binding. Similarly whilst demyelination occurs in foetal rat brain aggregate cultures on treatment with anti-MOG antibody and complement, in the absence of complement anti-MOG fails to induce demyelination (Kerlero de Rosbo et al, 1990).

Macrophages have the capacity to secrete a barrage of soluble substances which are potentially destructive of myelin just as they are of the more usual targets of foreign organisms, virally-infected cells and tumour cells. Activation of macrophages, whether by cytokines, C3b binding or Fc receptor ligation, unleashes some of these agents which include reactive oxygen intermediates (superoxide anion, hydrogen peroxide), nitric oxide, proteases, lipases and cytokines (TNF-α, IL-1, IL-6) (Colton and Gilbert, 1987; Zielasek et al, 1992; Giulian et al, 1986a; Woodroffe et al, 1991). Superoxide (O$_2^-$) causes membrane damage by lipid peroxidation and reacts with hydrogen peroxide (H$_2$O$_2$) to form the hydroxyl radical (·OH). Nitric oxide (NO') is potentially damaging itself but it also reacts with superoxide to form peroxynitrite (ONOO') and subsequently the highly reactive hydroxyl radical (Hogg et al, 1992). These products are strong oxidising agents and as such could considerably affect the stability of the myelin membrane. The demyelinative potential of reactive oxygen intermediates has been demonstrated in vivo in the periphery (Hartung et al, 1988).

Phospholipase A$_2$ (PLA$_2$), another product of activated macrophages, effectively degrades myelin in vitro at pH 4.5. However at higher pH values, such as those found in vivo, this enzyme is
ineffective. Lactic acid, another macrophage product, secreted into the extracellular space may however temporarily lower the pH thereby permitting attack of the myelin sheath by PLA2 (Glynn and Linington, 1989). There is some evidence also that proteases such as plasminogen activator and neutral metalloproteases secreted by activated macrophages may contribute to extracellular degradation of myelin components (Glynn and Linington, 1989). TNF (one of the cytokine products of macrophages) is myelinotoxic in organotypic cultures, perhaps by direct action on the myelin or axonal membrane (Selmaj and Raine, 1988; Brosnan et al, 1988). The result is that the myelin sheath is abnormal but not actually degraded. Other cytokines such as IFN-γ (produced by activated T cells) may be involved in the processes of demyelination. IFN-γ injected into the subarachnoid space of rat brain potentiates the demyelinating effect of co-injected anti-MOG (Vass et al, 1992). The mechanism by which IFN-γ enhances antibody-mediated demyelination in this case is unclear but the activation of macrophages is a possibility.

Having broken down the myelin sheath into ‘bite-sized’ pieces with various secreted agents, macrophages may then engulf the fragments by receptor-mediated phagocytosis (FcR and CR3) and in fact, electron microscopy reveals the presence of phagocytosed myelin within coated pits and vesicles (Epstein et al, 1983).

1.3.4 Other mechanisms of myelin damage

Complement is an absolute requirement for antibody-mediated demyelination of CNS explants by chronic relapsing EAE (CREAE) sera (Bradbury et al, 1984). Apart from its role in opsonisation of myelin membrane and activation of macrophages, complement is involved in
other aspects of myelin damage. Activation of complement on the myelin surface leads to generation of complement membrane attack complexes (MAC) which form tiny pores in the lamellae (Podack and Tschopp, 1976). Myelin will activate Cl to formation of and binding of MAC in vitro in the absence of antibody. The pores created by MAC are sufficiently large (30-100 Å diameter) to permit entry of extracellular fluid and ions into cytoplasmic spaces in the myelin sheath. Proteases and other macrophage secretory products gaining access to the cytoplasmic proteins and Ca\(^{2+}\) dependent phospholipases, believed to be located on the cytoplasmic side of the myelin membrane, could be activated by the increased Ca\(^{2+}\) concentration. Thus the sheath could be subject to attack from the cytoplasmic side as well as the extracellular attack.

1.3.5 The role of microglia in inflammation in the CNS

Based on evidence described previously it seems likely that microglia function as the chief APC of the CNS. These cells are also sources of inflammatory cytokines (TNFα, IL-1 and IL-6). Injection of IFN-γ or TNFα into the spinal cord of healthy rats results in severe episodes of inflammation and perivascular cuffing (Simmons and Willenborg, 1990). Besides cytokines, microglia / brain macrophages secrete various other inflammatory mediators.

Both microglia and astrocytes are capable of producing arachidonic acid metabolites, termed eicosanoids. These include prostaglandins and thromboxanes derived from the cyclo-oxygenase pathway and leukotrienes and hydroxyeicosatetraenoic acids (HETEs) generated by the lipooxygenase pathway (Hartung et al, 1992). These arachidonic acid derivatives are induced by LPS, IL-1β and TNFα
stimulation and are involved in generation and maintenance of inflammatory responses - functioning as chemoattractants for neutrophils and monocytes / macrophages; enhancing adherence of platelets and leukocytes to the endothelium; enhancing vascular permeability and promoting oedema; regulating T and B lymphocyte and macrophage activities (Hartung et al, 1992).

1.4 MICROGLIA - THE RESIDENT CNS MACROPHAGE

In view of the evidence supporting a role for microglia in both inflammatory and demyelinating events in the CNS, further investigation of the functional properties of these cells is warranted. The nature of these cells, their ontogeny and functions in the CNS is discussed below.

1.4.1 Macrophage populations of the brain

A number of resident macrophage populations have been identified in brain tissue. The most extensively studied and largest population of brain macrophages is the microglia. Other putative macrophage populations including supraependymal macrophages, epiplexus cells and meningeal macrophages (reviewed in Jordan and Thomas, 1988) are of very limited distribution. Indeed the chief distinction between microglia and other brain macrophages is one of location, and it is unclear whether these cells constitute distinct populations or are part of a spectrum of morphologies adopted by a single macrophage population which includes resting microglia.
1.4.2 Microglia - the major resident macrophage of the brain

Microglia were first recognised as a morphologically distinct cell population in the brain by Rio Hortega (1932). There has always been some debate concerning the origin of these cells. Rio Hortega and others since believed them to be part of what is now termed the mononuclear phagocyte system, deriving perhaps from monocytes. An opposing camp held that microglia were neuroglial cells derived from the neuroectoderm (Fujita et al, 1981; Oehmichen, 1982). With recent improvements in immuocytochemical techniques and the availability of specific antibodies to cell markers, the evidence available appears to support a monocytic origin of microglia (Perry et al, 1985; Perry and Lund, 1989; Ling et al, 1982).

In studies using bone marrow chimeras the hematopoietic progenitor cells in an animal’s bone marrow are destroyed by irradiation and replaced by transplanting bone marrow from a donor animal. There being differences in the major histocompatibility antigens of donor and recipient animals, this antigen can then be traced in the chimeric animal (Hickey and Kimura, 1988). Immunocytochemical analysis shows that donor cells, shown to enter the CNS, adopt the morphology of microglia (Hickey and Kimura, 1988). Since rat microglia normally express barely detectable levels of MHC antigen, it is usually necessary in such experiments to upregulate expression, for example by inducing a graft versus host reaction.

One of the major arguments against a monocytic origin for microglia was based on the absence of common cell markers. Microglia were not labelled with antibodies which stained monocytes and other macrophage populations (Oehmichen et al, 1979). However tissue macrophages are heterogenous and it is not unusual for there to
be differences in the expression of antigens on resident macrophages and activated macrophages or monocytes. With the development of more antibodies specific to cell markers it is apparent that microglia share a number of markers with other macrophage populations, including Fc receptors, F4/80 (macrophage-restricted) antigen on mouse, complement type 3 receptors and leukocyte common antigen (leukocyte-restricted). Perry et al (1985) traced the F4/80 marker through various morphological stages from monocytes invading the CNS to microglia in the adult. Fc and complement type 3 receptors are also retained during this conversion.

Further evidence that microglia derive from monocytes comes from experiments in which embryonic mice retinas are transplanted into the midbrain of newborn rats. The transplants become integrated to a degree such that functional neuronal connections are formed (Klassen and Lund, 1987). Under these circumstances it is observed that all the microglia in the transplant have originated from host leukocytes and macrophages, i.e. host monocytes have invaded the mouse tissue and differentiated into microglia (Perry and Lund, 1989).

It is now thought that monocytes invade the CNS during the late embryonic and early post-natal period, both before and after development of the vascular system (Jordan and Thomas, 1988). From the results of Hickey and Kimura (1988) using bone marrow chimeras, there appears to be some chemotactic signal released by dying microglia or by other cells in the CNS. Microglia in the adult have a very slow turnover rate (Schultze and Korr, 1981; McCarthy and Leblond, 1988). Movement of monocytes into the developing CNS also appears to occur despite the intact BBB and again some signal must be involved in the recruitment of monocytes from the circulation, though the nature of this is uncertain. Such a signal may
be one of those inducing proliferation and neuronal growth or may be released by dying cells (as perhaps is the case in incidents of brain trauma in the adult). There is in fact a correlation between natural cell death and invasion of the CNS by monocytes (Perry et al, 1985).

1.4.3 Microglial cell morphology and general properties

Two principal forms of microglia have been described. So-called "ramified" microglia are small oval cells with a variable number of long, branching processes. They have little cytoplasm, containing a number of inclusion bodies (possibly lysosomes). These cells appear during the late post natal period and persist through adult life. They are located in interstitial spaces among neuronal somata and glia and in regions of fibres and neuropil. Though they are ubiquitous and tend to form regular arrays, their distribution is not entirely uniform. They are typically present in greater numbers in gray matter than in white matter (Perry and Gordon, 1988). Lacking hydrolytic enzymes and the ability to engulf particles (Oemichen, 1983), ramified microglia are commonly considered to be quiescent. Perhaps the most striking feature of ramified microglia is their large surface area [up to 7x that of a typical Kupffer cell in the liver (Lawson et al, 1990)]. Thus, despite the apparent quiescent nature of ramified microglia considerable resources must be employed in maintaining the plasma membrane. Cammermeyer (1970) suggested that this unique morphology results from adaptation of the cell to make use of limited extracellular space. However, recently Perry and Gordon (1991) proposed that this morphology may arise to maximise binding of receptor(s) on the microglial cell to ligand(s) in the CNS.
Amoeboid microglia are morphologically similar to monocytes and macrophages (Oemichen, 1983; Ling, 1981). They are irregular, possessing pseudopodia and thin filopodia-like processes but not the long branching processes of ramified microglia. They have many cytoplasmic vacuoles and lysosomes, and, in contrast to ramified microglia, they have considerable phagocytic and secretory capacities (Giulian and Baker, 1986). Amoeboid microglia appear in the CNS during late embryogenesis, at sites of axonal growth and glial proliferation, and disappear by the late postnatal period. They reappear in adults at times of brain trauma (Brierley and Brown, 1982; Murabe and Sano, 1982). Evidence suggests that amoeboid microglia are the principal scavengers on the brain during neuropathological conditions (Ling, 1981; Oemichen, 1983).

There are reports of another transient microglial form termed ‘reactive’ (Murabe et al, 1981) which has been associated with injury. These cells have been described as small, round/rod-shaped with membrane ruffles but no processes, pseudopodia or filopodia and containing lipid vacuoles and lysosomes. However the distinction between reactive and amoeboid microglia is disputed and may only be temporal; many investigators use these terms interchangeably. (Reactive and amoeboid microglia are assumed to be one and the same for the purposes of this discussion.)

Another area of dispute is the relationship between ramified and amoeboid microglia. There is evidence that amoeboid microglia convert into ramified microglia in vivo during the postnatal period (Ling, 1981; Murabe and Sano, 1982) and in vitro (Giulian and Baker, 1986). This is consistent with the view of amoeboid microglia as a phagocytic cell involved in tissue modelling in the developing brain and ramified microglia as the resting state in adult brain. However
the question is, do the amoeboid/reactive cells evident on injury to the brain, derive from ramified cells (i.e. a reversal of the post-natal process) or some other precursor such as circulating monocytes? Support for the conversion of ramified to amoeboid microglia is found in the work of Brierley and Brown (1982) but this is contradicted by Murabe and Sano (1982) who suggest that a precursor other than the ramified microglia gives rise to amoeboid/reactive microglia which, after playing its part in the response to injury, then converts to the ramified form. Evidence that numbers of ramified microglia increase at the site of injury (Giulian, 1987; Woodroffe et al, 1991) supports either or both of these scenarios and it has been proposed that in fact a likely explanation is the interconversion of ramified (quiescent) and amoeboid (reactive) forms according to the tissue status (Jordan and Thomas, 1988). Chamak and Mallat (1991) report that in vitro the differentiation of amoeboid microglia towards a ramified, resting phenotype is encouraged by the presence of fibronectin. This effect is reversed by laminin or serum. Thus the balance of these extracellular matrix proteins may influence the morphological changes observed during development or on occasion of injury. Furthermore these findings suggest that undefined serum components, gaining access to the CNS via a leaky BBB, could also influence the activation / differentiation state of microglia. The exact inter-relationship of the different microglial forms requires further clarification.

1.4.4 Role of brain macrophages in the developing CNS

Macrophages have been observed in the process of phagocytosing dying cells, however there is no evidence that macrophages contribute to death of neurons or glia despite the in
vitro demonstration of respiratory burst activity (Perry et al, 1985). Amoeboid microglia may aid tissue modelling and organisation in the developing brain by engulfing misplaced axons or transient axons which are retracted once they have served their purpose (Innocenti et al, 1983 a,b; Perry and Gordon, 1991). It would appear that microglia / macrophages are capable of removing axons without causing the death of the neuronal cell body.

The extracellular matrix of immature brain contains components which are absent in the adult brain (Cohen et al, 1986). Proteinases secreted by macrophages may be responsible for the degradation of these after they have served their purpose. In vitro stimulated amoeboid microglia produce TNFα and IL-1, inducers of astrocyte proliferation in vivo and in vitro (Selmaj et al, 1990; Giulian et al, 1986a,b; Giulian et al, 1988). Phagocytosis of debris may activate amoeboid microglia in vivo to likewise produce these cytokines and hence induce astroglial proliferation. TNF and IL-1 may also stimulate angiogenesis (Leibovich et al, 1987). Macrophages are also capable of producing nerve growth factor (NGF) which may stimulate neuronal growth, and plasminogen activator which may indirectly affect neuronal migration (Moonen et al, 1982).

Thus by virtue of their powerful phagocytic capacity and their wide array of secretory products, amoeboid microglia and other macrophages may play an important part in development of the CNS.

1.4.5 Role of brain macrophages in the response to CNS injury

Penetrative injury to the adult CNS provokes a rapid accumulation of inflammatory cells (Ling, 1981; Giulian, 1987). This is followed by the development of a 'gliotic' scar, a dense network of
astroglial processes (Latov et al, 1979). Such a wound involves damage to the BBB and one of the earliest responses is the recruitment of monocytes and polymorphonuclear cells from the circulation. A large proportion of the inflammatory cells surrounding the lesion site are of microglial origin (Woodroofe et al, 1991) and these, along with infiltrating cells, are the likely source of IL-1 and IL-6 detected at the wound site (Woodroofe et al, 1991; Giulian and Lachman, 1985). Restoration of the BBB must be of crucial importance following a penetrative injury. This makes the observation of Giulian and co-workers (1988a), that injection of IL-1 into mammalian brain induces both astrogliosis and neovascularisation, particularly interesting. Astrocytes will also proliferate in vitro in response to IL-1 (Giulian and Lachman, 1985). Thus evidence suggests that microglia and infiltrating macrophages, as potent sources of IL-1 and other cytokines, play an important part in the response to injury, inducing astrocyte proliferation and regeneration of damaged vasculature. Aside from this, microglia / macrophages are probably responsible for removal of debris from the wound site. In vivo administration of colchicine which among other effects reduces the number of mononuclear phagocytes at the site of a penetrative wound, results in a slower rate of clearance of debris and a reduction of both astrogliosis and neovascularisation (Giulian et al, 1988a).

Where a nerve fibre is damaged, axons which have been separated from their cell body, and the myelin sheaths of these axons, degenerate. This is termed “Wallerian degeneration“. In such a case damage to the BBB is limited to the site of injury (not along the full length of the degenerating axons) and recruitment of monocytes from the circulation occurs at a much lower level than in instances of stab wounds. There are however activated microglia along the length of
the degenerating fibre (Perry et al, 1987). The inability of most CNS axons to regenerate has been linked to oligodendrocytes and the presence of proteins in myelin which inhibit growth of axons (Caroni and Schwab, 1988 a, b; Schnell and Schwab, 1990). It is postulated that clearance of myelin debris from the degenerating fibre would aid regrowth of axons, however activation of resident macrophages, with this aim in mind, may be counter-productive since subsequent astrogliosis may inhibit axonal regrowth (Reier, 1986).

1.4.6 Properties of microglia / brain macrophages relevant to demyelination and inflammation

From the discussion of mechanisms of demyelination and of the process of inflammation, particular characteristics of microglia are of interest in an investigation into the role of microglia in these events. Clearly the phagocytic capacity of microglia (FcR and CR3 expression), secretion of reactive oxygen intermediates and cytokines etc. are properties which are especially relevant to the process of demyelination. Similarly the capacity of these cells to present antigen and produce cytokines is of interest in considering their role in inflammation in the CNS.

1.4.6.1 Phagocytosis

For most tissue macrophages their chief functions as effector cells in the response to injury and infection and in development and tissue homeostasis, rely heavily on their phagocytic capabilities and it is this property which largely characterises macrophages. Giulian and Baker (1986) observed a dramatic reduction in the phagocytosis of latex beads by microglia in vitro on differentiation from an amoeboid to a
'process-bearing' form which approached that of ramified microglia in vivo. This is an example of 'non-specific' phagocytosis i.e. it is not Fc or complement receptor mediated and occurs in the absence of IgG or complement (C) (Oehmichen, 1982). In contrast phagocytosis as mediated by these receptors has a requirement for IgG and/or complement. This has been termed 'immunological' phagocytosis by Oehmichen (1982) and is often studied using antibody- or complement-coated targets such as erythrocytes.

Oehmichen (1982) reports little evidence of Fc and complement receptor-mediated phagocytic capacity in the parenchyma of healthy mature brain tissue. However this activity was demonstrated in cells within the subarachnoid and perivascular spaces and in the lateral ventricles (Oehmichen, 1976). In contrast to Oehmichens's observations, Perry et al (1985) identified Fc and CR3 receptors on the cell surface of microglia in normal brain. Fc and complement receptor-mediated phagocytosis by microglial cells is observed in instances of brain injury (Oehmichen, 1982), as in active multiple sclerosis plaques (Nyland et al, 1980) and in the developing CNS (Perry and Gordon, 1988). Many such studies have employed IgG-coated erythrocytes (EA) and IgG and complement-coated erythrocytes (EAC) for detection of Fc and C receptors.

Macrophages which are phagocytically active usually contain lysosomes. However in resting microglia these are scarce and acid phosphatase activity (a lysosomal marker) is weak (Oehmichen, 1982). Consequently resting microglia have been assumed phagocytically inactive (Cammermeyer, 1970). In contrast amoeboid (reactive) microglia which increase in number in the damaged CNS do contain lysosomes and strong acid phosphatase activity (Oehmichen, 1982) and are phagocytically active (Perry and Gordon, 1988). Thus microglia
respond to injury to the CNS by conversion from the quiescent non-phagocytic form to the highly phagocytic amoeboid form.

A number of in vitro studies have been performed using amoeboid microglia. The observations of Giulian and Baker (1986), mentioned above, bear out the apparent relationship between differentiation state and activity noted in microglia in situ. Microglia in vitro have been shown to express Fc receptors (Woodroffe et al, 1989; Loughlin et al, 1992) and complement receptors (Suzumura et al, 1987; Frei et al, 1987). Expression of these receptors is subject to modulation by a wide range of cytokines (Loughlin et al, 1991), reactive oxygen metabolites (Gresham et al, 1988) and immune complexes (Mannhalter et al, 1986).

(a) Fc receptors (FcR)

Binding of particle-bound immunoglobulins to macrophages initiates not only phagocytosis of the particle but the simultaneous secretion of peroxide and arachidonate metabolites. Fc receptors have been referred to as ‘unregulated receptors’ as binding constitutively and immediately elicits these responses (Wright and Silverstein, 1986).

In humans, FcyRI, II, and III (analogous to FcγRI, IIb and IIa respectively in rodents) comprise the group of receptors which recognise IgG (reviewed by Kinet, 1989). FcyRI has a high affinity for monomeric IgG. FcyRII and III have a low affinity for monomeric IgG but can bind immune complexes by multiple receptor-ligand interactions. FcyRI, II and III and the analogous rodent receptors are all monomeric and contain a transmembrane domain (though a form of FcyRIII exists in humans which lacks this domain). The high affinity receptor for IgE (FcεRI) contains three subunits. There is also a low-affinity receptor for IgE (FcεRII) (Kinet, 1989). The use of IgG-opsonised erythrocytes for analysis of FcR expression does not permit distinction
between subtypes of FcγR. However there are numerous studies in which FACS analysis is used to distinguish between subtypes.

Unligated Fc receptors diffuse rapidly in the plane of the plasma membrane and do not appear to be linked to the cytoskeleton (Michl et al, 1983). There is evidence to suggest that there is an initial influx of Na⁺ on ligation of FcRII (Wright and Silverstein, 1986).

(b) Complement receptors (CR)

CR1 and CR3 both recognise cleavage products of C3, namely C3b and C3bi respectively. In contrast to Fc receptors, ligand binding to CR1 and CR3 does not automatically induce phagocytosis, there being inactive and active states of these receptors. Ligation to the CR1 or CR3 receptor initiates phagocytosis but unlike the Fc receptors there is no release of peroxide or arachidonic acid (Wright and Silverstein, 1986).

The ability of CR3 receptors to promote phagocytosis has been correlated with their mobility in the plane of the plasma membrane (Wright and Silverstein, 1986). C3 (CR1 and CR3) receptors on resident peritoneal macrophages are inactive and relatively immobile. However cytokine treatment activates the receptors and the resultant phagocytosis correlates with the increase in receptor mobility observed. CR1 and CR3 on unstimulated human monocytes and macrophages are mobile but not active indicating some other requirement for activation of these receptors. The switch to the active state can be induced by for example T cell derived factors, fibronectin or phorbol esters binding to the respective receptors (Griffin and Griffin, 1979; Wright et al, 1983; Wright and Silverstein, 1982), perhaps by a reversible phosphorylation or methylation of the complement
receptor. Continuous presence of the activating factor is necessary, activation occurring within 10 minutes in murine and human cells.

Though ligation of both Fc and C3 receptors promotes phagocytosis, release of reactive oxygen intermediates and arachidonate metabolites occurs only on Fc receptor ligation. Binding of the Fc portion of immunoglobulin to the receptor, rather than the event of phagocytosis itself, appears to be the signal for release of these products. The fact that Fc and C3 receptors have one but not all responses in common raises an interesting question concerning the signalling systems which bring about these responses. Either the two receptors share a common messenger system for induction of phagocytosis in which case Fc receptor ligation must induce a second signal for release of superoxide etc.; or there are two distinct signalling mechanisms which produce the phagocytic response, one of which also induces secretion of reactive products. This second possibility is supported by evidence that macrophages adopt different morphologies during phagocytosis of IgG- and C3-coated targets (Kaplan, 1977).

1.4.6.2 Antigen presentation

With increasing evidence challenging its immunologically privileged status, considerable effort has been put into identification of the potential antigen-presenting cells (APC) in the brain. The presence of APCs would be a prerequisite for initiation of an immune response within the CNS, as T cells recognise antigen in association with the MHC class II molecule. Candidates for the role of APC in the brain have included endothelial cells, astrocytes and microglia. In vitro studies indicate that MHC class II can be induced (in particular by IFN-γ) in all three of these cell populations (Male et al, 1987; Frohman et al,
1988; Suzumura et al, 1987; Woodroffe et al, 1989). IFN-γ-treated astrocytes and microglial cells successfully present antigen to class II-restricted antigen-specific T helper cells in vitro (Fontana et al; Fierz et al, 1985; Frei et al, 1987). In in vivo-mimicking conditions (IFN-γ at less than 100 U/ml), Matsumoto et al (1992) have demonstrated that whereas isolated microglia will present MBP to T cells, astrocytes in fact suppress T cell proliferation. They further observed that primary mixed glial cell cultures, consisting largely of astrocytes and microglia, only had weak antigen presenting capacity and they suggest that despite their capacity to present antigen, microglia in vivo may fail to do so.

Immunocytochemical studies to assess expression of MHC class II in brain have also been performed. MHC class II antigen has been detected on both astrocytes and brain macrophages in multiple sclerosis (MS) and in experimental allergic encephalitis (EAE) (Traugott et al, 1985; Hofman et al, 1986; Hayes et al, 1987). The immunocytochemical evidence supporting a role for microglia as an antigen presenting cell has already been discussed (1.3.1). Together with the considerable in vitro evidence, these findings suggest a role for microglia in antigen presentation in vivo.

1.4.6.3 Microglia secrete cytokines

Stimulated microglia produce IL-1, IL-6 and TNFα in vitro (Woodroffe et al, 1991; Giulian et al, 1986a). IL-1 and IL-6 production is detected during viral infections (Frei et al, 1989; Le and Vilcek, 1989) and TNFα and IL-1 have been identified in MS brain tissue (Hofman et al, 1989; Wucherpfenig et al, 1991). Astrocytes also produce IL-1 and TNF in vitro but at much lower levels than microglia are capable of (Giulian et al, 1986a; Sawada et al, 1989).
Immunocytochemical evidence suggests that IL-1 and IL-6 detected in the brain following mechanical trauma is produced by microglia rather than astrocytes (Woodroffe et al, 1991; see chapter 2). IL-1 in particular plays an important part in initiation of an immune response. By the nature of the cytokine network a cascade of events can arise from the production of a single cytokine. IL-1 produced at a stab wound is mitogenic for astrocytes. The resultant astrogliosis is an important part of the CNS response to injury. Cytokines in the brain affect normal functions as diverse as sleep (Krueger et al, 1984) and food intake (McCarthy et al, 1985).

Clearly as a potential source of cytokines in the CNS, microglia may function as part of the homeostatic mechanisms as well as in response to various insults. Of course microglia are themselves subject to regulation by the prevailing cytokines and their functional properties discussed here (including cytokine production) are modulated by cytokines in vitro and by extrapolation in vivo.

1.4.6.4 Microglia are capable of producing nitric oxide and superoxide

In vitro stimulation of microglia with lipopolysaccharide (LPS; a component of bacterial cell walls) or IFN-γ results in the production of nitrite, one of the products of the nitric oxide (NO; N=O⁻) pathway, commonly used as a measure of its activity (Zielasek et al, 1992). Similarly IFN-γ stimulation of microglia increases production of superoxide anion by these cells (Woodroffe et al, 1989).

Nitric oxide is a relatively unstable radical, being highly susceptible to oxidation. Small amounts of nitric oxide produced constitutively play a part in normal physiological functions such as vasodilation in the periphery. Activation of cells during infection by foreign microbes or parasites or in the presence of tumour cells induces production of
larger amounts of nitric oxide by means of an inducible NO synthase enzyme. In these circumstances nitric oxide is toxic to the foreign bodies and tumour cells, however it may also damage healthy tissue. Nitric oxide may also have immunoregulatory roles (Kolb and Kolb-Bachofen, 1992).

Superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are highly reactive oxygen intermediates produced by monocytes, macrophages and neutrophils. Superoxide production has been demonstrated in microglia in culture (Colton and Gilbert, 1987; Woodroofe et al, 1989) but there is as yet no evidence that microglia produce superoxide anion in vivo. Reactive oxygen intermediates produced in vivo may protect against infective organisms in the CNS however, like nitric oxide, they may also have deleterious effects on healthy CNS tissue. The potential of superoxide to damage peripheral tissues such as the lung has been demonstrated (Fox, 1984).

Although the production of superoxide and nitric oxide in vivo by microglia remains to be demonstrated, one is tempted to suggest that through these products microglia may not only function as part of the defence against invading organisms and tumour cells but also in initiating autoimmune diseases such as multiple sclerosis.

1.5 CYTOKINES - an introduction

The cells which participate in inflammatory and demyelinating events in the CNS are subject to regulation by and are also potential sources of cytokines. Thus the functional properties of brain macrophages / microglia discussed previously may be modulated by
these factors. On another level cytokines such as TNF may be involved more directly in myelin damage as mentioned earlier.

These low molecular weight proteins or glycoproteins generally act locally in an autocrine or paracrine fashion and are involved in homeostatic control of the immune system and in interactions between the immune system and the neural and endocrine systems. One of the most interesting and perplexing features of cytokines is the complex network which they and other growth factors form and which involves the production and action of cytokines by/on a wide array of cells. Cytokines generally have a wide range of often overlapping activities, producing varied responses in different cell types or in the same cell in different functional states (Balkwill, 1989).

The cytokine network is such that cytokines interact at a variety of levels, modulating expression of other cytokines or their receptors as well as influencing their activity. In the body a cell is exposed to a cocktail of cytokines, growth factors, hormones and other mediators and the cell response to a particular cytokine may be modulated by these agents. Other factors influencing the response include the local cytokine concentration, previous exposure to other mediators and the length of time during which the cell is exposed to the cytokine. There are a number of naturally occuring cytokine inhibitors capable of modulating cellular responses. These include interleukin-1 receptor antagonist (IL-1ra) which shares considerable homology with IL-1 and occupies the IL-1 receptor but cannot trigger signal transduction via this receptor (Dinarello and Thompson, 1991). There are also a number of cytokine receptors which exist in a soluble form, for example the TNF receptor. These receptors in the extracellular matrix compete with membrane-located forms for the particular cytokine
thereby reducing the effective concentration of cytokine available for signalling.

1.5.1 Cytokine receptors and signal transduction

Often receptors recognise more than one cytokine. For example TNFα and lymphotoxin (LT) or the α and β forms of IL-1 compete for the same receptors. Cytokines may regulate cellular events directly or indirectly at the level of gene transcription, mRNA processing and translation or processing of protein products. However little is known about the mechanisms whereby the cytokine-receptor ligation event is transduced to influence intracellular events. Cytokine receptors generally do not possess any enzymatic activity, suggesting a prerequisite for other molecule(s) (Foxwell et al, 1992).

The pleiotropic nature of cytokines poses a problem in explanation of cytokine receptor signalling, similar to that discussed in relation to Fc and C3 receptor signalling. How does a single cytokine generate a number of widely varied responses in a cell? Part of the explanation may be that there are a number of receptors which recognise the cytokine as with TNF or IL-1 receptors. If these receptors are expressed in different cells or at different times [and there is some evidence that this is the case with IL-1 receptors (Foxwell et al, 1992)], this could explain the variety of responses generated by a single cytokine in different cell types. Overlap in regulatory activities of cytokines is particularly striking in the case of IL-1 and TNFα or LT. Thus two cytokines recognised by different receptors generate overlapping cellular responses. Each cytokine may generate a unique signal, triggering their various responses ie. the same effect can be induced by two different second messenger systems. Alternatively
each cytokine may generate more than one signal and any overlapping activities would result from activation of a common second messenger system.

1.5.2 Cytokines in the central nervous system

Current evidence suggests that cytokines in the circulation can enter the brain at sites where the BBB is 'leaky', such as the circumventricular organs (Coceani et al, 1988) or when the BBB is damaged eg. by mechanical injury. Cytokines may not necessarily have to cross the BBB in order to exert an effect on central functions. For example IL-1 induces synthesis of nitric oxide in brain endothelial cells (Garthwaite, 1991) which may act as an IL-1 transducing agent, mediating effects within the CNS (Rothwell, 1991). However receptors for cytokines, including IL-1 and IL-2, have been identified in brain tissue (Haour et al, 1990; Hofman et al, 1986) using autoradiographic and immunocytochemical techniques. There is evidence also suggesting that cells within the CNS are capable of producing cytokines. Blood monocytes and lymphocytes gaining access to the CNS via a leaky/damaged BBB are potential sources of cytokines in the brain. However as already mentioned, resident brain cells such as microglia and astrocytes have been demonstrated to be capable of cytokine production (eg IL-1, IL-6 and TNF) on stimulation in vitro (Giulian et al, 1986a; Fontana et al, 1982). Immunohistochemical studies have identified IL-1, IL-2 TNF and IFN-γ in lesions in MS brain tissue (Hofman et al, 1986; Hofman et al, 1989; Selmaj et al, 1991a). There is evidence also that IL-6 is produced in the brain (Rothwell, 1991). Giulian et al (1988a) eluted IL-1 activity from biopsies of brains which had received stab wounds and attributed the astrogliosis and
neovascularisation which results from such a wound to the actions of this cytokine. There is strong evidence that IL-1 and IL-6 produced in response to mechanical damage originate primarily from microglia (Woodroofe et al, 1991; see chapter 2).

These findings suggest that cytokines are principally associated with disease and injury. However, mRNA for IL-1β has been detected in normal rat brain (Farrar et al, 1981) and the protein product has been detected immunohistochemically in rat and human brain tissue (Lechan, 1990; Breder et al, 1988). Cytokines acting directly on the CNS have effects on the neuroendocrine system and on neuronal function with consequences for a wide range of behavioural and metabolic functions such as sleep and diet (Rothwell, 1991).

A number of the cytokines are of particular interest in this study, because they modulate microglial activity or have been identified in the CNS. These are discussed briefly in the following section.

1.5.2.1 Interleukin-1 (IL-1)

The range of potential central effects of cytokines is best illustrated with IL-1 (see Rothwell, 1991, for review). IL-1β has been linked to fever and increased metabolic rate (Rothwell, 1991), activation of the hypothalamic-pituitary-adrenal (HPA) axis (Sternberg, 1989), and behavioural properties such as sleep and exploration (Tazi et al, 1990). Aside from these effects, IL-1 has effects locally in the brain including astrogliosis and neovascularisation in response to injury (Giulian et al, 1988a; Giulian and Lachman, 1985) and in the developing brain (Giulian et al, 1988b).
IL-1 is produced by almost every nucleated cell type (Balkwill, 1989). As one of the first cytokines released at a site of injury or infection, IL-1 plays a key role in macrophage and T-lymphocyte activation and, along with IL-6 and colony-stimulating factors, it enhances endothelial cell adhesiveness thereby encouraging cell recruitment. IL-1β is the principal secreted form of IL-1. Production of IL-1α and β is inducible by other cytokines (TNF, IFN-α, β and γ), by cell damage, by LPS, and by antigens and viral toxins. IL-1, positively and negatively (the latter through induction of prostaglandin E₂), regulates expression of its own gene (Dinarello et al, 1987; Warner et al, 1987). IL-1ra has an affinity for the IL-1 receptors similar to that of IL-1α and β but it does not induce signal transduction (Dripps et al, 1991). With regard to IL-1 signal transduction there are reports of the involvement of a G protein with elevation of cAMP levels and subsequent activation of protein kinase A (PKA) and of the involvement of NFκB transcription factor (Mizel, 1990). However these findings are disputed elsewhere (O'Neill et al, 1990).

1.5.2.2 Tumour necrosis factor (TNF)

The primary sources of TNF are macrophages and T or B lymphocytes. As mentioned previously there is considerable overlap in the activities of IL-1 and TNF. Like IL-1, TNF plays a role in defence against foreign organisms and viral infection (Wong and Golddel, 1986), exhibiting proinflammatory and cytotoxic activity. The two forms of TNF termed TNFα and TNFβ (also known as lymphotoxin, LT) are very similar in their activities but whilst TNFα is predominately of myeloid origin, TNFβ is produced mainly by T cells. TNF co-operates with IL-2 in stimulation of T cell proliferation and alone it enhances B cell proliferation and antibody secretion. Like
many cytokines, TNF induces secretion of other cytokines (IL-1, IL-6, colony-stimulating factors) and its own secretion (Balkwill, 1989).

As has been mentioned previously, microglias and astrocytes produce TNFα on stimulation in vitro. Both IL-1 and TNFα individually and synergistically induce differentiation of astrocytes and proliferation of both astrocytes and microglia when added to mixed glial cultures (astrocytes, microglia and oligodendrocytes) (Merrill, 1991). Autoimmune demyelination in the animal model of MS, experimental autoimmune encephalomyelitis (EAE), is inhibited by intra-peritoneal administration of a polyclonal anti-TNFα antibody (Selmaj et al, 1991b) suggesting that this cytokine plays a part in demyelination in such cases. A key role for TNF in inflammation is also likely. Direct injection of TNFα into healthy rat spinal cords induces inflammation and perivascular cuffing (Simmons and Willenborg, 1990).

1.5.2.3 Interferon-γ (IFN-γ)

The principal source of IFN-γ is activated T lymphocytes. There is evidence that several second messenger systems are involved in IFN-γ signal transduction, including cAMP, Ca²⁺ and protein kinase-C (PK-C). The result is that expression of many proteins is regulated at the level of transcription and translation. There appears to be a requirement for an accessory factor to ensure successful signalling (Hibino et al, 1992).

IFN-γ induces secretion of several cytokines including TNF, IL-1, colony-stimulating factors and IFN-α. It has a cytostatic effect on many normal and transformed cells. With regard to the CNS, it is of interest to note that IFN-γ activates microglial cells in culture to express MHC class II (Frei et al, 1987) and Fc receptors (Woodroofe et al,
IFN-γ-treated astrocytes and microglia successfully function as antigen-presenting cells (APCs) in vitro (Fontana et al, 1987) though there are contradictory reports concerning astrocytes (Matsumoto et al, 1992). Sasaki et al (1990) demonstrated that class II expression could be induced in astroglia and in microglia but whereas this effect in astroglia could be negatively regulated by cAMP and PK-C, expression in microglia was not affected by manipulation of these factors. These results illustrated a differential regulation of MHC class II expression in the two cell types.

1.5.2.4 Transforming growth factor-β (TGFβ)

TGFβ1, produced by activated T cells, activated macrophages and a number of other cell types, is a potent suppressor of the immune response (Tsunawaki et al, 1988; Wahl et al, 1988). TGFβ2 appears to have similar functions. Both TGFβ1 and TGFβ2 are homodimers. A heterodimeric form TGFβ1.2 also exists. There are at least three glycosylated TGFβ-binding proteins, however little is known about TGFβ receptor signalling.

TGFβ1 and β2 both suppress presentation of autoantigen (myelin basic protein) by astrocytes in vitro and antagonise induction by IFN-γ of MHC class II antigen expression on astrocytes (Schluesener, 1990). TGFβ1 also inhibits the production of IL-1, IFN-γ and TNFα by macrophages in vitro (Espevik et al, 1987). These results hint at a role for TGFβ as a regulator of inflammation in the CNS and elsewhere. In an interesting study by Shull et al (1992), the mouse TGFβ1 gene was selectively disrupted, resulting in TGFβ1-deficient mice. These animals develop a wasting syndrome and demonstrate multifocal inflammation. Merrill and Zimmerman (1991) report that the natural and IFN-γ-stimulated cytotoxicity towards oligodendrocytes
demonstrated by rat microglia in vitro is inhibited by TGFβ. This observation is particularly interesting in the context of MS where there is evidence that microglia are involved in destruction of myelin at lesion sites.

1.5.2.5 Colony-stimulating factors

Colony-stimulating factors (CSFs) include interleukin-3 (IL-3), granulocyte-macrophage-CSF (GM-CSF) and macrophage-CSF (M-CSF) among others. IL-3 and GM-CSF partially cross-compete for their receptors which display high and low affinity. These two cytokines induce tyrosine phosphorylation of a similar set of cytoplasmic proteins (Isfort and Ihle, 1990). Giulian and Ingeman (1988) infused various of these factors into the cerebral cortex of rats and 48 hours later observed a large increase in the number of activated intrinsic mononuclear phagocytes at the injection site. IL-3, GM-CSF (Giulian and Ingeman, 1988) and M-CSF (Stanley, 1985) are mitogenic for brain macrophages in vitro whereas IL-1, IL-2, IFN-γ, TNF and G-CSF are not. Astrocytes are a potential source of CSF-1 in the brain and IL-1 and TNF-α stimulate its production by astrocytes in vitro (Thery et al, 1992).

1.5.2.6 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS), a component of bacterial cell walls, is not a cytokine but it is a potent inducer of IL-1, IL-6 and TNFα production by monocytes, macrophages, microglia and astrocytes (Woodroofe et al, 1991; Giulian, 1986a). The nature of the interaction between LPS and these host inflammatory and immune cells is unclear though there is evidence that LPS may bind to the leukocyte function-associated antigen (LFA-1) and a number of LPS-binding
proteins have been identified. The interaction between LPS and the cell membrane appears to occur in two stages (Hamilton and Adams, 1987). There is a rapid temperature-independent attachment followed by a slower temperature-dependent event which may involve the integration of the lipid portion of LPS into the membrane. LPS suppresses IFN-γ-induced MHC class II expression (Loughlin et al, 1992; Hamilton and Adams, 1987) whilst IFN-γ enhances LPS-induced expression of TNF mRNA (Hamilton and Adams, 1987). Inhibition of IFN-γ-induced class II by LPS is blocked on treatment with cyclohexamide indicating a requirement for de novo protein synthesis for successful inhibition by LPS.

1.5.3 Macrophage / microglial activation

The events which render macrophages fit for the execution of complex functions such as in inflammation and demyelination are loosely termed 'macrophage activation'. The activation status of macrophages is assessed primarily on the basis of levels of expression of key cell surface markers such as MHC class II antigen and LFA-1 and functional properties such as superoxide production and phagocytic activity (Hamilton and Adams, 1987). The response of the cell to extracellular signals depends not only on the nature of the particular signal but on the presence of any other signal whether stimulatory or inhibitory, on the recent history of the macrophage and, where there is more than one signal, the relative doses and sequence of exposure of the cell to these signals. Responses also differ in different types of macrophage, depending on their functions and state of differentiation.

The best characterised macrophage activating factor (MAF) is IFN-γ (Schreiber and Celada, 1983). In the scenario proposed by
Hamilton and Adams (1987), IFN-γ pushes macrophages from a responsive to a primed state and a second signal, such as LPS, pushes this primed state towards a fully activated state. The different properties of cells at these stages reflect their ability to perform particular functions. Depending on the particular characteristic, IFN-γ and LPS can have opposing or cooperative effects. For instance whilst LPS suppresses the induction of MHC class II antigen by IFN-γ, IFN-γ enhances the expression of TNF mRNA initiated by LPS (Hamilton and Adams, 1987). LPS suppression of IFN-γ-induced MHC class II expression is inhibitable by cyclohexamide, indicating a requirement for de novo protein synthesis.

The regulatory effects of IFN-γ and LPS on macrophages appear to some extent to be exerted at the level of mRNA synthesis or stability as there is a good correlation between expression / secretion of the end product and levels of the appropriate mRNA (Hamilton and Adams, 1987).

1.5.4 Cytokines in trauma and disease in the CNS

Monocytes which enter the CNS via a leaky BBB, and activated microglia or astrocytes are potential sources of IL-1 in the CNS as described earlier. Astrocyte proliferation in response to IL-1 and other growth factors promotes the formation of an astrocytic scar in damaged and diseased CNS tissue (Guilian and Lachman, 1985; Giulian et al, 1988a). IL-1 and TNFα, both products of microglia/macrophages or monocytes, may stimulate the production of M-CSF by astrocytes (Thery et al, 1992) which in turn stimulates microglial cell proliferation (Giulian and Ingeman, 1988). IL-1 also enhances
macrophage and T-lymphocyte activation thereby promoting the inflammatory response.

Activated T cells produce IL-2 and glial growth promoting factor (GGPF) both of which encourage proliferation of oligodendrocytes, suggesting potential for repair and remyelination at lesion sites. However IL-2 production and oligodendrocyte proliferation is inhibited by prostaglandin E (PGE) secreted by microglia/macrophages and astrocytes. Thus a balance exists between inflammation / demyelination and repair processes which is determined by the levels of these cytokines and other mediators.

The importance of cytokines in the CNS has already been discussed in terms of inflammation and demyelination. Whilst IFN-γ and TNFα appear to have chiefly deleterious effects with regard to inflammation or demyelination (Selmaï and Raine, 1988; Selmaï et al, 1991b; Simmons and Willenborg, 1990; Vass et al, 1992), there is evidence to suggest that TGFβ1 may play a protective role with regard to inflammation (Shull et al, 1992).

1.5.5 Cytokine manipulation in vivo - therapeutic potential

There has been considerable optimism that cytokines could be of therapeutic benefit. Many cytokine genes have been cloned and the recombinant proteins are widely available. However, the difficulties involved in administration of a cytokine for therapeutic purposes are considerable. The pleiotropic nature of cytokines means that targeting of a single effect is difficult. Furthermore cytokines generally act locally and administration via a systemic route could have unforeseen consequences and exposes the cytokine to degradation and dilution. It is becoming clear that a less direct approach may be appropriate.

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The existence of various natural cytokine inhibitors such as IL-1ra and the soluble receptors for eg. TNFα, IFN-γ, IL-2 and IL-4, provide a possible means of controlling the activity of particular cytokines. Manipulation of existing control mechanisms such as the production of a natural antagonist or an inhibitory cytokine or regulation of cytokine receptor expression / activation may prove to be therapeutically valuable (for review see Feldmann, 1991).

1.6 THE PURPOSE OF THIS STUDY

The following approach has been adopted to clarify some of the issues concerning microglial cell involvement in inflammation and demyelination, and the importance of cytokines in these events.

(1) Some microglial properties, namely MHC class II expression, Fc receptor expression, cytokine production and superoxide production, were identified as particularly relevant to the processes of inflammation and demyelination.

(2) The capacity of isolated microglia to produce cytokines (IL-1, IL-6, TNF) on stimulation in vitro has been assessed. Microdialysis techniques were used to monitor cytokine production in vivo in response to injury to the CNS and immunocytochemical analysis of the tissue provided information on the cellular response.

(3) The regulatory influences of cytokines on the microglial properties indicated in (1) were investigated in vitro, exploring cytokine interactions and modulatory effects of non-cytokine mediators such as glucocorticoids and neurotransmitters.
In an attempt to approach the \textit{in vivo} situation where cells of the CNS are in intimate contact with various other cell types and under the influence of the mediators which these cells produce, experiments have been performed using a 3-dimensional aggregate culture system. These aggregates contain myelinating neurons and the various cell types found in the mammalian CNS. The aggregate culture system provided a means of assaying demyelination. Cytokine-mediated and anti-MOG-mediated demyelination have been compared using this system. The role of macrophages in inflammation and demyelination has been explored using aggregate cultures enriched with macrophages.
CHAPTER 2

CYTOKINE PRODUCTION BY ISOLATED MICROGLIA IN VITRO AND IN THE CNS IN VIVO IN RESPONSE TO INJURY

2.1 INTRODUCTION

It is widely accepted that cytokines play an important part in embryonic development of the brain and during instances of infection, inflammation and other insult such as mechanical damage. In particular interleukin-1 (IL-1) has been recognised as a principle mediator during inflammatory incidents or after injury to the central nervous system (CNS), as it is in the periphery. In the periphery IL-1 acts as a growth factor, stimulating the proliferation of lymphocytes and fibroblasts at sites of inflammation (Dinarello, 1984). Injury to the CNS provokes the rapid appearance of inflammatory cells (Ling, 1981; Giulian, 1987), including blood monocytes, no longer hindered by the blood-brain barrier (BBB), and resident microglia / macrophages. This is followed by the proliferation of astroglia such that a gliotic 'scar', a dense network of astroglial processes, develops near the wound site (Latov et al, 1979). This cellular response is believed to inhibit the regeneration of neurons (Reier et al, 1986) and the formation of myelin membrane (Barchi et al, 1982). Stab wounds to the cerebral cortex contain significant levels of IL-1 (Giulian and Lachman, 1985). Furthermore, injection of IL-1 into mammalian brain stimulates astrogliosis and growth of new blood vessels (Giulian et al, 1988a). This evidence suggests that IL-1 may be the growth factor responsible for the astrocyte proliferative reaction at sites of injury in the CNS and
indeed, astrocytes proliferate in vitro in response to IL-1 (Giulian and Lachman, 1985).

Do cytokines in the CNS originate in the periphery and subsequently cross the BBB, or are they produced within the CNS, and if so, by which cells? Evidence suggests that the BBB is impermeable to IL-1 except in areas such as the circumventricular organs where the barrier is 'leaky' (Coceani et al, 1988). When the BBB is damaged or permits entry of circulating blood monocytes (and other cells), these may be sources of cytokines in the CNS. However microglia and astrocytes are also potential sources. Both secrete IL-1 when stimulated with LPS in vitro, though microglia can be induced to release much greater quantities of IL-1 than astrocytes (Giulian et al, 1986a). The capacity of microglia to produce IL-1 and IL-6 in vitro on stimulation by LPS and the effect of interferon-γ (IFN-γ) on this response is described in this chapter. A comparison is made between microglia and peritoneal macrophages in this regard.

There are disadvantages in using tissue biopsies (Giulian et al, 1988a), to assess in vivo cytokine production in response to injury.

1. In order to obtain information on the time course of cytokine production a different animal is required for each time point, thereby introducing the factor of variation between individuals.

2. The tissue biopsy contains undamaged tissue and cytokine secretion by the tissue represents an 'averaged-out' level of production, not the local level of cytokine production at the site of injury.

3. A delay is necessary for successful elution/extraction of the cytokine.

4. The process of excision of tissue biopsies may itself induce further cytokine production or risk of contamination from the circulation.
The method described here uses a microdialysis technique which permits repeated sampling from the same conscious, freely moving animal. Insertion of the microdialysis probe itself results in localized brain injury. The extent of injury is dictated by the necessarily large dimensions of the dialysis probe and is thereby much greater than that inflicted in typical stab-wound experiments. This technique has the advantage that cytokines detected in the dialysate are representative of local levels at the site of injury at the noted sampling time.

In the study described here in vivo production of IL-1 and IL-6 was measured using this microdialysis technique. Dialysate samples were collected within 2 - 3 h of implantation of the probe and subsequently at 1, 2 and 7 days post-implantation. In a parallel study, IL-1 and IL-6 content was determined in CSF of animals which had a probe implanted. The brains of these animals were analysed using immunocytochemical techniques to determine the cellular response to injury.

2.2 MATERIALS AND METHODS

2.2.1 CNS tissue

After decapitation of 30 day old Sprague-Dawley rats of both sexes (Charles River, UK) cerebrae were dissected out and meninges removed by rolling on methanol-sterilised filter paper (Whatman, Maidstone, Kent) under sterile conditions. This tissue was used for isolation of microglial cells for in vitro studies.
2.2.2 Media

Cell dissociation and separation were carried out in Earles balanced salt solution (EBSS; Gibco BRL Ltd., Paisley, Renfrewshire, U.K.). Resident peritoneal cells were collected in EBSS without calcium and magnesium (EBSS-Ca,Mg; Gibco BRL). Cells were cultured in Dulbecco’s Modified Eagles medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated foetal calf serum (ICN Flow Labs., High Wycombe, Bucks., U.K.), 1mM sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin solution (Gibco BRL) [DMEM+10%FCS].

2.2.3 Isolation of microglia from rat brain

The procedure for isolation of microglia from rat brain as developed in this laboratory (Hayes et al, 1988) has been further optimised. The results of experiments performed to optimise the procedure are detailed in the results section and the following is the revised method.

A mixed glial cell suspension was prepared by subjecting chopped brain tissue from Sprague-Dawley rats to enzyme digestion. Microglia were then isolated from this suspension by rosette formation with opsonised erythrocytes via the Fc receptor, and subsequent separation of rosetted cells on a density gradient. Approximately 50g of brain tissue (from 40 rats) was processed at a time in 4 batches of 10-15g each. An average yield of $1 \times 10^6$ cells/g brain was obtained.
2.2.3.1 Enzymes

All enzymes were obtained from Sigma Chemicals, Dorset, U.K. 10 x stock solutions of trypsin (1.25% w/v; type III), collagenase (1000 U/ml; type XI) and deoxyribonucleate 5'-oligonucleotidohydrolase (200 µg/ml; DNase I, type II) were prepared in EBSS. The stock solutions were passed through a 0.45µm, then a 0.22µm filter (ICN Flow Labs., Rickmansworth, U.K.) to sterilise and stored in aliquots at -20°C.

2.2.3.2 Haemagglutination titration

A dilution series of anti-human erythrocyte membrane antibody (Dako, U.K.) was prepared in duplicate in EBSS to give final volumes of 250µl in a 96 well round-bottomed microtitre plate (Nunc, Gibco BRL Ltd., U.K.). The series covered a range from 1/2 to 1/4096 in 1:1 dilution steps and was expanded in a range of 1/385 to 1/1389 using 5:1 dilution steps. To each well containing 250µl of diluted antibody was added 5µl of erythrocytes packed by centrifugation at 200g, to give a 2% v/v erythrocyte concentration. As a control a 2% suspension of erythrocytes in EBSS alone was included. The plate was incubated for 30 min at room temperature and wells were examined over a light box to select the sub-agglutinating dose with which to prepare EA for use in microglial cell isolation and the Fc receptor assay (3.2.5).

2.2.3.3 Opsonisation of erythrocytes (EA)

Human peripheral venous blood was collected by venepuncture in heparinised tubes and spun at 200g for 10 min at 4°C. The plasma and buffy-coat overlying the red cell pellet were removed and the erythrocytes were washed twice in Alsevers solution (Gibco BRL Ltd., U.K.) at 4°C, by centrifugation (200g for 10 min). Erythrocytes were stored in Alsevers solution at 4°C until used. EA was prepared by
incubating a 2% v/v suspension of human erythrocytes (packed by centrifugation at 200g for 10 min) in EBSS including the selected dose of antibody for 30 min at RT with constant mixing. Erythrocytes were pelleted and washed twice in EBSS. EA for use in microglial cell isolation was resuspended at 4% in EBSS.

2.2.3.4 Preparation of mixed glial cell suspension

All procedures were carried out in laminar flow hoods (Hepaire, U.K.) under sterile conditions, and the tissue kept at 4°C throughout, unless otherwise stated. Brain tissue was chopped in two 90° planes in 0.4mm slices on a McIlwain chopper (The Mickle Laboratory Engineering Co., Mill Works, Gomshall, Surrey), and placed in sterile pre-weighed 250ml glass bottles. 1ml of each stock enzyme solution (trypsin, collagenase and DNase I; see 2.2.3.1) and 7ml EBSS were added per gram of tissue and the mixture stirred at 37°C for 15 min. The digestion was halted by addition of 1ml newborn bovine serum (NBS; ICN Flow Labs.) per gram original tissue and dilution of the partially digested tissue to approximately twice its original volume in ice-cold EBSS-Ca/Mg. This suspension was centrifuged at 140g, for 10 min at 4°C. The enzyme digestion step was repeated and the pellet resuspended in EBSS and passed through methanol-sterilised 132μm and 80μm nylon mesh (John Staniar and Co., U.K.). The resultant mixed glial cell suspension was made up to 5ml/g starting tissue in EBSS.

2.2.3.5 Isolation of FcR+ microglia from the mixed glial cell suspension

Equal volumes of mixed glial cell suspension, at 5ml/g starting tissue, and the 4% EA suspension were mixed and incubated at 37°C for 30 min, then pelleted (200g for 10 min) and gently resuspended in
EBSS-Ca/Mg to give 10ml/g starting tissue. Rossetted cells were isolated using Percoll (Pharmacia, U.K.) which was adjusted to 1.086g/ml using 10x concentrated BME (10xBME; Gibco BRL Ltd.) and sterile distilled water in the ratio, 62ml Percoll : 28ml water : 10ml 10xBME. 25ml quantities of the cell suspension were layered over an equal volume of this Percoll preparation in 50ml centrifuge tubes and centrifuged at 700g for 20 min at 18°C.

The pellets containing rosetted cells were collected and gently resuspended to approximately 1ml/g starting tissue in EBSS-Ca/Mg. The resultant suspension was divided into 5ml quantities in 50ml tubes. Erythrocytes were lysed by hypotonic shock. Sterile distilled water (40ml) at 4°C was added to each 5ml aliquot of rosetted cells and mixed for 45s. Tonicity was restored with the addition of 4.5ml of 10xBME. The cells were centrifuged at 140g for 10 min at 4 °C. Pellets were pooled and resuspended to approximately 0.5ml/g starting tissue in EBSS-Ca/Mg and 5ml aliquots were subjected to a second 30s lysis step, as above. After centrifugation the pellets, containing microglia, were pooled and resuspended in 3ml DMEM+10%FCS for counting purposes. Viable cells were enumerated by 0.2% eosin dye exclusion in a haemocytometer.

Cells were plated into 96 well flat-bottomed plates (Nunc, Gibco BRL) at 10^5 cells/well in 150μl DMEM+10%FCS and maintained at 37°C in a 95% air/5% CO₂ incubator (Heraeus, Philip Harris, U.K.). The media was changed after 1 and 2 days and cells were maintained in culture for 5 days prior to cytokine treatment.
2.2.4 Isolation of peritoneal macrophages

Peritoneal macrophages (Mφ) were obtained from Sprague-Dawley rats by peritoneal lavage with 50ml of ice-cold EBSS-Ca/Mg. Cells were pelleted by centrifugation, 140g at 4°C for 10 min, washed once in EBSS-Ca/Mg and resuspended in DMEM+10%FCS. Mφ were plated into 96 well flat-bottomed plates at 10^5 cells/well in 150μl DMEM+10%FCS.

2.2.5 Lipopolysaccharide stimulation of microglia and Mφ in vitro

Lipopolysaccharide (LPS) from E.coli (Sigma Chemical Co. U.K.) was stored at -20°C as a stock solution (1mg/ml in EBSS). Three days after plating of microglia and Mφ, LPS at a range of concentrations up to 10μg/ml in DMEM+10%FCS was added to triplicate wells. After 48h, supernatants were harvested and the triplicate wells pooled. Samples were stored at -70°C until assayed for cytokine content.

2.2.6 Microdialysis

2.2.6.1 Animals

Adult male Sprague-Dawley rats (280-320g, Charles River, U.K.) were housed individually under a 12h light-dark cycle (lights on 06-00) and allowed food (RRF diet, Charles River, U.K.) and water ad libitum.

2.2.6.2 Cannula construction

The dialysis cannula (see Fig. 2.1) was constructed by inserting a 100μm diameter steel wire into a 16mm length of hollow dialysis fibre (Diaflow Hollow Fiber; 100,000 molecular weight cut off; 300μm
Fig. 2.1

Construction of microdialysis cannula for continuous sampling of extracellular fluid in the brain of a freely moving rat.
diameter; Amicon, Danvers, U.S.A.). The two ends of the dialysis fiber were inserted into the tips of two 5cm lengths of polyethylene tubing (Portex, Hythe, U.K.) and sealed with epoxy resin. The U-shape was made by gentle flexing of the fibre around a bent hypodermic needle and the shape maintained by sealing the polyethylene tubing with epoxy resin. The wire insert served to prevent collapse of the dialysis membrane during implantation of the probe into the brain. Probes were kept overnight in sterile artificial cerebrospinal fluid (ACSF; 125mM NaCl, 2.5mM KCl, 1.18mM MgCl₂, 1.26mM CaCl₂) containing 1% rat serum.

2.2.6.3 In vitro recovery of IL-1 and IL-6

Initial recovery experiments for IL-1 and IL-6 across the dialysis probe were performed in vitro using a crude rat cytokine preparation. Flow rates of 0.5μl/min and 2μl/min were used and probes were washed through for 2h before sample collection. Samples were collected for the following 2h and stored frozen at -70°C until assayed for cytokine content.

2.2.7.4 Surgical and sampling procedures

Following anaesthesia with sodium pentobarbitone (Sagatal; BDH, Dagenham, U.K.; 60mg/kg) rats were placed in a stereotaxic frame and a 3mm burr hole trephined (AP -0.8, L 3.5 relative to bregma; according to Paxinos and Watson, 1982). An incision of the dura was made with the tip of a 27 gauge hypodermic needle and the probe placed to V 2.0 from the interaural line. Stainless steel screws (Plastics One, Roanoke, U.S.A.) were mounted in the skull, adjacent to the probe and the whole assembly secured with cranioplastic cement.
The location of the probe is indicated in Fig. 2.2.

The first dialysate sample was collected 2-3h after implantation following perfusion with ACSF containing 0.01% rat albumin at 2μl/min for 2h. Subsequent dialysate samples were taken from conscious freely moving rats at the same perfusion rates and at sampling times of 1, 2 and 7 days following implantation. In a separate group of rats, 50-60μl of cisternal cerebrospinal fluid (CSF) samples, collected at the same time points, were rapidly frozen in solid CO\textsubscript{2} and kept at -70°C until required for cytokine analysis.

2.2.7 Cytokine assays

Cytokine bioassays were performed by Meenu Wadhwa at the National Institute for Biological Standards and Controls (NIBSC).

2.2.7.1 IL-1 Bioassay

IL-1 levels were determined using the NOB-1 cell line, a subclone of the murine thymoma line, EL4 (Gearing et al., 1987). For assay, samples were distributed at appropriate dilutions in 100μl volumes in 96 well plates. 100μl of NOB-1 cells (1x10\textsuperscript{6}/ml) were added to each well in RPMI 1640 (Gibco BRL Ltd., U.K.) containing 5% FCS and the plates incubated for 24h at 37°C in a humidified CO\textsubscript{2} incubator. 50 μl supernatant was removed from the wells and transferred to a separate 96 well plate. 5 x 10\textsuperscript{3} CTLL-2 cells in 50 μl of RPMI 1640 containing 10% FCS were added to each well and the plates incubated overnight at 37°C in a CO\textsubscript{2} incubator. 0.5 μCi of tritiated thymidine was added to each well and after 4h, the cells were harvested onto filter mats and the radioactivity incorporated into DNA determined by
The cannula was positioned using a stereotaxic frame, at AP -0.8 and L 3.5 relative to bregma and inserted to V -8.0 (from the skull surface). [Taken from Paxinos and Watson, 1982]

Distances are in mm.
AP = anterior-posterior.
L = lateral.
V = ventral.
liquid scintillation counting. A titration of the International Standard for IL-1 (86/632), in which 1 unit corresponds to approximately 10pg of human IL-1, was included in each assay.

2.2.7.2 IL-6 Bioassay

IL-6 levels were measured by a proliferation assay using the murine hybridoma cell line B9 (Brakenhoff et al, 1987). For assay, samples were distributed as a two-fold dilution series in 100µl volumes in 96 well plates. 100 µl of B9 cells (5 x 10^4/ml) were added to each well in RPMI 1640 containing 5% FCS. After 72h of incubation at 37°C in a humidified CO₂ incubator 10µl of the tetrazolium salt, MTT (3, 4, 5-dimethyl thiazol-2-ys) -2, 5 diphenyltetrazolium bromide; 5mg/ml in PBS) was added to each well followed 4h later by 25µl of acid sodium dodecylsulphate (10% w/v SDS dissolved in 0.02M HCl). The plates were left in darkness at RT for approximately 1h and the absorbance of each well determined at 620nm using a Titertek ELISA reader. A titration of the NIBSC reference reagent for IL-6 (88/514), where 1 unit corresponds to approximately 200pg of human rIL-6, was included in each assay.

2.2.7.3 TNF Bioassay

TNF levels were determined in a cytotoxicity assay using murine L929 cells (Meager et al, 1987). For assay, 100µl of L929 cells (3 x 10^5/ml) in RPMI 1640 containing 5% FCS were distributed in 96 well plates. The plates were incubated overnight at 37°C in a humidified CO₂ incubator. In a separate plate, samples were distributed as a two-fold dilution series in 100µl volumes of medium containing actinomycin D (2µg/ml) and transferred to the plate containing cells. After 24h incubation, the supernatants were discarded and the cells
stained for 30 min with 100μl/well of 0.05% amido black. The stain was discarded and 100 μl of formalin/acetate fixative was added for 15 min. The fixative was discarded and the wells washed in running tap water. After air-drying, the stain was dissolved in 0.38% sodium hydroxide and the optical density of each well at 620nm was determined using a Titertek ELISA reader (Labsystems, ICN Flow). A titration of the NIBSC interim reference reagent for TNF (87/650), where 1 unit corresponds to approximately 25pg of human rTNF, was included in each assay.

2.2.8 Immunocytochemistry

2.2.8.1 Immunocytochemistry of brains with implanted probe

When brains were to be processed for immunocytochemistry the wire was removed from the U-loop after implantation of probes to allow easier sectioning. At days 1 and 7 after implantation, brains were carefully dissected out and snap frozen in isopentane cooled on liquid nitrogen. Cryostat sections (10μm thick) were collected on gelatin coated slides and those containing the probe region were processed for immunocytochemistry using the avidin-biotin peroxidase method (Vector Labs., Peterborough, U.K.). Incubations were performed at RT in a humidified box to prevent drying of the sections. PBS was used throughout for washing and 1%BSA/PBS was used for dilution of antibodies.

Fixation of sections was performed depending on the primary antibody in use (see below). Sections were incubated for 20 min with 50μl 0.2% normal horse serum in PBS to block non-specific binding of biotin conjugated horse immunoglobulins. Excess serum was drained from the slides and replaced with 50μl of the first antibody diluted in
PBS. Controls were included where the first antibody was replaced with myeloma protein of the same immunoglobulin subclass and concentration. Following a 1h incubation with the first antibody, the slides were washed for 10 min in 3 changes of PBS with constant stirring. Excess PBS was removed by draining and careful blotting of the slide and 50μl of the biotin conjugated anti-mouse IgG diluted 1:200 in PBS, was added to each section. After a 30 min incubation with this solution slides were washed and blotted as before. The avidin biotin peroxidase complex solution was mixed 30 minutes prior to use to permit complex formation. Washed sections were incubated with 50μl of this solution for 1h and washed as before.

Peroxidase activity was detected using 3’3-diaminobenzidine (DAB) (Sigma Chemical Co., Poole, Dorset), 0.5 mg/ml in PBS, 0.01% H₂O₂. The substrate solution was commonly prepared using 10ml aliquots of a 25mg/ml stock solution of DAB. Slides were incubated in the peroxidase substrate solution for 2 to 5 min depending on the staining intensity. Slides were then washed in tap water, and mounted in glycerol/PBS (50% v/v). Slides were viewed on a Leitz Dialux microscope and photographed with Ilford HP5 film.

The following first antibodies were used for immunocytochemical analysis of probe brain. MAb OX42 (Serotec, Oxford, UK) which stains the CR3 receptor on microglia, macrophages and neutrophils was used at a concentration of 1:100 after ethanol fixation for 1 min at RT. MAb ED1 (Serotec, Oxford, UK) for circulating monocytes and macrophages was used at a concentration of 1:100 after tissue fixation for 10 min with ice cold acetone. MAb 5.2E4 for GFAP staining of astrocytes was used at 1:500 after similar acetone fixation (Newcombe et al, 1986).
2.2.8.2 Immunocytochemical staining of microglia in culture

For purposes of immunocytochemical staining microglia were plated out in 8-well glass chamber slides (Labtech, Gibco BRL) at 2x10⁵ cells/well and maintained as described above. Prior to staining the slides were fixed with fresh 4% paraformaldehyde in PBS for 15 min at RT and, after washing 3 times in PBS, were blocked with 1%BSA/PBS for 1h at 37°C or overnight at 4°C. The plastic walls of the chamber slides were removed leaving the gasket in place to prevent mixing of reagents in each well. The avidin biotin peroxidase complex method as described above was used to stain for specific cell markers. Peroxidase activity was detected as for staining of sections. Slides were mounted in glycerol/PBS (50% v/v).

Microglia in culture were also labeled using fluorescently labeled acetylated low density lipoprotein (dil-ac-LDL; Molecular Probes Inc., Eugene, Oregon, U.S.A.) which binds to and is internalised via LDL receptors on microglia (Berg et al, 1986). Cells in chamber slides were incubated with 50μl dil-ac-LDL (1:20 in DMEM) per well for 4h at 37°C in the dark. The cells were then washed four times in PBS and fixed in fresh 4% paraformaldehyde at RT for 15 min. After washing, slides were mounted in glycerol/PBS (50% v/v).

2.3 RESULTS

2.3.1 Optimisation of the microglial isolation procedure

The procedure for isolation of microglia described in Hayes et al (1988) was further optimised to allow processing of larger quantities of tissue and in an attempt to improve yields. Speed of isolation and cost factors were also considered. The critical stage in determination of the
yield was deemed to be the rosetting stage when the mixed glial cell suspension was incubated with IgG-coated erythrocytes (EA). A number of variables in this step were adjusted. The possibility of increasing the density of cells in the suspension for centrifugation through Percoll was explored and this stage was performed in 50ml tubes instead of the usual 10ml tubes. In all these experiments comparisons were made with single preparations which were split and treated as indicated.

(1) Increasing the size of the tube used at the Percoll density gradient stage from 10ml to 50ml does not affect the separation and does not adversely affect yields. For two preparations, a and b:

<table>
<thead>
<tr>
<th>Tube vol. (ml)</th>
<th>Yield (x10^5 / g tissue)</th>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.1</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>2.7</td>
<td>8.6</td>
<td></td>
</tr>
</tbody>
</table>

(2) Yields were reduced on increasing the cell density at the layering stage. The advantages of reduced requirement for Percoll and increased speed of isolation do not outweigh the disadvantages of reduced yields.

<table>
<thead>
<tr>
<th>Yield (x10^5 / g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>usual density (see 2.2)</td>
</tr>
<tr>
<td>2 x density</td>
</tr>
</tbody>
</table>

(3) Yields were considerably improved by increasing the ratio of EA:glial cells during rosetting. Erythrocytes were opsonised as usual, ie. as a 2% (v/v) suspension of packed erythrocytes, and resuspended
to give 2%, 5% and 10% (v/v) suspensions of the opsonised erythrocytes (EA) for use.

<table>
<thead>
<tr>
<th>%EA (v/v)</th>
<th>Yield (x10^5 / g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6.2</td>
</tr>
<tr>
<td>5</td>
<td>13.4</td>
</tr>
<tr>
<td>10</td>
<td>23.6</td>
</tr>
</tbody>
</table>

However a correspondingly large amount of anti-erythrocyte antibody was required for these preparations.

(4) The possibility of performing the opsonisation of erythrocytes at a greater cell density to reduce the amount of anti-erythrocyte antibody required was explored. A comparison was made of two different batches of 10% EA suspensions: one in which a 10% v/v suspension of erythrocytes had been used for the opsonisation stage [10], and one prepared from EA that had been opsonised as a 2% v/v suspension which was then resuspended after washing to give a 10% v/v suspension [2-10]. The concentration of opsonising antibody was the same in both cases.

<table>
<thead>
<tr>
<th>EA</th>
<th>Yield (x10^5 / g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[10]</td>
<td>a 3.8 2.2</td>
</tr>
<tr>
<td>[2-10]</td>
<td>6.8 7.6</td>
</tr>
</tbody>
</table>

As one might expect, the efficiency of opsonisation of erythrocytes, and consequently the yield, was reduced by increasing the erythrocyte concentration to 10% during opsonisation.
In view of the results in (3) and (4) a compromise of a 4% EA suspension, opsonised as a 2% suspension, was chosen. On average this improved yields by approximately 50%.

(5) Yields were further improved by increasing the temperature for the rosetting stage.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temp</th>
<th>Yield (x10^5 / g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>4°C</td>
<td>4.8</td>
</tr>
<tr>
<td>60</td>
<td>RT</td>
<td>5.2</td>
</tr>
<tr>
<td>30</td>
<td>37°C</td>
<td>8.4</td>
</tr>
</tbody>
</table>

There was considerable ingestion of erythrocytes by microglia after 60 min at 37°C so the incubation time was reduced to 30 min to minimise this.

As a result of these studies the rosetting stage of the isolation procedure was revised as follows. An improvement in yields of approximately 70% was achieved.

(a) Rosetting stage - Hayes et al (1988)

The glial cell suspension was mixed with a 2% EA suspension in the ratio 1:1. After standing on ice for 30 min, the cells were spun down and kept on ice for a further 20 min after which the pellet was resuspended for the Percoll gradient step.

(b) Rosetting stage-Modified

The glial cell suspension was mixed with a 4% EA suspension (opsonisation of E as a 2% suspension) in the ratio 1:1. The mixture was incubated at 37°C for 30 min with occasional mixing. Following centrifugation the pellet was resuspended for the Percoll gradient step.
Microglia isolated using this method were labeled using OX42 MAb (Serotec) against CR3 and dil-ac-LDL which binds to and is internalised via the LDL receptor, according to the procedure described in 2.2.8.2. In Fig. 2.3 the typical morphology of microglia after several days in culture is demonstrated.

2.3.2 In vitro production of cytokines by rat microglia and Mφ

Isolated rat microglia, and resident peritoneal Mφ, both plated at $10^5$ cells/well were cultured in the presence of LPS (in a range from 1ng/ml to 10µg/ml) with and without IFN-γ (10 U/ml). After 48h the pooled supernatants of triplicate wells were assayed for IL-1, IL-6 and in some instances, TNFα content. Supernatants of unstimulated (medium treated) controls were assayed for IL-1 and IL-6 content. In the case of microglia this contained no detectable IL-1 and only background levels of IL-6 (200 pg/ml) (means of 5 experiments). Medium harvested from unstimulated Mφ contained 65 ± 36 pg/ml IL-1 (n=3) and 7.2 ± 2.0 ng/ml IL-6 (n=5).

LPS stimulation induced production of IL-1 and IL-6 by both microglia and Mφ. The pattern of IL-1 and IL-6 production with increasing LPS, was consistent but the actual amounts of the cytokines varied greatly between experiments. Levels of IL-1 and IL-6 produced by both cell types are illustrated in Figs. 2.4(a) and (b) respectively. These are the results of single experiments and illustrate the trends observed. In Fig. 2.4(a), there is a progressive increase in IL-1 production by microglia with increasing LPS dose up to 5µg/ml. Mφ produced a greater amount of IL-1 at all LPS concentrations, reaching a
Cells were plated in glass chamber slides and kept in culture for 4-5 days before staining with (a) OX42 (recognises the CR3 receptor; magnification x380) and (b) Dil-ac-LDL (binds to the LDL receptor; magnification x1070).
Fig. 2.4
Production of IL-1 and IL-6 by microglia and peritoneal macrophages in vitro.

(a) IL-1 production

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Microglia; +IFN-γ</th>
<th>Macrophage; +IFN-γ</th>
<th>Macrophage</th>
<th>Microglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>50</td>
<td>200</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>0.01</td>
<td>100</td>
<td>160</td>
<td>160</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>1</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>10</td>
<td>1500</td>
<td>1500</td>
<td>1500</td>
<td>1500</td>
</tr>
</tbody>
</table>

(b) IL-6 production

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Microglia; +IFN</th>
<th>Macrophage; +IFN-γ</th>
<th>Macrophage</th>
<th>Microglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>5</td>
<td>20</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>0.01</td>
<td>10</td>
<td>16</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>0.1</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
</tbody>
</table>

Microglia or peritoneal Mφ, plated at 10⁵ cells / well, were treated with LPS (1 ng/ml) in the presence and absence of IFN-γ (10 U/ml) for 48h. Supernatants of triplicate wells were pooled and assayed for IL-1 (pg/ml) and IL-6 (ng/ml). The results of a single experiment demonstrating observed trends are shown.
maximum at the lower concentration of 1μg/ml LPS [Fig. 2.4(a)]. The increase in IL-6 production was similar for both cell types in response to lower concentrations of LPS. However, at higher doses (1 - 10μg/ml), there was little further increase in IL-6 production by microglia whilst production by Mφ continued to rise [Fig. 2.4(b)].

Both microglia and Mφ produced greater amounts of IL-6 than IL-1 at all LPS concentrations. At an LPS concentration of 5μg/ml mean levels of IL-1 and IL-6 produced by microglia were 270 ± 244 pg/ml (n=4) and 17.4 ± 12.4 ng/ml (n=5), respectively. Mφ, treated with the same concentration of LPS, produced 448 ± 332 pg/ml (n=3) and 32.8 ± 17.6 ng/ml (n=8) of IL-1 and IL-6 respectively.

Figs. 2.4(a) and (b) also show the effect on IL-1 and IL-6 production of inclusion of 10 U/ml IFN-γ during stimulation by LPS. The presence of IFN-γ appears to reduce the level of both IL-1 and IL-6 production by microglia and Mφ in response to LPS. This effect is further illustrated by the mean amount of IL-1 [Figs. 2.5(a),(b)] and IL-6 [Figs. 2.5(a),(b)] produced in response to 1μg/ml LPS in the presence or absence of 10 U/ml IFN-γ (mean of 3 - 5 experiments). IFN-γ (10 U/ml) has itself no effect on IL-1 or IL-6 production in either microglia or Mφ. There is insufficient data to determine statistical significance but IFN-γ does appear to inhibit production by both cell types.

TNF and IL-1 content of supernatant from medium and LPS-treated microglia and Mφ cultures was determined in two experiments. Mφ treated with 100ng/ml LPS produced four times as much TNF as microglia treated in the same fashion (2000 and 500 pg/ml respectively). IL-1 production by the same cultures was also approximately four-fold higher in Mφ than in microglia (200 and 58 pg/ml respectively). Thus there is a good correlation between IL-1 and TNF production by both microglia and Mφ.
Fig. 2.5

Effect of IFN-γ on LPS-induced cytokine secretion by microglia and macrophages

(a) IL-1 production in vitro

(b) IL-6 production in vitro

In 3-5 experiments, cells (10^5/well) were incubated with LPS (1µg/ml) and/or IFN-γ (10 U/ml). Supernatants of triplicate wells were pooled and assayed for IL-1 (pg/ml) and IL-6 (ng/ml). Mean IL-1 (a) and IL-6 (b) concentrations +/- SEM are indicated.
2.3.3  *In vitro recovery of IL-1 and IL-6 across dialysis probes*

The efficiency of recovery of IL-1 and IL-6 across the dialysis membrane was assessed by placing dialysis probes in crude rat cytokine preparations and pumping through ACSF containing 0.01% rat albumin at flow rates of 0.5 and 2μl/min. Table 2.1 details the mean recoveries of IL-1 and IL-6 in 3-6 experiments (concentration in the dialysate is expressed as a % of the cytokine concentration outside the probe). Recovery of IL-1 was greater than that of IL-6, reflecting their relative molecular weights of 17.5 and 28 kDa respectively. Recovery was dependent on the flow rate, being approximately 3-4-fold higher at a flow rate of 0.5μl/min than that at a flow rate of 2μl/min. In the dialysis experiments a flow rate of 2μl/min was used. Note that in the following results, brain dialysate concentrations were not corrected for *in vitro* recoveries.

2.3.4  *In vivo production of IL-1 and IL-6 after mechanical insult to the brain*

CSF was collected from probe-implanted and control rats on days 0, 1, 2 and 7 following anaesthesia. Dialysate was collected from a separate group of freely moving rats 2-3h after implantation and at days 1, 2 and 7 following implantation. CSF and dialysate samples were assayed for IL-1 and IL-6 content.

2.3.4.1  *Microdialysis*

IL-1 and IL-6 levels (U/ml) in brain dialysate over 7 days are shown in Fig.2.6(a) and (b) as means for 4 and 7 animals respectively. IL-1 was significantly elevated in dialysate at 1 - 2 days post
Table 2.1

IL-1 and IL-6 recovery in vitro across the dialysis membrane

<table>
<thead>
<tr>
<th>Flow rate</th>
<th>IL-1</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 µl/min</td>
<td>3.5 +/- 0.3 % (3)</td>
<td>1.9 +/- 0.4 % (5)</td>
</tr>
<tr>
<td>2.0 µl/min</td>
<td>0.9 +/- 0.2 % (3)</td>
<td>0.5 +/- 0.2 % (6)</td>
</tr>
</tbody>
</table>

In 3 - 6 experiments dialysis probes were placed in crude rat cytokine preparations and ACSF containing 0.01% rat albumin was pumped through at 0.5 and 2 µl/min. Values indicated are mean recoveries +/- SEM (concentration in the dialysate is expressed as a % of the cytokine concentration outside the probe).
Dialysate from freely moving probe-implanted animals, collected 2-3 h after implantation (day 0) and at days 1, 2 and 7 thereafter, was assayed for IL-1 (a) and IL-6 (b) content. Values indicated are mean concentrations of IL-1 and IL-6 +/- SEM for 4 and 7 animals respectively.
implantation and was maximal at day 1 (17 ± 4 pg/ml; n = 4). IL-1 levels detected in dialysate dropped by day 7 [Fig. 2.6(a)]. In contrast to IL-1, IL-6 was detected in the dialysate at a level of 220 ± 60 pg/ml within 2-3h of implantation of the probe [Fig. 2.6(b)]. The level of IL-6 in dialysate 24h later was only marginally higher (240 ± 60 pg/ml) and thereafter the level detected fell to 35% and 17% of this at day 2 and 7 respectively [Fig. 2.6(b)].

2.3.4.2 CSF study

Note that both IL-1 and IL-6 were detectable in these bioassays at concentrations ≥1 pg/ml. CSF from all control animals (n=5) was found to contain no IL-1 or IL-6. IL-1 was not detectable in the CSF from probe implanted animals at all time points. IL-6 was detectable at a level of 20 ± 2 pg/ml in CSF within 2-3h of implantation [Fig. 2.7]. Within 24h there was a 50% increase in IL-6 in the CSF but, by day 2, levels fell to near the detectable limit.

2.3.5 Immunocytochemistry of probe brain

Species and subclass specific controls demonstrated virtually no background staining. There was an increase in the number and staining intensity of OX42+ cells, one day after implantation of the probe, compared to control tissue. These cells could be categorised according to their morphology:

(i) neutrophils, lobed nucleii
(ii) rounded macrophage
(iii) microglia, ramified (2-3 processes), often bipolar.

Neutrophils and rounded macrophage cells were identified close to the probe and beyond these, at a distance from the probe, were microglial cells [Fig. 2.8(a)]. Microglia of the injured hemisphere
IL-6 content of CSF following probe implantation

CSF collected from probe-implanted animals on days 0, 1, 2 and 7 was assayed for IL-1 and IL-6. Values indicated are mean IL-6 concentrations +/- SEM for 5 animals. IL-1 was below detectable limits (ie. < 1pg/ml) at all time points.
Immunocytochemistry of probe brain on day 1 (a,b; magnification x95) and day 7 (c,d; magnification x370): OX 42 (a,c) and ED1 (b,d) staining.

Increased OX 42 staining on day 1 (a) suggests activation of microglia. OX 42+ cells in the vicinity of the probe are rounded but, distal to the probe they have a ramified morphology. By day 7, OX 42+ cells have increased in number and staining intensity (c). ED1+ cells (b,d) have a rounded morphology and are found close to the probe. By day 7 (d) these cells have increased in number.
demonstrated a further increase in expression of OX42 by day 7 post-implantation [Fig. 2.8(c)].

ED1 staining identified round macrophage cells in the vicinity of the probe. The number of ED1+ cells increased in the 7-day period but there was no noticeable increase in staining intensity or change in morphology [Figs. 2.8(b) and (d)]. ED1+ cells were also found in the ventricles and were associated with small blood vessels close to the probe.

Astrocytes, positively stained with an anti-GFAP antibody, were identified throughout the brain. At day 1 there was no difference in the GFAP staining between the control and probe-damaged hemispheres in the same rat. The number of astrocytes and the intensity of their GFAP staining increased by day 7 post-implantation [Figs. 2.9(a) and (b)].

2.4 DISCUSSION

From results of in vitro recovery experiments, it is apparent that the 100,000 molecular weight cut off dialysis membrane presents considerable steric hindrance to the movement of IL-1 (17.5kDa) and IL-6 (28kDa). Under the flow rate conditions used here the IL-1 and IL-6 content of the dialysate represents 0.5-1% of the external concentration in vitro. In fact, in vivo recovery will be less than this as diffusion of molecules is impeded by cells and the diffusion pathway is prolonged (Benveniste, 1989). Despite the low recovery, this technique permits examination of the kinetics of cytokine production in vivo in response to injury. Dialysate levels demonstrated definite changes over the 7 day period following probe implantation. IL-1 was scarcely detectable on day 0 (2-3h after
GFAP staining of probe brain on day 1 (a) and on day 7 (b) by immunocytochemistry [magnification x535].

On day 1 (a) there is a low level of GFAP. By day 7 (b) astrocytes are strongly positive for GFAP and have increased in number.
implantation) but rose significantly within 24h. IL-1 levels were still high after 48h but had dropped considerably by day 7. In contrast, there was considerable IL-6 in the dialysate at day 0 and a slight increase on this level 24h later but by day 7 levels had declined. It is not however possible to pinpoint peak IL-6 production on the basis of these results.

The high level of IL-6 detected immediately following implantation is likely to be the result of damage to the BBB. Circulating levels of IL-1 and IL-6 are negligible in normal rats but within hours of surgical intervention there is a 100-fold increase in IL-6 (Van Gool et al, 1990). According to Benveniste et al (1984) the integrity of the BBB is re-established within 30 min of insertion of a 300 - 600μm diameter probe, such that it is impermeable to an inert amino acid. Measurement of local cerebral blood flow, an indicator of tissue reaction caused by implantation of the probe, indicates that disturbances in tissue metabolism are negligible 24h after implantation (Benveniste, 1989). Since the integrity of the BBB is restored by day 1 post-implantation and since IL-1 has a half-life of only 30 min in the circulation (Newtown et al, 1988), the IL-1 detected in the dialysate on day 1 does not originate primarily in the circulation. It is unlikely that CSF in contact with the dialysis membrane contributed significantly to cytokine levels in the dialysate since IL-1 and IL-6 levels in the CSF were less than a tenth of those in the dialysate, which in turn (due to poor recovery across the membrane) reflects less than 1% of actual interstitial concentrations. It is reasonable to conclude, therefore, that IL-1 in the dialysate 24-48h after probe implantation is due to local production of the cytokine at the lesion site. Levels of IL-6 in the CSF after implantation of the probe mirror the temporal pattern of IL-6 in
the dialysate and may indicate diffusion of the cytokine from the injury site into the CSF.

Giulian et al (1988a) measured IL-1 secretion, during a 15h incubation in medium at 37°C, by tissue biopsies taken at 0, 1, 2, 5 and 20 days after infliction of a stab wound to the cerebral cortex and found that peak secretion occurred at 2 days post-injury. Thus these findings are in agreement with those described here in which microdialysis techniques were employed. An alternative method for sampling the extracellular fluid in the CNS in vivo makes use of the push-pull cannula (Benveniste, 1989). This cannula is similar in construction to those used in microdialysis but it lacks a dialysis membrane. ACSF is pumped in one side and fluid is pumped out at the same rate from the other side of the cannula. Without a dialysis membrane, recovery is vastly improved and this method is consequently more sensitive than microdialysis. The cannula is much smaller than that used here and is therefore less injurious to the tissue. It can also be positioned accurately in areas of interest in the brain. The push-pull method is however technically difficult as, if there is any pressure difference between the inwards and outwards pumps, fluid may accumulate at the site of insertion or tissue may be pulled into and block the cannula. The microdialysis method used here has the advantage that it provides a cell-free dialysate. Samples collected by the push-pull method will contain cells and these may produce cytokines. The samples must therefore be centrifuged as soon as possible to remove cells.

The assays used here to measure IL-1 and IL-6 content of dialysate or culture medium provide indications of the levels of biologically active cytokine. ELISA and IRMA cytokine assays have a major drawback in that they do not distinguish between for example
cytokine which has been sequestered by binding to a soluble receptor form and is therefore inactive, and free active cytokine. Furthermore the presence of naturally occurring antagonists such as IL-1ra will be reflected in a decrease in the detectable amount in a biological assay for IL-1 but not in an ELISA for this cytokine.

Giulian et al (1988a) report that peak IL-1 secretion coincides with the appearance of large numbers of mononuclear phagocytes at the site of the stab wound. In this study, the temporal patterns of IL-1 and IL-6 detection in the dialysate suggests that cells present at the lesion site 24h after implantation of the probe are the most likely source of cytokines.

An immunocytochemical analysis of probe-damaged tissue was performed using OX42 which recognises microglia, circulating macrophages and neutrophils, and ED1, which recognises monocytes and most macrophage subpopulations but not microglia. On day 1 post-implantation there was increased OX42 expression in the damaged area and in adjacent apparently unaffected areas.

Most of these OX42+ cells, including those near the lesion, are microglia as only a small proportion of these are ED1+. There is evidence to suggest that microglia can be induced to re-express ED1 antigen (Graeber et al, 1990), so some of these ED1+ cells may be of microglial origin. Whilst the accumulation of these inflammatory cells is evident during peak cytokine secretion, by day 7 when secretion has decreased, their number has further increased. Using an anti-GFAP antibody, astrocytes, evident throughout the brain in probe-damaged and contralateral hemispheres on day 1, were observed in increased numbers and with increased GFAP expression by day 7. Thus the astrocyte response to injury appears after peak cytokine production. Hozumi et al (1990) report that GFAP+ glia increase in
number and staining intensity following a forebrain wound, reaching a maximum after 7 days, which is in agreement with the observations made here.

Both microglia and astrocytes have been shown to produce IL-1 and TNF in vitro. Microglia demonstrate the greater capacity for production of both these cytokines (Giulian et al, 1986a; Sawada et al, 1989). If as the evidence seems to suggest, microglia are the major source of cytokines produced in response to CNS injury, why does cytokine production fall as the number of microglia / macrophages continues to increase by day 7? Though it can be a problem in tissue microdialysis, blocking of the probe by adherence of cells is not likely to be the explanation since the decrease in cytokine levels in the dialysate is mirrored by decreased IL-6 in the CSF. It is possible that cytokine synthesis decreases between day 2 and day 7 due to negative feedback mechanisms. Injection of IL-1 into mammalian brain induces reactive astrogliosis (Giulian et al, 1988a). Thus a plausible scenario would be that microglia which invade/proliferate in damaged CNS tissue are the major source of IL-1 and IL-6 detected at elevated levels within 24h and subsequent proliferation of astrocytes is in response to this IL-1.

The potential of microglia to produce IL-1 and IL-6 has been demonstrated here in vitro. Microglia were isolated from the brains of 30 day-old rats. At this age microglia in situ have the ramified morphology of adult cells. Parallel studies were performed on peritoneal macrophages. On stimulation with LPS both microglia and macrophages secrete IL-1, IL-6 and TNF. Peritoneal macrophages secrete higher levels of these cytokines than do microglia. Interestingly, whilst IFN-γ itself has no effect on cytokine production by microglia or macrophages it inhibits the response to LPS. Perhaps,
in vivo. IFN-γ produced by activated T cells at a site of inflammation acts in a similar way to downregulate cytokine production by activated microglia and infiltrating macrophages. Such a mechanism may explain the drop in cytokine levels detected between day 2 and day 7 in the microdialysis study.

In a study of cellular responses to mechanical injury to the mammalian brain, immunocytochemical evidence suggests that the major producers of IL-1 and IL-6, secreted at the lesion site, are microglia. With breaching of the BBB, monocytes and other blood cells infiltrate the tissue and the presence of these cells and an increased number of activated microglia is observed within 24h of injury. Much later there is a proliferative response on the part of astrocytes, apparently induced by IL-1 secreted by activated microglia and infiltrating macrophages. Increases in IL-1 and IL-6 have also been observed during viral infections (Frei et al, 1989; Le and Vilcek, 1989) and IFN-γ, TNF and IL-1 have all been identified in MS brain tissue (Hofman et al, 1989; Wucherpffenig et al, 1991).

Almost every kind of insult to the CNS including trauma, infection, infarction and demyelination precipitates a response by amoeboid microglia. These cells migrate to the site of injury, proliferate, secrete cytokines and engulf tissue debris. Some of these responses have been illustrated here.

This study demonstrates the important role of microglia and the cytokines which these cells secrete in the CNS response to injury and illustrates the potential of the microdialysis technique for continuous monitoring of cytokine production in the CNS in response to injury.
CHAPTER 3

FUNCTIONAL PROPERTIES OF MICROGLIA AND THEIR REGULATION IN VITRO BY CYTOKINES

3.1 INTRODUCTION

Substantial evidence implicates microglia in both inflammatory and demyelinating events in multiple sclerosis (MS) (Hayes et al, 1987; Prineas et al, 1984). Functional properties of microglia relevant to these events, and to macrophage function in general, have been investigated in vitro. These include the phagocytic potential of the cells (expression of Fc receptors), the capacity to present antigen to T-cells (MHC class II expression) and the production and release of oxygen radicals and lysosomal hydrolases. What effect do cytokines have on these functional properties of macrophages and do microglia differ from other macrophage populations in this respect? In an in vitro study, isolated rat microglia and peritoneal macrophages were treated with individual cytokines (IFN-γ, TNFα, IL-1, IL-3, GM-CSF, IL-4 and TGFβ1) or LPS and the effect on these functional properties assessed.

Cells in vivo are likely to be exposed to more than one cytokine at any given time or over a period of time. In a further study cells were treated with combinations of two cytokines, added either simultaneously or consecutively, to elucidate some of the interactions between cytokines and to investigate whether these depended on the timing of additions. Of particular interest were cytokines capable of down-regulating a response and, in view of their reported inhibitory properties (Te Velde et al, 1990; Abramson and Gallin, 1990;
Tsunawaki et al, 1988; Schluesener, 1990), these studies focused on IL-4 and TGFβ1.

Knowledge of the interactions between the neuroendocrine and immune systems suggests that other mediators may influence cell behaviour: for example, glucocorticoids produced in response to stimulation of the hypothalamic-pituitary-adrenal axis (HPA-axis) and neurotransmitters of the sympathetic nervous system (Leonard et al, 1991). The potential of mediators such as noradrenaline and the glucocorticoids dexamethasone and corticosterone to influence the activation state of microglia and macrophage has been investigated.

3.2 MATERIALS AND METHODS

3.2.1 Cytokines and other mediators

Stock solutions of all cytokines were prepared under sterile conditions at the following concentrations and stored as described. Recombinant rat IFN-γ (1 x 10^5 U/ml in 1% BSA/PBS; Holland Biotechnology, The Netherlands) and recombinant mouse IL-1α (10 ng/ml in 0.1% BSA/PBS; Genzyme, UK) were stored at -70°C. Recombinant mouse IL-1β (5 μg/ml in 1% BSA/PBS; British Biotechnology, Cowley, Oxford, U.K.), recombinant mouse IL-3 (10^5 U/ml in 1% BSA/PBS; Gibco BRL, U.K.) and recombinant mouse GM-CSF (5 x 10^3 U/ml in 1% BSA/PBS; New Brunswick Biologicals, Hatfield, Herts., U.K.) were stored at -20°C. Recombinant human TNFα (2 x 10^4U/ml in 1% BSA/PBS), natural human TGFβ1 (1ng/ml in 0.1% BSA/4mM HCl) and recombinant murine IL-4 (5 x 10^5 U/ml in 0.1% BSA/PBS), all from British Biotechnology Ltd., were stored at
4°C. LPS (1 mg/ml in EBSS; Sigma Chemical Co., Poole, Dorset, U.K.) was stored at -20°C.

A stock solution of 10 mM noradrenaline (NA; Sigma) in 10 mM ascorbic acid was filter-sterilised and stored at 4°C for up to 2 weeks. Stock solutions of 10 mM dexamethasone (DEX; Sigma) and 10 mM corticosterone (CS; Sigma) were prepared in dimethylsulphoxide (DMSO; Sigma) and stored at 4°C. Dilutions of DEX and CS in DMEM+10%FCS were filter-sterilised before use.

3.2.2 Isolation of microglia and peritoneal macrophages

Microglia and peritoneal macrophages (Mφ) were isolated as described in chapter 2 (2.2.1 - 2.2.4). In order to test whether the rosetting stage of the microglial cell isolation activated the cells or influenced their response to cytokines in some way, freshly isolated Mφ were subjected to a similar rosetting stage. A suspension of Mφ at approximately 10^6 cells/ml in EBSS was mixed with an equal volume of 4% EA, prepared as described in 2.2.3.3. The incubation and separation over Percoll and lysis of erythrocytes were carried out as per the microglial cell isolation.

All cells were counted and plated in 96 well plates (Nunc, Gibco BRL) at 0.5x10^5 or 10^5 cells/well in DMEM + 10%FCS and maintained at 37°C in a 95% air/5% CO2 incubator. Media was changed after 1 and 2 days and cells were maintained in culture for 5 days prior to cytokine treatment.
3.2.3 Treatment of cells with cytokines and other mediators

All incubations were in DMEM + 10% FCS which included indomethacin (1μg/ml; Sigma, U.K.). Indomethacin was present to inhibit LPS- or cytokine-induced synthesis of prostaglandins which may down-regulate cytokine effects.

3.2.3.1 Incubation of cells with individual cytokines

On the fifth day after plating, the cells (microglia and Mφ) were treated with varying concentrations of individual cytokines. Assays were performed in triplicate wells. All cytokines were incubated with the cells for 72h before assaying for MHC class II or Fc receptor expression or superoxide anion production.

3.2.3.2 Incubation of cells with combinations of cytokines

In initial experiments, cells were treated for 72h with two cytokines present simultaneously. In later experiments, comparisons were made of simultaneous and sequential additions of the cytokines. Thus, 4-6 days after plating, cells were subjected to two 72h treatments with medium alone, an individual cytokine or a combination of cytokines. Treatments were performed in duplicate. After the second 72h period cells were assayed for Fc receptor and MHC class II expression.

3.2.3.3 Incubation of cells with IFN-γ and NA, DEX or CS

All studies on the effect of NA, DEX or CS on IFN-γ activation of MG or Mφ 4 - 6 days after plating, involved simultaneous treatment with the mediators. Cells were treated for 72h with medium, IFN-γ,
NA, DEX or CS alone or IFN-γ plus NA, DEX or CS. All treatments were performed in duplicate.

Incubations containing NA, and medium and IFN-γ-treated controls for these, also contained 100 nM ascorbic acid which prevented the otherwise rapid degradation of NA in solution. After the 72h incubation cells were assayed for Fc receptor and MHC class II expression.

3.2.4 ELISA for MHC Class II

After incubation with cytokines, medium was flicked out and plates rinsed in warm PBS (200μl/well) and fixed for 15 min at RT with 4% paraformaldehyde in PBS (150μl/well). Plates were then washed four times with PBS and stored overnight at 4°C in 1% BSA/PBS to block. BSA/PBS was flicked out and cells incubated for 60 min at 37°C with 50μl of the first antibody, OX6 (Serotec Ltd., Oxford, U.K.) ascites fluid, specific for MHC Class II, diluted 1:100 in BSA/PBS. As a subclass-specific control, cells were incubated in identical conditions with mouse IgG1 myeloma protein (Sigma, U.K.) diluted 1:100 in 1% BSA/PBS. After incubation with the first antibody, plates were washed six times with 1% BSA/PBS (200μl/well). Cells were then incubated for 60 min at 37°C with 50μl/well of peroxidase-conjugated rabbit anti-mouse immunoglobulin diluted 1:1000 in 1% BSA/PBS after which they were washed six times by flooding with PBS and finally once in tap water. 50μl substrate solution [1mg/ml 2, 2'‐azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) in freshly prepared 0.05M phosphate-citrate buffer containing 0.014% H₂O₂ (reagents from Sigma)] was added per well and plates were incubated at 37°C for 30 min. Plates were then read in a Titertek reader (Labsystems Ltd.,
Basingstoke, Hants., U.K.) at 405 nm. Results were analysed by computer (ELISA+ software, Meddata Inc., N.Y., USA) and mean OD 405nm for duplicate or triplicate wells were calculated.

3.2.5 **Fc receptor (FcR) assay**

The pseudoperoxidase activity of opsonized erythrocytes (EA) bound and ingested by cells in culture was used as a measure of FcR expression (Jungi, 1985) since a good correlation has been observed between the relative number of Fc receptors expressed and phagocytosis of EA by Mφ following cytokine stimulation (Becker and Daniel, 1990).

3.2.5.1 **Opsonisation of erythrocytes for FcR assay**

EA was prepared as for microglial cell isolation, described in Chapter 1. EA for use in the FcR assay was resuspended at 2% in EBSS.

3.2.5.2 **Incubation of cells with EA/E**

After incubation with cytokines, medium was flicked out and plates washed once by flooding with PBS at 37°C. A 2% EA suspension in EBSS, prepared as described above, or a 2% suspension of unopsonised erythrocytes (E) in EBSS was mixed with an equal volume of 1% BSA/PBS to give a final concentration of 1% EA or E. All assays contained test and control wells which had been treated identically. Test wells received 50µl 1% EA while control wells received 50µl 1% E. Plates were incubated for 90 min at 37°C and the number of opsonised and unopsonised erythrocytes bound and/or ingested was determined.
3.2.5.3 Binding and/or ingestion of EA/E

EA or E which had not been bound or ingested by the cells after the incubation period were washed off by flooding plates six times in PBS (37°C) and blotting. 100μl of 0.3% SDS (aq) was added per well to solubilise the cells and release the pseudoperoxidase activity of bound and ingested erythrocytes.

3.2.5.4 Preparation of a standard curve

A standard curve was prepared in quadruplicate by serial dilution of the 1% EA or E suspensions in PBS. 100μl of EA or E in the first well of a 96 well V-bottomed plate was mixed by pipetting and 50μl transferred to the second well containing 50μl of PBS. This was repeated across the plate resulting in a 1 in 2 dilution series. The standard curve plate was covered and incubated for 90 min at 37°C with the test plates. After this period it was centrifuged for 10 min at 140g in a Sorvall RT6000B centrifuge (Du Pont Ltd., Stevenage, Herts., U.K.) using a 96 well plate carrier, to pellet the erythrocytes. The supernatant was flicked out and the cells were solubilised in 100μl of 0.3% SDS (aq) and transferred to a flat-bottomed 96-well plate before detection of pseudoperoxidase activity.

3.2.5.5 Detection of pseudoperoxidase activity

The substrate used for detection, diaminobenzidine tetrahydrochloride (DAB; Sigma, U.K.) was stored as a 25 mg/ml stock solution in PBS at -20°C. The substrate solution was prepared fresh by mixing 8 ml of stock DAB with 500 ml PBS and adding 2ml hydrogen peroxide. 200μl of this solution was added per well and the absorbance of the pseudoperoxidase catalysed product (OD 450nm) was
read at a noted time (0 - 15 min). The standard curve plate was treated identically and read at the same time(s).

3.2.5.6 Calculation of results

Absorbance readings were analysed by computer (ELISA+ software, Meddata Inc., N.Y.). For each chosen reaction time the absorbance readings of the standard curve were meaned and plotted against the number of erythrocytes present.

The volume of erythrocytes following centrifugation at 200g of human blood, termed the packed cell volume (PCV), is 40.6% of the total volume and there are $4.8 \times 10^{12}$ erythrocytes per litre of blood. Hence a 1% (v/v) suspension of packed erythrocytes contains $1.18 \times 10^{10}$ erythrocytes per 100ml and 50μl of 1% EA or E contains $5.91 \times 10^6$ red cells. For the linear portion of the standard curve erythrocyte number was plotted against optical density of peroxidase catalysed substrate. Examples of typical standard curves are depicted in Fig. 3.1.

Absorbances of triplicate wells were meaned and, using the standard curve, converted to actual number of erythrocytes per well. To calculate the specific Fc receptor-mediated binding and ingestion, E binding (non-specific) was subtracted from EA binding (total specific and non-specific) (EA-E). Results are expressed as increases or decreases in EA-E over basal levels.

3.2.6 Assay for superoxide anion production

Superoxide anion production was measured by the method of Pick and Mizel (1981). Adherent cells were washed in DMEM without phenol red (Gibco BRL Ltd., U.K.) and incubated at 37°C with ferricytochrome c (100μM; Sigma Chemical Co.) and phorbol-12-
Mean absorbance (of quadruplicate wells) of pseudoperoxidase catalysed product was plotted against number of erythrocytes per well. Standard curve plates were read at the same times (following addition of DAB substrate) as test plates and the appropriate standard curve used to convert test absorbances to erythrocyte number.
myristate-13-acetate (PMA, 20nM; Sigma) in this medium. Superoxide dismutase (300 U/ml; Sigma) was included for controls. Plates were read at 0 and 60 min at 550 nm in a Titertek reader. Mean absorbances were calculated and the amount of superoxide anion (O$_2^-$) produced was calculated according to the following equation:

$$\text{nmol O}_2^- \text{produced/well/hr} = \frac{(D \times 100)}{6.3}$$

where $D = \text{Test OD}_{550} - \text{Control OD}_{550}$

3.2.7 MTT assay

Cell viability was assessed by the capacity of the cells to convert the tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2,yl]-2,5-diphenyltetrazolium bromide) to a coloured product.

After incubation of cells with cytokines, 30µl of a 5mg/ml solution of MTT in PBS was added to each well and plates were incubated for 4h at 37°C. 25µl of acid sodium dodecylsulphate (10% w/v SDS in 0.02 M HCl) was added to each well and plates kept in darkness at RT for approximately 1h before reading the plates at 620nm using a Titertek ELISA reader. The mean OD 620nm of duplicate or triplicate wells was calculated.

3.2.8 Statistical Analyses

Statistical significance was determined using the students 1 and 2 sample t-tests. A significance level of $P \leq 0.05$ was taken to be statistically significant.
3.3 SECTION A

3.3.1 Results (A)

Initial studies were performed to determine the effect of individual cytokines (IFN-γ, TNFα, IL-1, IL-3, GM-CSF, IL-4, TGFβ1) and LPS on functional properties of rat microglia and Mϕ, namely Fc receptor and MHC class II expression and, in some instances, superoxide production. The procedure followed and results obtained are detailed in this section.

3.3.1.1 Effect of cytokines on cell number

Microglia and Mϕ (10^5/well) were cultured for 72h in the presence of cytokines as indicated in table 3.1. The effect of these treatments on cell number was assessed using the MTT assay. The values in Table 3.1 represent mean optical densities read at 620nm ± SEM. At the chosen doses all of the cytokines and LPS did not significantly affect cell number.

3.3.1.2 Binding/ingestion of EA by microglia and Mϕ is sensitive to a wide range of cytokines and to LPS

Microglia and Mϕ (0.5x10^5 or 10^5 cells /well as indicated) were cultured for 72h in the presence of cytokines: IFN-γ (1-10 U/ml), TNFα (1-300 U/ml), IL-1α (10-200 pg/ml), IL-1β (100-500 pg/ml), GM-CSF (1-100 U/ml), IL-3 (1-100 U/ml), IL-4 (10-50 U/ml), TGFβ1 (1-5 ng/ml) and LPS (10-250 ng/ml). The effect of these treatments on Fc receptor expression on microglia is illustrated in Fig. 3.2(a) and that on Mϕ in Fig. 3.2(b). Values are increases of [EA-E] over that observed in the absence of cytokine (see 3.2.5.6).
Table 3.1

Effect of cytokines and LPS on cell number

<table>
<thead>
<tr>
<th>Cytokine (U/ml)</th>
<th>0</th>
<th>1.0</th>
<th>2.5</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG</td>
<td>0.16 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.18 ± 0.01 (5-17)</td>
</tr>
<tr>
<td>Mφ</td>
<td>0.16 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.16 ± 0.01 (7-15)</td>
</tr>
<tr>
<td>TNF-α (U/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG</td>
<td>0.19 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>0.20 ± 0.02 (3)</td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>0.15 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>0.18 ± 0.01 (7-10)</td>
<td></td>
</tr>
<tr>
<td>IL-1α (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>0.18</td>
<td>0.19</td>
<td>0.20</td>
<td>(2)</td>
</tr>
<tr>
<td>GMCSF (U/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG</td>
<td>0.14 ± 0.01</td>
<td>0.15 ± 0.02</td>
<td>0.16 ± 0.02</td>
<td>0.15 ± 0.01 (5)</td>
</tr>
<tr>
<td>Mφ</td>
<td>0.16 ± 0.01</td>
<td>0.15 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.02 (8-14)</td>
</tr>
<tr>
<td>LPS (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG</td>
<td>0.17 ± 0.02</td>
<td>0.18 ± 0.03</td>
<td>0.20 ± 0.03 (3)</td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>0.15 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.19 ± 0.01 (9)</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean absorbances at 620nm ± SEM where appropriate (ie. for n≥3). Figures in parentheses are n values.
Fig. 3.2
Effect of cytokines on Fc receptor expression on microglia and peritoneal macrophages.

(a) Microglia

(b) Peritoneal Macrophages

Microglia (a) and peritoneal macrophages (b) were incubated for a period of 72h with cytokines as indicated. Macrophages were plated at $10^5$ / well and microglia were at $10^6$ / well in all cases with the exception of those treated with IL-4 which were at $0.5 \times 10^6$ /well. Values represent mean increases ($n= 5-20$) in the number of opsonised erythrocytes bound via the Fc receptor ([EA-E], $x 10^3$ rbc / well) +/- SEM. Increases / decreases are significant (P≤0.05) with a few exceptions (Δ).
Thus Fc receptor expression on both cell types is sensitive to a wide range of cytokines. IFN-γ, TNFα, IL-1α and β, IL-3, GM-CSF, IL-4 and LPS are all stimulatory resulting in increased FcR expression. Although basal levels of expression of Fc receptors varied between cell preparations, the increases observed upon treatment with cytokines were consistent. Constitutive FcR expression [EA-E] in the absence of cytokine was in general higher in microglia than in Mφ (100 ± 14 x 10^3 compared with 37 ± 5 x 10^3 RBC per 10^5 cells plated).

IFN-γ induced a much greater increase in FcR expression in microglia than in Mφ, and in both cases the increases were significant (P<0.05). TNFα significantly increased FcR expression by both cell types, although the effect was more pronounced with microglia than with Mφ. FcR expression in microglia decreased significantly at 300 U/ml TNFα compared with 100 U/ml (P<0.05).

Stimulation of microglia and Mφ with LPS produced a similar pattern, with significant increases in FcR expression, and in both cases the decrease in expression between 50 ng/ml and 250 ng/ml LPS was significant. Treatment of microglia with 50 pg/ml IL-1α produced a significant increase in FcR expression in microglia (P<0.01) approximately twice that found in Mφ.

There is a significant increase in FcR expression in both microglia and Mφ on treatment with IL-4 at 10 U/ml and a slightly greater response to IL-4 at 50 U/ml. [Note that the microglial response to IL-4 indicated in Fig. 3.2(a) represents that of microglia plated at half the cell density used for all other assays.] Of all the cytokines tested here, only TGFβ1 (5 ng/ml) reduces FcR expression relative to the basal level. Again the response of microglia to this cytokine is greater than that of Mφ.
Microglia therefore, proved more responsive than Mϕ to IFN-γ, TNFα, LPS, IL-4, IL-1α and β and TGFβ1 with respect to FcR expression. It would appear that the extent of this microglial response varies much more between cytokines than does the Mϕ response.

3.3.1.3 IFN-γ, TNFα and LPS increase non-specific as well as specific FcR-mediated binding / ingestion of EA by Mϕ but not microglia

The effect of cytokines on non-specific binding [E] as opposed to specific Fc receptor mediated binding [EA-E] was observed in both cell types (Table 3.2). Microglia demonstrated no change in the non-specific binding/ingestion of erythrocytes on treatment with IFN-γ, TNFα, IL-1α, IL-1β, GM-CSF, IL-3 and LPS. However Mϕ demonstrated increased E-binding/ingestion on treatment with IFN-γ, TNFα and LPS. In the case of IFN-γ this increase was comparable to the increase in FcR-mediated binding/ingestion induced by IFN-γ [Fig. 3.2(a) and (b)]. LPS and TNFα -induced increases in E binding/ingestion by Mϕ were less than the corresponding increases in FcR-mediated binding/ingestion.

3.3.1.4 Effect of a range of cytokines and LPS on MHC class II expression on microglia and Mϕ

Microglia and Mϕ were cultured with cytokines as above. Expression of MHC class II was assessed and the results are detailed in Table 3.3. All microglial cultures contained 10^5 cells/well except those treated with IL-4 when 0.5 x 10^5 cells were plated/well.

Microglia consistently have less constitutive MHC class II expression than Mϕ. After 7-8 days in culture (the last 72hr including 1 µg/ml indomethacin in the culture medium), microglia expressed a level of MHC class II 4-fold less than in Mϕ [0.02 ± 0.01 and 0.08 ± 0.01]
Table 3.2

Effect of cytokine treatment on binding / ingestion of unopsonised erythrocytes (E) by peritoneal macrophages and microglia.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Concentration (U/ml)</th>
<th>Mϕ</th>
<th>MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>0.1</td>
<td>38 ± 8 (15)*</td>
<td>2 ± 1 (13)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>53 ± 10 (20)*</td>
<td>2 ± 1 (17)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>17 ± 10 (21)*</td>
<td>3 ± 2 (20)</td>
</tr>
<tr>
<td>TNFα</td>
<td>1.0</td>
<td>19 ± 4 (8)*</td>
<td>1 ± 1 (7)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>32 ± 7 (12)*</td>
<td>4 ± 1 (16)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>21 ± 8 (11)*</td>
<td>6 ± 2 (21)</td>
</tr>
<tr>
<td>IL-1α</td>
<td>10.0</td>
<td>12 ± 6 (5)</td>
<td>1 ± 0 (9)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>6 ± 2 (13)</td>
<td>1 ± 0 (9)</td>
</tr>
<tr>
<td></td>
<td>200.0</td>
<td>7 ± 5 (12)</td>
<td>1 ± 0 (6)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>10.0</td>
<td>3 (2)</td>
<td>1 ± 0 (4)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>7 ± 1 (3)</td>
<td>2 ± 2 (3)</td>
</tr>
<tr>
<td></td>
<td>200.0</td>
<td>10 ± 3 (5)</td>
<td>1 ± 1 (7)</td>
</tr>
<tr>
<td>GMCSF</td>
<td>1.0</td>
<td>12 ± 1 (3)</td>
<td>0 ± 0 (6)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>10 ± 4 (4)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>6 ± 4 (4)</td>
<td>0 ± 1 (7)</td>
</tr>
<tr>
<td>LPS</td>
<td>1.0</td>
<td>3 ± 5 (9)</td>
<td>1 ± 1 (3)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>22 ± 6 (12)*</td>
<td>2 ± 1 (13)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>16 ± 4 (11)</td>
<td>2 ± 1 (14)</td>
</tr>
</tbody>
</table>

Values are mean increases ± SEM (over basal levels) in number of unopsonised erythrocytes (E; x10⁻³/well) bound or ingested by microglia (MG) or peritoneal macrophages (Mϕ) [1x10⁵/well]. Where values were significantly different from zero (P<0.05), this is indicated by an asterix (*). Figures in parentheses are n values.
**Table 3.3**

Effects of cytokines and LPS on expression of MHC class II on microglia (MG) and peritoneal macrophages (Mφ), measured by ELISA.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Concentration (Units/ml)</th>
<th>Microglia (MG)</th>
<th>Peritoneal macrophages (Mφ)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IFN-γ</strong></td>
<td>0</td>
<td>0.01 ± 0.01 (24)</td>
<td>0.08 ± 0.02 (14)*</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.08 ± 0.02 (14)*</td>
<td>0.21 ± 0.03 (23)*</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.21 ± 0.03 (23)*</td>
<td>0.32 ± 0.05 (24)*</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.32 ± 0.05 (24)*</td>
<td>0.45 ± 0.06 (24)*</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>0</td>
<td>0.01 ± 0.01 (11)</td>
<td>0.00 ± 0.02 (8)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.00 ± 0.02 (8)</td>
<td>0.04 ± 0.01 (10)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>0.04 ± 0.01 (10)</td>
<td>0.00 ± 0.02 (6)</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>0.00 ± 0.02 (6)</td>
<td>0.00 ± 0.02 (6)</td>
</tr>
<tr>
<td><strong>IL-1α</strong></td>
<td>0</td>
<td>0.02 ± 0.03 (7)</td>
<td>0.03 ± 0.02 (6)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>0.03 ± 0.02 (6)</td>
<td>0.03 ± 0.04 (3)</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>0.03 ± 0.04 (3)</td>
<td>0.01 ± 0.03 (6)</td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
<td>0</td>
<td>0.05 ± 0.02 (8)</td>
<td>0.05 ± 0.02 (8)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>0.05 ± 0.02 (8)</td>
<td>0.06 ± 0.02 (7)</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>0.06 ± 0.02 (7)</td>
<td>0.15 ± 0.04 (6)*</td>
</tr>
<tr>
<td><strong>GMCSF</strong></td>
<td>0</td>
<td>0.03 ± 0.01 (6)</td>
<td>0.03 ± 0.01 (6)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.03 ± 0.01 (6)</td>
<td>0.03 ± 0.01 (6)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>0.03 ± 0.01 (6)</td>
<td>0.03 ± 0.01 (6)</td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td>0</td>
<td>-0.01 ± 0.05 (8)</td>
<td>0.06 ± 0.07 (3)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.06 ± 0.07 (3)</td>
<td>-0.02 ± 0.06 (8)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>0.06 ± 0.07 (3)</td>
<td>-0.02 ± 0.06 (8)</td>
</tr>
<tr>
<td><strong>TGFβ1</strong></td>
<td>0</td>
<td>0.15 ± 0.05 (9)</td>
<td>-0.08 ± 0.02 (7)*</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>-0.08 ± 0.02 (7)*</td>
<td>0.15 ± 0.05 (9)</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>0.03 ± 0.02 (6)</td>
<td>0.06 ± 0.02 (5)*</td>
</tr>
<tr>
<td><strong>LPS</strong></td>
<td>0</td>
<td>0.01 ± 0.02 (9)</td>
<td>0.00 ± 0.02 (8)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.00 ± 0.02 (8)</td>
<td>0.02 ± 0.01 (8)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>0.02 ± 0.01 (8)</td>
<td>0.02 ± 0.01 (8)</td>
</tr>
<tr>
<td></td>
<td>250.0</td>
<td>0.03 ± 0.01 (8)</td>
<td>0.03 ± 0.01 (8)</td>
</tr>
</tbody>
</table>

Values represent mean absorbances at 405nm ± SEM. Figures in brackets are number of experiments. With the exception of IL-4-treated microglia which were plated at 0.5x10^5 cells / well, cells were plated at a density of 10^5 cells /well. All treatments were for a period of 72h. Where absorbances were significantly different from that of the medium treated controls (0) (P≤0.05), this is indicated with an asterix (*).
OD units, respectively (P<0.05)]. Treatment with IFN-γ produced significant increases in MHC class II expression (P<0.01 at all concentrations) in both microglia and Mφ, with a greater response in Mφ than in microglia. There was a small but significant increase (P<0.05) in MHC Class II expression in Mφ on treatment with 200 pg/ml IL-1α, but no increase was observed in microglia. The effect of IFN-γ and IL-1α on MHC class II expression on both cell types is illustrated in Fig. 3.3. IL-4 (50 U/ml) and TGFβ1 (5 ng/ml) both significantly (P<0.05) reduced class II on Mφ but had no effect on microglia. TNFα (1-300 U/ml), LPS (1-250 ng/ml), IL-1β (10-500 pg/ml) and GM-CSF (1-50 U/ml) had no significant effect on MHC class II expression by either microglia or Mφ.

3.3.1.5 Rosetting Mφ does not affect their response to IFN-γ, TNFα, or LPS

In order to assess the effect that the rosetting and Percoll separation stages of the microglial cell isolation have on the activation state of the cells or their response to cytokines, a number of experiments were performed in which freshly isolated Mφ were subjected to a similar treatment before plating out.

The effect of IFN-γ, TNFα or LPS stimulation in vitro on FcR and MHC class II expression on rosetted Mφ was determined in several experiments. The results of these experiments, shown in Table 3.4, suggest that rosetted Mφ behave similarly to non-rosetted Mφ with regard to Fc receptor or MHC Class II expression on treatment with IFN-γ, TNFα or LPS.

This evidence demonstrates that the procedure used to isolate microglia does not affect the response of these cells to cytokines in vitro.
Fig. 3.3

Effect of IFN-γ and IL-1α on MHC class II antigen expression on microglia and peritoneal macrophages.

Microglia (MG) and peritoneal macrophages (Mφ) were plated at 10 cells/well. All treatments lasted 72h and were performed in triplicate. Values represent mean absorbances at 405nm +/- SEM (no. of experiments, n = 14-36 for IFN-γ; n = 3-8 for IL-1α).

(*) denotes significantly different from medium treated control.
Table 3.4
Comparison of response of rosetted and non-rosetted peritoneal macrophages to cytokines.

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ (U/ml)</th>
<th>TNF-α (U/ml)</th>
<th>LPS (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td><strong>FcR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>0</td>
<td>36 ± 12</td>
<td>92 ± 19 (11)</td>
</tr>
<tr>
<td>Ros. Mφ</td>
<td>0</td>
<td>62</td>
<td>104 (1)</td>
</tr>
<tr>
<td><strong>Class II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>0.08 ± 0.01</td>
<td>0.47 ± 0.06</td>
<td>0.45 ± 0.06 (10)</td>
</tr>
<tr>
<td>Ros. Mφ</td>
<td>0.07</td>
<td>0.28</td>
<td>0.33 (2)</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>0</td>
<td>-</td>
<td>71 ± 16 (9)</td>
</tr>
<tr>
<td>Ros. Mφ</td>
<td>0</td>
<td>-</td>
<td>71 (1)</td>
</tr>
<tr>
<td><strong>Class II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>0.11</td>
<td>0.08</td>
<td>0.03 (2)</td>
</tr>
<tr>
<td>Ros. Mφ</td>
<td>0.06</td>
<td>0.10</td>
<td>0.07 (1)</td>
</tr>
<tr>
<td><strong>LPS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>0</td>
<td>99 ± 18</td>
<td>24 ± 10 (10)</td>
</tr>
<tr>
<td>Ros. Mφ</td>
<td>0</td>
<td>65</td>
<td>13 (1)</td>
</tr>
<tr>
<td><strong>Class II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mφ</td>
<td>0.09 ± 0.03</td>
<td>0.05 ± 0.04</td>
<td>0.03 ± 0.01 (8)</td>
</tr>
<tr>
<td>Ros. Mφ</td>
<td>0.07</td>
<td>0.02</td>
<td>0.03 (1)</td>
</tr>
</tbody>
</table>

Rosetted macrophages (Ros.Mφ; 10⁵ /well) were treated with IFN-γ, TNFα or LPS for 72h and assayed for FcR and MHC class II expression. For comparison the effect of these treatments on the same parameters in non-rosetted macrophages (Mφ) is included. Values are means ± SEM as described previously. Numbers in parentheses are n values.
3.3.1.6 Superoxide production

Mφ (10^5 cells/well) were treated with IFN-γ (1 - 10 U/ml), TNFα (1 - 50 U/ml) or LPS (1 - 50 ng/ml) for 72h before assaying for production of superoxide. Fig. 3.4 illustrates the amount of superoxide anion (nmol/well/h) produced by Mφ. IFN-γ, TNFα and LPS treatments all increase superoxide anion production. There is a 2-fold increase in superoxide production with 50 U/ml TNFα and with 10 U/ml IFN-γ; and with 10 ng/ml LPS there is a 1.6-fold increase.

3.3.2 Discussion (A)

The results described here demonstrate the effect of LPS and a wide range of cytokines on some of the functional properties of microglia and macrophages in vitro. Fc receptor and MHC class II expression were the two properties studied in greatest detail and clearly cytokine regulation of these differs widely. The effect of a more limited range of cytokines on production of superoxide anion by peritoneal macrophages in response to PMA stimulation is commented on.

Table 3.5 summarises the effects of LPS and the various cytokines tested on FcR and MHC class II expression on both cell types. Phagocytic capacity (FcR-mediated binding and ingestion of EA) of microglia is sensitive in varying degrees, to all of the cytokines investigated here, whereas the capacity of microglia to present antigen (MHC class II expression) is sensitive to IFN-γ only. Peritoneal macrophages differ in that class II expression in these cells in vitro is also regulated by IL-1β, TGFβ1 and IL-4. With the exception of TGFβ1, which reduces FcR expression in microglia and macrophages to below basal levels, all the cytokines tested increase FcR expression in both cell
Fig. 3.4

Effect of IFN-γ, TNF-α and LPS on production of superoxide anion by peritoneal macrophages

Peritoneal macrophages (10^5/well) were treated with IFN-γ (n=24-27), TNF-α (n=5-7) or LPS (n=4-6) as indicated for 72h before assay (performed in triplicate). Results are presented as mean production of superoxide anion (nmol / well / h) +/- SEM.
### Table 3.5

**Summary of the effects of a range of cytokines and LPS on Fc receptor and MHC class II expression on microglia and peritoneal macrophages.**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>FcR Microglia</th>
<th>FcR Macrophage</th>
<th>MHC class II Microglia</th>
<th>MHC class II Macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LPS</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-1</td>
<td>++</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>IL-4</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>- -</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

+ and - denote respectively an increase and a decrease relative to basal levels

0 denotes no change
types. IFN-γ and IL-1β both upregulate MHC class II expression on macrophages whereas expression is reduced on treatment with IL-4 or TGFβ1. TGFβ1 is a broad spectrum immunosuppressive cytokine and this is borne out by the results discussed in section B. IL-4 in contrast has both inhibitory and stimulatory effects on macrophage function.

Constitutive and stimulated increases (with TNFα, LPS, IL-1 and IFN-γ) in FcR expression in microglia are higher than in macrophages. In contrast microglia have a lower level of constitutive and IFN-γ-stimulated class II expression than do macrophages. These observations may reflect a comparatively high tendency to, or inducibility of, phagocytic activity on the part of microglia in vitro. MHC class II expression appears to be more tightly regulated than FcR expression and this is particularly striking in the case of microglia where class II is induced by IFN-γ alone, of the cytokines tested. Thus class II expression on microglia is dependent on a product of antigen-stimulated T cells and presentation of antigen to T cells at a developing site of inflammation may depend strictly on the presence of this immunomodulator.

LPS has been extensively studied as an activating signal, inducing effector functions such as complement-mediated phagocytosis (Griffin and Mullinax, 1990) and nitric oxide production (Zielasek et al, 1992) and the production of IL-1 and TNF by microglia and macrophages as described in chapter 2. It has been noted here that both IL-1 and TNFα increase Fc receptor expression on microglia and macrophages and this observation is supported by reports of increased ingestion of IgG-coated E by adherent human polymorphonuclear leukocytes (PMN) on treatment with IL-1β or TNF-α (Moxey-Mims et al, 1991). The increase induced by LPS treatment may in fact be mediated by the production of these cytokines, though this cannot be
concluded from these observations. Similarly the increase in nitrite production by microglia stimulated in vitro with LPS (Zielasek et al, 1992) may be the result of induction of NO synthetase by IL-1β and/or TNFα (Pfeilschifter and Vosbeck, 1991) produced in response to LPS.

3.3.2.1 MHC class II expression

The restriction of MHC class II antigen induction on microglia to IFN-γ is in line with other studies. (Warren and Vogel, 1985; Alvaro-Gracia et al, 1990; Yong et al, 1991). Investigating the effect of cytokines on MHC class II expression on synoviocytes, Alvaro-Gracia et al (1990) found that TNFα, IL-1β, IL-3, IL-4 and GM-CSF all failed to affect expression. Similarly Yong et al (1991) report that IL-1, TNFα and LPS have no effect on MHC class II expression on astrocytes. The evidence regarding the effect of TNFα, in particular on MHC antigen expression, is various, with reports of induction of class II on macrophage cell lines (Watanabe and Jacob, 1991; Chang and Lee, 1986) but not on peritoneal macrophages (Zimmer and Jones, 1990; Frendl and Beller, 1990) or astrocytes (Yong et al, 1991). TNFα had no effect on MHC class II expression on either of the macrophage populations in this study. Reports suggest that IL-1 does not affect MHC class II on synoviocytes, astrocytes or macrophages (Alvaro-Gracia et al, 1990; Yong et al, 1991; Freendl and Beller, 1990) and whilst no effect was observed here on expression on microglia there was a small increase in expression on peritoneal macrophages.

In contrast to our observations of minimal IL-4 effects on MHC class II expression on microglia and macrophages (a small decrease in the latter case), there are reports of IL-4 increasing MHC class II on monocytes (Donnelly et al, 1991) and peritoneal macrophages (Crawford et al, 1987; Cao et al, 1989). However, as mentioned
previously, IL-4 has been found to have no effect on MHC class II expression on synoviocytes (Alvaro-Gracia et al, 1990). An explanation of this apparent discrepancy may be found in the observation by Cao et al (1989) that IL-4-induced MHC class II expression on peritoneal macrophages returns to basal levels within 72h, the incubation time used in this study.

TGFβ1 reduces MHC class II expression in macrophages but not in microglia which already have a very low level of constitutive expression. In agreement with these findings, Czarnecki et al (1988) report a reduction in MHC class II on macrophages treated with TGFβ1 while rat astrocytes, which do not express constitutive class II, are unaffected by this treatment (Schluesener, 1990).

3.3.2.2 Fc receptor expression

The effect of IFN-γ on Fc receptor expression is well catalogued for monocytes (Jayram et al, 1989; Arend et al, 1987) and macrophages (Woodroffe et al, 1989; Jungi et al, 1989; Politis and Vogel, 1996). IFN-γ also increases FcR expression on rat microglia as demonstrated here. The range of cytokines which influence FcR expression on microglia and macrophages in vitro is extensive. The observation of increased binding and ingestion of EA by IL-3 or GM-CSF-treated cells is supported by the increased phagocytosis of polystyrene microspheres demonstrated by similarly treated microglia (Giulian and Ingeman, 1988).

TGFβ1 reduced, to below basal levels, the FcR-mediated phagocytic capacity of both microglia and macrophages. There is however evidence that TGFβ1 enhances expression of the low affinity FcγRIII in peripheral blood monocytes and phagocytes (Welch et al, 1990; Wahl et al, 1992). In the experiments described here IL-4
increased FcR expression on both microglia and macrophages. This is compatible with observations of increased ingestion of IgG-opsonised erythrocytes by mature monocytic phagocytes (Sampson et al, 1991) and increased FcγRII-dependent binding of IgG complexes to bone marrow macrophages in response to IL-4 (Crawford et al, 1987). However there is evidence, using antibodies specific for various Fc receptor subtypes and FACS technology, that IL-4 reduces expression of FcγRI and II on mononuclear phagocytes (Becker and Daniel, 1990). Sampson et al (1991) found that IL-4 enhanced phagocytosis by Mφ of not only IgG but also complement-coated targets. It appears that IL-4 may have an effect on the general ingestive mechanism apart from any effect it may have on expression of specific receptors. IL-4 therefore increases FcR-mediated binding / ingestion by macrophages of IgG-opsonised targets or IgG complexes in a functional assay which does not discriminate between receptor sub-types, such as used here. However, as the apparently conflicting results show, such observations may disguise the more complex range of effects, on different FcR subtypes, of IL-4 (Te Velde et al, 1990; Becker and Daniel, 1990) and indeed any of the other cytokines.

Microglia have a very low level of non-specific binding (E-binding) and this is not affected by cytokine treatment. However macrophages respond to IFN-γ, TNFα and LPS stimulation with increases in non-specific as well as FcR-mediated binding and ingestion of erythrocytes and in the case of IFN-γ-stimulation, these increases are of comparable proportions. These observations may reflect a difference in the number and/or variety of cell-surface adhesion molecules expressed on peritoneal macrophages and microglia. Alternatively microglial cell function in the CNS may be
subject to tighter regulation in this respect, as with cytokine regulation of class II expression, than are peritoneal macrophages.

3.3.2.3 Superoxide anion production

The capacity of peritoneal macrophages to produce superoxide anion on stimulation with PMA, was enhanced on treatment with IFN-\(\gamma\), TNF-\(\alpha\) and LPS, as described in 3.2.1.6. Treatment of microglia with IFN-\(\gamma\) increases superoxide production (Woodrooffe et al, 1989) and Colton and Gilbert (1987) report that microglia do secrete superoxide in response to PMA. In agreement with the findings reported here, Szefler et al (1989) demonstrated increased superoxide release by human monocytes on pretreatment with IFN-\(\gamma\) or LPS. Interestingly, they found that the LPS effect was not inhibited by hydrocortisone which would prevent synthesis or secretion of IL-1 or TNF in response to LPS stimulation. Thus, they concluded that the effect was due to some direct action of LPS itself and not mediated by cytokines. Microglia also have the capacity to produce and release nitric oxide (NO) and do so in response to LPS or IFN-\(\gamma\)-stimulation (Zielasek et al, 1992). Nitric oxide and superoxide anion are extremely reactive products and damage surrounding tissue when released at a site of inflammation

On consideration of the results discussed here and the work of others it becomes apparent that the effect of various cytokines on the functional properties of cells can be difficult to predict. A particular cytokine may produce quite different responses in two different cell types and even in the loosely-termed 'macrophage' population, the response may depend on the tissue of origin, the state of differentiation and maturation of the cells and the exposure time
3.4 SECTION B: cytokine interactions

In initial studies, the effect on the functional properties of microglia and M\(\phi\), of co-stimulation with IFN-\(\gamma\) and TNF\(\alpha\), IL-1\(\alpha\) or LPS was investigated. Subsequently a more elaborate study compared the effects of treatment with IL-4 or TGF\(\beta\)1 and IFN-\(\gamma\) or TNF\(\alpha\) where cells were exposed to the two cytokines sequentially and simultaneously. The interaction between TNF\(\alpha\) and IFN-\(\gamma\) was similarly investigated.

3.4.1 Results (B)

3.4.1.1 Effect of TNF\(\alpha\), IL-1\(\alpha\) or LPS in combination with IFN-\(\gamma\) on functional properties of microglia and M\(\phi\)

Microglia and peritoneal M\(\phi\), both at 10\(^5\) cells/well, were treated for 72h with an individual cytokine, IFN-\(\gamma\), TNF\(\alpha\), IL-1\(\alpha\) or LPS, or a combination of IFN-\(\gamma\) and TNF\(\alpha\), IL-1\(\alpha\) or LPS

Fc Receptor expression

Fig. 3.5(b) shows the effect of IFN-\(\gamma\) on FcR expression by M\(\phi\) in combination with either 50 U/ml TNF\(\alpha\), 50ng/ml LPS or 10 pg/ml IL-1\(\alpha\). Individually IFN-\(\gamma\) and IL-1\(\alpha\) produce increases in FcR expression, and in combination these doses have an additive effect on FcR expression. Co-treatment of M\(\phi\) with IFN-\(\gamma\) plus TNF\(\alpha\) or LPS produces net reductions in FcR expression by M\(\phi\). In contrast to the additive effect of IFN-\(\gamma\) and IL-1\(\alpha\) on M\(\phi\), similar treatment of
Fig. 3.5

Effect of TNFα, IL-1α and LPS in combination with IFN-γ on Fc receptor expression on microglia and peritoneal macrophages

(a) Microglia

(b) Macrophages

Microglia (a) and peritoneal macrophages (b), both plated at 10^5 cells / well, were treated for 72h with IFN-γ (10 U/ml), TNFα (50 U/ml), IL-1α (10 pg/ml), LPS (50 ng/ml) or a combination of IFN-γ and TNFα, IL-1α or LPS at these doses. All treatments were performed in duplicate. Values indicated are mean increases over basal levels of [EA-E] +/- SEM (n = 4-11), as described before.
Microglia had no effect on the level of expression induced by IFN-γ, although the response of microglia to 10 pg/ml IL-1α alone was comparatively low [Fig. 3.5(a)]. Microglia responded similarly to Mφ on treatment with IFN-γ in combination with TNFα or LPS. Individually these cytokines all stimulate Fc receptor expression in microglia, but the combination of IFN-γ with TNFα or LPS produced net decreases in expression.

**MHC Class II expression**

As described earlier, IL-1α, TNFα and LPS have no effect on MHC class II expression by Mφ at the concentrations used here (10 pg/ml, 50 U/ml and 50 ng/ml, respectively). However, both TNFα and LPS depress the response of Mφ to IFN-γ with respect to MHC class II expression [Fig. 3.6(b)]. IL-1α appears to give a small increase in the response of Mφ to IFN-γ, but this increase is not significant. Similarly, microglial class II expression, as induced by 10 U/ml IFN-γ, is reduced when 50 U/ml TNFα or 50 ng/ml LPS is included, as shown in Fig. 3.6(a). As with Mφ, microglia showed no significant change in class II expression when IL-1α was included with IFN-γ.

3.3.1.2 **Effect of TNFα in combination with IL-1β on functional properties of microglia and Mφ**

The effect of IL-1β on Fc receptor expression by microglia in combination with TNFα is shown in Fig. 3.7. Individually IL-1β (500 pg/ml) and TNFα (50 U/ml) increase FcR expression, and in combination these doses result in an additive response. Neither IL-1β nor TNFα affected MHC class II expression on microglia individually, and co-treatment with IL-1β and TNFα had no effect on basal levels of MHC class II (not shown).
Fig. 3.6

Effect of TNFα, IL-1α and LPS in combination with IFN-γ on MHC class II expression on microglia and peritoneal macrophages.

(a) Microglia

(b) Peritoneal macrophages

Microglia (a) and peritoneal macrophages (b), both plated at $10^5$ cells / well, were treated for 72h with IFN-γ (10 U/ml), TNFα (50 U/ml), IL-1α (10 pg/ml), LPS (50 ng/ml) or a combination of IFN-γ and TNFα, IL-1α or LPS at these doses. All treatments were performed in duplicate. Values indicated are mean absorbance at 405nm +/- SEM (n = 4-19).
Fig. 3.7

Combined effect of TNF-α and IL-1β on Fc receptor expression on microglia.

Microglia (10^5/well) were treated with TNFα (50 U/ml), IL-1β (500 pg/ml) or a combination of these for 72h. The effect of these treatments (performed in duplicate) on Fc receptor expression was assessed. Indicated values are mean increases in [EA-E] over basal levels +/- SEM (n = 5).
3.4.1.3 Sequential and simultaneous treatments with IFN-γ and TNFα: effects on FcR and MHC class II expression on microglia and Mφ

TNFα (50 U/ml) inhibits IFN-γ-induction of MHC class II on microglia and Mφ when both cytokines are present. There is also a mutual antagonism between the the two cytokines with regard to FcR expression. These observations have been described in 3.4.1.1. It was of interest to know whether the exposure of the cells to first IFN-γ, then TNFα (the likely sequence at site of inflammation) produced the same responses.

Microglia (0.5 x 10^5/well) or Mφ (10^5/well) were cultured for an initial 72h period with medium or IFN-γ (1-10 U/ml) followed by a second 72h period with TNFα (50 U/ml) or IFN-γ + TNFα. Cells were then assayed for FcR and MHC class II expression.

**FcR expression**

Fig. 3.8 illustrates the effect of IFN-γ (10 U/ml) and TNFα (50 U/ml) alone and in combination, on FcR expression on Mφ. As shown previously IFN-γ and TNFα individually increase expression but when present together they antagonise each other. When cells are treated with IFN-γ first and TNFα second there is also some antagonism (significantly less than IFN-γ alone; P ≤ 0.05) though this is less pronounced.

In a small number of experiments using microglia, treatment with IFN-γ followed by TNFα, appears to produce an additive increase in expression [Fig. 3.9], though microglia responded similarly to Mφ on co-treatment with IFN-γ and TNFα (3.4.1.1).

**MHC class II expression**

Fig. 3.10 illustrates the effect of IFN-γ (10 U/ml) and TNFα (50 U/ml), alone and in combination, on MHC class II expression on Mφ (mean of 2-4 experiments). As shown previously TNFα alone has no
Peritoneal macrophages (10^5/well) were subjected to two consecutive 72h treatments (1 and 2 as indicated above) with medium (---) or IFN-γ followed by TNFα and/or IFN-γ. All treatments were performed in duplicate. Results are expressed as mean increases in [EA-E] over basal levels +/- SEM (n=5-6). (*) significantly different from 0; (+) significantly different from IFN-γ-treated control (P≤0.05).
Microglia (0.5 x 10^5/well) were treated first with IFN-γ (1 - 10 U/ml) for 72h followed by either medium [circles] or TNFα (50 U/ml) [filled circles] for a further 72h before assay (performed in duplicates). Results are expressed as mean increases in [EA-E] over basal levels +/- SEM (n = 3-4).
**Fig. 3.10**

Combined effects of IFN-γ and TNFα on MHC class II expression on peritoneal macrophages.

Peritoneal macrophages (10⁵/ well) were subjected to two consecutive 72h treatments (1, 2 above) with medium (---), IFN-γ and/or TNFα, as indicated, before assay (performed in duplicate). Results are presented as means of absorbance at 405nm +/- SEM (n = 3-4).
effect on MHC class II expression but it inhibits induction of expression by IFN-γ when present during stimulation. Treatment with TNFα following IFN-γ stimulation also reduces class II expression compared to cells which were cultured with medium only following IFN-γ stimulation.

3.4.1.4 Sequential and simultaneous treatments with IL-4 and IFN-γ: effects on FcR and MHC class II expression on microglia and Mφ

Microglia (0.5 x 10⁵/well) and Mφ (10⁵/well) were cultured for an initial 72h period with either medium alone, IFN-γ (at 1 and 10 U/ml) or IL-4 (50 U/ml) followed by a second 72h treatment with the individual cytokines or a combination of IFN-γ with IL-4. Cultures were then assayed for Fc receptor and MHC class II expression and a comparison made of the effects of sequential treatment versus co-treatment with the cytokines.

FcR expression

As described earlier IFN-γ induces FcR expression on microglia and Mφ. However, while this increased expression persists in microglia up to 72h after removal of IFN-γ [Fig. 3.11(a)], expression in macrophages returns to basal levels within this period [Fig. 3.11(b)]. In the absence of IFN-γ, IL-4 at 50U/ml also induces FcR expression on microglia and macrophages and 72h after removal of the IL-4, expression is still elevated [Fig. 3.11(a) and (b)].

Although IL-4 and IFN-γ individually induce FcR expression, the combination of 50 U/ml IL-4 and 10 U/ml IFN-γ results in a level of expression in microglia and Mφ significantly lower than that induced by IFN-γ alone and in the case of Mφ there is no significant increase at all [Fig. 3.11(a) and (b)]. A lower dose of IFN-γ (1 U/ml) in combination with IL-4 (50 U/ml) produces a response in microglia
Fig. 3.11

Combined effects of IL-4 and IFN-γ on Fc receptor expression on microglia and peritoneal macrophages.

Microglia (0.5x10^5 / well) and peritoneal macrophages (10^5 / well) were subjected to two consecutive 72h treatments (1 and 2 above) with IL-4 (10-50 U/ml) and/or IFN-γ (1-10 U/ml) before assay (performed in duplicates). Panels illustrate the effect of IL-4 added during, before and after IFN-γ stimulation. For clarity error bars are omitted. Results are presented as mean increases in [EA-E] over basal levels (microglia: n=3-9; Mφ: n=3-12). (*) significantly different from 0; (+) significantly different from IFN-γ-treated control, (P≤0.05).
which is approximately additive. In Mφ this combination is not significantly different from IL-4 or IFN-γ alone.

Microglia demonstrate an additive increase in FcR expression after sequential treatment with 50 U/ml IL-4 followed by 1 U/ml IFN-γ but at the higher IFN-γ concentration (10 U/ml) though the response is less than additive, the mutual antagonism observed with co-treatment is not seen. A similar profile is observed when microglia are first stimulated with IFN-γ (at 1 U/ml) then IL-4. In the case of Mφ, even though expression due to IFN-γ is absent 72h after its removal, the observed inhibition of IL-4-induced FcR expression during this period is dependent on the concentration of IFN-γ in the first treatment i.e., at 10 U/ml IFN-γ there is a greater inhibitory effect with IL-4 than at 1 U/ml IFN-γ [Fig. 3.11(b)]. The degree of inhibition of FcR expression increases with increasing concentration of IL-4 when IFN-γ is present. In the case of Mφ the inhibition is virtually complete even at 10 U/ml IL-4. Note that even at half the cell density of Mφ, microglia bind or ingest many more EA, illustrating their impressive phagocytic potential.

**MHC Class II expression**

Fig. 3.12(a) and (b) illustrate that while class II remains elevated in Mφ 72h after removal of IFN-γ, it does not do so in microglia. Treatment of microglia with IL-4 at 50 U/ml for 72h in the absence of IFN-γ has no effect on class II expression. In macrophages however, IL-4 significantly downregulates expression (P≤0.05) [Fig. 3.12(a) and (b)]. When microglia or Mφ are simultaneously treated with IL-4 and IFN-γ, IL-4 significantly decreases the IFN-γ-induced class II expression in both cell types [Fig. 3.12(a) and (b)]. Pretreatment of microglia with IL-4 before IFN-γ stimulation results in a small but significant decrease in the response to IFN-γ at 1 U/ml [Fig. 3.12(a)].
Combined effects of IL-4 and IFN-γ on MHC class II expression on microglia and peritoneal macrophages.

Microglia (0.5×10^6 / well) and peritoneal macrophages (10 / well) were subjected to two consecutive 72h treatments (1 and 2 above) with IL-4 (10-50 U/ml) and/or IFN-γ (1-10 U/ml) before assay (performed in duplicates). Panels illustrate the effect of IL-4 added during, before and after IFN-γ stimulation. For clarity error bars are omitted.

Results are presented as means of absorbance at 405nm (microglia: n=3-9; Mφ: n=3-12). (*) significantly different from medium treated control, (+) significantly different from IFN-γ-treated control; (P≤0.05).
Similar treatment of Mφ also seems to depress the response to IFN-γ but this does not test as statistically significant [Fig. 3.12(b)]. MHC Class II expression induced in microglia by IFN-γ returns to basal levels after 72h and treatment with 50 U/ml IL-4 during this period has no effect on expression [Fig. 3.12(a)]. Treatment of Mφ which have elevated class II, due to IFN-γ-stimulation, with 50 U/ml IL-4 results in a large decrease in class II expression [Fig. 3.12(b)].

The degree of inhibition of MHC class II expression on microglial by IL-4 present before or during IFN-γ stimulation is dependent on the IL-4 concentration.

3.4.1.5 Sequential and simultaneous treatments with TGFβ1 and IFN-γ: effects on FcR and MHC class II expression on microglia and Mφ

Microglia (0.5 x 10^5 or 1 x 10^5 /well as indicated) and Mφ (1 x 10^5 /well) were cultured with TGFβ1 (5ng/ml) and/or IFN-γ (1 - 10 U/ml) as described above (3.4.1.4).

FcR expression

The effect of TGFβ1 and IFN-γ on FcR expression on microglia and Mφ is depicted in Fig. 3.13(a) and (b) respectively. TGFβ1 (5ng/ml) significantly reduced FcR expression in both cell types and this effect persists up to 72h after removal of the TGFβ1 (bars C and D). TGFβ1 also antagonises induction of expression by IFN-γ when both cytokines are present (bar E), and in the case of microglia, FcR is reduced to below basal level. A comparable inhibition of IFN-γ-induced increases is achieved by pretreatment of microglia with TGFβ1 (bar F). In Mφ the extent of inhibition by TGFβ1 is greater when cells are exposed to TGFβ1 before (bar F) rather than during treatment with IFN-γ (bar E). When microglia are first stimulated with IFN-γ (10 U/ml) and then treated with TGFβ1 it does not significantly affect IFN-γ-induced FcR
Fig. 3.13
Combined effects of TGFβ1 and IFN-γ on Fc receptor expression on microglia and macrophages.

(a) Microglia

(b) Peritoneal macrophages

Microglia and peritoneal macrophages (10^5 cells / well) were subjected to two consecutive 72h treatments (1 and 2 above) with IFN-γ (10 U/ml) and/or TGFβ1 (5 ng/ml) as indicated. All treatments were performed in duplicate. Results are mean increases in [EA-E] over basal levels +/- SEM (n = 3-8). (*) significantly different from 0; (+) significantly different from relevant IFN-γ-treated control.
expression (bar G) although there is a small apparent decrease in expression. In the case of Mφ, where FcR expression has already returned to basal levels after 72h (bar B), treatment with TGFβ1 following IFN-γ-stimulation results in a comparable decrease in expression to that obtained by treatment with TGFβ1 alone (bar G and bar C).

MHC class II expression

TGFβ1 (5ng/ml) has a similar effect to IL-4 on MHC class II expression, i.e. it causes a small but significant decrease in expression on Mφ (P<0.05) after 72h [Fig. 3.14(b)]. TGFβ1 had no effect on microglial class II expression [Fig. 3.14(a)]. Fig. 3.14(a) and (b) illustrate the effect, on microglia and Mφ respectively, of simultaneous and sequential treatment with IFN-γ (10 U/ml) and TGFβ1 (5ng/ml). In all cases there is a significant decrease (P<0.05) in class II expression on Mφ compared to the IFN-γ control (bars F and G vs Bar B and bar H vs bar C). A similar effect was observed on microglia, though the decreases were not statistically significant.

3.4.1.6 Sequential and simultaneous treatments with IL-4 or TGFβ1 and TNFα: effects on FcR expression on microglia and Mφ

Microglia (0.5 x 10^5/well) and Mφ (10^5/well) were cultured for an initial 72h period with medium, TNFα (10 - 50 U/ml), IL-4 (50 U/ml) or TGFβ1 (5ng/ml) followed by medium, one of these cytokines or a combination of TNFα with IL-4 or TGFβ1 for a further 72h.

Cultures thus treated were assayed for Fc receptor expression and the results are depicted in Fig. 3.15(a) and (b) [TGFβ1 and TNFα; Mφ and microglia] and Fig. 3.16 [IL-4 and TNFα; Mφ].

The increase in the FcR expression induced by TNFα appears to persist, 72h after removal of the cytokine, on Mφ cultures [Fig. 3.15(b);
Combined effects of TGFβ1 and IFN-γ on MHC class II expression on microglia and macrophages.

Microglia (a) and peritoneal macrophages (b) (10 cells / well) were subjected to two consecutive 72h treatments (1 and 2 above) with IFN-γ (10 U/ml) and/or TGFβ1 (5 pg/ml) as indicated. All treatments were performed in duplicate. Results are means of absorbance at 405nm +/- SEM (n = 3-11). (*) significantly different from medium treated control [A]; (+) significantly different from relevant IFN-γ-treated control.
Fig. 3.15

Combined effects of TNFα and TGFβ1 on Fc receptor expression on microglia and peritoneal macrophages.

(a) Microglia

(b) Peritoneal macrophages

Microglia (a) and peritoneal macrophages (b) (0.5x10^5/well and 10^5/well respectively) were subjected to two consecutive 72h treatments (1 and 2 above) with medium (---), TGFβ1 (5 ng/ml) and/or TNFα (50 U/ml) before assay (performed in duplicates). Results are expressed as mean increases in [EA-E] over basal levels +/- SEM (microglia: n=3-4; Mφ: n=3-6).
Fig. 3.16

Combined effects of TNFα and IL-4 on Fc receptor expression on peritoneal macrophages.

Peritoneal macrophages (10 /well) were subjected to two consecutive 72h treatments (1 and 2 above) with medium (---), TNFα (50 U/ml) and/or IL-4 (50 U/ml) before assay (in duplicate). Results are expressed as mean increases in [EA-E] over basal levels +/- SEM (n = 3).
bar B] and at a somewhat reduced level on microglial cultures (not shown). The effect of TGFβ1 on FcR expression appears to be greater 72h after its removal than immediately after treatment (bars D and C). There are some differences in the response of microglia and Mφ to TNFα and TGFβ1. In the case of microglia, treated simultaneously with TNFα and TGFβ1 or treated with TGFβ1 before TNFα (bars E and F), the effect on FcR is approximately additive, i.e., the difference between these responses and those of the relevant TGFβ1 controls is similar to the increase in expression produced by TNFα alone. In contrast there appears to be a genuine inhibition of expression on Mφ treated with TGFβ1 either before, during or after TNFα stimulation.

There is evidence of antagonism between IL-4 and TNFα where FcR expression is concerned [Fig. 3.16]. Treatment of Mφ with IL-4 and TNFα together results in a response which is intermediate between the responses to the individual cytokines. Similarly pretreatment with IL-4, though it elevates expression, reduces the response to subsequent TNFα stimulation. IL-4 also successfully depresses FcR expression on Mφ, previously stimulated with TNFα.

3.4.2 Discussion (B)

This study in which cells are exposed to more than one cytokine at a time or over a period of time, illustrates some of the interactions between cytokines and the importance of timing, relative cytokine concentration and cell type in consideration of results.
3.4.2.1 Effect of combinations of IFN-γ, TNFα, IL-1 and LPS on functional properties

In initial experiments cytokines were added simultaneously to microglia or macrophages in culture. An incubation time of 72h was chosen as this was sufficient to obtain a measurable effect on FcR expression with individual cytokines and permitted comparison with the results described in section A. Whilst LPS and TNFα have no effect alone on MHC class II expression, when either of these factors is included with IFN-γ, they antagonize induction of MHC class II by IFN-γ on both microglia and macrophages. In contrast, IL-1 has no effect on IFN-γ induction of class II in either cell type. All the cytokines, IFN-γ, IL-1, TNFα and LPS, induce expression of Fc receptors on microglia and macrophages. However, whereas IFN-γ and IL-1 additively increase expression, IFN-γ in combination with either TNFα or LPS produces a net decrease in expression. Co-stimulation of microglia with TNFα and IL-1 produced an additive increase in FcR expression and had no effect on MHC class II expression. These observations are summarised in Table 3.6.

Thus LPS (which induces TNFα and IL-1 production by both cell populations) and TNFα have similar effects in combination with IFN-γ. These observations suggest that in an in vivo situation it would be TNFα which would predominate over IL-1, overriding any additive effect of IL-1 and IFN-γ and perhaps preventing an increase in antigen-presenting capacity or phagocytic properties of microglia. Arend et al (1987) have suggested that the LPS inhibition of IFN-γ-induced FcR expression in human monocytes is primarily caused by IL-1 produced in response to LPS stimulation and, to a lesser extent, by TNFα, having observed that IL-1 and TNFα are both inhibitory. In this study there is no evidence that IL-1α inhibits IFN-γ-induced FcR expression and it
Table 3.6

Summary of effects of TNFα, LPS, IL-1 and IFN-γ on Fc receptor and MHC class II expression on microglia (MG) and macrophages (Mφ)

<table>
<thead>
<tr>
<th>Cytokine(s)</th>
<th>Fc receptor</th>
<th>MHC class II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MG</td>
<td>Mφ</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>TNFα</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>LPS</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>IL-1</td>
<td>++</td>
<td>+</td>
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<tr>
<td>IFN-γ + TNFα</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ + LPS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ + IL-1</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

+ denotes an increase above basal levels; - denotes a decrease below basal levels.
0 denotes no change observed.
appears that the inhibitory effect of LPS is due to TNFα rather than IL-1. Hoffman and Weinberg (1987) also observed an antagonism by TNFα of an IFN-γ-induced effect on murine peritoneal exudate cells, in this case a reduction in the number of macrophages bearing Fc receptors to IgG2b.

There are a number of apparently conflicting reports concerning the effect of TNFα on IFN-γ induction of MHC class II. Hoffman and Weinberg (1987) found that whilst TNFα synergistically increased the MHC Class II response to IFN-γ in a malignant murine macrophage cell line, it partially inhibited this effect in murine peritoneal macrophages. There is evidence of a synergistic effect on MHC class II on islet cells (Pujol-Borrell et al, 1987) and of antagonism on synoviocytes (Alvaro-Gracia et al, 1990). Tanaka and McCarron (1990) also observe that the effect of TNFα on MHC Class II induction by IFN-γ depends on the cell type; IFN-γ-induction of this antigen on cerebral endothelial cells is antagonised by TNFα whilst TNFα enhances this response on astrocytes. Furthermore they find that IL-1 also inhibits MHC Class II induction by IFN-γ on cerebral endothelial cells, which contrasts with the observation made here that TNFα but not IL-1 markedly inhibits IFN-γ activation of microglia. In agreement with the observation that LPS inhibits induction of MHC Class II by IFN-γ, Frendl and Beller (1990) report a similar effect on thioglycollate-elicited peritoneal exudate cells when LPS is present at a concentration upwards of 50 ng/ml. Interestingly they noted that at low doses (0.05 - 0.5 ng/ml), LPS enhanced IFN-γ-induced MHC Class II expression. The relative doses of cytokines or other modulators determines not only the degree of a cellular response but in some instances the nature of the response. This dose-dependency is apparent from
investigations of the effect of IL-4 and IFN-γ on macrophage properties as discussed later.

Conflicting reports regarding the effect of combinations of modulatory cytokines demonstrate the importance of considering the state of differentiation and maturation of cells and the length of time they are exposed to cytokines when making comparisons. For example, Watanabe and Jacob (1991) observed that TNFα enhances IFN-γ-induced MHC class II expression in undifferentiated cells, whereas in mature macrophage populations expression of this antigen is reduced. This offers a possible explanation of some of the inconsistencies described here. Zimmer and Jones (1990) found that the effect of co-treatment with TNFα and IFN-γ depends on how long the cells are exposed to the cytokines. They observed that TNFα blocks the increase in MHC class II and FcR expression on peritoneal macrophages caused by exposure to IFN-γ after 72h, which is consistent with this study.

The mutual antagonism between TNFα and IFN-γ reported by Alvaro-Gracia et al (1990) required simultaneous addition of IFN-γ and TNFα. However, in experiments described here there is evidence of antagonism between the two cytokines when macrophages are exposed to IFN-γ before TNFα, albeit less dramatic than that observed with simultaneous addition.

It is interesting to note that TNFα has a more dramatic effect on FcR expression than on MHC class II expression in IFN-γ-treated microglia or macrophages, reflecting perhaps a more critical control of phagocytic rather than antigen-presenting capability in vivo. This may be significant in diseases such as MS, in which the functional deficit is caused by demyelination. The antagonism between TNFα and IFN-γ observed here suggests that, in vivo, TNFα produced
subsequent to IFN-γ induction of MHC class II expression on macrophages may act as a control mechanism to attenuate MHC class II expression and down-regulate the capacity of these cells to present antigen. TNFα might induce phagocytic properties in microglia when IFN-γ levels are low and vice versa; however when both cytokines are present the result may be down-regulation of these properties.

3.4.2.2 Effects on functional properties of simultaneous and consecutive treatment with IL-4, TGFβ1, TNFα, IFN-γ

Table 3.7 summarises the main observations on the effect of IL-4 and TGFβ1 on FcR and MHC class II expression in combination with IFN-γ or TNFα. IL-4 causes an increase in FcR expression in both macrophages and microglia and a small decrease in MHC class II expression in macrophages as described in Section A. The increase in FcR expression on both cell types is a simple additive one when treated with IL-4 followed by IFN-γ. However, when both cytokines are present simultaneously an additive stimulatory effect on FcR expression switches to a mutually antagonistic one, with an increasing concentration of IFN-γ. Likewise, there is evidence of antagonism at higher doses of IFN-γ when microglia and macrophages are treated with IL-4 after IFN-γ stimulation. A similar effect has been noted by Sternberg et al (1987), who found that serotonin (5-HT) augmented phagocytosis induced by low concentrations of IFN-γ but suppressed this effect at higher concentrations of IFN-γ. These observations serve to illustrate the difficulties of extrapolating results based on limited dose response experiments with single cytokines to the in vivo situation.

Though IL-4 alone has a minimal effect on MHC class II expression when present during IFN-γ stimulation it inhibits
**Table 3.7**

Summary of effect of IFN-\(\gamma\), TNF\(\alpha\), IL-4 and TGF\(\beta1\) on expression of Fc receptors and MHC class II by microglia (MG) and macrophages (M\(\phi\)).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Fc receptor</th>
<th>MHC class II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MG</td>
<td>M(\phi)</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TNF(\alpha)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TGF(\beta1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFN-(\gamma) + IL-4</td>
<td>+/- (a)</td>
<td>+/-</td>
</tr>
<tr>
<td>IFN-(\gamma) + TGF(\beta1) (b)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TNF(\alpha) + IL-4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TNF(\alpha) + TGF(\beta1)</td>
<td>0/-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ stimulatory
- inhibitory
0 no change

\(a\) Can be either stimulatory or inhibitory depending on order of addition or concentration.

\(b\) TGF\(\beta1\), added before or during activation, is inhibitory.
induction of MHC class II expression on both cell populations. Treatment of microglia or macrophages with IL-4 before IFN-γ stimulation also inhibited MHC class II induction by IFN-γ but to a much smaller extent. IL-4 considerably reduced MHC class II expression in macrophages previously stimulated with IFN-γ. It appears that as an inhibitor of IFN-γ-induced effects, IL-4 is most effective when present during or after IFN-γ-stimulation.

There is also evidence of antagonism between IL-4 and TNFα with respect to FcR expression on macrophages, on both sequential and simultaneous treatments. Note that there are similarities between TNFα and IL-4 with regard to their effects on IFN-γ-induced responses. Both TNFα and IL-4 will individually increase FcR expression themselves but each proves mutually antagonistic towards IFN-γ.

There are other reports of inhibition by IL-4 of IFN-γ-driven Fc receptor expression (Te Velde et al, 1990, Becker and Daniel, 1990) but not of the additive effect of IL-4 and IFN-γ at lower doses of IFN-γ as reported here. This balance between stimulatory and down-regulatory effects suggests that in vivo cellular events would be subject to a fine control by the relative concentrations of cytokines produced locally.

These experiments also provide interesting data on the effect on MHC class II or FcR expression of removal of a particular cytokine, i.e., how long-lived are its effects? FcR expression on microglia remains elevated 72h after removal of IFN-γ but that on macrophages has returned to basal levels within this period. However whilst FcR expression due to IFN-γ and IL-4 is absent on macrophages 72h after removal of the cytokine, the antagonism between IFN-γ and IL-4 is still evident with inhibition by IL-4 added during this period. IFN-γ-induced MHC class II expression persists in macrophages but not in microglia. The increase in FcR expression induced by IL-4 in both
microglia and macrophages is persistent for at least 72h. The long-lived effect of IL-4 has been noted in other studies (Abramson and Gallin, 1990; Te Velde et al, 1990) and suggests that the continued presence of IL-4 is not always required to sustain a response or influence the response of the cell to subsequent treatments.

Since modulation of MHC class II and phagocytic capacity does not require the simultaneous presence of IL-4 and IFN-γ, the cytokines would appear not to interfere or block each other directly. There is evidence also that IFN-γ modulation of MHC class II expression requires synthesis of an intermediate protein (Fertsch et al, 1987). Cyclohexamide prevents the inhibition by IL-4 of IFN-γ-induced synthesis of TNFα mRNA, suggesting that IL-4 also mediates this activity via synthesis of another protein (Gautam et al, 1992). Long exposure of macrophages to IL-4 is necessary to induce maximal phagocytosis as the effect is dependent on the endogenous production of IFN-α/β (Sampson et al, 1991). IFN-β inhibits IFN-γ-induced expression of MHC class II antigen on murine macrophages (Ling et al, 1985) and is believed to act pre-translationally to reduce mRNA levels either directly, affecting transcription, or indirectly, antagonising the induction or action of some factor responsible for MHC class II induction (Fertsch et al, 1987).

If IFN-γ and IL-4 do both act through induction/suppression of other factors, this would explain the ‘carry-over’ effects observed here. For example these cytokines modulate the effect of subsequent cytokine on FcR expression though they have been removed and the response to them has apparently worn off. It seems likely that modulation of MHC class II expression occurs at the level of transcription (Fertsch et al, 1987; Ling et al, 1985) and that IL-4 effects may be mediated indirectly by induction of IFN-α/β. Less is known
about regulation of phagocytic function although Sampson et al (1991) concluded that the induction of phagocytosis by IL-4 could be mediated by endogenously produced IFN-α/β.

TGFβ1, as described in Section A, reduces FcR expression on microglia and macrophages and MHC class II levels on macrophages. Both effects are still evident 72h after removal of TGFβ1. This cytokine is also a potent inhibitor of IFN-γ and TNFα-induced FcR expression and of IFN-γ-induced MHC class II on macrophages. Microglia behave similarly except that although TGFβ1 reduces FcR expression in TNF-α-treated cells, the cytokines appear to cancel each other out rather than antagonise each other. Inhibition of IFN-γ-induced effects by TGFβ1 is most effective when it is present before or during IFN-γ stimulation. However TGFβ1 will inhibit macrophage FcR expression due to TNFα whether it is present before, during or after TNFα stimulation. There is considerable data supporting a role for TGFβ as an immunosuppressive cytokine (Tsunawaki, 1988; Schluesener, 1990; Kuruvilla et al, 1991). Various reports of the down-regulatory effects of TGFβs suggest that these cytokines are most effective when used before or during stimulation (Nelson et al, 1991; Pfeilschifter and Vosbeck, 1991) which is in agreement with our findings. Schluesener (1990) also reports that this is the case for reduction of IFN-γ-induced MHC class II on astrocytes by TGFβ1 and β2. Of particular relevance to CNS inflammation is the observation that IFN-γ or PMA-induced cytotoxic effects of microglia on oligodendrocytes can be successfully inhibited by TGFβ1 in vitro (Merrill and Zimmerman, 1991).

Though, of all the cytokines tested in section A, only IFN-γ affected MHC class II expression on microglia, it is apparent that TNFα, IL-4 and TGFβ1 are all capable of influencing expression indirectly by
modulation of the response to IFN-γ. The phagocytic capacities of microglia and macrophages are upregulated by an extensive range of cytokines in vitro. However it is clear that although cytokines such as IFN-γ, IL-4 and TNFα, are each stimulatory, in combination they can produce a very different response. TGFβ1 is a more straightforward inhibitory cytokine, capable of downregulating the response to IFN-γ or TNFα. Thus in an in vivo situation, the functional properties of the cell will be influenced by the spectrum of cytokines and, as we have seen, their relative concentrations and the sequence in which they are produced/secreted in the local environment. It is in this context which in vitro results must be viewed and apparent disparities between different studies may be explicable in these terms. Furthermore, in interpretation of results of such experiments, it must be borne in mind that cells such as macrophages, from different sources, or at different differentiation stages do not always respond to cytokines in a similar fashion (Watanabe and Jacob, 1991; Becker and Daniel, 1990).

3.5 SECTION C

The potential of non-cytokine mediators such as noradrenaline and the glucocorticoids dexamethasone and corticosterone to influence the activation state (basal and cytokine-induced) of microglia and macrophages is considered in this section.
3.5.1 Results (C)

3.5.1.1 Effect of noradrenaline on induction of FcR and MHC class II expression on microglia and Mφ

Microglia (0.5 x 10^5/well) or Mφ (10^5/well) were cultured for 72h in the presence of either IFN-γ (1 - 10 U/ml), noradrenaline (NA; 10 - 100μM) or IFN-γ plus NA and assayed for FcR and MHC class II expression. All incubations in which NA was present and the relevant media and IFN-γ-treated controls, included 100nM ascorbic acid, to inhibit degradation of noradrenaline. Note that μM concentrations of noradrenaline are commonly used in in vitro studies of Mφ function (Hu et al, 1991).

FcR expression

Fig. 3.17(a) and (b) illustrate the effect of NA on FcR expression on microglia and Mφ respectively. NA has no significant effect on expression on microglia but appears to decrease expression on Mφ. NA at 100μM does however reduce IFN-γ-induced expression on both cell types. At 10μM, NA is ineffective in this respect. From the results of a single experiment (not shown) where Mφ were treated with TNFα (10 - 50 U/ml) with and without NA (10μM), there is some suggestion that NA inhibits induction of FcR by TNFα, although this requires confirmation.

MHC class II expression

Fig. 3.18 illustrates the effect of NA on IFN-γ induction of MHC class II on microglia. NA alone has no effect on expression of this antigen and at a concentration of 10μM, NA has no significant effect on induction of MHC class II by IFN-γ. However 100μM NA significantly inhibits IFN-γ-induced expression (P≤ 0.05). Thus NA has
Fig. 3.17

Effect of noradrenaline on IFN-γ-induced Fc receptor expression on microglia and peritoneal macrophages.

Microglia (a) and peritoneal macrophages (b) (0.5x10⁵/well and 10⁵/well respectively) were treated for 72h with IFN-γ (1-10 U/ml) and/or noradrenaline (NA; 10-100 μM) before assay (performed in duplicates). Results are expressed as mean increases in [EA-E] over basal levels +/- SEM (microglia: n=6; Mφ: n=4).
Fig. 3.18

Effect of noradrenaline on IFN-γ-induced MHC class II expression on microglia

Microglia (0.5x10^5/well) were treated for 72h with IFN-γ (1-10 U/ml) and/or noradrenaline (NA; 10-100μM) before assay (performed in duplicates). Results are expressed as means of absorbance at 405nm +/- SEM (n = 5). (*) significantly different from medium control, (+) significantly different from IFN-γ-treated control; (P≤0.05).
a dose dependent effect. Similar treatment of macrophages with NA did not affect the MHC class II response to IFN-γ (not shown).

3.5.1.2 Effect of dexamethasone and corticosterone on induction of FcR and MHC class II expression on microglia and Mφ

Microglia (0.5 x 10⁵/well) or Mφ (10⁵/well) were cultured for 72h in the presence of either IFN-γ (1 - 10 U/ml), DEX (1-10 μM), CS (1-10 μM) or a combination of IFN-γ and DEX or CS; and assayed for FcR and MHC class II expression. Note that in vivo serum CS levels are in the region of 0.1-0.5 μM (J. Leonard, personal communication). Local concentrations in the tissue could be expected to be higher.

**FcR expression**

DEX alone (1μM) reduced FcR expression on microglia (P<0.05) and inhibited induction of expression when present [Fig. 3.19(a)] (P<0.05) during IFN-γ-stimulation. FcR expression on Mφ was reduced slightly by treatment with DEX (1μM). There was also a reduction in IFN-γ-induced FcR expression by Mφ, but this is not significant. CS (1μM) reduces FcR expression by microglia and macrophages (P ≤ 0.05) and significantly reduces IFN-γ-induced FcR expression in Mφ (P ≤ 0.05). Microglia also show a decrease in FcR expression on co-treatment with IFN-γ and CS relative to an IFN-γ-treated control [Fig. 3.19(a)].

**MHC class II expression**

DEX (1μM) or CS (1μM) had no effect on MHC class II expression on microglia but both caused a reduction in expression on Mφ [Fig. 3.19(b)]. Dex (1μM) significantly inhibited induction of expression by IFN-γ (10 U/ml) on microglia and macrophages [(Fig. 3.19(b)] (P ≤ 0.05). Likewise, corticosterone (1μM) reduced IFN-γ-induced (10 U/ml)
Combined effects of IFN-γ and corticosterone or dexamethasone on Fc receptor and MHC class II expression on microglia and peritoneal macrophages.

(a) Fc receptor expression

(b) MHC class II expression

Microglia (0.5x10⁶/well) and peritoneal macrophages (10⁶/well) were treated for 72h with individual mediators or a combination of IFN-γ and DEX or CS [10 U/ml IFN-γ; 1µM CS; 1µM DEX] before assessing the effect of these treatments on Fc receptor (a) and MHC class II (b) expression. FcR values are mean increases in [EA-E] over basal levels +/- SEM (n=2-6). MHC class II values are means of absorbance at 405nm +/- SEM (n=3-8). (*) significantly different from 0 or medium control, (+) significantly different from corresponding IFN-γ-treated control; (P≤0.05).
expression on both cell types. CS (1μM) significantly reduced expression on microglia induced by 1 U/ml IFN-γ (not shown).

3.5.2 Discussion (C)

Non-cytokine mediators influence cellular responses to cytokines and this study demonstrates that the sympathetic neurotransmitter noradrenaline and the glucocorticoids, dexamethasone and corticosterone, are capable of down-regulating IFN-γ-induced responses in microglia and macrophages. Noradrenaline inhibits IFN-γ-driven increases in phagocytic capacity and MHC class II expression on both microglia and macrophages in vitro, although by itself it has no effect on either functional property. Inhibition by noradrenaline of IFN-γ-induction of MHC class II on astrocytes is mediated via β2 adrenergic receptor signal transduction (Frohman et al, 1988). The β2 transduction pathway appears to be important in macrophage activation (Hetier et al, 1988) but there is little data on how it affects phagocytic function in particular. The cytotoxic actions of macrophages in vitro, as exemplified by TNF production, is significantly inhibited by noradrenaline (Hu et al, 1991).

Corticosterone is an endogenous glucocorticoid and dexamethasone a synthetic drug with approximately an 8-fold higher affinity for the glucocorticoid receptor and a much more potent anti-inflammatory agent in clinical use. Both glucocorticoids reduced the phagocytic capacity of microglia and, to a lesser extent, macrophages but had no effect on MHC class II expression and both reduced IFN-γ-induced Fc receptor and MHC class II expression on microglia and macrophages, in keeping with their known anti-inflammatory actions in vivo. Despite the much higher affinity of dexamethasone for the
glucocorticoid receptor, there is little difference between the responses to corticosterone and dexamethasone at the doses used in this study, although differences may have been evident at lower doses. In agreement with these findings, Pan et al (1990) report that dexamethasone partially blocks the induction of FcγRI on U-937 cells and neutrophils by IFN-γ, and hydrocortisone, another endogenous corticosteroid, has been shown to inhibit the binding of opsonised red blood cells to neutrophils (Klempner and Gallin, 1978) and monocytes (Tolone et al, 1979). Elsewhere, dexamethasone has been reported to synergistically increase IFN-γ-induction of Fc receptors on monocytes (Pan et al, 1990) and elicited peritoneal macrophages (Warren and Vogel, 1985) although MHC class II induction by IFN-γ is suppressed (Salkowski and Vogel, 1992; Warren and Vogel, 1985; Fertsch et al, 1987). In an in vivo study, Kiefer and Kreutzberg (1991) found that administration of dexamethasone two days before facial nerve axotomy, selectively inhibits the induction of MHC class II expression on microglial and perivascular cells without affecting other activation markers or cell morphology.

Glucocorticoids down-regulate the immune response by blocking induction of cytokine genes and by inhibiting the actions of these cytokines. They may modulate cell responses to cytokines by induction of lipocortins, small peptides which bind to and inhibit the activity of phospholipase A₂. On the other hand, cytokines may modulate sensitivity of cells to glucocorticoids. For example IFN-γ increases the number and affinity of glucocorticoid receptors in macrophages (Salkowski and Vogel, 1992). Thus, in vivo, cytokines which amplify the immune response can also sensitize macrophages to feedback inhibition by glucocorticoids. Furthermore, IL-1, IL-6 and TNFα have been shown to induce glucocorticoid secretion upon

Thus the response of macrophages to cytokines is modulated by non-cytokine mediators such as noradrenaline and glucocorticoids. The cytokine mediators of the immune system cannot therefore be viewed in isolation but in terms of interactions with the agents of the neural and endocrine systems.
CHAPTER 4

MYELINATION AND DEMYELINATION IN AGGREGATING CELL CULTURES OF FOETAL RAT TELENCEPHALON CELLS: EFFECTS OF CYTOKINES AND THE ROLE OF MACROPHAGES

4.1 INTRODUCTION

Dissociated foetal rat telencephalon cells under constant rotation in chemically defined medium form three-dimensional aggregates. In these conditions cells develop and differentiate on a time scale and in a fashion similar to that in the developing brain (Almazan et al, 1985). Under rotation the foetal cells rapidly aggregate in a haphazard fashion, forming clusters, and over a period of about 30 days these clusters develop into regular spheres of highly organised and differentiated brain cells (Honegger, 1985). Cellular proliferation and/or differentiation is enhanced in these cultures by several hormones and growth factors including epidermal growth factor and triiodothyronine which stimulate astrocyte and oligodendrocyte differentiation respectively (Almazan et al, 1985 a,b; Almazan et al, 1986). Examination of subcellular fractions by electron microscopy demonstrates the presence of myelin membranes which, on analysis, are chemically similar to those in normal brain tissue (Matthieu et al, 1979).

These aggregate cultures provide a useful system for studying myelination and demyelination. The MBP content correlates with the extent of myelination as assessed by electron microscopy. Thus a radioimmunoassay (RIA) for basic protein is a comparatively reliable means of evaluating the effect of various treatments or conditions on
myelination / demyelination in the aggregates. In the presence of complement, demyelination can be induced in these cultures by a monoclonal antibody directed against myelin / oligodendrocyte glycoprotein (MOG) (Kerlero de Rosbo et al, 1990).

In view of the evidence that brain macrophages / microglia are responsible for destruction of myelin and phagocytosis of debris in MS and the impact which cytokines appear to have on the functional properties of these cells, investigation of the effects of cytokines on myelination / demyelination is warranted. Cytokines may influence these events in various ways, perhaps by direct action on oligodendrocytes or the myelin sheath or indirectly, by induction of other mediators or activation of effector cells.

The effect of cytokines (IFN-γ, TNFα, IL-1α, IL-2) and LPS on myelinating aggregate cultures has been examined and the results are detailed below. As well as recording the MBP content of the cultures, cyclic nucleotide phosphohydrolase (CNPase) activity and glutamine synthase (GS) activity, measures of oligodendrocyte numbers and astrocyte differentiation respectively, have been assessed. Cytokine treatment lasted four days, during which time cultures were re-fed once. Peak myelination in untreated aggregates is achieved by day 20 to 25 of the culture period so cytokine treatment was commenced around this time. The effect of cytokines and LPS on anti-MOG-mediated demyelination has also been investigated.

In order to elucidate the role played by macrophages in myelination / demyelination, aggregates were enriched with macrophages by co-culturing the cells with freshly isolated peritoneal macrophages from the start of the culture period. The effect of cytokines and of anti-MOG on these macrophage-enriched cultures has been noted. Besides biochemical analysis of these cultures, the
aggregates have been examined using immunocytochemical techniques.

4.2 MATERIALS AND METHODS

4.2.1 Myelinating aggregate culture system

Serum-free, rotation-mediated aggregating cell cultures were prepared from foetal (15 - 16 days gestation) Sprague-Dawley rat telencephalon.

4.2.1.1 Media

Unless otherwise stated, all supplements were obtained from Sigma Chemical Co., Poole, Dorset, UK. Mechanical dissociation and washes of foetal telencephalon cells were carried out in D1 [modified Puck's solution D1 (Gibco BRL, Paisley, U.K.), pH7.2, 340 mosm containing 138 mM NaCl, 5.4 mM KCl, 0.17 mM Na$_2$HPO$_4$, 0.22 mM KH$_2$PO$_4$, 5.55 mM D-glucose and 58.43 mM sucrose] supplemented with gentamycin-sulphate (25mg/l; Gibco BRL) and phenol red (5mg/l; Gibco BRL). Cells were cultured in serum-free DMEM (Gibco BRL), supplemented with transferrin (1µg/ml), insulin (800nM), triiodothyronine (30nM), hydrocortisone-21-phosphate (20nM), 1xBME vitamins (Gibco BRL), vitamin B$_{12}$ (1.36mg/l), retinol (5.0 mg/l), DL-α-tocopherol (10.0 mg/l) and L-carnitine/HCl (2.0 mg/l). Gentamycin sulphate (25mg/l) was used as an antibiotic.

4.2.1.2 CNS tissue

The foetuses were removed from pregnant Sprague-Dawley mothers after 15 - 16 days gestation and placed in D$_1$ medium on ice.
The brains were dissected out and placed on a cold-plate for dissection of the telencephalon. These were collected in D_1 in a 50ml tube on ice.

4.2.1.3 Mechanical dissociation of telencephalon cells

The dissociation step was performed on ice. Tissue was washed twice in D_1 by decanting the medium and then sieved through a 200µm porosity nylon mesh by gently mashing with a sterile glass rod and collected in D_1 in a centrifuge tube on ice. This suspension was subsequently filtered through a nylon mesh, porosity 150µm, under gravity. The cell suspension, in D_1, was centrifuged for 15 min at 300g at 4°C with the brake off. The pellet was washed once in this fashion and resuspended in complete serum-free DMEM medium. 4ml aliquots (2 x 10^7 cells) of the cell suspension were seeded into 25ml De Long flasks and kept under constant rotation (70 - 80 rpm; Kuhner shaker, Philip Harris, U.K.) at 37°C in a humified atmosphere of 10% CO_2 / 90% air (Heraeus incubator, Philip Harris).

4.2.1.4 Maintenance of cultures

On the second day in vitro (DIV=2) cultures were transferred to 50ml De Long flasks and 4ml complete DMEM added making a total volume of 8ml/flask. Medium was replenished by exchange of 5ml DMEM per flask at DIV = 3, 6, 9, 12, 15, 18 and every other day thereafter. This was achieved by tilting the flask and allowing aggregates to settle before pipetting off 5ml of medium. On DIV = 20 half of the contents of the flasks were transferred to a fresh 50ml De Long flask containing 4ml of pre-warmed medium and 4ml medium was added to each of the original flasks to give 8ml/flask.
4.2.2 Antibody-induced demyelination in aggregating cultures

Demyelination was induced in aggregating brain cell cultures by addition of a monoclonal antibody (α-MOG), derived from clone 8-18C5 (Linington et al, 1984) directed against myelin/oligodendrocyte glycoprotein (MOG) in the presence of complement (guinea-pig serum). When medium was being exchanged on DIV = 26, 250μl of anti-MOG (1mg/ml) and 200μl of guinea-pig serum (both filter-sterilised) were added to cultures. Control flasks received PBS with or without guinea-pig serum. These additions were repeated on DIV = 28. Aggregates were harvested for biochemical analysis on DIV = 30.

4.2.3 Mφ enrichment of aggregating cultures

Peritoneal Mφ isolated from Sprague-Dawley rats according to the method described in chapter 2 were used to enrich the Mφ component of aggregating cultures. Mφ from a number of animals were pooled and viable cells counted by eosin exclusion. Mφ were suspended in a suitable volume of complete serum-free DMEM such that addition of 100μl of this suspension to flasks of dissociated telencephalon cells resulted in the desired ratio of Mφ : telencephalon cells. For example addition of 100μl of Mφ suspended at 10^7 cells/ml resulted in a 5% Mφ content (10^6 Mφ/flask). Freshly isolated Mφ were added within 4h of seeding. Thereafter macrophage-enriched cultures were treated in an identical fashion to controls.
4.2.4 Treatment of aggregate cultures with cytokines

Stock solutions of the following cytokines were prepared under sterile conditions and stored as described. Rat recombinant IFN-γ (10^5 U/ml in H2O; Holland Biotechnology, The Netherlands); human recombinant TNFα (2x10^4 U/ml in 0.1% BSA/PBS; British Biotechnology, Oxford, U.K.) were stored at -20°C. Murine recombinant IL-1α (25μg/ml in 0.1% BSA/PBS; Genzyme U.K.) was stored at -70°C. Human recombinant IL-2 (3x10^4 U/ml in 1% BSA/PBS; British Biotechnology) was kept at 4°C. Lipopolysaccharide (5mg/ml in PBS; Sigma Chemicals) was stored at -20°C. IFN-γ, human recombinant TNFα, IL-1α and LPS stock solutions were all aliquoted before freezing to avoid deterioration through freeze-thaw cycles. Murine recombinant TNFα (2x10^5 U/50μl; Genzyme) was thawed on arrival and refrozen in suitable aliquots.

Aggregate cultures were treated with cytokines on DIV = 26 and again on DIV = 28. All cytokine dilutions were prepared in filter-sterilised complete DMEM containing 1.6% w/v BSA such that exchange of 0.5ml of cytokine solution for 0.5ml of medium / flask resulted in the desired cytokine concentration and a final concentration of 0.1% BSA. Control cultures received BSA alone. Aggregates were harvested for analysis on DIV = 30.

4.2.5 Harvesting aggregate cultures for biochemical analysis

On DIV = 30 the contents of each flask were transferred to 10ml centrifuge tubes on ice and the aggregates allowed to settle under gravity. The supernatant was removed and aggregates were washed twice in this fashion with ice cold PBS. The aggregates were
transferred in a small amount of PBS to eppendorf tubes and as much
of the PBS was removed as possible. The pellets were then frozen on
dry ice and eppendorfs stored at -70°C until homogenisation.

Eppendorfs containing the frozen aggregates were placed on ice
and homogenised with 300μl of homogenisation buffer (2mM
K$_2$HPO$_4$, 1mM EDTA adjusted to pH 6.8 with 2mM KH$_2$PO$_4$, 1mM
EDTA) using a close fitting pestle. Homogenates were sonicated for
10s using a microtip (intensity 3) and the volume measured before
preparing aliquots for biochemical analyses. The homogenates were
vortexed vigorously prior to removing an aliquot. The MBP and
glutamine synthase assays required 100μl aliquots of homogenate. For
the CNPase assay 20μl of homogenate was added to 80μl of 1% Triton-
X-100 and for the Lowry protein assay 20μl of homogenate was added
to 500μl of 0.05M NaOH. Aliquots were stored at -70°C until they were
assayed.

4.2.6 Measurement of Protein concentration

Protein concentration was measured by the Folin phenol
method (Lowry et al, 1951) using bovine serum albumin as the
standard. All samples and standards were assayed in duplicate. The
aliquot of homogenate (20μl homogenate + 500μl 0.05M NaOH = 1/26
dilution) was thawed, sonicated in a waterbath and vortexed. 50μl of
each sample was diluted with 100μl H$_2$O. To this was added 50μl of
0.4 M NaOH and 1ml of solution A (prepared by mixing 2% Na$_2$C$_3$O$_3$,
0.02% dipotassium tartrate in 0.1 M NaOH with 1% CuSO$_4$ . 3H$_2$O in
the ratio 99:1 immediately before use) and all tubes were vortexed.
After 15 min 100μl of Folin solution (diluted 1:1 with H$_2$O) was added
and the tubes placed in the dark for a further 30 min after which time
the absorbance at 750nm was determined using a UV spectrophotometer. Using a standard curve (0 - 20μg BSA), absorbance readings were converted to μg protein / sample. The protein concentration in the homogenate was calculated as follows:

\[
\frac{(M \times D)}{V} = \mu g \text{ protein } / \mu l \text{ in original homogenate}
\]

\[
= mg \text{ protein } / ml
\]

where:

- \( M \) = mean μg protein/sample
- \( D \) = dilution of homogenate in 0.05M NaOH (ie. 26)
- \( V \) = volume (μl) used per assay (ie. 50)

4.2.7 Radioimmunoassay for MBP

The MBP content of homogenates was measured by radioimmunoassay (Kerlero de Rosbo et al, 1990).

4.2.7.1 Reagents for RIA

Stocks of the following solutions were stored at 4°C: saline solution containing 140mM NaCl, 2.9mM KCl, 1.15mM CaCl\(_2\), 1.15mM MgCl\(_2\) and a 2M Tris-acetate, pH 7.7 solution. Lab-trol E, a control serum (Baxter Dade AG, Dudingen, Switzerland), was included in all reactions and was prepared as two solutions. The first, ‘Lab-trol’ in Table 4.1, was a 0.5% v/v solution of Lab-trol E in saline solution. The second solution also contained a detergent, 0.025% w/v Hexadecyltrimethylammonium bromide (CTMB), and a further 0.05 M NaCl. Solution B, added to all reactions, contained histones from calf thymus (Sigma Chemical Co.) at 10mg/ml in 2M tris-acetate buffer pH 7.7 to which was added 5% newborn bovine serum. Solution C was used for dilution of standards and homogenates and was prepared by
mixing solution B, H₂O and 0.4% CTMB, in saline solution plus 0.8M NaCl in the ratio 1 : 4 : 5.

The anti-MBP antiserum (donated by J.-M. Matthieu), stored as a 1/10 dilution at -70°C, was further diluted 1/50 in 0.5% NaCl containing a protease inhibitor (Trasylol, 800 IU/ml; Bayer, Switzerland). A blank received the Trasylol/NaCl solution without antiserum. Solution D contained the ¹²⁵I-labelled BP (1×10⁶ cpm/ml) in a mixture of solution B, solution C and H₂O in the ratio 9 : 9 : 82. All solutions except those containing CTMB (which precipitates at 5°C) were kept on ice during preparations.

4.2.7.2 RIA reactions

Homogenates were thawed and sonicated briefly. Dilutions of a standard homogenate [60ng myelin basic protein (BP)/µl; donated by J.-M. Matthieu] in solution C were used as a standard curve, covering a range of 1 - 60ng BP in 50µl volumes. Homogenate samples for analysis were also diluted in solution C, typically 1 part in 16 in these experiments. The reaction mixtures for blanks, standards and homogenate samples were all prepared in duplicate in 2ml tubes, making additions in the order indicated in Table 4.1 and vortexing after each addition.

The tubes were covered and placed in a shaking water bath at 37°C for 1h after which they were placed on ice and 20µl of solution D containing the ¹²⁵I-labelled BP (2×10⁴ cpm) was added per tube. They were then incubated at 4°C for approximately 42h after which time 570µl of cold ethanol was added to each tube. They were gently vortexed and left at 4°C for 30 min to precipitate the proteins. Tubes were centrifuged at 900g for 90 min at 4°C. Supernatants were removed to fresh tubes and both the pellets and supernatants were
Table 4.1

Preparation of reaction mixtures for myelin basic protein RIA (section 4.2.7).

<table>
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<td>Lab-trol+CTMB+NaCl</td>
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<tr>
<td>(1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>Soltn. B (Histones)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>(2)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>antiserum (inTrasylol+NaCl)</td>
<td>-</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Trasylol+NaCl</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Homogenate (in Soltn. C)</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Soltn. C</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(4)</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

All volumes are in µl; HOMOG = Homogenate.

The amount of Lab-trol+CTMB+NaCl solution used was calculated as (400 / D) where D is the dilution factor of the homogenate. The amount of Lab-trol was adjusted accordingly such that the volume at this stage (1) was 400µl.

A dilution of 1/16 of the test homogenate (in Solution C) was generally suitable in these experiments. For more concentrated samples a higher concentration of detergent CTMB was required.
counted (2 min count, $^{125}\text{I}$ program on LKB Wallac, Minigamma counter).

**4.2.7.3 Calculation of results**

All duplicate counts were meaned.

For the blank:

\[ B = \frac{(\text{SN} + 1)}{\text{P}} \times 100 \]

where SN = counts in supernatant

P = counts in pellet

and for samples or standards:

\[ X = \frac{(\text{SN} + 1)}{\text{P}} \times 100 - B \]

A standard curve of $X$ versus \log[ng BP] was plotted and used to convert homogenate (HO) sample $X$ values to ng BP/tube.

\[ \text{ng BP/\text{\textmu}l homogenate} = \frac{\text{ng BP/tube} \times 16 \text{ (dilution factor)}}{50 \text{ (vol of diluted HO/reaction)}} \]

The BP content of cultures is expressed as $\mu$g BP/mg protein, calculated by dividing the above figure by protein content (mg/ml).

**4.2.8 CNPase assay**

$2', 3'$ - cyclic nucleotide $3'$ - phosphohydrolase (CNPase; EC 3.1.4.37) activity was determined by the method of Sogin (1976). The assay is based on a two step process during which $2'-3'$ cyclic NADP is converted to NADP and subsequently reduced to NADPH which is detectable by measuring the absorbance at 340nm. The first step is catalysed by CNPase and the second requires a second substrate
glucose-6-phosphate (G-6-P) and the enzyme glucose-6-phosphate dehydrogenase (G-6-PDH).

4.2.8.1 Reagents for CNPase assay

All reagents were obtained from Sigma Chemical Co. A stock of 0.274M MES, 41.12mM MgCl₂, pH6.0 (Sogin solution) was kept at 4°C. The two substrates, 11.1mM 2' - 3' cyclic NADP (Sigma N5257) and 55.5mM glucose-6-phosphate (G-6-P; Sigma G 7879) were aliquoted and stored at -20°C. Glucose-6-phosphate dehydrogenase (G-6-PDH; Sigma G 7878), 0.06 U/ml in 0.2% BSA/Sogin solution, was stored for up to 1 month at 4°C.

4.2.8.2 Protocol for CNPase assay

Homogenate aliquots (20μl neat homogenate + 80μl 1% Triton X-100) were thawed and sonicated briefly. A standard homogenate (donated by J.-M. Matthieu) was used to prepare a dilution series (1/50 - 1/400 in 1% Triton X-100). Samples to be tested were diluted in 1% Triton X-100 before use (generally 1/10). All standards and test samples were assayed in duplicate. Reaction mixtures were prepared in 3ml tubes on ice and constituted 800μl Sogin solution, 100μl G-6-P solution, 100μl cNADP solution, 10μl G-6-PDH and 60μl H₂O. A Pye Unicam spectrophotometer was programmed to measure increase in absorbance at 340nm after a 99 s delay, reading 20 times at 10 s intervals, the program lasting approximately 5 min. Thus reactions were started at 5 min intervals. The substrate mixture was prewarmed to 31-32°C by placing in a waterbath for 5 min. 40μl of the diluted homogenate (test/standard) was added to the prewarmed mixture, vortexed and transferred immediately to the glass cuvette and the program was started. Meanwhile the reaction mixture for the
next sample was placed in the waterbath. A blank in which the homogenate was replaced with 40μl H₂O was also measured.

4.2.8.3 Calculation of results

If values were too high, i.e. outside range of standards, the homogenates were diluted further in 1% Triton X-100 before assay. The CNPase activity in the original neat homogenate was calculated according to the following formula:

\[
\text{CNPase activity} = \frac{(OD_{\text{test}} - OD_{\text{blank}}) \times \text{dilution}}{\text{Vol. assayed} \times [\text{Protein}]} \rightarrow \mu\text{mol/min/mg protein (mg/ml)}
\]

4.2.9 Glutamine synthetase assay

Glutamine synthetase (GS; EC 6.3.1.2) activity was assayed by a modification of the method of Pishak and Phillips (1979) using [1-\(^{14}\)C]glutamic acid (New England Nuclear-DuPont, Boston, MA, USA) as the glutamine precursor and phosphoenolpyruvate/pyruvate kinase as the ATP regenerating system enabling conversion of \(^{14}\)C-glutamic acid to \(^{14}\)C-glutamine by GS.

4.2.9.1 Reagents for GS assay

All reagents were obtained from Sigma Chemical Co. Suitable aliquots of [1-\(^{14}\)C] glutamic acid were stored lyophilised in 4ml tubes and the GS cocktail was prepared in these tubes such that 10μl of the GS cocktail contained 1x10⁵ cpm. The cocktail was prepared by adding pyruvate kinase (50 U/ml), ATP (sodium salt, 10mM) and 2-mercaptoethanol (20mM) to a Imidazole-HCl buffer (pH7.4) containing
glutamic acid (potassium salt, 50mM), MgCl$_2$.6H$_2$O (12.5mM), NH$_4$Cl (4mM), phosphoenolpyruvate (sodium salt, 13mM), ouabain (1mM) and Triton X-100 (0.16%).

4.2.9.2 Glutamine synthetase reaction

Homogenate samples and standards were thawed and sonicated. Two dilutions of all samples were made in homogenisation buffer (4.2.5) [1:3 and 1:1 to give a volume of 40μl]. The blanks (in duplicate) contained 40μl of homogenisation buffer only. All samples, standards and blanks received 10μl of GS cocktail containing $^{14}$C-glutamic acid (1x10$^5$ cpm). Tubes were vortexed and placed in a 37°C water bath for 20 min after which time the reaction was stopped by addition of 1ml of ice cold water and tubes were placed on ice. $^{14}$C-Glutamic acid which had not been converted to glutamate was removed from the reaction mixture on Dowex AG1-X8 200-400 mesh columns (5.5 x 0.5 cm). The columns were prepared for this separation step by rinsing through with acetic acid (5N) followed by H$_2$O. The contents of each reaction tube were poured onto a column and rinsed out with 1ml cold dH$_2$O. The ‘run-through’ was collected in scintillation vials. Samples were washed through with 5 x 1ml cold volumes of dH$_2$O, also collected in the vials. 10ml of scintillation fluid was added to each vial, the vials were capped and vortexed thoroughly. These vials were then counted for 10 min each on a $^{14}$C program (Searle Analytic Mark III, Denley Instruments Ltd., Daux Rd., Billinghamurst, Sussex). A ‘total count’ was obtained by addition of 10ml scintillant to 10μl GS cocktail. Columns were regenerated after use by washing through 3 x 2ml volumes of acetic acid (5M) followed by 3 x 2ml volumes of dH$_2$O.
4.2.10 **Immunocytochemical staining of aggregate cultures**

In order to study the cellular composition and configuration of aggregates, including those supplemented with peritoneal macrophages, they were harvested, snap frozen and cryostat sectioned before immunocytochemical analysis using a panel of antibodies.

4.2.10.1 **Harvesting aggregate cultures for immunocytochemistry**

The contents of the culture flask were transferred to a 10ml centrifuge tube. The aggregates were allowed to settle under gravity and the supernatant was removed. Aggregates were washed twice in PBS at RT in this fashion. They were then transferred in a little PBS to one half of a gelatin capsule and using a pasteur pipette the PBS was removed quickly before the capsule became too soft. The capsule was then carefully filled with O.C.T. compound (Merck Ltd., Poole, Dorset, U.K.) such that the pellet of aggregates was not dispersed but kept as compact as possible. The capsule containing the aggregates in O.C.T. was then snap frozen in isopentane cooled on liquid nitrogen and transferred at once to a -70°C freezer.

4.2.10.2 **Sectioning and staining of snap frozen aggregates**

The gelatin capsule was peeled off the frozen O.C.T.. 10µm thick cryostat sections (Slee, UK) were cut and picked up on gelatin-coated glass slides, air-dried and stored at -70°C until required for staining. In preparation for immunocytochemistry, slides were warmed to RT and sections ringed using a gelatin pen (Dako, UK). Before blocking of sections, all except those for which OX42 was the primary antibody were fixed for 10 min in ice-cold acetone. Sections for OX42 staining were fixed for 2 min in ethanol at RT.
The following primary antibodies were employed. MAb OX42 (Serotec, Oxford, U.K.) recognising the CR3 receptor on microglia, macrophages and neutrophils (Robinson et al, 1986) was used at a concentration of 1:50 following fixation. MAb 5.2E4 for GFAP staining of astrocytes was used at 1:200 (Newcombe et al, 1986). An anti-galactocerebroside (anti-GC) MAb (from this laboratory) was used at a concentration of 1:100 to label oligodendrocytes. Anti-MBP polyclonal antibody (from this laboratory) was used at a concentration of 1:200. The MAb ED1 (Serotec) for monocytes and macrophages was used at 1:50. All dilutions were made in 1% BSA/PBS. Species and sub-class specific controls were substituted for primary antibodies to assess non-specific staining.

The avidin-biotin peroxidase staining procedure was followed as described in 2.2.8.1. In some cases sections were counterstained with haematoxylin following the peroxidase reaction. Rinsed slides were placed in Mayers haematoxylin (Merck Ltd.) for 4-5 min and rinsed under running tap water for 5 min. All sections were dehydrated by passing through absolute ethanol, 50% ethanol, 50% ethanol : 50% xylene and xylene in that order. A drop of DPX mountant (Merck Ltd.) was placed on each section and a coverslip positioned on top. Slides were viewed on a Leitz Dialux microscope and photographed with Ilford HP5 and Kodak Ektachrome 160T film.
4.3 RESULTS

4.3.1 Effect of cytokines and anti-MOG on biochemical parameters of aggregates

Aggregate cultures were maintained and treated with cytokines on day 26 and 28 as described in the methods section. Aggregates were harvested and homogenised on day 30 in culture and homogenates subsequently assayed for BP content and CNPase and GS activity. All treatments were performed in triplicate and the means of the various parameters calculated. The means of 2-4 experiments are expressed as proportions of the control (Table 4.2). Note that though there was some variation in the absolute values of individual experiments, within each group relative effects were consistent.

Treatment with IFN-γ (rat recombinant), TNFα (both murine and human recombinant), IL-1α (murine recombinant) and LPS caused a reduction in the BP content of the cultures whilst CNPase activity was only slightly reduced. Thus myelin loss occurred without significant loss of oligodendrocytes. There is also a small drop in GS activity with treatments in particular with IFN-γ. The extent of demyelination caused by the various cytokine treatments ranged from 45 to 60% loss. Murine TNFα was ineffective at a dose of 100 U/ml but myelin loss was considerable at 500-1000 U/ml. IL-1α and human recombinant TNFα demonstrated a flat rate of demyelination, apparently independent of the increasing cytokine concentration. The result with LPS is interesting because at higher doses (250-1000 ng/ml) there appears to be less myelin loss than at lower doses (10-50 ng/ml), perhaps due to feedback inhibition by LPS-induced cytokines or a toxic effect of LPS on an effector cell.
Table 4.2

Effect of treatment of myelinating aggregate cultures with cytokines and LPS

<table>
<thead>
<tr>
<th>Medium control</th>
<th>BP</th>
<th>CNPase</th>
<th>GS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ (U/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.65 ± 0.07</td>
<td>0.91 ± 0.12</td>
<td>0.78 ± 0.04</td>
</tr>
<tr>
<td>50</td>
<td>0.58 ± 0.00</td>
<td>0.95 ± 0.03</td>
<td>0.79 ± 0.05</td>
</tr>
<tr>
<td>100</td>
<td>0.57 ± 0.05</td>
<td>0.96 ± 0.09</td>
<td>0.79</td>
</tr>
<tr>
<td>200</td>
<td>0.62 ± 0.15</td>
<td>1.01 ± 0.08</td>
<td>0.79 ± 0.03</td>
</tr>
<tr>
<td>TNFαm(U/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.10 ± 0.05</td>
<td>1.02 ± 0.13</td>
<td>ND</td>
</tr>
<tr>
<td>500</td>
<td>0.71 ± 0.17</td>
<td>1.01 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>TNFαh(U/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.53 ± 0.01</td>
<td>0.80 ± 0.01</td>
<td>0.90</td>
</tr>
<tr>
<td>500</td>
<td>0.56 ± 0.04</td>
<td>0.92 ± 0.08</td>
<td>0.88</td>
</tr>
<tr>
<td>IL-1α(U/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.52 ± 0.10</td>
<td>0.90 ± 0.08</td>
<td>0.93</td>
</tr>
<tr>
<td>50</td>
<td>0.57 ± 0.02</td>
<td>0.86 ± 0.07</td>
<td>0.96</td>
</tr>
<tr>
<td>LPS (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.54 ± 0.06</td>
<td>0.91 ± 0.08</td>
<td>0.92</td>
</tr>
<tr>
<td>1000</td>
<td>0.52 ± 0.10</td>
<td>0.90 ± 0.08</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Additions were made on days 26 and 28 in vitro and cultures were harvested on day 30 in vitro. BP content and CNPase and GS activity were assayed and are expressed as a proportion of the control. Results represent the means of 1 - 4 experiments ± SEM where appropriate. ND = not determined.
Table 4.3 shows the results of parallel experiments (sharing the same controls) in which anti-MOG and guinea-pig serum (complement) were also present in untreated and cytokine-treated cultures. In the absence of any cytokine, anti-MOG plus complement elicited a decrease in BP content, a slight decrease in CNPase activity and a small increase in GS activity. Demyelination mediated by anti-MOG and complement was enhanced in the presence of IFN-γ, TNFα, IL-1α and LPS. Again there was comparatively little effect on CNPase activity and GS activity.

The effect of IL-2 on myelination and oligodendrocyte cell number was investigated in a single experiment (triplicate flasks) in which aggregates were treated throughout the culture period (DIV= 1 to 30) with 50 U/ml IL-2. This treatment had no effect on myelination, gauged by BP content (Table 4.4).

4.3.2 Effect of Mφ enrichment on biochemical parameters of aggregate cultures

Table 4.5 details the results of two experiments (each in quadruplicate) in which telencephalon cell cultures were supplemented with Mφ at the beginning of the culture period. Flasks which contained Mφ produced a greater number of aggregates than the control flasks. These aggregates were also visibly larger in size than control aggregates. Biochemical analysis revealed a greater degree of myelination (BP content) in Mφ-enriched cultures compared to control cultures and this increase was dependent on the Mφ : telencephalon cell ratio. Where Mφ were added as approximately 6.5% and 5% of the cells by number, BP content was respectively 3 fold and 1.5 fold that of the control. There was a small reduction in CNPase and GS activity
Table 4.3

Comparison of the effect of cytokines and LPS on aggregate cultures in the presence and in the absence of anti-MOG monoclonal antibody and complement (α-MOG+C).

<table>
<thead>
<tr>
<th></th>
<th>no (α-MOG+C)</th>
<th>plus (α-MOG+C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BP</td>
<td>CNPase</td>
</tr>
<tr>
<td>Medium control</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IFN-γ (U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.57</td>
<td>0.96</td>
</tr>
<tr>
<td>200</td>
<td>0.61</td>
<td>1.03</td>
</tr>
<tr>
<td>TNFαm (U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.15</td>
<td>1.15</td>
</tr>
<tr>
<td>500</td>
<td>0.54</td>
<td>1.05</td>
</tr>
<tr>
<td>TNFαh (U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.54</td>
<td>0.79</td>
</tr>
<tr>
<td>500</td>
<td>0.60</td>
<td>0.84</td>
</tr>
<tr>
<td>IL-1α (U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.52</td>
<td>0.90</td>
</tr>
<tr>
<td>50</td>
<td>0.57</td>
<td>0.86</td>
</tr>
<tr>
<td>LPS (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.54</td>
<td>0.91</td>
</tr>
<tr>
<td>250</td>
<td>0.69</td>
<td>1.06</td>
</tr>
</tbody>
</table>

All additions were made on days 26 and 28 in vitro and cultures were harvested on day 30 in vitro. BP content and CNPase and GS activity were assayed and are expressed as a proportion of untreated control values. Results represent means of parallel experiments (n=1-2; triplicate flasks).
**Table 4.4**

The effect of continuous treatment with IL-2 on BP content and CNPase and GS activity of aggregate cultures.

<table>
<thead>
<tr>
<th>medium</th>
<th>BP</th>
<th>CNPase</th>
<th>GS</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IL-2 (50 U/ml)</td>
<td>1</td>
<td>0.78</td>
<td>0.94</td>
</tr>
</tbody>
</table>

In a single experiment IL-2 (50 U/ml) was included in the medium of test flasks from day 1 in vitro until day 30 when the cultures were harvested. Results are expressed as a proportion of the control values.
### Table 4.5

The effect of macrophage enrichment of aggregate cultures on BP content and CNPase and GS activity.

<table>
<thead>
<tr>
<th></th>
<th>BP μg/mg protein</th>
<th>CNPase μmol/min/mg protein</th>
<th>GS μmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.42 (1)</td>
<td>2.98 (1)</td>
<td>221.1 (1)</td>
</tr>
<tr>
<td>+Mϕ (~6.5%)</td>
<td>7.21 (2.98)</td>
<td>2.64 (0.89)</td>
<td>146.7 (0.7)</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.81 (1)</td>
<td>2.04 (1)</td>
<td>162.2 (1)</td>
</tr>
<tr>
<td>+Mϕ (~5%)</td>
<td>2.66 (1.47)</td>
<td>1.77 (0.87)</td>
<td>135.7 (0.8)</td>
</tr>
</tbody>
</table>

In two experiments, freshly isolated peritoneal macrophages (Mϕ) were added to culture flasks (in triplicate) containing dissociated foetal rat telencephalon cells such that they comprised approximately 6.5% (A) and 5% (B) of the total number of cells per flask. Control flasks received no macrophages and all cultures were maintained and harvested as usual. Means of actual values are indicated and are also represented in parentheses as a proportion of controls.
in Mφ-enriched cultures compared to control aggregates.

4.3.3 Effect of cytokines and anti-MOG on Mφ-enriched cultures

In a single experiment (triplicate flasks) the effect of IFN-γ, TNFα and LPS on biochemical parameters of Mφ-enriched (approximately 6.5% Mφ on day 0) and normal aggregate cultures was investigated. Cytokine treatments were as above. The results, presented in Table 4.6, indicate that IFN-γ, TNFα, and LPS reduce BP content of these Mφ-enriched cultures as with the normal aggregates and the percentage decrease is similar in both cultures.

A second study compared anti-MOG-mediated demyelination in normal and Mφ-enriched (5% Mφ) aggregate cultures (Table 4.7). Anti-MOG and guinea-pig serum were present from DIV = 27 to 29 when aggregates were harvested. The degree of myelin loss, compared to relevant controls, was greater in the Mφ-enriched aggregates than in the normal aggregates when anti-MOG and complement were present. In both cases this treatment elicited a small decrease in CNPase activity and a slight increase in GS activity.

4.3.4 Immunocytochemical analysis of aggregate cultures

Immunocytochemical techniques have been used to identify the various cell types in the aggregates and to observe any changes in the morphology of these cells on cytokine treatment. Note that appropriate species and subclass specific controls showed negligible background staining.
Table 4.6

Effect of cytokines and LPS on normal and macrophage-enriched aggregate cultures.

<table>
<thead>
<tr>
<th></th>
<th>no Mφ</th>
<th></th>
<th>plus Mφ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BP</td>
<td>CNPase</td>
<td>GS</td>
<td>BP</td>
</tr>
<tr>
<td></td>
<td>µg/mg</td>
<td>µmol/min/ mg protein</td>
<td>µmol/min/ mg protein</td>
<td>µg/mg</td>
</tr>
<tr>
<td>medium control</td>
<td>2.42±0.26</td>
<td>2.98±0.07</td>
<td>221.1±8.5</td>
<td>7.21±1.00</td>
</tr>
<tr>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
</tr>
<tr>
<td>IFN-γ (U/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.42±0.14</td>
<td>3.06±0.06</td>
<td>185.8±8.7</td>
<td>4.72±0.25</td>
</tr>
<tr>
<td>(0.59)</td>
<td>(1.03)</td>
<td>(0.84)</td>
<td>(0.65)</td>
<td>(0.97)</td>
</tr>
<tr>
<td>200</td>
<td>1.54±0.28</td>
<td>2.95±0.26</td>
<td>178.3±10.6</td>
<td>5.39±0.54</td>
</tr>
<tr>
<td>(0.64)</td>
<td>(0.99)</td>
<td>(0.81)</td>
<td>(0.75)</td>
<td>(1.13)</td>
</tr>
<tr>
<td>TNFαm (U/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2.54±0.32</td>
<td>2.66±0.10</td>
<td>ND</td>
<td>5.06±0.61</td>
</tr>
<tr>
<td>(1.05)</td>
<td>(0.89)</td>
<td>(0.32)</td>
<td>(0.70)</td>
<td>(0.84)</td>
</tr>
<tr>
<td>500</td>
<td>2.12±0.24</td>
<td>2.34±0.79</td>
<td>ND</td>
<td>2.32±0.20</td>
</tr>
<tr>
<td>(0.88)</td>
<td>(0.79)</td>
<td>(0.32)</td>
<td>(0.75)</td>
<td>(1.13)</td>
</tr>
<tr>
<td>LPS (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.72±0.61</td>
</tr>
<tr>
<td>(0.65)</td>
<td>(0.98)</td>
<td>(1.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.60±0.79</td>
</tr>
<tr>
<td>(0.64)</td>
<td>(1.03)</td>
<td>(1.19)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values indicated are actual means of quadruplicate flasks from a single experiment ± SEM and figures in parentheses are these values expressed as proportions of the relevant (+ Mφ) controls.
Table 4.7

α-MOG-mediated demyelination in normal and macrophage-enriched aggregates

<table>
<thead>
<tr>
<th></th>
<th>BP μg/mg protein</th>
<th>CNPase μmol/min/mg protein</th>
<th>GS μmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>no Mφ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR</td>
<td>1.81 ± 0.08</td>
<td>2.04 ± 0.13</td>
<td>162.2 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
</tr>
<tr>
<td>+α-MOG</td>
<td>0.98 ± 0.05</td>
<td>1.69 ± 0.01</td>
<td>259.9 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>(0.54)</td>
<td>(0.83)</td>
<td>(1.6)</td>
</tr>
<tr>
<td>+ Mφ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR</td>
<td>2.66 ± 0.33</td>
<td>1.77 ± 0.22</td>
<td>135.7 ± 19.6</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
</tr>
<tr>
<td>+α-MOG</td>
<td>0.73 ± 0.10</td>
<td>1.06 ± 0.03</td>
<td>163.2 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>(0.27)</td>
<td>(0.60)</td>
<td>(1.2)</td>
</tr>
</tbody>
</table>

α-MOG and guinea-pig serum (complement) were added to normal (no Mφ) and macrophage-enriched (+ Mφ) cultures on day 27 in vitro and aggregates harvested on day 29. Macrophage-enriched cultures were supplemented with approximately 5% peritoneal macrophages (by cell number) from day 0 of culture period. Values indicated are actual means of 3-6 flasks from a single experiment ± SEM and figures in parentheses are fractions of the relevant (± Mφ) controls.
Fig. 4.1 and 4.2 illustrate the pattern of staining observed in normal and Mφ-enriched aggregates in the absence of any cytokine treatment. In Fig. 4.1 OX42 (a,b) and ED1 (c,d) MAbs were used. OX42 stains microglia and macrophages whereas ED1 recognises macrophages but not microglia. OX42+ and ED1+ cells are scarce in normal aggregates [Fig. 4.1(a), (c)] but in those cultures which have been supplemented with Mφ there are many more OX42+/ED1+ cells, indicating that these cells become integrated in the aggregates [Fig. 4.1(b), (d)]. Fig. 4.2 illustrates BP (a, b), GFAP (c, d) and GC (e) staining in normal and Mφ-enriched aggregates. There is a slight increase in staining intensity of BP in Mφ-enriched cultures which is in agreement with the observed increase in BP content on biochemical analysis [Fig. 4.2 (a), (b)]. BP staining is widespread and diffuse with frequent intensely stained patches. This contrasts with the pattern of isolated clumps of strongly positive cells obtained with the anti-GC antibody [Fig. 4.2 (e)]. GFAP+ astrocytes are distributed fairly uniformly throughout the aggregate and there appears to be no difference in staining of this marker in normal and Mφ-enriched cultures [Fig. 4.2 (c), (d)].

Fig. 4.3 depicts BP staining on both normal and Mφ-enriched aggregates which were treated with IFN-γ (200 U/ml) (c, d) and the relevant untreated controls (a, b). The intensity of BP staining in both cultures appears to decrease on treatment with IFN-γ, again in line with the biochemical analysis of BP content. The effect of this same treatment on GFAP staining is illustrated in Fig. 4.4. In both cultures the branching network formed throughout the aggregate by astrocytic processes is clearly visible. There appears to be more GFAP+ astrocytes in the Mφ-enriched aggregates [Fig. 4.4(b)] than in normal aggregates [Fig. 4.4(a)] where both have been treated with IFN-γ (200 U/ml).
There are virtually no OX42+ (a) or ED1+ (c,e) cells in normal untreated aggregates. Expression of CR3 (demonstrated by OX42) and the ED1 antigen is increased in cultures which are enriched in Mφ [(b) and (d,f) respectively] reflecting the successful integration and survival of these cells in the aggregate cultures. ED1-stained sections were counterstained in haematoxylin. [a,b: magnification x260; c,d: magnification x 60; e,f: magnification x 285].
Fig. 4.2
Demonstration of BP, GFAP and GC expression on normal and Mφ-enriched aggregate cultures by immunocytochemistry.

Normal (a, c, e) and Mφ-enriched (b, d) aggregates.

There is a slight increase in BP staining intensity in Mφ-enriched cultures [compare (a) and (b)]. Whereas BP staining is widespread (a, b), GC positive staining forms more discrete, intense patches (e; normal culture). GFAP in normal (c) and Mφ-enriched (d) cultures is uniform. Sections were counterstained in haematoxylin. [a,b,c,d: magnification x285; e: magnification x60].
Fig. 4.3
Immunocytochemical demonstration of the effect of IFN-γ on BP levels in normal and Mφ-enriched aggregate cultures.

Normal (a,c) and Mφ-enriched (b,d) aggregates.

There is reduced BP staining in both normal and Mφ-enriched aggregates on treatment with IFN-γ (200 U/ml) (c,d) compared to untreated cultures (a,b). Note that the increased BP in Mφ-enriched cultures is apparent in IFN-γ-treated as in untreated cultures. Sections were counterstained in haematoxylin.

[a,b: magnification x285; c,d: magnification x60]
Fig. 4.4

Demonstration by immunocytochemistry of GFAP expression on normal and Mφ-enriched aggregate cultures treated with IFN-γ.

Mφ-enriched aggregates (b) contain more GFAP+ astrocytes than do normal aggregates (a) on treatment with IFN-γ (200 U/ml). Sections were counter-stained in haematoxylin. [magnification x285; see Fig. 4.2 (c) and (d) for untreated controls].
Normal and Mϕ-enriched aggregates which were treated with murine-recombinant TNFα (500 U/ml) are compared with untreated controls in terms of OX42 staining in Fig. 4.5. Treatment with TNFα seems to induce expression of CR3 in normal aggregates [Fig. 4.5 (a), (c)]. These weakly positive cells have a ramified/bipolar morphology suggestive of microglia. There also appears to be an increase in the intensity of OX42 staining in Mϕ-enriched cultures on treatment with TNFα [Fig. 4.5 (b), (d)].

4.4 DISCUSSION

This study demonstrates that LPS and the cytokines IFN-γ, TNFα and IL-1α are all potent inducers of demyelination in myelinated aggregate cultures during the period of peak myelination. Continuous treatment with IL-2 (50 U/ml) for the whole culture period has no effect on myelination. Antibody-mediated demyelination has been demonstrated in this system in confirmation of previous reports (Kerlero de Rosbo et al, 1990), by use of anti-MOG in the presence of complement and these results indicate that the extent of demyelination can be further increased by simultaneous treatment with LPS, IFN-γ, TNFα or IL-1α. The considerable demyelination resulting from these treatments is not accompanied by oligodendrocyte cell death. There is a small increase in the astrocyte marker (GS) following anti-MOG-mediated demyelination but not with LPS, IFN-γ, TNFα or IL-1α treatment. Thus these results point to a selective destruction of myelin without incurring oligodendrocyte cell death or affecting other cells such as astrocytes.

Immunocytochemical analysis of cultures to which peritoneal macrophages were added at the start of the culture period indicates
Fig. 4.5
Immunocytochemical demonstration of the effect of TNFα on CR3 expression in normal and Mφ-enriched aggregate cultures.

Normal (a,c) and Mφ-enriched (b,d) aggregates.

There is increased expression of CR3, as demonstrated by OX42 staining, on microglia-like cells in normal aggregates treated with TNFα (500 U/ml) [(c) compared to (a)]. Mφ in untreated Mφ-enriched aggregates are OX42+ (b) and on treatment with TNFα (d) these express somewhat higher levels of this marker. [magnification x260].
that these macrophages become successfully integrated into the aggregate structures. Normal aggregates contain very few OX42+ cells however expression of CR3 appears to be moderately upregulated on treatment of these cultures with TNFα, suggesting activation of microglia / macrophages by this cytokine. The higher GFAP in Mφ-enriched cultures which have been treated with IFN-γ compared to similarly treated normal aggregates may be caused by IL-1 produced by Mφ. IL-1 is an astroglial growth factor (Giulian et al, 1988a) and it stimulates astroglial proliferation following brain injury (Giulian and Lachman, 1985). Thus this response to IFN-γ may be mediated by IL-1.

On biochemical analysis macrophage-enriched aggregates were found to contain more BP/mg protein indicating a more extensive myelination. Demyelination could be induced in these macrophage-enriched cultures, as with the normal aggregates, by treatment with anti-MOG in the presence of complement or with LPS, IFN-γ or TNFα. Surprisingly LPS and these cytokines produced a degree of demyelination comparable to that achieved in normal aggregates. Thus there is no evidence that cytokine-induced activation of macrophages is the key to demyelination in this case. Perhaps this demyelination results from some direct action of the cytokines on myelin or oligodendrocytes. In contrast anti-MOG-mediated demyelination is proportionately greater in the macrophage-enriched aggregates indicating perhaps that macrophage availability for Fc and/or complement receptor-mediated ingestion of myelin is a limiting factor in this demyelination.

Antibody-mediated demyelination has been investigated in depth in both in vitro and in vivo systems though less is known about the role of cytokines in demyelination. Sera from animals with demyelinating EAE contains antibodies directed against MBP,
proteolipid protein (PLP) and galactocerebroside and causes demyelination in CNS cultures (Lassmann and Linington, 1987). Vass et al (1992) report that though anti-MOG when injected into the subarachnoid space of rat brain causes only modest demyelination, co-injection of IFN-\(\gamma\) (itself ineffective) potentiated this effect. In the in vitro results described here, treatment with IFN-\(\gamma\), TNF\(\alpha\) or IL-1\(\alpha\) alone induced demyelination and when anti-MOG was also present there was an additive or moderately synergistic effect. The effect of TNF on myelinated cultures has been reported elsewhere. Selmaj and Raine (1988) found that TNF (but not IFN-\(\gamma\) or IL-2) induced delayed-onset necrosis of oligodendrocytes and myelin dilatation where the myelin sheath was abnormal but not degraded. It has been suggested that this pathology results from direct action of TNF\(\alpha\) on myelin or the axonal membrane (Brosnan et al, 1988).

In chronic EAE vesiculation and swelling of the myelin sheath are early signs of demyelination (Dal Canto et al, 1975). Another feature is myelin stripping by macrophages. Macrophages and microglia express both Fc and C3b receptors and the presence of phagocytosed myelin in coated pits and vesicles of macrophages suggests that ingestion of myelin is receptor-mediated (Epstein et al, 1983). As well as being involved in the clearing-up of myelin debris it seems likely that macrophages are involved in the initial stages of myelin damage. Macrophages produce potentially very destructive agents including proteases, lipases, reactive oxygen intermediates, cytokines and glycosidases. Released by the macrophages adjacent to the myelin sheath these agents would effectively degrade myelin. The cellular processes of mononuclear cells adjacent to the myelin in demyelinating lesions in EAE lack organelles, reflecting perhaps considerable recent secretory activity (Lampert, 1983).
One might have expected that supplementing the aggregate cultures with macrophages would have made them more susceptible to demyelination induced by IFN-γ, TNFα and IL-1α since, as described in chapter 3, these cytokines all upregulate Fc receptor expression and IFN-γ and TNFα induce superoxide secretion by macrophages. In fact the extent of demyelination in macrophage-enriched cultures was similar to that in normal cultures. Perhaps the demyelination resulting from cytokine treatment is not mediated by macrophages but is a direct effect of the cytokine on the myelin or oligodendrocyte such as observed by Selmaj and Raine (1988) or Merrill and Zimmerman (1991). Alternatively, the number of macrophages / microglia present in the normal aggregates may be sufficient to produce maximal cytokine-induced demyelination and increasing their number would fail to further enhance demyelination. Macrophage-enrichment of the cultures did however render them more susceptible to anti-MOG-mediated demyelination. This suggests that in this case the availability of macrophages is a determining factor and may be indicative of an Fc and/or C3b receptor-mediated mechanism. The differences between cytokine and antibody-induced demyelination and their relation to each other requires further clarification but on the basis of initial studies quite different mechanisms appear to be involved.

The enhanced myelination in untreated macrophage-enriched aggregates in comparison to normal aggregates demonstrates the other side of the coin. Clearly a balance exists between enhancement of myelination versus demyelination by macrophages. Macrophages play a part in tissue modelling in the developing CNS. They phagocytose dying and apoptotic cells (Perry and Gordon, 1991) and thereby facilitate axonal growth and myelination in the developing CNS (see
Clearance of myelin debris by macrophages at a site of demyelination is necessary before remyelination can occur. Macrophages produce various growth factors including epidermal growth factor (EGF), acidic and basic fibroblast growth factor (FGF), insulin-like growth factor-1, platelet-derived growth factor 1 and 2 (PDGF) and transforming growth factor (TGF) as well as IL-1 and other cytokines. It seems likely then that macrophages may influence both growth and differentiation of many cell types in the CNS. In particular PDGF influences progenitor cell and oligodendrocyte proliferation and differentiation (Wolswijk et al, 1991) and may influence myelination. Aggregates were treated with IL-2 in the expectation that, as a promoter of oligodendrocyte proliferation, this cytokine would affect the extent of myelination. However there was no effect at the single dose of IL-2 tested.

Further investigation is necessary to determine fully how macrophages participate in or influence the processes of myelination and demyelination.
CHAPTER 5

CONCLUSION

The functional properties of microglia, in particular their phagocytic and lytic capabilities as well as antigen presenting capacity, appear to ideally qualify them for participation in the processes of inflammation and demyelination in the central nervous system. The regulation of these properties has been one of the chief areas of interest in this study. Like virtually every mammalian cell type, microglia are subject to regulation by cytokines. As has been demonstrated, microglia themselves secrete cytokines on stimulation in vitro and in vivo. The wide variety of cytokines, naturally occurring inhibitors and other mediators and the many different variables in in vivo situations makes the study of cell regulation a complex matter.

From the results of this investigation, where a largely in vitro approach has been adopted, it is apparent that microglial functional properties are differentially regulated by cytokines and that these cytokines do not act independently but interactively. A cytokine may affect the response of a cell to subsequent cytokine treatments as well as modulating the effect of those present simultaneously. Although microglia are similar in many respects to other macrophage populations such as peritoneal macrophages, the responses of these cell types to cytokines vary. Other variables which determine cell responses include relative doses of cytokines and the timing / sequence of exposure to the cytokines. Microglial function is also subject to regulation by components of the neural and endocrine systems and interactions between these and the immune system in vivo cannot be ignored.
The use of the aggregate culture system facilitated study of demyelination \textit{in vitro}. Demyelination can be induced in these cultures by cytokines, however involvement of macrophages / microglia in this process remains to be demonstrated. The observation that enrichment of these cultures with macrophages leads to enhanced myelination is particularly interesting. Brain macrophages / microglia may play a part in both destruction of myelin and in myelination / remyelination during development or following myelin loss due to disease. A balance may exist between these activities and cytokines are likely to be critical in determining which way this balance tips.

Further investigation of ways in which microglial activities could be modulated are necessary, in particular a number of approaches to counteracting or neutralising the effect of stimulatory cytokines on microglia should be explored. These include the use of neutralising antibodies to cytokines, their intermediates or their receptors. Other possibilities include naturally occurring inhibitors and soluble receptor forms; inhibitors of transcription of the cytokine gene or of translation of the mRNA; inhibitors of the processing of a cytokine precursor or of secretion of the cytokine. The use of inhibitory cytokines such as TGF-β1 to downregulate cytokine-induced microglial activation also deserves further investigation. The interaction between IFN-γ and IL-4 is interesting because the nature of the microglial response to these cytokines depends not only on the order in which the cells are exposed to the cytokines but also on their relative concentrations. This suggests that it may be possible to influence cellular activity by making comparatively subtle changes to levels of expression of cytokines.

The aggregate culture system employed here provides an ideal means of studying the process of myelination, demyelination and
remyelination in vitro. In future studies, identification of the putative macrophage-derived factors capable of influencing myelination would be of primary interest. The use of Northern blotting techniques to detect message for any macrophage-derived factors, basic protein, CNPase etc. would complement the biochemical analysis used here and aid the determination of those factors involved in demyelination as well as investigation of the process of remyelination following cytokine or antibody-mediated demyelination.
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