The Modulatory Effect of Cytokines on Cell Proliferation in C6 Glioma Cells

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List of Abbreviations

ACTH	adrenocorticotrophic hormone
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
APRF	the acute phase response factor
BBB	blood-brain barrier
Cal C	calphostin C
cAMP	cyclic adenosine 3′,5′ monophosphate
CDMEM	complete Dulbecco's modified Eagle medium
cGMP	cyclic guanosine 3′,5′ monophosphate
CNP	2′,3′-cyclic nucleotide 3′-phosphohydrolase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
cPKCs	Ca^{2+}-dependent or conventional PKCs
CPM	counts per minute
CRPMI Medium	complete Roswell Park Memorial Institute 1640 medium
CSF	colony stimulating factor
DAG	diacylglycerol
dbcAMP	N^6-2′-O-dibutyryl cyclic adenosine-3′,5′-monophosphate
dbcGMP	N^6-2′-O-dibutyryl cyclic guanosine-3′,5′-monophosphate
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
EAE	experimental allergic encephalomyelitis

EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid
EPO	erythropoietin
G-CSF	granulocyte colony stimulating factor
G-Protein	guanine nucleotide-binding protein
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte/macrophage colony stimulating factor
GS	glutamine synthetase
HEPES	N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]
Her A	herbimycin A
ICAM	intercellular adhesion molecule
IFN	interferon
IGF-I	insulin-like growth factor-I
IL	interleukin
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-6	interleukin-6
iNOS	inducible NOS
$LaCl_3$	lanthanum chloride
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MHC	major histocompatibility complex
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NAME	N^W-nitro-L-arginine methyl ester

NGF	nerve growth factor
NMA	N^G-methyl-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
nPKCs	Ca^{2+}-independent or novel PKCs
O-2A	oligodendrocyte-type-2 astrocyte
OD	optical density
PBS	phosphate buffered saline
PDA	phorbol-12,13-diacetate
PDGF	platelet-derived growth factor
PKC	protein kinase C
PMA	phorbol 12 -myristate 13-acetate
PtdSer	phosphatidyserine
Ro31-8220	3-{1-[3-(amidinothio)propyl]-3-indolyl}-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione methanesulfonate
RPMI Medium	Roswell Park Memorial Institute 1640 medium
SDS	sodium dodecylsulfate
SNP	sodium nitroprusside
SP	substance P
ST	staurosporine
TGF	transforming growth factor
TK	tyrosine kinase
TNF-α	tumor necrosis factor-alpha
TYR	tyrphostin

Abstract

Cytokines are a group of protein cell regulators that are mainly produced by a wide variety of cell types, especially cells of the immune system. Recently, it has been shown that cytokines can induce immune responses on the cells of central nervous system. Since cytokines play an important role in the communication between cells of the immune and nervous systems, it is of considerable interest to study their effects on C6 glioma cells, which represent a good model system for studying the conditions and factors which regulate the proliferation and differentiation of glial cells and the signalling pathways that might be involved in these processes.

Results of the present study indicated that cytokines such as TNF-α, IL-1α, IL-1β and LIF stimulate [^{3}H]-thymidine incorporation in C6 cells, in a dose- and time-dependent manner. Not only DNA synthesis was increased but the growth of the C6 cells was also markedly enhanced by these cytokines. In addition, lipopolysaccharide (LPS), a well-known potent inducer of cytokine secretion, was found to induce significant proliferation in C6 cells.

The present study demonstrated that both TNF-α– and LIF-induced proliferation in C6 cells involve protein kinase C (PKC), Ca^{2+}

and tyrosine kinase. The involvement of PKC mediating the action of TNF-α was demonstrated by observations that potent PKC inhibitors, Ro31-8220, staurosporine and calphostin C, inhibited both the TNF-α– and LIF-stimulated proliferation in C6 cells; whereas PKC activators, PMA and PDA, induced C6 cell proliferation. In addition, the proliferative effects of PMA and PDA were also blocked by the three PKC inhibitors tested.

Since some PKC isoforms are calcium-dependent, the effects of A23187, a calcium ionophore, as well as some calcium channel blockers on cytokine-induced proliferation were investigated. A23187 alone stimulated C6 cell proliferation; and in the presence of a sub-optimal dose of cytokines such as TNF-α, IL-1α, IL-1β and LIF, A23187 further enhanced the proliferation induced by these cytokines. The involvement of calcium in mediating the proliferative effects of TNF-α and LIF is further supported by observations that calcium channel blockers--$LaCl_3$, verampil and nifedipine, inhibited the proliferative effects of these two cytokines in a concentration-dependent manner. In addition, EGTA, a Ca^{2+} chelator, has similar inhibitory action on the cytokine-induced C6 cell proliferation.

Since tyrosine kinase is a well known second messenger in cytokines function, its involvement in cell proliferation was also examined in this project. It was found that C6 cell proliferation induced by TNF-α and LIF was sensitive to the presence of selective

tyrosine kinase inhibitors, herbimycin A and tyrphostin, which could block the proliferative effects of PMA and PDA.

In addition, the present study also showed that nitric oxide synthase inhibitors, N^G-methyl-L-arginine and N^W-nitro-L-arginine methyl ester, were capable of inhibiting the proliferative effect of TNF-α or LIF on C6 cells. On the other hand, the exogenous nitric oxide donor, sodium nitroprusside, did not show any proliferative effect on C6 glioma cells. Though the nitric oxide formation was induced by cytokines including TNF-α, LIF, IL-1α and IL-1β, the increase was much lower than the reported data.

Finally, the involvement of cyclic nucleotides and β-adrenergic receptor in cytokine-induced C6 cell proliferation was also investigated. It was found that cyclic nucleotides, including dbcAMP and dbcGMP, and a β-adrenergic agonist, isoproterenol, significantly increased the proliferation of C6 cells. Likewise, propranolol, an antagonist of β-adrenergic receptor, was shown to reduce greatly the stimulatory effect of TNF-α or isoproterenol on [^{3}H]-thymidine incorporation in C6 cells at a concentration of 25 μM. The exact mechanism(s) by which cyclic nucleotides and β-adrenergic receptor might modulate C6 cell proliferation remains to be elucidated.

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Chapter 1 INTRODUCTION

1.1 Cytokines in the Central Nervous System

1.1.1 Basic Properties of Cytokines

Cytokines are a group of protein cell regulators, which are produced by a wide variety of cells in response to various inducing stimuli, and may have effects on cells distant from the cells of origin. Additionally, they are defined as a class of inducible, water-soluble, heterogeneous proteinaceous mediators of animal origin with molecular weights greater than 5,000 that exercise specific, receptor-mediated effects in target cells, or in mediator-producing cells themselves (Meager, 1990).

Cytokines are usually subdivided into well-characterized classes including "growth factors", "colony stimulating factors (CSF)", "interleukins (IL)", "tumor necrosis factors (TNF)", "interferons (IFN)" and "chemotactic factors", etc. The properties of some major cytokines are given in Table 1.1. Cytokines may be considered to have hormone-like activities. Since they mostly exhibit direct mitogenic effect and act at short range in local cellular environments, they have been described as a new category of

intercellular mediators, even though, sometimes, they act at a distance when released into the blood. Secondly, they are pleiotropic mediators, which can act on many different cell types, or on cells of the same lineage at different stages of development. Moreover, cytokines can be produced by specialized and unspecialized cells of many tissues and organs, and their receptors are usually found on many cell types other than the predicted target cells. Furthermore, their biological activities are often highly overlapped. In brief, all the above properties of cytokines are different from those of hormones.

Table 1.1 Properties of Some Major Cytokines*

Cytokine	No of A. A.	MW(kD)	Receptor types	Principal sources
TNF-α	157	51 (trimer)	55 kD/75 kD types	macrophages
LIF	179	38-67	α-chain: 200 kD β-chain: 130 kD (gp130)	T lymphocytes; carcinoma cells
IL-1α	159	17.5	80 kD/68 kD types	monocytes and many other cell types
IL-1β	153	17.3	80 kD/68 kD types	monocytes and many other cell types
IL-6	184	21	α chain: 80 kD β chain: 130 kD	T lymphocytes; monocytes; many other cell types

* From Clemens, 1991; Nicola, 1994 and Pimentel, 1994.

Following receptor occupancy by cytokines, intracellular signaling pathways are activated to effect metabolic changes, gene induction, and structural alterations leading to proliferative, differentiating, or functional responses of the target cells. The genes coding for cytokines appear to be tightly regulated. A specific stimulus is usually required for induction or de-repression of cytokine genes. In many instances, a number of these substances and entities act by binding to the cell surface, in a way much resembling the binding of cytokines to their cell receptors, to stimulate cytokine synthesis. For example, part of the cell wall of pathogenic Gram-negative bacteria known as, lipopolysaccharide (LPS), binds to the cell surface of macrophages and induces the synthesis of a number of cytokines (Lee *et al.*, 1993b).

It is well-documented that cytokines have important roles in the growth, development, and maintenance of advanced multicellular, multi-organ species where the orderly assembly and function of cells is vital to normal morphogenesis, not only during embryogenesis and neonatal development but also through to adolescence and adulthood (Nicola, 1994). In addition, cytokines play a large number of physiological roles, such as control of host defenses against infections, cell proliferation and differentiation, regulate hematopoiesis, immune responses and inflammatory responses and fever, wound healing and tissue remodelling, and influence on cellular metabolism etc. (Clemens, 1991).

1.1.2 The General Characteristics of Glial Cells

The first account of neuroglia was cited by Dutrochet in 1824, who noticed the existence in the central nervous system (CNS) of non-neuronal components made up of spindle-shaped cells which were morphologically distinct from neurons (Virchow, 1860). These cells were considered as a form of connective tissue within the CNS, and were called "neuroglia", which means nerve glue. There are two broad sub-groups of glial cells: the macroglia, which consists of astrocytes, oligodendrocytes and ependymal cells, and microglia. The basic properties of astrocytes, oligodendrocytes and microglia are described below.

1.1.2.1 Astrocytes

The astrocyte is the most abundant cell type in the CNS, outnumbering neurons by about 10:1. They are, in reality, a lineage representing a large family of cells that share certain biochemical and morphological specialization, while diverging in certain functional capabilities. Classically, there are two principal types of astrocytes, the protoplasmic (type-1) and fibrous (type-2) astrocytes, which are classified according to their morphological properties, antigenic phenotypes, kinetic development, appearance, and responses to the growth factors. The protoplasmic astrocytes are characterized by

thick, branched processes with spiny projections and are localized primarily within the gray matter. The fibrous astrocytes, in contrast, consist of relatively long, thin processes with few branches and are the predominant type in the white matter.

There are fundamentally three characteristic features that define astrocytes in the mammalian CNS. The most important is the cytoplasmic inclusion of 6- to 9-nm intermediate filaments whose major structural component is a 49-kD protein, the glial fibrillary acidic protein (GFAP) (Eng *et al.*, 1971). The two other features are the presence of glycogen granules observed by electron microscopy (Revel *et al.*, 1960), and membrane-associated orthogonal-particle complexes by freeze-fracture studies. These complexes are strictly unique to astrocytes and are not found on myelin, neurons or other glial cells (Dermietzel, 1974). Recently, other biochemical markers in astrocytes are also found, such as glutamine synthetase (GS), the calcium binding protein, S-100, and glutathione-S-transferase subtype Y_b (Cammer *et al.*, 1989; Norenberg & Martinez-Hernandez, 1979; Waniewski & Martin, 1986).

Along with the great variety of astrocyte types, there is an equal diversity of specified functions that can be ascribed to particular astrocytes based upon their positional relationships within the neuraxia and some of them are listed in Table 1.2.

Table 1.2. Some Known Astrocyte Functions*

1. Neuronal support
2. Guidance for developing neurons
3. Regulation of the extracellular environment
4. Synaptic encapsulation
5. Regulation of synaptic modulation
6. Participation in node of Ranvier formation
7. Regulation of cerebral blood flow by K^+ siphoning
8. Induction of blood-brain barrier properties in endothelial cells
9. Scar formation and repair of CNS following injury
10. Response capability to a variety of transmitters and peptides

* From Frohman *et al.*, 1989b.

1.1.2.2 Oligodendrocytes

Oligodendrocytes arise from the oligodendrocyte-type-2 (O-2A) progenitor cell. The function of the oligodendrocyte is to produce myelin. Myelin sheaths are cytoplasmic projections which extend from the oligodendrocyte cell body to wrap around nerve fibers in a spiral fashion. There are three kinds of oligodendrocyte-specific markers. The most common one is the galactocerebroside, which is unique to oligodendrocytes and is the major glycolipid of myelin (Raff *et al.*, 1978). The other type of marker is the protein markers, which include myelin basic protein, proteolipid protein and myelin-associated protein (Lees & Brostoff, 1984). Additionally, oligodendrocytes have several cell-specific enzymes, such as 2′,3′-

cyclic nucleotide 3'-phosphohydrolase (CNP) and glycerol-3-phosphate dehydrogenase (de Vellis *et al.*, 1967; Drummond *et al.*, 1962).

1.1.2.3 Microglial

Microglia constitute approximately 10% of the total glial cell population, which are considered as the resident macrophages of the brain (Benveniste, 1992b). According to their variable morphology, there are several major subtypes of microglia including ramified, ameboid and perivascular microglia. The major function of microglia is the phagocytosis of cellular debris (Perry & Gordon, 1988). Microglia can be identified by a number of cell markers, such as immunoglobulin Fc receptor (Perry *et al.*, 1985), type 3 complement receptors (Giulian & Baker, 1986), β_2-integrins (Akiyama & McGeer, 1990), nonspecific esterase (Suckling *et al.*, 1983) and lectin *Ricinus communis* agglutinin I (Mannoji *et al.*, 1986). However, all these known phenotypic markers for microglia are shared with other cell types.

1.1.3 The Effects of Cytokines on Neural Cells

Cytokines, many of which have been discovered in studies of the hematopoietic system, are well known to regulate immune cell development and functions. Interestingly enough, a number of

cytokines, such as TNF-α, IL-1α, IL-1β, IL-6 and LIF, have also been found to have the ability to regulate neural cell proliferation and gene expression. These cytokines are known to affect the survival, growth, and gene expression in various types of neurons and glial cells in culture (Bartfai & Schultzberg, 1993). Moreover, several of these cytokines are either normally expressed in the nervous system or are up-regulated in the central or peripheral nervous system after injury or in neurological disease (Merrill & Chen, 1991; Patterson, 1994). In view of the importance of these cytokines as signals in neuroimmune interactions, the basic characteristics of these cytokines and their modulatory effects on neuronal cells will be further discussed in the following sections.

1.1.3.1 TNF-α and Neural Cells

TNF-α is a 17 kD protein produced by macrophage in response to a wide variety of stimuli including mitogens, cytokines, bacteria, viruses and parasites (Spriggs *et al.*, 1992). TNF-α has been shown to take part in altering vascular endothelial cell functions during inflammation. For instance, TNF-α enhances their permeability (Brett *et al.*, 1989), induces their expression of adhesion molecules (Prober *et al.*, 1986) and increases local adhesion of neutrophils, monocytes or lymphocytes to endothelial cell surfaces (Pohlman *et al.*, 1986). TNF-α is known to stimulate other cell types to produce cytokines, including IL-1, IL-6 , CSFs and TNF-α itself (Aggarwal & Vilcek, 1992; Beutler,

1992). TNF-α can also regulate immune responses by modulating the expression of class I and II major histocompatibility complex (MHC) molecules on a variety of cell types. TNF-α is also an endogenous pyrogen which acts on cells in the hypothalamic regions of the brain to induce fever (Beutler & Cerami, 1989). In addition, TNF-α plays a role in multiple sclerosis, and is involved in autoimmunity and in bacterial, parasitic, and viral infection (Beutler, 1992).

TNF-α has been shown to mediate myelin damage *in vitro* (Selmaj & Raine, 1988), and has cytotoxic activity against rat oligodendrocytes, which results in cell death (Robbins *et al.*, 1987). These observations suggest that TNF-α contributes to the demyelination process in neurological diseases. TNF-α also has multiple effects on astrocytes which are noncytotoxic in nature, and may function in an autocrine manner as astrocytes express specific high affinity TNF-α receptors (Benveniste *et al.*, 1989), and secrete TNF-α upon activation by a variety of stimuli (Chung & Benveniste, 1990; Lieberman *et al.*, 1989). Astrocytes also respond to TNF-α by secreting IL-6, granulocyte colony stimulating factor (G-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF) (Benveniste *et al.*, 1990; Frei *et al.*, 1989; Malipiero *et al.*, 1990). Additionally, mouse microglia produce TNF-α in response to LPS or IFN-γ (Frei *et al.*, 1987). In primary astrocytes, TNF-α increases class I MHC and intercellular adhesion molecule-1 (ICAM)-1 expression, and enhances class II MHC expression induced by IFN-γ or virus

(Frohman *et al.*, 1989a; Massa *et al.*, 1987). This indicates that astrocyte can function as an antigen presenting cell (APC) within the CNS. TNF-α has a direct mitogenic effect on both primary astrocytes (Selmaj *et al.*, 1990) and human astroglioma cell lines (Bethea *et al.*, 1990; Lachman *et al.*, 1987), which is thought to contribute to the reactive astrogliosis associated with various neurological diseases. On the other hand, TNF-α also targets on the neurons of hypothalamus in fever (Dinarello *et al.*, 1986). In peripheral tissues, such as pancreatic cells, TNF-α has been shown to induce insulin secretion (Southern *et al.*, 1990). These observations confirm the diverse functions of TNF-α in the body.

1.1.3.2 LIF and Neural Cells

LIF is a basic and heavily glycosylated monomeric protein whose molecular weight is about 32-62 K (Hilton, 1992). LIF has been shown to be involved in the regulation of proliferation, differentiation and other functions in a wide diversity of cell types (Kurzrock *et al.*, 1991; Metcalf, 1991). Some examples are: hematopoietic stem cell growth and differentiation (Escary *et al.*, 1993), megakaryocyte maturation (Burstein *et al.*, 1992) and embryonic tissue development (Conquet & Brulet, 1990). Also, LIF can induce the production of IL-6 or other acute phase proteins in the liver (Murray *et al.*, 1990). Through the induction of a variety of cytokines, LIF can modulate inflammation, immune responses, and

connective tissue metabolism, and can act as a pathogenetic mediator in different disease states (Villiger *et al.*, 1993).

In the nervous system, LIF promotes the transition from noradrenergic to cholinergic function in cultured sympathetic neurons, concomitantly decreasing tyrosine hydroxylase activity and inducing the activity of choline acetyltransferase and vasoactive intestinal polypeptide (Murphy *et al.*, 1991; Sendtner *et al.*, 1990). Also, LIF affects the survival or differentiation of motor and sensory neurons (Murphy *et al.*, 1991; Sendtner *et al.*, 1990). When applied to peripheral nerves *in vivo*, this cytokine is retrogradely transported, and rescues damaged sensory neurons (Cheema *et al.*, 1994; Hendry *et al.*, 1992) as it does in cultured neurons (Murphy *et al.*, 1993). LIF also alters the phenotype of sensory neurons in culture (Fan & Katz., 1993; Nawa *et al.*, 1990). There was evidence that LIF can be produced by glial cells in culture (Patterson & Chun, 1974; Shadiack *et al.*, 1993). In addition, recombinant LIF can duplicate the effects of nerve injury in the induction of particular neuropeptides in sympathetic neurons (Jonakait, 1993; Patterson & Nawa., 1993; Sun *et al.*, 1994). In the brain, LIF level rises after injury (Jonakait, 1993). Collectively, these results indicate that LIF production in the nervous system is induced by injury, and plays an important role in neuronal responses during injury.

1.1.3.3 IL-1 and Neural Cells

IL-1 is a cytokine responsible for mediating a variety of processes in the host response to microbial and inflammatory diseases. There are two forms of IL-1, IL-1α and IL-1β, which are the products of two different genes (Pimentel, 1994). Although these two forms of IL-1 have less than 30% structural homology to one another, they both bind to the same surface receptor, and have essentially identical biologic activities (Arai *et al.*, 1990; de Giovine & Duff, 1990). IL-1 is the major co-stimulator for T-cell activation via the augmentation of both IL-2 and IL-2 receptor expression. These effects allow antigen-stimulated T cells to proliferate rapidly and expand in number. IL-1, in cooperation with other cytokines, enhance the growth and differentiation of B cells (Arai *et al.*, 1990; de Giovine & Duff, 1990). IL-1 is a principal participant in inflammatory reactions through its induction of other inflammatory metabolites, such as prostaglandin, collagenase, and phospholipase A_2 (Arai *et al.*, 1990; de Giovine & Duff, 1990). Similar to TNF-α, IL-1 acts on endothelial cells to promote leukocyte adhesion and stimulates numerous cell types to produce various cytokines, such as IL-6, TNF-α, CSFs and IL-1 itself (Arai *et al.*, 1990; de Giovine & Duff, 1990).

IL-1 affects a wide range of target cells in the nervous system. Purified IL-1 was shown to have a stimulatory activity for astrocyte growth *in vitro* (Giulian & Lachman, 1985), and when injected into the

brain, IL-1 can stimulate astrogliosis (Giulian *et al.*, 1988a). Recombinant IL-1 has also been shown to stimulate the proliferation of a human astrocytoma cell line, U373, and to modulate gene expression in astrocytes (Lachman *et al.*, 1987). Moreover, IL-1 can increase the expression of ICAM-1 adhesion molecule on human astrocytes (Frohman *et al.*, 1989a). This is particularly important because the expression of ICAM can enhance the ability of an APC to present antigen when the number of MHC molecules on the cell surface is low. This suggests that the combined expression of ICAM and MHC may be synergistic in eliciting a more potent immune response (Demaine, 1989).

IL-1 is also a potent inducer of other cytokine production in astrocytes. The stimulation of primary cultured rat astrocytes by IL-1 results in the secretion of TNF-α (Chung & Benveniste, 1990) and IL-6 (Benveniste *et al.*, 1990; Frei *et al.*, 1989), while in human astroglial cell lines CSFs (Tweardy *et al.*, 1990), TNF-α (Bethea *et al.*, 1991), and IL-6 (Yasukawa *et al.*, 1987) are produced in response to IL-1. The production of IL-1 can be induced by LPS in C6 glioma cell line (Fontana *et al.*, 1982), murine astrocytes and microglia (Giulian *et al.*, 1986; Malipiero *et al.*, 1990). Additionally, IL-1 acts as an autocrine stimulator of astrocytes, as they can secrete and respond to this cytokine.

In neurons, IL-1 has been shown to stimulate the synthesis of nerve growth factor (NGF) during peripheral nerve injury (Bartfai & Schultzberg, 1993; Berkenbosch *et al.*, 1987). IL-1β mRNA has been detected in the rat hippocampal pyramidal cell layer (Bandtlow *et al.*, 1990), and IL-1 receptor is observed in the dentate gyrus of hippocampus in rat (Farrar *et al.*, 1987) and mouse brains (Haour *et al.*, 1990; Takao *et al.*, 1990). Further studies on purified, homogeneous cell populations of neural crest origin, such as PC12 cells, also support the claim that neuronal cell types may synthesize IL-1 (Alheim *et al.*, 1991). These studies suggest that IL-1 is synthesized by neurons.

The effects of IL-1 on the neuroendocrine system include regulation of corticosterone and gonadal hormone production, induction of slow-wave sleep, fever, corticotrophin-releasing factor and gastric acid secretion (Bartfai & Schultzberg, 1993).

1.1.3.4 IL-6 and Neural Cells

IL-6, along with IL-1 and TNF-α, are pleiotropic cytokines involved in the regulation of inflammatory and immunologic responses (Kishimoto, 1989). IL-6, a 26 kD molecule, is secreted by a wide range of cells. Depending on the cell types, the synthesis of IL-6 is induced by a variety of agents, including the cytokines IL-1, TNF-α and IFN-γ (Le & Vilcek, 1989). The two better known actions of IL-6

are on hepatocytes and B cells. In these cells, IL-6 can stimulate hepatocytes to produce several plasma proteins, such as fibrinogen and C-reactive protein, which contribute to the acute phase response (van Snick, 1990). In the immune system, IL-6 is the principal cytokine for inducing terminal differentiation of activated B cells into immunoglobulin-secreting plasma cells (van Snick, 1990). A minor function of IL-6 is as co-stimulator of T cells and thymocytes (van Snick, 1990).

IL-6 has been demonstrated to have multiple effects on astrocytes, and functions in an autocrine manner as astroglioma is found to express specific high-affinity receptors for IL-6 (Taga *et al.*, 1987). There are two endogenous CNS sources for IL-6, the astrocyte and microglia. Primary rat and murine astrocytes can secrete IL-6 in response to a variety of stimuli including virus, IL-1, TNF-α, IFN-γ plus IL-1, LPS and calcium ionophore (Benveniste *et al.*, 1990; Frei *et al.*, 1989; Lieberman *et al.*, 1989), while transformed microglia clones also produce IL-6 (Rigi *et al.*, 1989). IL-6 has direct mitogenic effect on astrocytes (Selmaj *et al.*, 1990) and the astrocytes secrete nerve growth factor in response to IL-6, which induces neural differentiation (Frei *et al.*, 1989). IL-6 has been found to induce the differentiation of PC 12 cells, as well as to increase the number of voltage-dependent Na^+ channel (Satoh *et al.*, 1988). Moreover, IL-6 has also been shown to inhibit TNF-α production by monocytes (Aderka *et al.*, 1989). As astrocytes can secrete TNF-α, and TNF-α induces IL-6 production by

astrocytes, this may represent a negative regulatory pathway for controlling TNF-α expression in the CNS.

In addition to the above effects on differentiation and cytokine production, IL-6 has many actions on the pituitary gland, such as stimulation of ACTH, gonadotropins and prolactin release (Naitoh *et al.*, 1988). Recently, it has been shown that IL-1β induced an increased release of IL-6 from cultured rat anterior pituitary (Yamaguchi *et al.*, 1990).

1.1.4 Immune Responses in the Central Nervous System

From the above discussion, it is clear that cytokines play an important role in inducing immune responses on the cells of the central nervous system. However, the CNS has traditionally been considered as an "immunologically privileged site" for three major reasons. Firstly, the CNS lacks for the most part a lymphatic system that drains the tissues and captures potential antigens. Secondly, the CNS is protected from the blood by the blood-brain barrier (BBB), which is basically impermeable to many soluble substances, including cytokines, and restricts the migration of lymphoid cells into CNS. Lastly, the cells of the CNS express very low levels of antigens encoded by the MHC genes, whose products play a fundamental role in the induction and regulation of immune response (Booss *et al.*, 1983; Peters *et al.*, 1976; Wong *et al.*, 1984).

Despite the above observation, recent work has shown that T cells in very low numbers are found within normal brain tissue, and also in normal cerebrospinal fluid (Booss *et al.*, 1983). However, the presence of lymphatic-like capillaries in the brain provides a possible natural, untraumatized, route for lymphoid cells into the CNS (Prineas, 1979). Another evidence is that pathological events within the CNS often result in a breakdown of BBB, which permits cells of the peripheral immune system to enter this site. During human CNS diseases, such as viral encephalitis (Prineas, 1979), multiple sclerosis (Hauser *et al.*, 1983, Prineas & Wright, 1978; Traugott *et al.*, 1983), AIDS dementia complex (Navia *et al.*, 1989) and animal models of CNS diseases, such as experimental allergic encephalomyelitis (EAE), inflammatory infiltrates composed of activated T cells, B cells, and macrophages are found in the brain. Furthermore, glial cells, especially astrocytes, can secrete immunoregulatory molecules that influence immune cells, as well as the glial cells themselves. Table 1.3 shows the involvement of some cytokines in various diseases in the brain (Morganti-Kossmann *et al.*, 1992; Pimental, 1994). Thus, astrocytes, which have been demonstrated to act as intracerebral antigen presenting cells (Frohman *et al.*, 1989b), may play a crucial role in the immune response in the CNS via soluble cytokines.

Table 1.3 Cytokines and Neuropathogenesis*

Cytokine	Disease/Infection	Probable Sources
IL-1	multiple sclerosis, Alzheimer's disease injury	macrophages, microglia, astrocytes
TNF-α	multiple sclerosis, bacterial meningitis, HIV, injury	macrophages, astrocytes
TNF-β	multiple sclerosis,	T lymphocytes microglia, astrocytes
IL-6	viral or bacterial meningitis, HIV, meningoencephalitis, injury	macrophages, microglia, astrocytes
IFN-γ	multiple sclerosis,	lymphocytes
TGF-β	HIV, cytomegalovirus	macrophages, microglia, astrocytes

* From Morganti-Kossmann *et al.*, 1992; Pimental, 1994.

Although brain cells do not constitutively express class II MHC antigens, IFN-γ and TNF-α can induce the expression of these molecules in astrocytes and microglia (Fierz *et al.*, 1985; Fontana *et al.*, 1984; Pulver *et al.*, 1987; Suzumura *et al.*, 1987). Furthermore, viral infections, such as hepatitis and measles virus infection, also result in class II MHC antigen expression in the CNS (Massa & ter Meulen, 1987; Massa *et al.*, 1986). Like astrocytes, endothelial cells can produce class II MHC antigens upon treatment with IFN-γ (Male *et al.*, 1987; Pober *et al.*, 1983), and ICAM-1 is also presented on vascular endothelial cells and fibroblasts upon the stimulation with cytokines, such as IFN-γ, IL-1 and TNF-α etc. (Dustin *et al.*, 1986). After

induction, class II MHC and ICAM-1 positive endothelial cells could then act as the first APC encountered by blood-borne T cells, resulting in activation of these cells (Figure 1.1). These activated T cells would then be able to penetrate the BBB in a variety of fashions, such as passing through altered tight junctions between the endothelial cells, or dissolving the extracellular matrix produced by endothelial cells (Frohman, 1989b; Merrill, 1987). Activated T cells have been shown to secrete an endoglycosidase that digests the proteoglycans of the extracellular matrix (Naparstek *et al.*, 1984). Once in the brain, activated T cells release cytokines along with entering macrophages. These cytokines could exert a potent influence on astrocytes, thus creating an environment for local immune responses within the CNS. Additionally, astrocytes, oligodendrocytes, and microglia have been shown to constitutively express low levels of class I antigens (Wong *et al.*, 1984), which could be increased by cytokines, including IFN-γ, in a wide variety of species (rat, mouse and human) (Hirayama *et al.*, 1986; Suzumura *et al.*, 1986). The enhanced class I MHC antigen expression implicates that they can be rendered susceptible to lysis by class I-restricted cytotoxic T cells. In fact astrocytes were shown to serve as targets for class I MHC-restricted cytotoxic T cells *in vitro* (Skias *et al.*, 1985).

The subsequent immune response in the CNS is summarized in Figure 1.1. The proliferation of astrocytes is induced by TNF-α and IFN-γ from T cells, and IL-1 produced by astrocytes themselves. IL-2,

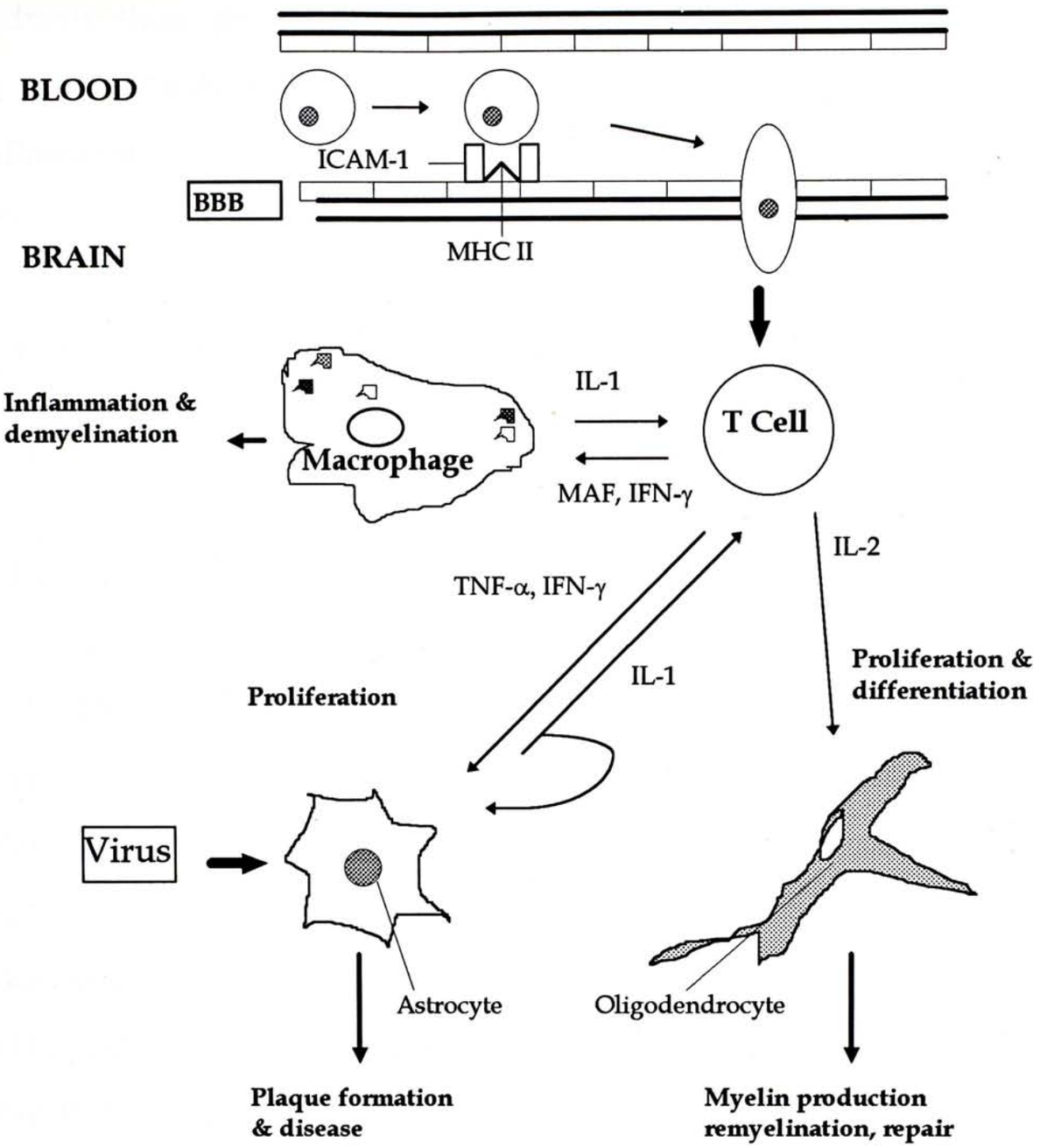


Figure 1.1 The blood T cells penetrate the BBB and initiate the immune response in the central nervous system. (Modified from Frohman, 1989b and Merrill, 1987).

produced by activated T cells, is specific for oligodendrocytes and induces their proliferation. In contrast, brain macrophages could cause demyelination. The further immune reactions result in inflammation, demyelination or remyelination, plaque formation etc., which are usually observed in the brain diseases (Merrill, 1987).

1.2 The C6 Glioma As a Model for the Study of Glial Cell Growth and Differentiation

1.2.1 The Rat C6 Glioma Cells

The term glioma describes the group of glial neoplasms including astrocytoma, glioblastoma, ependynoma, oligodendroglioma and mixed gliomas, such as oligoastrocytoma. Astrocytic gliomas, which contain fibrillary neoplastic cells, are the most common gliomas. Glioma cells are particularly easy to grow in tissue culture, thus in unselected material about 20 % of glioblastoma multiform cells can be grown as continuous cell lines (Westermark *et al.*, 1973). Although they may not be true representatives in all aspects of glioma or glial cells *in vivo*, the relative ease with which such cell lines can be established has made them widely used as model system for studies on the biology of glioma or glial cells. A number of rat brain tumors induced by N-nitrosomethylurea and consisting of more or less differentiated astrocyte-like cells are found to contain S-100 protein, a characteristic protein of glial cells. It was

shown that injection of newborn rats with these cultured tumor cells resulted in a high efficiency of tumor formation (Benda *et al.*, 1968). Five morphologically distinct clonal cell strains were established from these tumors, only one contains appreciable amounts of S-100, and this is the C6 glioma cell line (Benda *et al.*, 1968).

C6 glioma cells have provided a useful model to study glial cell properties, glial factors and sensitivity of glial cells to various substances and conditions. As a kind of transformed cell line, the growth characteristics of C6 cells are immortal, anchorage independent, loss of contact inhibition, high plating efficiency and shorter population doubling time. In addition to its homogeneous genetic properties, C6 cells are tumorigenic and angiogenic (Freshney, 1987). On the other hand, C6 cells express several glial-specific markers such as S-100, GFAP and GS, the markers for astrocytes, and CNP, an enzyme marker for oligodendrocytes, have been proved to be expressed in the C6 cell line (Kempski *et al.*, 1992; Vernadakis *et al.*, 1992). Additionally, the expression or activity of these markers in C6 cells could be induced. For instance, CNP is induced by neuron-derived factors, epidermal growth factor and fibroblast growth factor, whereas GFAP and GS are induced by insulin, cyclic AMP, platelet-activating factor, muscle-derived factors, chronic β-receptor activation and IL-4 (Brodie & Goldreich, 1994; Brodie & Vernadakis, 1991; Parker *et al.*, 1980; Vernadakis *et al.*, 1991). Interestingly, cytokines are known to be involved in the expression of

astrocytic and oligodendrocytic properties expression in C6 cells of early passages (Brodie & Goldreich, 1994).

C6 glioma cells have been found to respond to several cytokines, such as TNF-α, IL-6, IFN-γ and IL-4. The growth of C6 glioma cells is stimulated by TNF-α, IL-6 and IFN-γ (Munoz-Fernandez *et al.*, 1991; Munoz-Fernandez & Fresno, 1993). In addition, IL-4 exerts a biphasic effect on C6 cell proliferation, increasing cell proliferation at concentrations ranging from 10-50 ng/ml, and inhibiting at higher concentrations (Brodie & Goldreich, 1994). The inhibition of cell proliferation is associated with differentiation of the cells to express astrocytic phenotypes as evidenced by morphology, increased GFAP immunoreactivity and elevated GS expression (Brodie & Goldreich, 1994). IL-4 also induced the secretion of nerve growth factor by C6 glioma cells (Brodie & Goldreich, 1994). These results suggest that the C6 cell line represents a good cell model for the study of the proliferation and differentiation of glial cells *in vitro*.

1.2.2 The Differentiation and Proliferation of C6 Glioma Cells

It is well-known that the starting point for the cellular analysis of glial development was the optic nerve, the simplest part of the CNS (Figure 1.2). The first glial cell type to appear in the optic nerve during embryonic development is the type-1 astrocyte. The next two

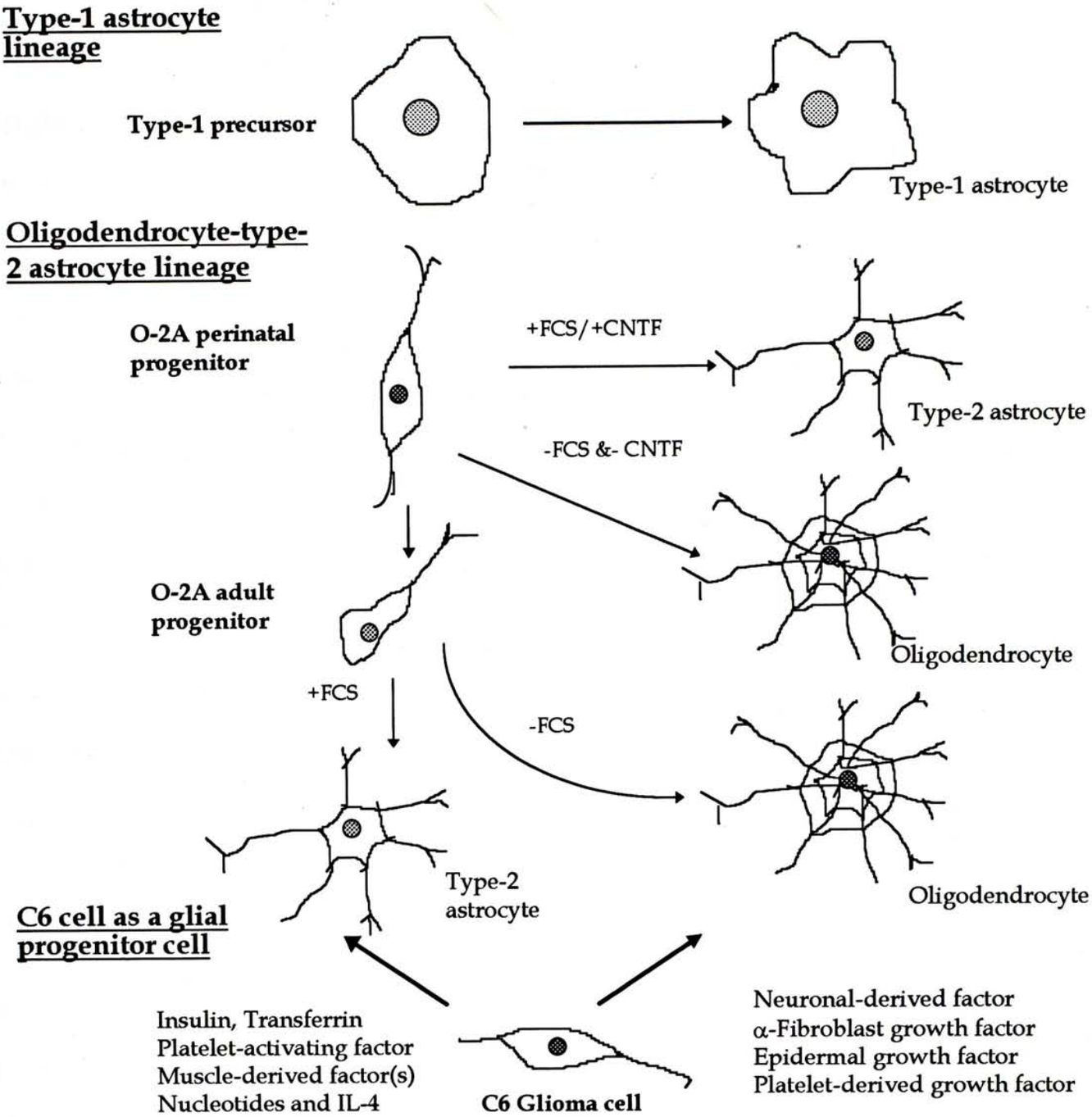


Figure 1.2 The development of glia and C6 glioma cell line (Modified from Linskey & Gilbert, 1995 and Vernadakis *et al.*, 1991).

glial cell types, type-2 astrocyte and oligodendrocyte, which appear during development of the rat optic nerve, are both derived from a single progenitor cell, the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell (Raff *et al.*, 1983), which migrates into the optic nerve from the optic chiasm during embryogenesis (Small *et al.*, 1987). *In vitro* studies suggest strongly that division of the O-2A progenitor cells is promoted by the type-1 astrocytes derived from the optic stalk, through the secretion of platelet-derived growth factor (Noble & Murray, 1984; Raff *et al.*, 1988). O-2A progenitors give rise to either type 2 astrocytes or oligodendrocytes, depending on the environmental influence. *In vitro* experiments have shown that the O-2A progenitor cell, in the presence of fetal calf serum, differentiates into a type 2 astrocyte, and in the absence of serum, into an oligodendrocyte. Another member of the O-2A lineage is the O-2A adult progenitor cell, which differs from its perinatal counterpart in many aspects, including antigen expression, morphology, cell-cycle length, motility, time-course of differentiation, in the manner in which it generates astrocytes and oligodendrocytes and seemingly also in its capacity for extended self-renewal (Wolswijk & Noble, 1989).

The influence of mitogenic factors and cytokines on macroglia development have also been studied. Mitogenic brain extracts from embryonic days 16-18 brain were found to induce a 4-fold increase in neonatal astrocytes *in vitro* (Morrison *et al.*, 1982). The astroglial

proliferation is due to the peptides released from ameboid microglia which act as glial promoting factors (Giulian, 1984; Giulian & Baker, 1985). One of these factors is IL-1, which is produced by ameboid microglia from rat cerebral and cerebellar cortex derived from the late embryonic period (Giulian *et al.*, 1986; Gordon & Hirsch, 1981). Thus, clusters of ameboid microglia found along growing axons in fetal corpus callosum may stimulate astroglial pathways which in turn determine the direction of axonal growth (Giulian *et al.*, 1988 a and b). Since this occurs during the critical developmental periods involving glial cell appearance, it has been suggested that substance P (SP), a neuro-transmitter, may play a role in cytokine production and maturation of glial and neuronal elements (Charnay *et al.*, 1983; Nomura *et al.*, 1982). Indeed, SP has been shown to stimulate astrocytes to produce IL-1, TNF-α and IL-6 (Kimball *et al.*, 1988; Lotz *et al.*, 1988). Conversely, IL-1 has also been demonstrated to induce SP production in sympathetic ganglia (Jonakait & Schotland, 1990). Thus, SP may influence glial cells during development in an autocrine loop involving various cytokines. In addition, several cytokines including IL-1 and TNF-α, as mentioned before, have effects on the proliferation and differentiation of glial cells. Since IL-1 and TNF-α are capable of co-inducing each other and that astrocytes produce IL-1 and TNF-α, both autocrine and paracrine events ensure proliferation and activation of astrocytes during development.

Early passage C6 glioma cells express the properties of the progenitor glial cells. Since they are GFAP, GS and CNP positive, C6 glioma cells have the properties of both astrocyte and oligodendrocyte. It has also been reported that C6 glioma cells exhibited differential enzyme expression with cell passage: the activity of CNP, an enzyme marker for oligodendrocytes, was markedly high and that of GS, an enzyme marker for astrocytes, was low in early passages (up to passage 26). However, this relationship was reversed in the late passages (beyond passage 70) (Parker *et al.*, 1980). In addition, early-passage cells express low level of GFAP immunoreactivity in contrast to late passage cells which express a high intensity of immunoreactive staining (Brodie & Vernadakis, 1991). These observations suggest that C6 glioma cells, as a bipotential progenitor, may give rise to oligodendrocytes at the early passages and to astrocytes at the later passages (Vernadakis *et al.*, 1991).

As mentioned before, a number of factors, such as insulin, cyclic AMP, platelet-activating factor, muscle-derived factors, transferrin and IL-4, can induce C6 cells to generate astrocytic phenotypes. On the other hand, factors including neuronal-derived factors, epidermal growth factor, fibroblast growth factor and platelet-derived growth factor can trigger the C6 cells to give rise to oligodendrocytic phenotypes (Figure 1.2). It has been known that many of these inducible factors, such as growth factors and

interleukins, are also involved in the inflammation and immune response in the CNS. These findings clearly indicate that various microenvironmental factors, including cytokines, play a prominent role in influencing the glial phenotypes of C6 glioma cells.

1.3 Signal Transduction Pathways in Cytokine-stimulated Glial Cells

1.3.1 Intracellular Signalling Pathways of Cytokines

Cytokines transmit their biological signals to responsive cells by interaction with specific high-affinity cell surface receptors. However, there is still relatively little knowledge of the mechanisms by which cytokines transmit signals via their receptors. At present, at least two signal-transmitting or activation pathways have been described that appear to be related directly to cytokine-mediated cellular response. One of these involves the calcium-binding protein calmodulin, guanine nucleotide-binding proteins (G-protein), and the enzymes adenylate cyclase and protein kinase A. In the other activation pathway, the receptor molecules themselves contain a tyrosine kinase which, when activated, subsequently activates phospholipases, possibly also through G-protein intermediates. The latter are responsible for the breakdown of plasma membrane inositol phospholipids to inositol triphosphate and diacylglycerol (DAG) which are active intermediates for intracellular Ca^{2+} release and

protein kinase C activation respectively (Meager, 1990). In fact, several mechanisms are involved in cytokine-triggered cell-specific responses. Among them, protein kinase C, tyrosine kinase, cyclic nucleotides and nitric oxide are highlighted here.

1.3.1.1 Protein Kinase C Pathway

Protein kinase C (PKC) comprises a family of related enzymes, which are α, β_I, β_{II}, γ, δ, ε, λ, ξ and θ. They possess regulatory and catalytic domains, with a site between them that is susceptible to proteolytic cleavage (Coussens *et al.*, 1986). Each molecule contains both conserved and variable sequence: C_1-C_4 are regions of conserved amino acids, V_1-V_5 are variable regions (Coussens *et al.*, 1986). Protein kinase C can be divided into two main groups: the Ca^{2+}-dependent, or conventional PKCs (cPKCs) and the Ca^{2+}-independent, or novel PKCs (nPKCs). The PKC isoforms α, β_I, β_{II} and γ, belong to the Ca^{2+}-dependent group, while the isoforms δ, ε, λ, ξ and θ, in which the C_2 conserved sequence is absent, belong to the Ca^{2+}-independent group (Hug & Sarre, 1993).

For the cPKCs, a model of activation was sufficient and convincing. It includes (1) the generation of diacylglycerol and inositol 1,4,5-triphosphate from plasma membrane-associated phosphatidylinositol 4,5-bisphosphate by the action of phospholipase C, (2) the release of Ca^{2+} from intracellular storage sites stimulated by

inositol 1,4,5-triphosphate, (3) the binding of Ca^{2+} to the C_2 conserved region of PKC and subsequent translocation of the enzyme to the plasma membrane where it is activated, via its C_1 conserved region, by diacylglycerol and phosphatidyserine (PtdSer), the latter being constitutively present in the membrane (Bell & Burn, 1991; Zidovetzki & Lester, 1992). In this model, phorbol ester, such as phorbol 12-myristate 13-acetate (PMA) and phorbol-12,13-diacetate (PDA), would mimic the action of DAG by their persistence in the cellular membrane, leading to a long-term activation of PKC (Gschwendt *et al.*, 1991). On the other hand, members of the nPKC group do not require Ca^{2+} for activation, but require either DAG/PtdSer, or PMA/PtdSer (Lee & Bell., 1991).

Cytokines, such as IFN-α, IL-1 and IL-3, have been reported to induce phosphatidylcholine hydrolysis (Cataldi *et al.*, 1990). The selective activation of PKC β and ε by IFN-α treatment of HeLa and Daudi cells respectively, have been demonstrated (Pfeffer *et al*, 1990, 1991). The activated PKC can alter the phosphorylation of proteins, which could trigger the further intracellular biological function.

1.3.1.2 Tyrosine Kinase Pathway

Cell proliferation appears to be regulated by the tyrosine kinases which phosphorylate tyrosines in specific protein substrates (Ullrich & Schlessinger, 1990). The stimulation of various cell types

by IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, erythropoietin (EPO) and ciliary neurotrophic factor (CNTF) has been shown to activate intracellular tyrosine kinases (Taga & Kishimoto, 1992). This kinase family includes Src, Ty2, JAK1 and JAK2 (Stahl & Yancopouls, 1993). Several cellular tyrosine kinases are also receptors for growth factors or cytokines, such as insulin-like growth factor-I, epidermal growth factor and macrophage colony stimulating factor (Aderem, 1993; Kishimoto *et al.*, 1994). In addition, some cytoplasmic tyrosine kinases that do not require ligand binding for activation are nevertheless often associated with the plasma membrane or cytokeleton of the cell (Eiseman & Bolen, 1990). Cytokine receptors themselves that do not possess kinase activity might regulate activity of associated non-receptor kinases. For example, the IL-3 receptor has no tyrosine kinase activity, but binding of IL-3 to sensitive cells causes increased tyrosine phosphorylation of several proteins (Linnekin & Farrer, 1990). One recent evidence showed that JAK2, a nonreceptor type tyrosine kinase, might be activated by this cytokine (Silvennoinen *et al.*, 1993). The protein substrates for tyrosine kinases have not been well defined. Often, the enzymes themselves become phosphorylated on one or more tyrosine residues, and cytokines receptors are often auto-phosphorylated after the binding of their ligands (Weiss & Littman, 1994). Thus, tyrosine phosphorylation may be the first event in a cascade of reactions leading to the many biological effects of the cytokine, as demonstrated for the insulin, IGF-I, platelet-derived growth factor (PDGF) and M-CSF receptors (Weiss & Littman, 1994).

1.3.1.3 Cyclic Nucleotide Pathway

The best characterized second messenger system in mammalian cells is the well-known adenylate cyclase/adenosine 3′,5′ monophosphate (cAMP) pathway. cAMP-dependent protein kinase mediates a wide variety of serine/threonine-specific protein phosphorylation events, directly or indirectly (Clemens, 1991). The wide range of cAMP-dependent protein kinase substrates explains why ligands that activate or inhibit adenylate cyclase have diverse effects, and is perhaps a precedent for understanding the multiple effects of other cytokines that use alternative pathways. Surprisingly, few cytokines appear to act directly through cAMP as a second messenger. Conversely, cAMP-dependent protein kinase can phosphorylate and regulate proteins involved in other signal transduction mechanisms. On the other hand, cyclic guanosine 3′,5′ monophosphate (cGMP) has a much more restricted second messenger role than cAMP. It is synthesized by guanylate cyclase and removed by phosphodiesterase-catalyzed hydrolysis to 5′GMP. As yet there are only a few reports suggesting possible involvement of cGMP in cytokine-stimulated signal transduction systems (Clemens, 1991).

1.3.1.4 Nitric Oxide Pathway

Nitric oxide (NO), a vascular and neuronal messenger and a cytotoxic and cytostatic agent, is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). Although multiple subtypes of NOS have been reported, they can be classified into two main categories (Nathan, 1992). One type of NOS is constitutively expressed in several cell types including neurons and endothelium, and can be activated by the reversible binding of calmodulin in a Ca^{2+}-dependent manner. The activity of the second type, whose expression can be induced in several cell types, including macrophages, hepatocytes, neutrophils, endothelial cells and astrocytes, is independent of Ca^{2+} and once expressed can be active for extended intervals. The latter one is described as inducible NOS (iNOS). This isoform requires NADPH and tetrahydrobiopterin as cofactors as well as flavoproteins. iNOS expression in nervous tissues can be induced not only by LPS but also by various cytokines, such as IFN-γ, IL-1β and TNF-α, alone or in combination (Hartung *et al.*, 1992; Kilbourn & Belloni, 1990).

A major action of NO is to stimulate soluble guanylate cyclase; in the CNS this would elevate cGMP levels in presynaptic nerve endings and glial cells. Cyclic GMP has been shown to decrease free calcium level within cells, possibly owing to stimulation of the Na^{+}/Ca^{2+} transporter (Furukawa *et al.*, 1991), and/or to inhibit the

formation of inositol phosphates evoked by agonists, perhaps by interaction with a G-protein (Hirata *et al.*, 1990). In astrocytes, it has been shown that agonists evoke inositol phosphates formation and induce calcium fluxes (McCarthy & Salm, 1991), thus NO-induced increases in cGMP level may serve to modulate these events. In this connection, it is interesting to note that nitric oxide has been shown to be involved in cytokine-induced growth of glial cells (Munoz-Fernandez & Fresno, 1993). These observations may indicate that NO associated with cGMP is seemingly both an intercellular messenger and a regulatory molecule in glial cells.

1.3.2 Intracellular Signalling Pathways in Cytokine-stimulated C6 glioma cells

There has been an increasing interest in the studies of the biological effects of cytokines on astrocytes and the possible signalling pathways that are involved. For example, IL-1β alone can induce NO production in astrocytes (Lee *et al.*, 1993a). However, NO production was markedly enhanced when the astrocytes were co-stimulated with IFN-γ and TNF-α (Lee *et al.*, 1993a). On the other hand, the NO releasing compound sodium nitroprusside (SNP) down-regulates, in a concentration-dependent manner, the IFN-γ-induced MHC II expression on astrocytes (Heuschling, 1995). It is known that C6 glioma cells can respond to the induction of cytokines including TNF-α, IFN-γ, IL-6 and IL-4 (Munoz-Fernandez *et al.*, 1991;

Munoz-Fernandez & Fresno, 1993). These indicate that similar signalling pathways may be triggered in cytokine-induced C6 glioma cells.

Similar to astrocytes, nitric oxide associated cGMP increase is found to act as a signal transducer in C6 glioma cells (Simmons & Murphy, 1993; Vigne *et al.*, 1993). It has been observed that C6 cells treated with LPS or LPS plus TNF-α show Ca^{2+}-independent and L-arginine-dependent NOS activity. The NOS induction may be dependent on new protein synthesis. In the presence of cofactors, such as NADPH, L-arginine and Ca^{2+} etc., the activated NOS could result in the increase in level of cGMP in C6 cells (Simmons & Murphy, 1992) and primary rat glial cultures (Galea *et al.*, 1992). On the other hand, cGMP production is significantly decreased by IL-4, IL-10 or transforming growth factor (TGF)-β_1 (Demerle-Pallardy *et al.*, 1993; Simmons & Murphy, 1993). One hypothesis regarding the mechanism of LPS induction is that it causes these cells to release cytokines which induce the enzyme directly. As expected, IFN-γ, IL-1β and TNF-α can induce L-arginine-dependent cGMP synthesis in C6 glioma cells whereas TGF-β_1 non-specifically decreased the induction (Simmons & Murphy, 1993). The observation that TNF-α, IFN-γ, and/or IL-1β and LPS plus TNF-α or IFN-γ increased NO production has supported the role of NO as a signal transducer triggered by cytokines in astroglial cells (Feinstein *et al.*, 1994b; Galea *et al.*, 1992). Moreover, NO is also likely to be involved in the

cytokine-induced growth of C6 cells, including TNF-α and IFN-γ (Munoz-Fernandez & Fresno, 1993).

The involvement of tyrosine kinase as a second messenger for the NOS production by C6 glioma has also been reported. Genistein and tyrphostin, the two selective inhibitors of tyrosine kinase (Akiyama *et al.*, 1987; Holen *et al.*, 1995), have been shown to prevent both LPS and cytokine-induced NOS activity in a dose-dependent manner (Feinstein *et al.*, 1994a). The results suggest that NOS induction in C6 glioma cells is tyrosine kinase dependent. It means that tyrosine kinase activation might be an upstream signal.

Significant PKC activity has been demonstrated in rat C6 glioma cells which correlated with the proliferation rate of the cells. Also, PKC can act as a kind of second messenger in A172, a human glioma cell line (Couldwell *et al.*, 1992). Furthermore, a PKC inhibitor, H-9, inhibited the TNF-α– and IFN-γ-induced C6 cell proliferation (Munoz-Fernandez & Fresno, 1993). These findings strongly suggest that activation of PKC is a potential common signal transduction mechanism induced by cytokines on glioma cells.

Besides the above mentioned signal transducer molecules, the existence of other signalling pathways, including cAMP, β_1-adrenergic receptor and phospholipase A_2, is also found in the C6 glioma cells. GFAP expression on C6 cells is induced by the addition

of dibutyryl cyclic adenosine monophosphate (dbcAMP), which can linearly increase the intracellular cAMP level. The result indicates that GFAP synthesis is regulated at the transcription level and that a cAMP-dependent mechanism determines its ultimate synthesis (Messens & Slegers, 1992). In addition, exposure of rat C6 glioma cells to either agonists or agents that increase cAMP levels leads to down-regulation of β_1-adrenergic receptor (Hosoda *et al.*, 1994). The regulation of β_1-adrenergic receptor mRNA occurs at the level of gene transcription and by the induction of a repressor of the β_1-adrenergic receptor gene (Hosoda *et al.*, 1994). More recently, it has been reported that treatment of the rat C6 glioma cell line with IL-1β resulted in an accumulation of cytosolic phospholipase A_2 mRNA (Ozaki *et al.*, 1994). Nevertheless, whether β_1-adrenergic receptor and phospholipase A_2 are directly involved in the cytokine-induced signal cascades has yet to be determined.

1.4 The Aims of This Project

Cytokines are a heterogeneous group of polypeptide mediators that have been associated classically with the activation of the immune system and inflammatory response. An increasing number of related mediators have been shown to act on a variety of cell types, including cells of the nervous system. As reviewed in the previous sections, it is clear that the cells of immune system and CNS can have similar functions: secretion of immunoregulatory cytokines, response

to cytokines and antigen presentation. The activation of astrocytes and microglia may contribute to either the initiation and/or propagation of intracerebral immune response. Recently, several lines of evidence show that cytokines, such as IL-1, IL-6, IFN-γ and TNF-α, are implicated as signals in neuroimmune interactions (Blalock, 1994; Roszman & Brooks, 1992). It is well-known that the cells of the CNS, particularly the glial cells, are modulated by various cytokines. However, little is known about the cellular and molecular mechanisms involved in the cytokine modulation of various neuronal cell types. To understand better the relationship and communication between the immune system and the nervous system, it is important to study the modulatory effects of various cytokines and their interactions in regulating the growth and differentiation of cells of the CNS. In addition, the elucidation of the nature of the second messenger signals involved in neuroimmune interactions can provide better insights into the molecular and cellular actions of cytokines in the CNS.

Although cytokines such as TNF-α and IFN-γ can induce glial cell proliferation and differentiation *in vitro* (Benveniste *et al.*, 1991; Selmaj *et al.*, 1990), whether or not these cytokines can interact with one another and the signalling pathways mediating the activities of these cytokines in glial cells remain to be established. Because of the homogeneity of the C6 glioma cells and their known responsiveness to various cytokines, they were used as a model to study the

modulatory effects of cytokines on the proliferation and differentiation of glial cells *in vitro*. In this project, cell proliferation assay was primarily used to investigate the regulatory effects of various cytokines on C6 cells. Moreover, the cytokine-induced C6 cell proliferation was used as a major parameter to monitor the second messenger systems that mediate the actions of cytokines.

In the first set of experiments, the modulatory effects of various cytokines, including TNF-α, IL-1α, IL-1β and LIF on C6 glioma cell proliferation were examined. Attempts were also made to determine whether combinations of these cytokines can act synergistically or antagonistically in C6 cell proliferation. In the second set of experiments, the involvement of second messenger systems in cytokine-induced C6 cell proliferation was studied, with particular reference to the activation of protein kinase C and tyrosine kinase, the roles of cyclic nucleotides and nitric oxide etc. PKC, a common second messenger system in many cell types, was first examined since its activity is very high in C6 glioma cells. Two PKC activators, 12-myristate 13-acetate (PMA) and phorbol-12,13-diacetate (PDA), were studied for their effects on C6 cell proliferation. Also, three PKC inhibitors, staurosporine, calphostin C and Ro31-8220, were tested if they could inhibit the proliferation of C6 cells triggered by cytokines or PKC activators. In addition, the possible involvement of tyrosine kinase, cyclic AMP or GMP, nitric oxide and β-adrenergic receptor in

cytokine-induced C6 cell proliferation was examined using specific inhibitors, agonists or antagonists.

In addition, the role of Ca^{2+} in cytokine-triggered C6 cell proliferation was elucidated. The intracellular Ca^{2+} level in C6 cells was raised by incubating C6 cells with calcium ionophore, and reduced by addition of calcium channel blockers, such as $LaCl_3$, verampil and nifedipine, or the calcium chelator, EGTA.

It is known that the C6 glioma cell line has bipotential glial progenitor properties. During its development, the early passage C6 cells can be induced to express the astrocytic or oligodendrocytic phenotype by different stimuli. By morphological study and GFAP/hematoxylin staining, the influence of cytokines on the differentiation the rat C6 glioma cells was also examined. By studying the modulatory effects of well-characterized cytokines on C6 cell proliferation and differentiation *in vitro*, it is hoped that this would provide valuable information on the biological role of cytokines in regulating glial cell growth in normal and pathological states.

Chapter 2 MATERIALS AND METHODS

2.1 Rat C6 Glioma Cell Culture

2.1.1 Preparation of Culture Media

2.1.1.1 Complete Dulbecco's Modified Eagle Medium

Dulbecco's modified Eagle medium (DMEM) with high glucose and L-glutamine was purchased from Gibco Lab. (Grand Island, NY, U.S.A.). The powdered DMEM for 1 liter solution and 3.7 g sodium bicarbonate ($NaHCO_3$) were dissolved in 1 liter of double-distilled water. The medium was adjusted to pH 7.2 and filtered (filter: 0.2 μm, Micro Filtration Systems, Dublin, CA, U.S.A.) immediately under suction. Then, horse serum (Gibco) and antibiotics (penicillin, 10,000 U/ml; streptomycin, 10,000 μg/ml; fungizone, 25 μg/ml; Gibco) were added, the complete DMEM (CDMEM) obtained contains 10% (v/v) horse serum and 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. This medium was stored at 4 °C until use.

2.1.1.2 Complete Roswell Park Memorial Institute 1640 Medium

Roswell Park Memorial Institute (RPMI) medium was prepared almost the same as DMEM except that 2.0 g sodium bicarbonate ($NaHCO_3$) was added. The complete RPMI (CRPMI) medium contains 10% (v/v) fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone. Because RPMI medium contains N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) as a buffering system, its pH is more stable than DMEM; hence, the CDMEM was used in the maintenance of cell culture while the CRPMI medium was used in assays.

2.1.2 Maintenance of the C6 Cell Line

Rat C6 glioma cells were obtained from the American Type Culture Collection (U.S.A.). They were cultured, under aseptic conditions, in CDMEM at 37 °C in a humidified incubator (Shel-Lab, Model 2400, Sheldon Manufacturing, Inc.) under an atmosphere of 5% CO_2/95 % air.

The cells were subcultured after 2-3 days in culture, at which time they had reached confluence. For the subculture procedure, the medium was discarded, and cells washed with sterilized phosphate buffered saline (PBS, 8.18 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.44 g $Na_2HPO_4 \cdot 2H_2O$ per liter double-distilled water, pH 7.4). The cells

were treated with 1 ml of 0.25% trypsin (Gibco) at 37°C for 5 minutes. Then 10 ml of CDMEM was added and the cells were dispersed by repeated pipetting. After the cells were washed twice with DMEM, they were adjusted to a cell density of 10^5 cells/ml and further cultured in a 75 cm^2 culture flask (Falcon, Becton Dickinson Labware, NJ, U.S.A.) containing 20 ml CDMEM.

For long-term storage, 1 ml of cells (10^7 cells) suspended in FBS with 5% dimethyl sulfoxide (DMSO, Sigma) was stored in liquid nitrogen in plastic ampoules (Nunc., Denmark). When required, aliquots were thawed rapidly in CDMEM at 37°C, subcultured and cells of passages 18-28 were used in this study. To ensure the purity of the cell line, cells were checked regularly for contamination under a microscope and histochemically with antibody against glial fibrillary acidic protein (see Section 2.9 for details).

2.1.3 Cell Preparation for Assays

As described above, cells of passages 18-28 were trypsinized, centrifuged and resuspended in CRPMI medium. For assays using 96-well microtiter plate (Corning Laboratory Sciences Company, U.S.A.), 0.1 ml of 2.5×10^4 cells/ml cell suspension was added to each well. For 24-well plate and 6-well plate (Corning, U.S.A.), 0.5 ml cells (3×10^4 cells/ml) or 1 ml cells (7.5×10^4 cells/ml) were seeded in each well respectively.

2.2 Determination of Cell Proliferation

2.2.1 Determination of Cell Proliferation by [^{3}H]-Thymidine Incorporation

Rat C6 glioma cells were treated and plated as described in Section 2.1.3. All procedures were performed under aseptic conditions in a cell culture hood (Biogard hood, the Baker Company, Inc.).

To study the effects of cytokines and/or drugs on C6 cell proliferation, cells were incubated in the presence, or absence of cytokine(s), with or without the addition of drug(s) for various time periods as indicated in the text. Then, 0.5 μCi [^{3}H]-thymidine (specific activity: 2 Ci/mmol; Amersham, U.K.) was added to each well and further incubated for 6 hours to estimate the incorporation of [^{3}H]-thymidine into DNA. At the end of incubation, cells were frozen and thawed once and harvested with a semi-automatic cell harvester (Flow Lab, U.K.). The radioactivity trapped by glass-fiber filters (GF/C, Whatman, U.S.A.) was determined in a liquid scintillation counter (Beckman, Model LS 1801, U.S.A.)

2.2.2 Measurement of Cell Viability Using Neutral Red Assay

To test for the viability of the cells following cytokine and/or drug treatment, cells were plated in a 96-well microtiter plate as described in section 2.2.1, except that the cell density was adjusted to 10^5 cells/ml. After 48 hours of incubation, the medium was removed, and the wells were washed with 150 µl normal saline (0.9 % NaCl) followed by 50 µl neutral red solution (0.5% neutral red in normal saline). Then the cells were incubated with 0.5% neutral red solution (100 µl per well) at 37 °C for 1 hour. After washing six times with normal saline, 100 µl of 1% sodium dodecylsulfate (SDS, Sigma) was added to each well to solublize the cells and to release the neutral red dye. The plate was shaken in a plate shaker (Lab-Line Instruments, Inc.) for 2 hours and the optical density (OD) at 540 nm was determined with a microplate reader (Bio-Rad, Model 3550).

2.2.3 Data Analysis

The cytokine or drug induced C6 cell proliferation was measured by the increase in the amount of [^{3}H]-thymidine incorporated and expressed as % stimulation. This was calculated by subtracting the counts per minute (CPM) determined in the absence of cytokine and/or drug (control) from the CPM determined in the presence of cytokine and/or drug (sample), then divided by the control CPM which is shown as follows:

$$\% \text{ Stimulation} = 100 \times (CPM_{sample} - CPM_{control})/CPM_{control}$$

The measurement of cell growth was expressed as % stimulation according to the formula below:

$$\% \text{ Stimulation} = 100 \times (OD_{sample} - OD_{control})/OD_{control}$$

All results were expressed as the arithmetic mean ± standard error of quadruplicate determinations. The data shown were representative of at least 2-3 similar experiments. The Student's "t" test was used to determine the confidence limits in group comparison. Normally $p < 0.05$ was regarded as significantly different.

2.3 Effects of Cytokines and Lipopolysaccharide on C6 Cell Proliferation

Recombinant human and mouse tumor necrosis factor-α (TNF-α, specific activity is 6×10^7 U/mg) were obtained from Boehringer Mannheim (Germany), whereas interleukin-1α (IL-1α, specific activity is 1×10^8 U/mg), interleukin-1β (IL-1β, specific activity is 1×10^8 U/mg), interleukin-6 (IL-6, specific activity is 5×10^6 U/mg) and leukemia inhibitory factor (LIF, specific activity is 1×10^7 U/mg) were obtained from R & D Systems (U.S.A.). Lipopolysaccharide

(LPS) was purchased from Sigma (U.S.A.). Cytokines were dissolved in RPMI medium containing 2% heat-inactivated (56°C for 30 minutes) FBS, while LPS was prepared in sterilized PBS. Cytokines were stored in aliquots at -20 °C, except LPS at -70 °C until use.

For the time course of cell proliferation triggered by various cytokines, cells were cultured in 96-well microtiter plate overnight, then the medium was removed and cells were exposed for 15, 30, 60, 90, 120 or 240 minutes to cytokines, after which cells were washed twice with CRPMI medium and further incubated until a total period of 48 hours. The [^{3}H]-thymidine incorporation was determined as described in Section 2.2.1.

2.4 Effects of Protein Kinase C Activators and Inhibitors on Cytokine-induced C6 Cell Proliferation

Two kinds of PKC activators: phorbol 12-myristate 13-acetate (PMA, Sigma) and phorbol-12,13-diacetate (PDA, Sigma), were dissolved in absolute ethanol at 1.62 mM and 2.23 mM respectively, and stored at -20 °C until use. On the other hand, three kinds of PKC inhibitors: staurosporine (ST, Sigma), calphostin C (Cal C, Sigma) and 3-{1-[3-(Amidinothio)propyl]-3-indolyl}-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione methanesulfonate (Ro31-8220, Roche Producs Ltd., U.K.), were dissolved in absolute ethanol, RPMI medium and DMSO,

respectively (Sigma) at concentrations of 100 μM, 10μM and 100 μM before use.

The effects of PMA and PDA on C6 cell proliferation and the effects of ST, Cal C and Ro31-8220 on TNF-α (100 U/ml) or LIF (1 ng/ml)-induced C6 cell proliferation were assayed as described in Section 2.2.1.

2.5 Effects of cAMP or cGMP on Cytokine-induced C6 Cell Proliferation

N^6-2′-O-dibutyryl cyclic adenosine-3′,5′-monophosphate (dbcAMP, Sigma) and N^6-2′-O-dibutyryl cyclic guanosine-3′.5′-monophosphate (dbcGMP, Sigma) were dissolved in RPMI medium at 10 mM and stored in -20 °C until use. The effects of dbcAMP or dbcGMP alone, or in combination with TNF-α or LPS on C6 cell proliferation were studied as described in Section 2.2.1.

2.6 Effects of Tyrosine Kinase Inhibitors on Cytokine-induced C6 Cell Proliferation

Two kinds of tyrosine kinase (TK) inhibitors: tyrphostin (TYR, Sigma) and herbimycin A (Her A, Sigma), were dissolved in RPMI medium and stored at -20 °C before use. C6 glioma cells were

exposed to different doses of TYR or Her A in the presence or absence of TNF-α (100 U/ml), or LIF (1 ng/ml) for 48 hours, and cell proliferation assay was studied as described in Section 2.2.1.

2.7 Effects of Calcium Ion on Cytokine-induced C6 Cell Proliferation

In these experiments, C6 cells were exposed to ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA, Sigma), a calcium ion chelator; lanthanum chloride ($LaCl_3$) (Sigma), verampil (Sigma) or nifedipine (Sigma), all are calcium ion channel blockers; or A23187 (Sigma), a calcium ionophore; in the presence or absence of various concentrations of TNF-α or LIF for 48 hours. Then, [^{3}H]-thymidine incorporation in C6 cells was examined as described in Section 2.2.1. Because some calcium drugs are light sensitive, experiments were carried out in the dark and the culture plates were wrapped with aluminum foil.

2.8 Effects of Nitric Oxide on Cytokine-induced C6 Cell Proliferation

2.8.1 Effects of Sodium Nitroprusside and Nitric Oxide Synthase Inhibitors on Cytokine-induced C6 Cell Proliferation

Sodium nitroprusside (SNP, Sigma), an exogenous nitric oxide donor, was used to investigate the effect of nitric oxide on C6 cell proliferation. SNP was freshly prepared in PBS (20 mM) and diluted in CRPMI medium before use.

On the other hand, N^G-methyl-L-arginine (NMA, 10 mM in RPMI medium, Sigma) and N^W-nitro-L-arginine methyl ester (NAME, 20 mM in RPMI medium, Sigma), both being nitric oxide synthase inhibitors, were added to C6 glioma cell cultures in the presence or absence of TNF-α (100 U/ml) or LIF (1 ng/ml). The method for [^{3}H]-thymidine incorporation was the same as described in Section 2.2.1.

2.8.2 Nitric Oxide Production Assay

Nitric oxide (NO) formation was detected by nitrite accumulation in the culture supernatant using Griess reaction (Green *et al.*, 1982). In aqueous solution, NO reacts rapidly with O_2 and accumulates in the culture medium as nitrite and nitrate ions. Nitrite forms a chromophore with the Griess reagent and thus its concentration can be determined by a colorimetric assay. C6 glioma cells were incubated at 37°C with different cytokines in a 96-well microtiter plate (Section 2.2.1). After 72 hours, 50 µl cell free supernatant was transferred to another 96-well microtiter plate. Then, an equal volume (50 µl) of freshly prepared Griess reagent was added to the well, and the plate was shaken at room temperature for 15

minutes. Griess reagent consists of one part of 0.1% napthylethylenediamine dihydrochloride in distilled water and one part of 1% sulfanilamide in 5% concentrated H_3PO_4. The absorbance was read at a wavelength of 540 nm by a microplate reader (Bio-Rad, Model 3550). The nitrite concentration can be quantified using sodium nitrite (0 - 50 μM) as a standard.

2.9 Effects of β-Adrenergic Receptor Agonist and Antagonist on C6 Cell Proliferation

Isoproterenol, a β-adrenergic receptor agonist, and propranolol, an antagonist of the β-adrenergic receptor, were purchased from Sigma Chem. Co. They were dissolved in double-distilled water at 250 mM and 10 mM respectively and stored at -20°C until use. C6 glioma cells were cultured for 48 hours in control medium, 1 μM of isoproterenol, or TNF-α (10 U/ml or 100 U/ml), plus various concentrations of propranolol (0 - 25 μM), and the [^{3}H]-thymidine incorporation was determined as described in Section 2.4.

2.10 Morphological Studies on Cytokine-Treated C6 Glioma Cells

All photographs were taken under the Axiophot microscope (Zeiss, 7082 Oberkocken, Germany). The cells were prepared and stained as described below.

2.10.1 Wright-Giesma Staining

C6 glioma cells were plated on 15 mm cover slips placed in 6-well plates and incubated with or without TNF-α (100 U/ml) or LIF (1 ng/ml), according to the protocols in Section 2.1.3. After 24, 48 or 72 hours of incubation, the cells were washed with PBS, stained with modified Wright-Giemsa staining solution (Sigma) for 30 seconds, followed by destaining. The air-dried cover slips were mounted with Canada Balsam mounting solution and the morphology examined under a light microscope.

2.10.2 Glial Fibrillary Acidic Protein Staining

C6 glioma cells were plated on 12 mm cover slips placed in 24-well plates and incubated with different doses of TNF-α or LIF. After 48 hours of incubation, cultures were fixed with 4% paraformadehyde in 0.1 M PBS for 15-30 minutes at room temperature. Then, the cells were exposed to primary antibody (rabbit IgG anti-glial fibrillary acidic protein (anti-GFAP, Sigma)) for 2 hours at room temperature, followed by secondary antibody (goat anti-rabbit IgG, Sigma) for 1 hour at room temperature. After the

immuno-reactions, the cells were treated with Vectastain Elite ABC Kit (Vector Laboratories) for 1 hour at room temperature, and then incubated with 3,3'-diaminobenzidine solution (10 mg/10 ml 0.05M Tris, pH 7.5) containing 0.01 % H_2O_2 for about 2-5 minutes to develop the color. Cells were stained with hematoxylin (see Section 2.10.3) and examined microscopically, and the percentage of GFAP positive cells were scored by counting at least 200 cells.

2.10.3 Hematoxylin Staining

C6 glioma cells plated on 12 mm cover slips were treated with cytokines, and stained with anti-GFAP antibody according to the methods described above (Section 2.9.2). Then, the cells were stained with Mayer's hematoxylin solution for 1-5 minutes, and washed in water. Excess stain was removed selectively by immersing the cover slips in acid alcohol (1% HCl in 70% alcohol) followed by bluing in Scott's tap water (alkaline ammonium water). After being washed in water, the stained cells were dehydrated stepwise in 70%, 80% and 95% alcohol quickly, and finally in 100% alcohol three times for five minutes each time.

Mayer's hematoxylin solution consists of the following ingredients: hematoxylin 5 g, ammonium alum 50 g, sodium iodate 0.3 g, distilled water 700 ml, glycerol 300 ml and glacial acetic acid 20 ml. For the preparation of the Mayer's hematoxylin solution,

ammonium alum was poured into distilled water, and the solution was boiled. Then, hematoxylin was added and the ammonium water was kept boiling for further 2 minutes. When sodium iodate was added, the solution was stirred and cooled down. As the last step, glycerol and glacial acetic acid were dissolved in the solution. Finally, the solution was filtered before use.

Chapter 3 RESULTS

3.1 Effects of Cytokines on C6 Cell Proliferation

Since cytokines play an important role in the communication between cells of the immune and nervous systems (Benveniste, 1992a; Martin & Tracey, 1992), it is of considerable interest to study their effects on astrocytes, the most abundant cell type in the CNS. Astrocytes are reported to express a number of different cytokine receptors which are known to be important in regulating the proliferation and differentiation of astrocytes (Benveniste, 1992a). Like astrocytes, C6 glioma (astrocytoma) cells also respond to a large number of cytokines, including TNF-α, IFN-γ, IL-4 and IL-6 (Brodie & Goldreich, 1994; Munoz-Fernandez & Fresno, 1993; Simmons & Murphy, 1992; 1993), thus they represent a good model system for studying the conditions and factors which regulate the development and differentiation of glial cells and the signalling pathways that might be involved in these processes (Mangoura *et al.*, 1989). In this thesis project, the effects of various cytokines in triggering DNA synthesis in C6 glioma cells were examined. In the first set of experiments, C6 glioma cells were treated with different cytokines, such as TNF-α, IL-1α, IL-1β, IL-6 and LIF, for various time periods, and the [^{3}H]-thymidine incorporation was measured according to the

methods described in Section 2.2.1. Moreover, since LPS is known to stimulate the production of cytokines (Lee *et al.*, 1993b), the effect of LPS on C6 cell proliferation was also investigated.

3.1.1 Effects of Cytokines on C6 Cell Proliferation

In this study, C6 glioma cells were cultured with different cytokines at various concentrations, including TNF-α, IL-1α, IL-1β, IL-6 and LIF, and the [^{3}H]-thymidine incorporation was measured. Fig. 3.1 showed the effects of recombinant mouse and human TNF-α on C6 cell proliferation. The proliferation of C6 cells was increased by mouse, or human, TNF-α in a dose-dependent manner and in case of mouse TNF-α, the proliferation also increased in a time-dependent manner. Moreover, the proliferative effect of mouse TNF-α was found to be more potent than that of the human one. With the mouse TNF-α, optimal stimulation (an increase of 62 %, in [^{3}H]-thymidine incorporation) was obtained with 100 U/ml TNF-α after 48 hours of incubation and the stimulatory effect declined at a higher concentration of TNF-α (500 U/ml). Because of these observations, mouse but not human TNF-α was used in all subsequent studies, and 48 hours incubation was chosen in the following experiments.

As it has been reported that TNF exerted its proliferative effect via the biosynthesis and/or secretion of other cytokines (Frei *et al.*, 1989), thus the effects of other cytokines were studied. Fig. 3.2

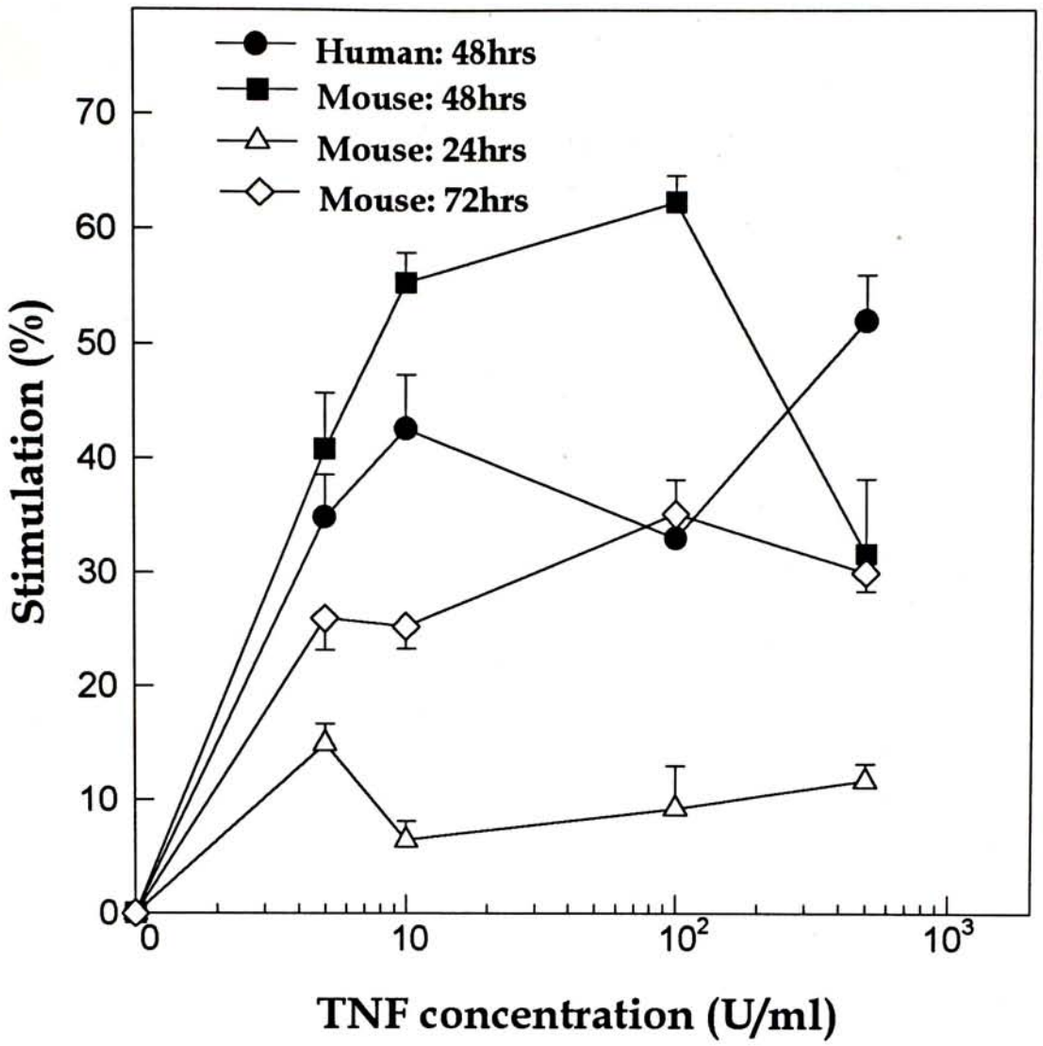


Fig. 3.1 The effects of mouse and human TNF-α on C6 cell proliferation. [^{3}H]-thymidine incorporation was determined according to the methods in Section 2.3 and expressed as percentage of stimulation as described in Section 2.2.3. Each point represents the mean ± standard error of four replicate wells. The X-axis was represented in a logarithmic scale.

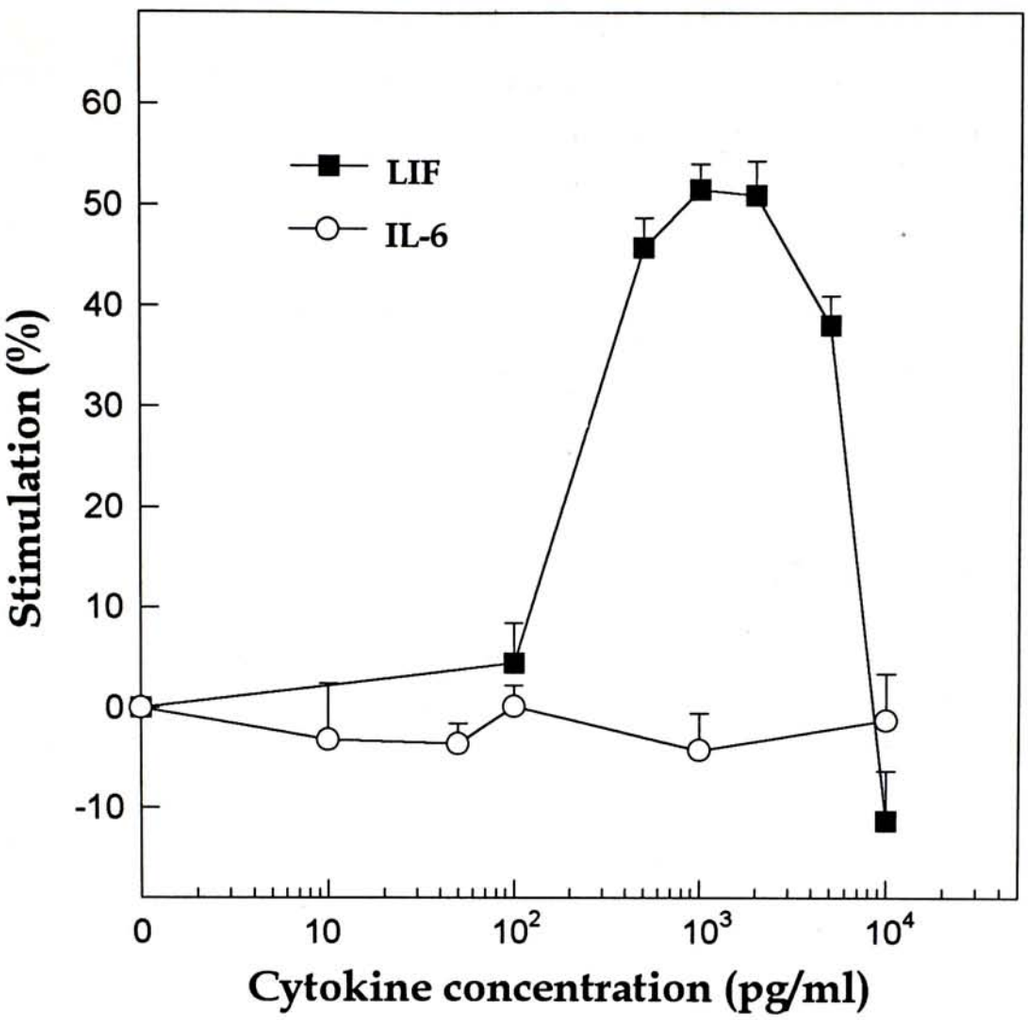


Fig. 3.2 The effects of LIF and IL-6 on C6 cell proliferation. C6 cells were cultured with various concentrations of LIF or IL-6 for 48 hours, and [^{3}H]-thymidine incorporation was determined as described in Section 2.3. Results were expressed as percentage of stimulation and each point represents the mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale.

showed the results of IL-6 and LIF on C6 cell proliferation. IL-6 at the range of 0.01-10 ng/ml had no significant effect on C6 cell proliferation. On the other hand, LIF was very effective at concentrations from 0.4 ng/ml to 5 ng/ml. Maximum stimulation (51% increase in [^{3}H]-thymidine incorporation) was obtained with 1 ng/ml of LIF. At concentrations lower than 0.1 ng/ml of LIF, no stimulation was observed, while slight inhibition was seen at 10 ng/ml. The latter may be due to the cytotoxic effect of LIF at higher doses.

In addition to TNF-α and LIF, IL-1α and IL-1β also showed dose-dependent stimulation on C6 cell proliferation, and maximum stimulation of 65 % and 67 % were observed with 1 ng/ml of IL-1α and 100 pg/ml of IL-1β, respectively (Fig. 3.3). These two interleukins appeared to be more potent than LIF as stimulation was observed with pg/ml of either IL-1, while ng/ml of LIF was required to attain similar stimulation. Additionally, IL-1β was found to be more effective than IL-1α, especially at lower concentrations.

3.1.2 The Time Course of Cytokine-induced C6 Cell Proliferation

Preliminary studies showed that 100 U/ml TNF-α or 1 ng/ml LIF could induce maximal proliferation of the C6 glioma cells, therefore these concentrations were used to investigate the time course for the

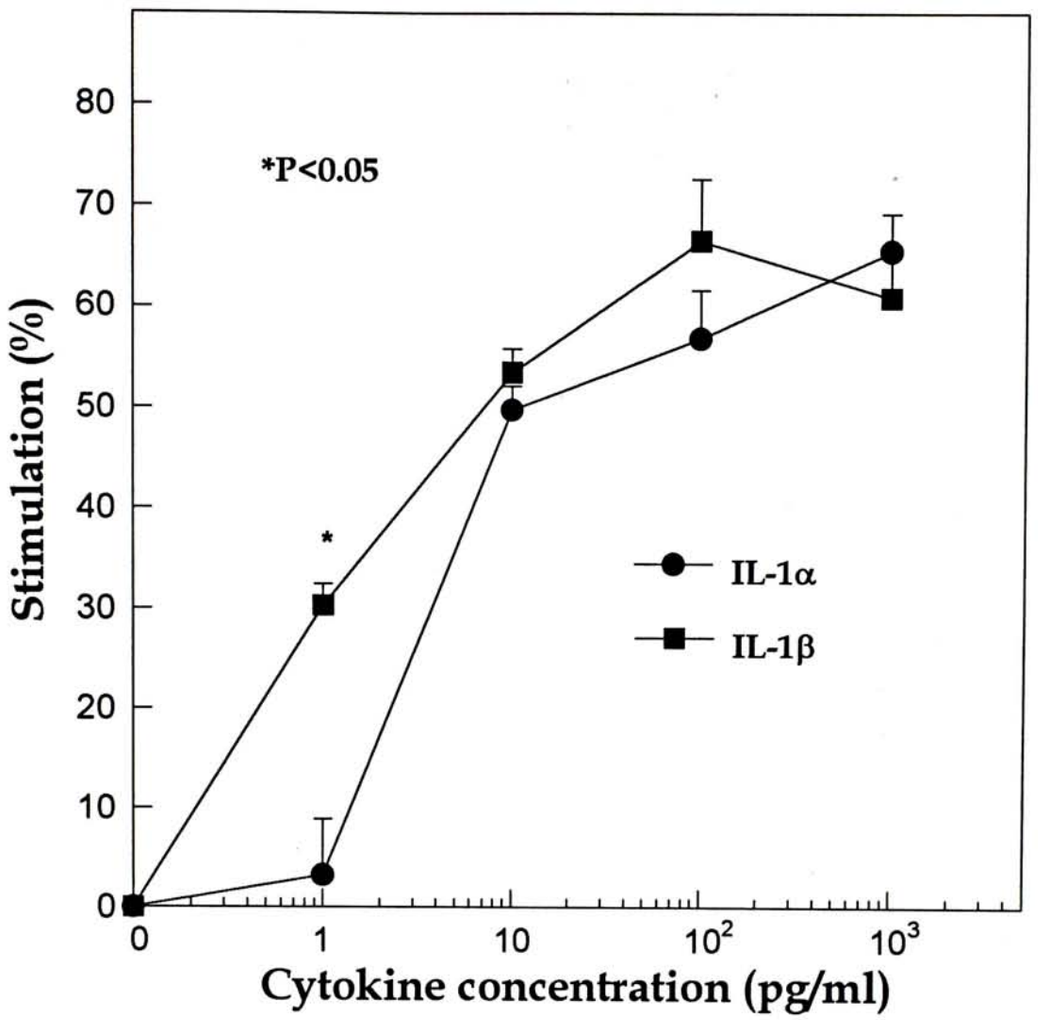


Fig. 3.3 The effects of IL-1α and IL-1β on C6 cell proliferation. C6 cells were cultured with various concentrations of IL-1α or IL-1β for 48 hours, and [^{3}H]-thymidine incorporation was determined as described in Section 2.3. Results were expressed as percentage of stimulation and each point represents the mean ± standard error of quadruplicate wells. Significant difference between the corresponding points of IL-1α and IL-1β ($P<0.05$) was indicated by an asterisk. The X-axis was represented in a logarithmic scale.

proliferative effect of these two cytokines. The experimental procedure used was as described in Section 2.3.

Fig. 3.4 showed the results of C6 cell proliferation after exposing the cells to 100 U/ml TNF-α or 1 ng/ml LIF for different durations. The proliferative effect induced by these two cytokines did not require the continuous presence of these cytokines in culture. Significant stimulation was observed after 60 minutes exposure to 100 U/ml of TNF-α, and higher stimulation was observed at longer incubation periods. On the other hand, treatment with 1 ng/ml of LIF for 15 minutes caused significant stimulation, which increased only slightly at longer incubation periods. Because of the rapid proliferative action of this cytokine, LIF was also included in the second messenger study (Section 3.3) in order to determine if TNF-α and LIF triggered different signalling pathways in C6 cells.

3.1.3 Effects of Lipopolysaccharide on C6 Cell Proliferation

LPS has been shown to induce cytokine production in human primary cultured astrocytes (Lee *et al.* 1993b), it was therefore of interest to examine if LPS could also affect cell proliferation in C6 cells. Fig. 3.5 showed the effect of various doses of LPS on C6 cell proliferation. The stimulatory effect of LPS was both dose-dependent and time-dependent, and maximum stimulation (50 % increase in

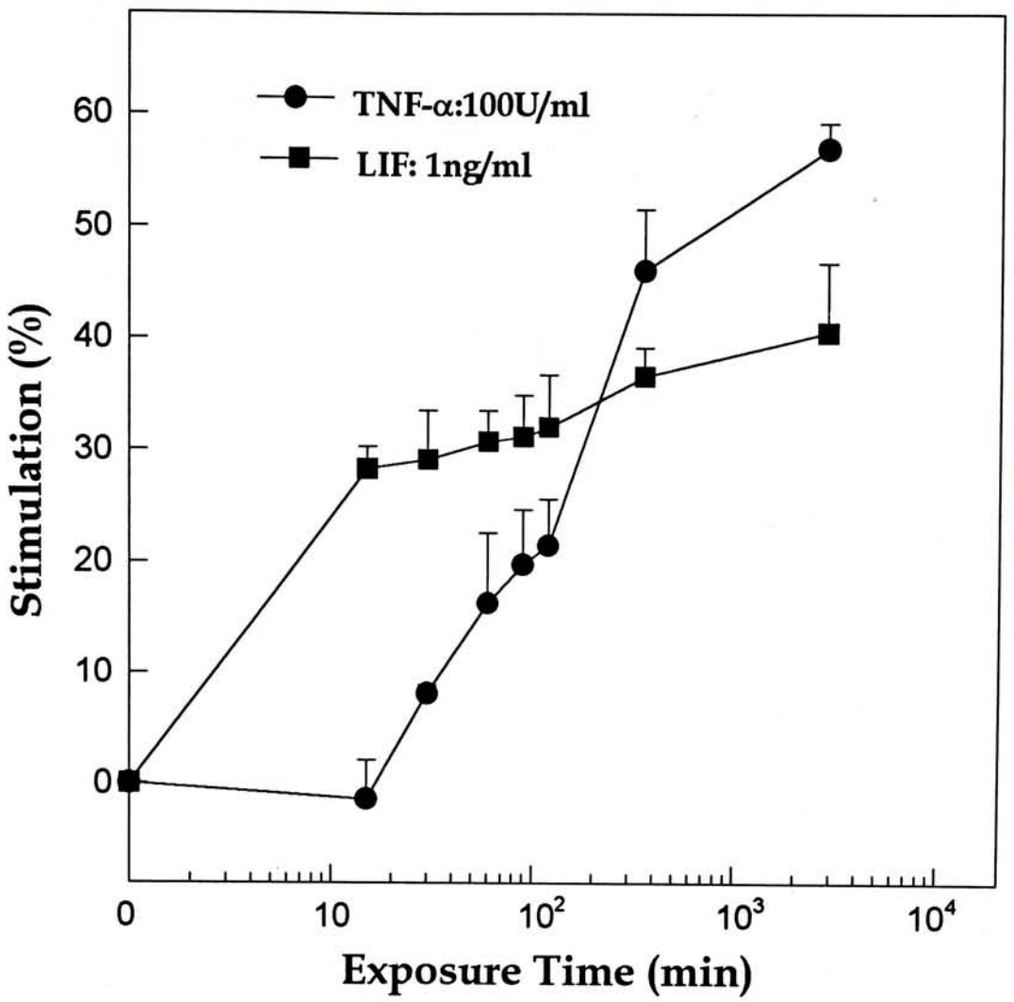


Fig. 3.4 The time course of C6 cell proliferation induced by TNF-α or LIF. The C6 glioma cells were seeded overnight and then incubated with TNF-α or LIF for different durations as shown, and [^{3}H]-thymidine incorporation was determined as described in Section 2.3. Results were expressed as percentage of stimulation and each point represents the mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale.

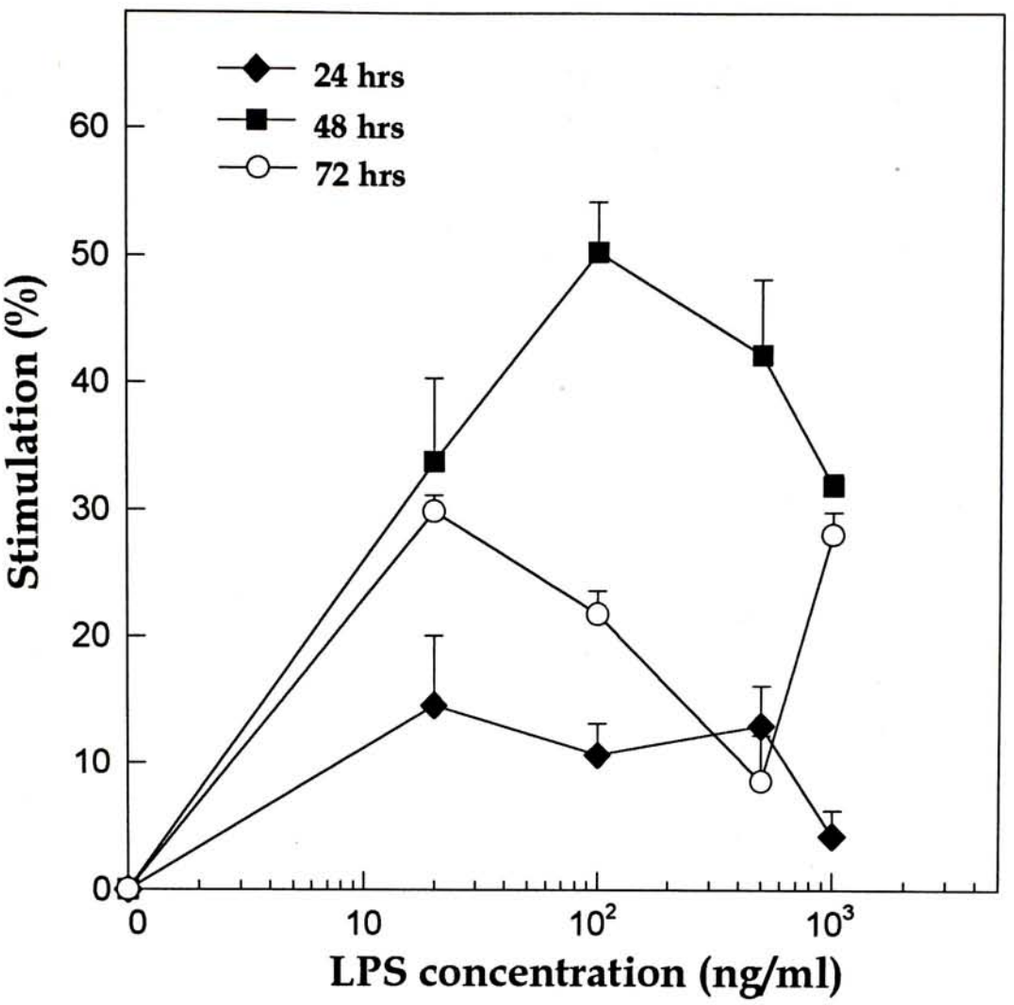


Fig. 3.5 Effects of exposure time and doses of LPS on C6 cell proliferation. [^{3}H]-thymidine incorporation was carried out as described in Fig 3.1 and results were expressed as the percentage of stimulation as described in the Section 2.3. The results were obtained in the presence of different doses of LPS for 1, 2 and 3 days. Values shown are mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale.

[^{3}H]-thymidine incorporation) was obtained with 100 ng/ml LPS after 48 hours of incubation.

3.1.4 Effects of Cytokines on the Growth of C6 Cells

In order to examine if cell growth was affected by different cytokines, the C6 glioma cells were cultured in the presence of different doses of cytokines, then the cell growth was monitored with the uptake of the vital dye neutral red as described in Section 2.2.2. After 48 hours of incubation, there was no significant change in cell number compared with the control (data not shown). However, after 72 hours of treatment with cytokines--TNF-α, LIF, IL-1α or IL-1β, the cell number was markedly increased (Fig. 3.6). Among the two forms of interleukin 1 studied, IL-1β appeared to be more effective than IL-1α in stimulating C6 cell growth (Fig. 3.6B).

3.2 Morphology and GFAP Expression in Cytokine-treated C6 Glioma cells

3.2.1 Effects of Cytokines on the Morphology of C6 cells

As discussed in the introduction, early passages of C6 glioma cells exhibit progenitor properties and can differentiate into either oligodendrocytic or astrocytic phenotype (Brodie & Vernadakis, 1991; Parker *et al.*, 1980). Moreover, cell morphology has been shown to be

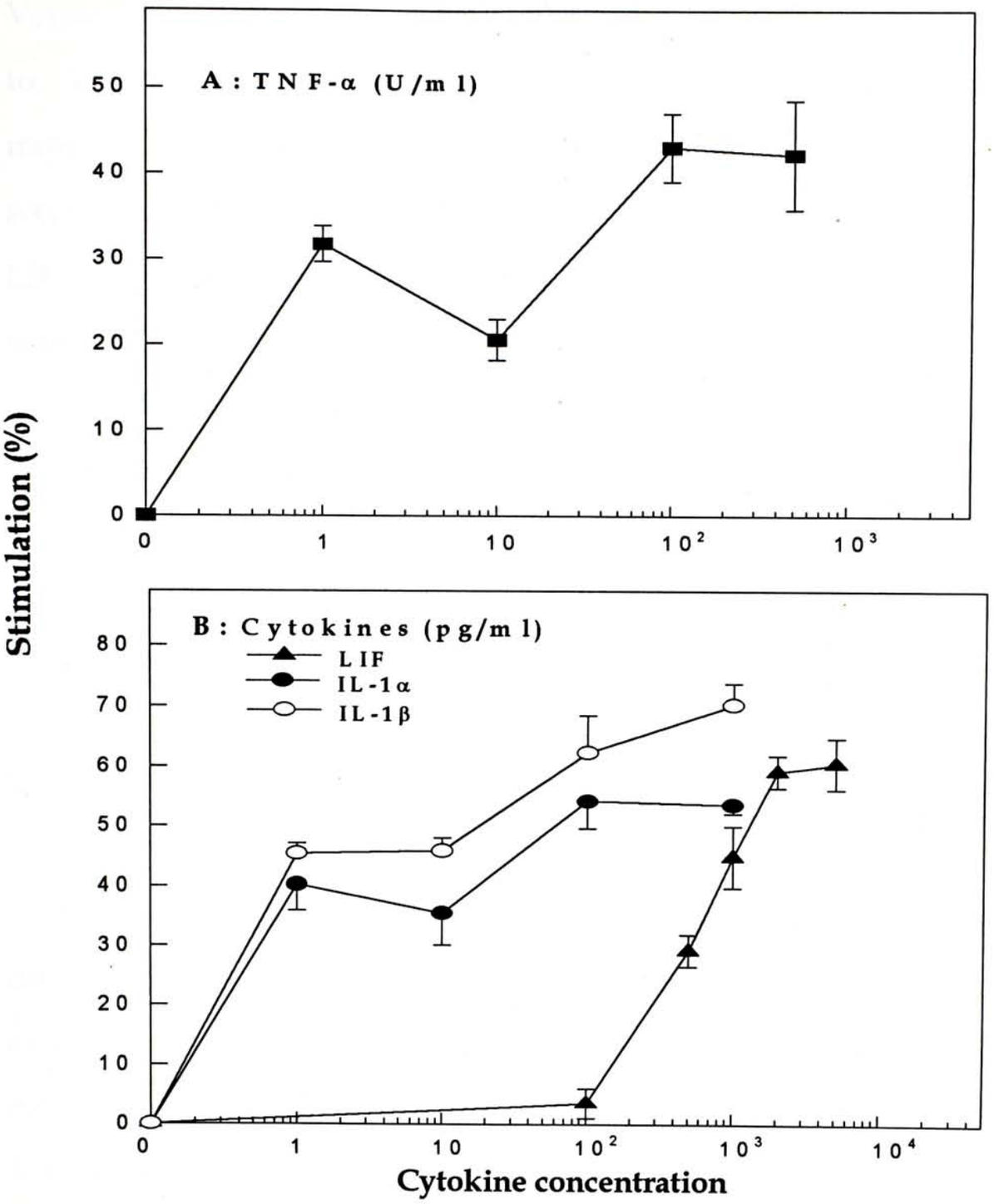


Fig. 3.6 Induction of C6 cell growth by TNF-α (A), LIF and IL-1 α or β (B). C6 cells were cultured for 3 days in the presence or absence of various concentrations of cytokines. The cell growth was estimated with neutral red staining as described in Section 2.2.2. Results were expressed as percentage of stimulation and each point represents the mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale.

a useful criterion for monitoring cell differentiation (Brodie & Vernadakis, 1991; Vernadakis *et al.*, 1991). Therefore, it is of interest to know whether treatment with cytokines can alter the morphological appearance of C6 cells. In this study, C6 glioma cells were exposed to cytokines, including 100 U/ml TNF-α and 1 ng/ml LIF 3 days before they were stained with the Wright-Giemsa stain. It was observed that these cytokines did not induce significant morphological changes except that the cells were smaller (Fig. 3.7) Although no significant changes in morphology were observed, there was a clear increase in the cell number in each case. This observation is in agreement with the results that these cytokines significantly increased the [^{3}H]-thymidine incorporation in C6 cells.

3.2.2 The Effects of Cytokines on GFAP Expression in C6 Glioma Cells

Since early passages of C6 glioma cells can be stimulated to differentiate into oligodendrocytic or astrocytic phenotype, the expression of GFAP was examined with anti-GFAP antibodies. Figure 3.8 showed C6 cells incubated with 100 U/ml of TNF-α or 1 ng/ml LIF for 48 hours, followed by exposure to anti-GFAP antibodies. No obvious changes in cell morphology could be observed except that the size of the cytokine-treated cells was smaller. Interestingly, it was found that the number of GFAP-negative cells in the LIF-treated cells was slightly higher than that of the untreated control cells, whereas

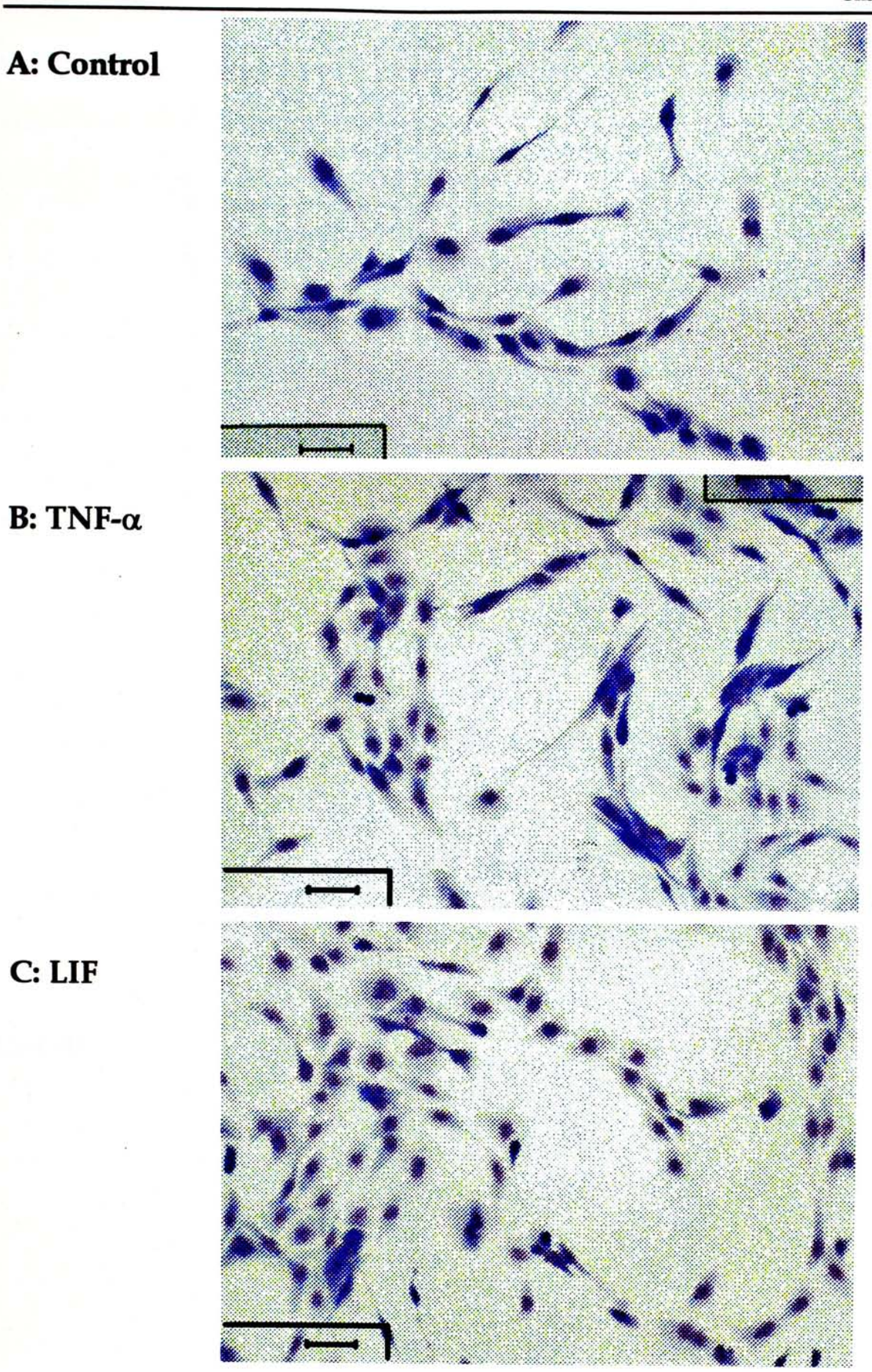
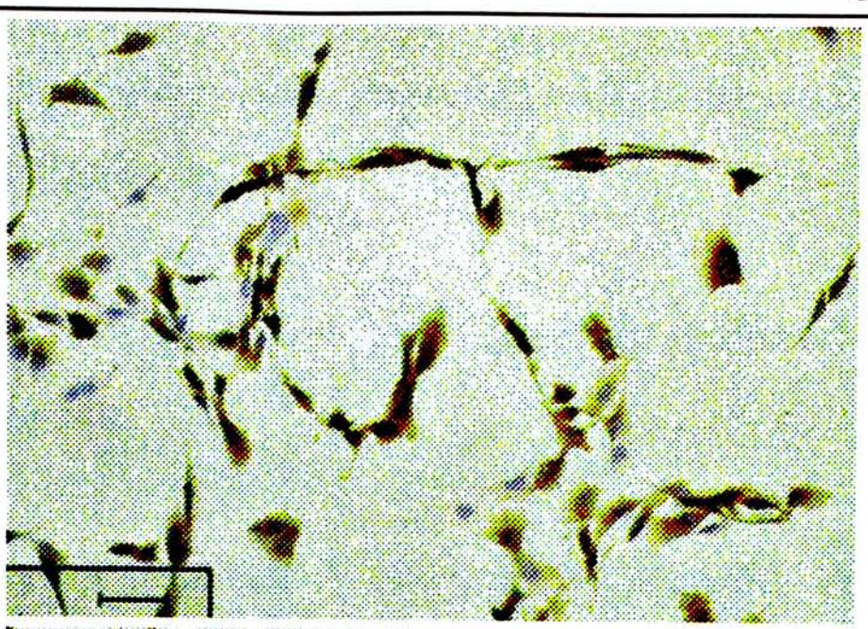
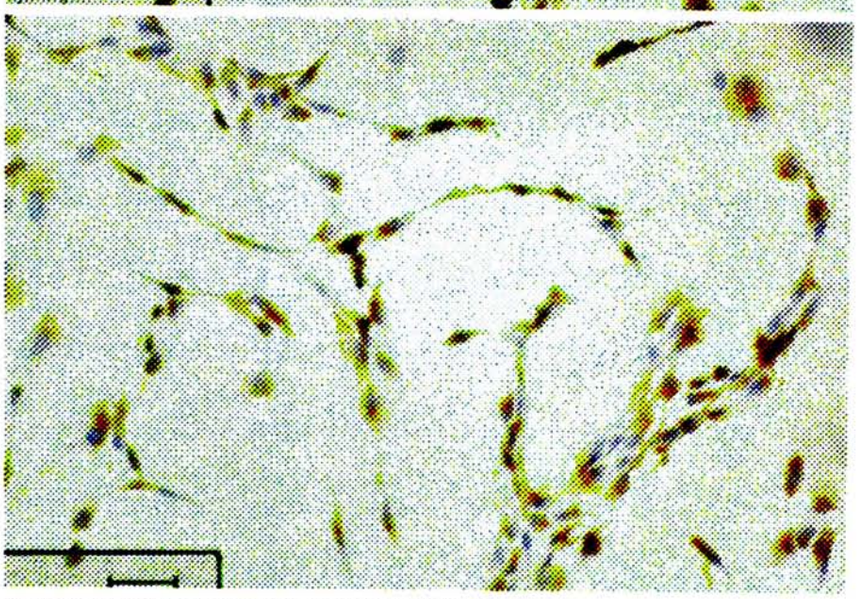


Fig. 3.7 Effect of TNF-α and LIF treatment on the morphology of C6 cells. C6 cells were cultured on a 15 mm cover slip in the presence of 100 U/ml TNF-α (B) or 1 ng/ml LIF (C) or in medium (A) for 3 days, then stained with Wright-Giesma stain as described in Section 2.10.1. The bar represents 50 μm.

A: Control



B: TNF-α



C: LIF

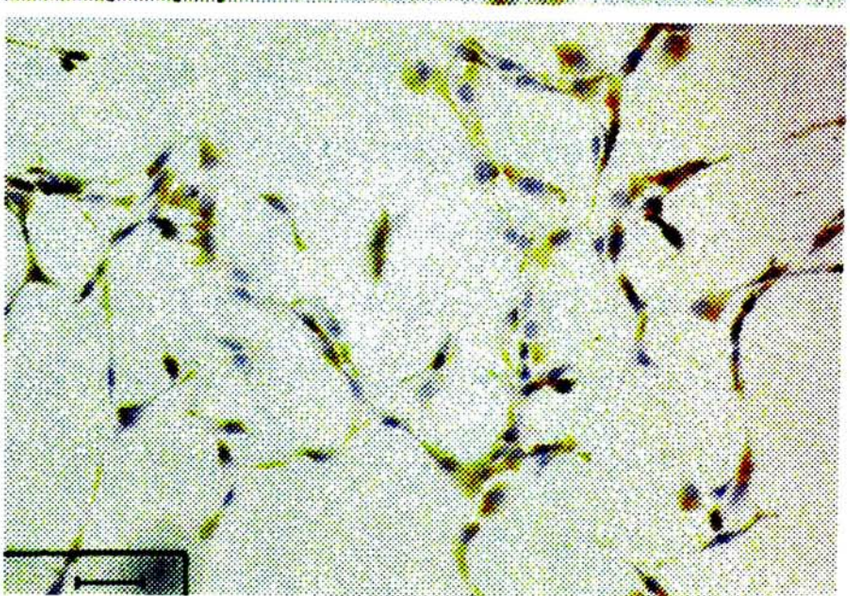


Fig. 3.8 Effect of TNF-α and LIF treatment on the morphology of C6 cells. C6 cells were cultured on a 12 mm cover slip in the presence of 100 U/ml TNF-α (B) or 1 ng/ml LIF (C) or in medium (A) for 2 days, then stained with GFAP/hematoxylin stain as described in Section 2.10.2 and 2.10.3. The bar represents 50 μm.

no significant difference was observed between the control and the TNF-α-treated cells (Fig. 3.9).

3.3 The Signalling Pathway of Cytokine-induced C6 Cell Proliferation

Experiments described in Section 3.1 showed that several cytokines could induce C6 glioma cell proliferation; however, the underlying mechanism remains unclear and very little is known about the signal-transduction pathways mediating the action of various cytokines. Since TNF-α and interleukin-1 have been reported to stimulate various second messengers in primary glial cell cultures (Norris *et al.*, 1994), the possible involvement of various signalling pathways in cytokine-induced proliferation in C6 cells was studied. The second messenger systems examined include protein kinase C (PKC), tyrosine kinase (TK), calcium ions, cyclic nucleotides, as well as the nitric oxide. As the β-adrenergic mechanism has been shown to stimulate proliferation in primary astrocyte cultures (Mantyh *et al.*, 1995), therefore, the effects of isoproterenol, a β-adrenergic agonist, on cytokine-induced proliferation in C6 cells were investigated to see if a relationship exists between β-adrenergic mechanism and the inflammatory cytokines.

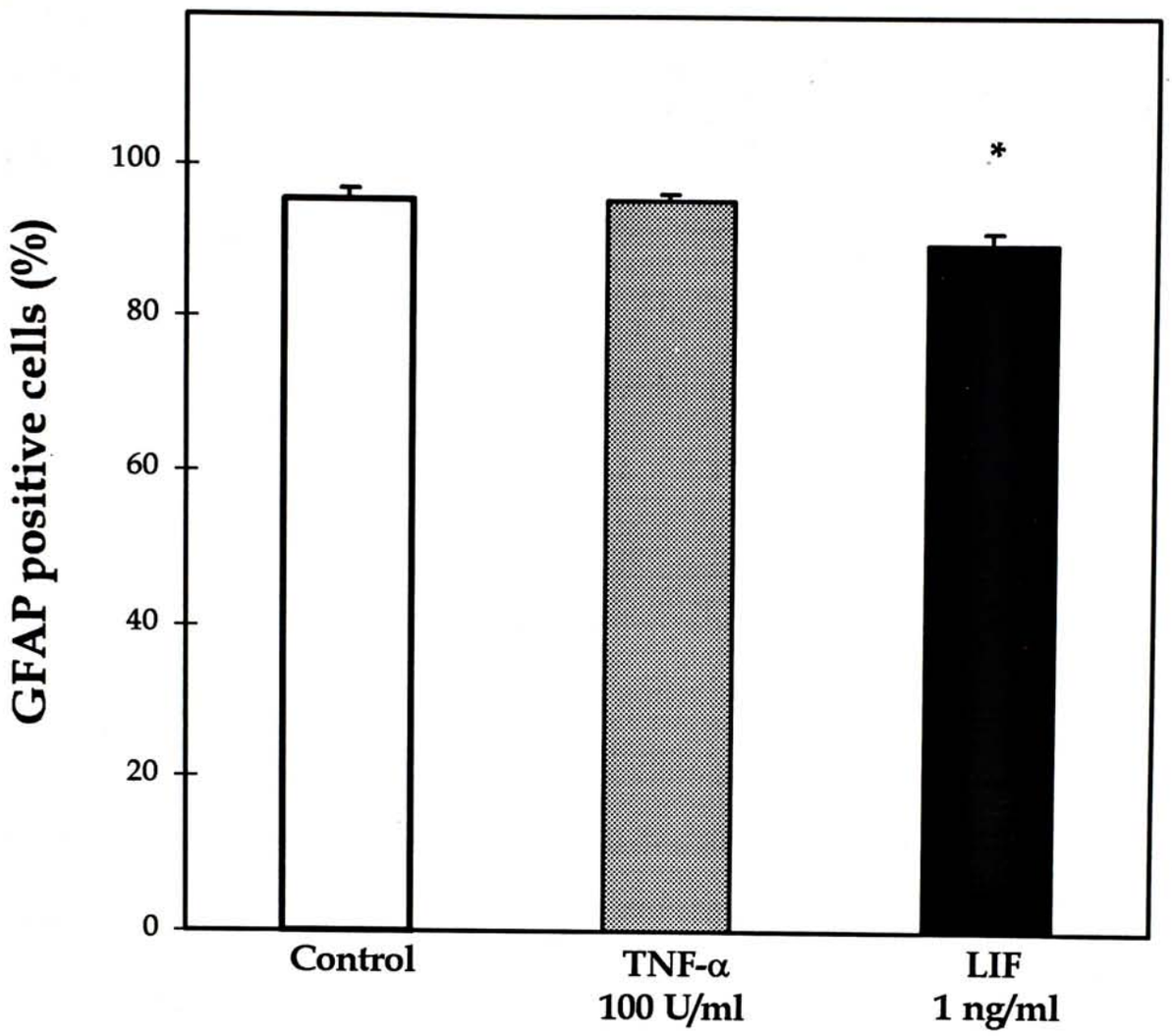


Fig. 3.9 Effect of cytokine treatment on GFAP expression in C6 cells. Cells were incubated with TNF-α (100 U/ml), or LIF (1 ng/ml) for 2 days, then exposed to GFAP antibodies and followed by hematoxylin staining as described in Section 2.10.2 and 2.10.3, and the percentages of the GFAP positive cells were counted. Values shown are means ± standard error of three determinations. *P<0.05, significantly different from control by using the Student's t-test.

3.3.1 The Involvement of Protein Kinase C in Cytokine-induced C6 Cell Proliferation

Since certain cytokines have been shown to induce phosphatidycholine hydrolysis (Cataldi *et al.*, 1990) and activate certain PKC isozymes (Pfeffer *et al.*, 1990), it is of interest to see if PKC is involved in mediating the proliferative effect of cytokines in C6 cells. In the present study, phorbol 12-myristate 13-acetate (PMA) and phorbol-12,13 diacetate (PDA), which are potent PKC activators (Hug & Sarre, 1993), were used to investigate whether the proliferative effect of cytokines is mediated through activation of PKC. In the range of 8.11 pM to about 1.62 nM PMA and 22.2 pM to about 2.23 nM PDA, either PKA activator was able to increase DNA synthesis in C6 cells. The maximum stimulation was observed with 162 pM PMA and 223 pM PDA respectively (Fig. 3.10).

To test if PKC mediated the proliferative effect of TNF-α and LIF, the effect of three PKC inhibitors on cytokine-induced C6 cell proliferation was investigated. The three PKC inhibitors examined were staurosporine, calphostin C and Ro31-8220, and all of them have been shown to be potent PKC inhibitors (Davis *et al.*, 1992; Hu & Fan, 1995; Tamaoki *et al.*, 1986). Incubation of the cells in any one of these three PKC inhibitors caused a slight (maximum 10 %) stimulation in C6 cells (Figs. 3.11-3.13), except at higher

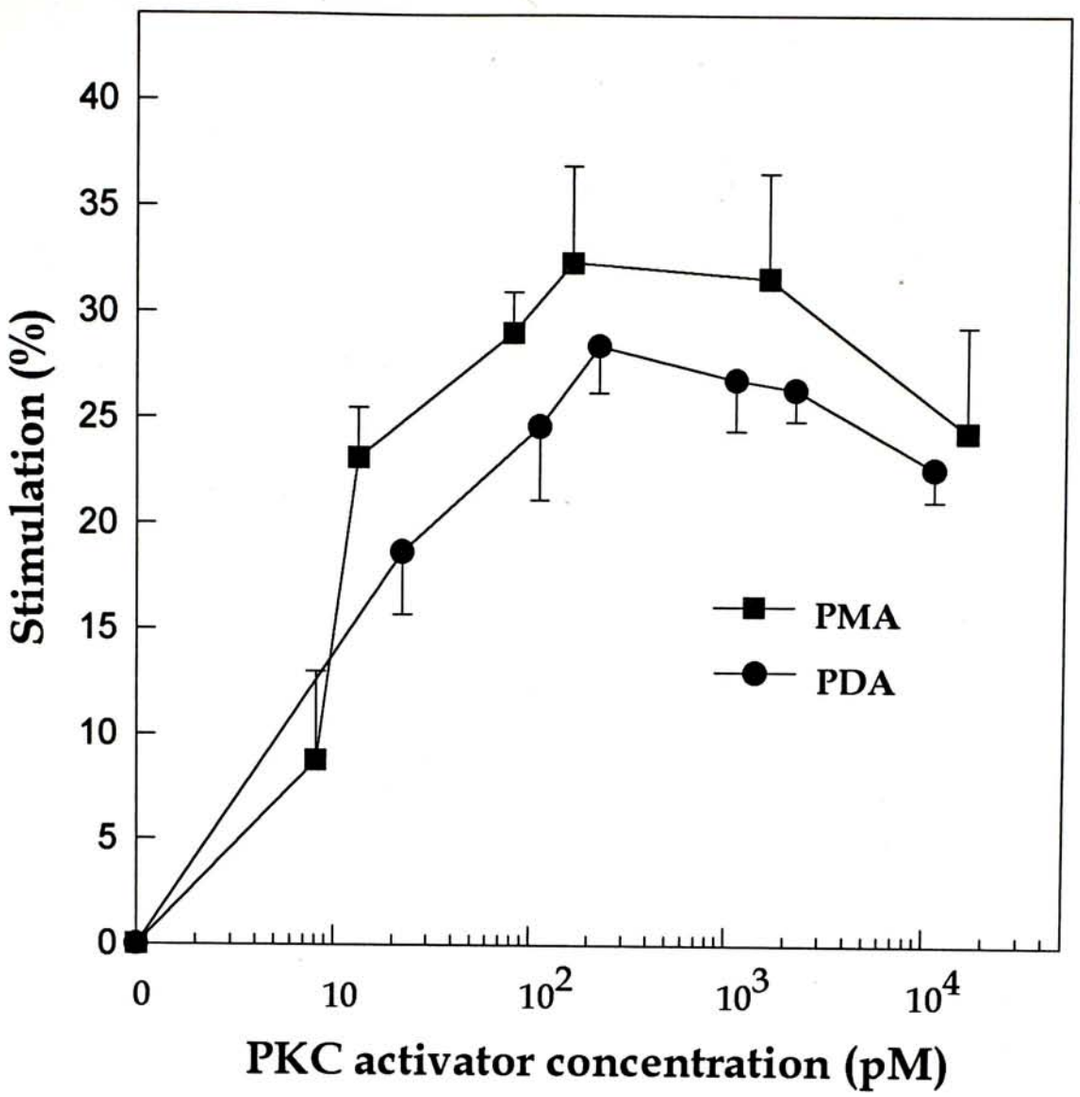


Fig. 3. 10 Effects of PKC activators on C6 cell proliferation. C6 glioma cells were treated with various concentrations of PMA or PDA for 48 hours, and proliferation was assayed as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. EC_{50} is the concentration of drug which causes 50 % of maximum stimulation. Estimated EC_{50}: PMA ≈ 12.3 pM, PDA ≈ 16.7 pM.

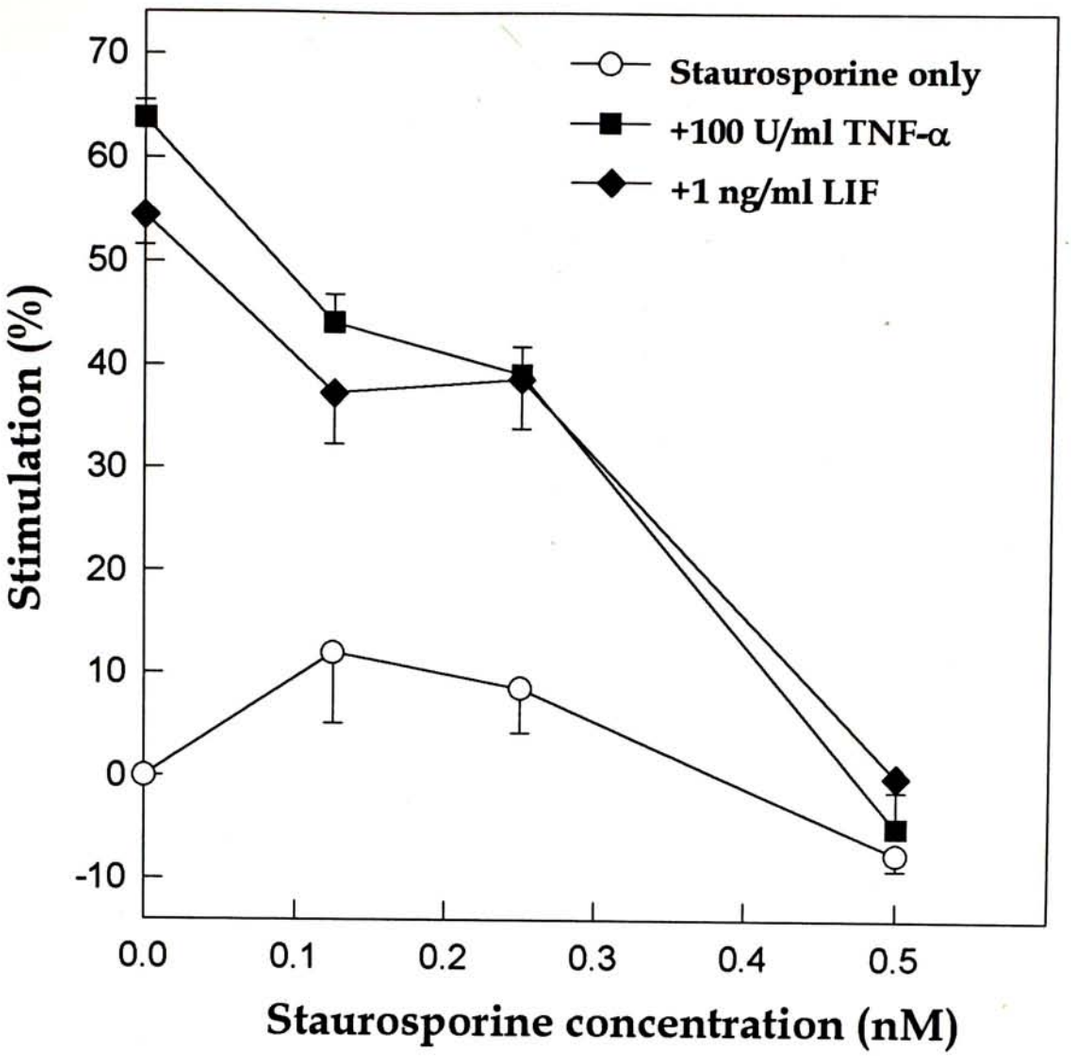


Fig. 3.11 Effects of various concentrations of staurosporine on TNF-α or LIF-induced proliferation of C6 cells. Cells were cultured for 48 hours with or without TNF-α (100 U/ml) or LIF (1 ng/ml) in the presence of indicated concentrations of staurosporine. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. IC_{50} is the concentration of the drug which inhibits 50 % of maximum stimulation. Estimated IC_{50} values: TNF-α ≈ 0.3 nM, LIF ≈ 0.34 nM.

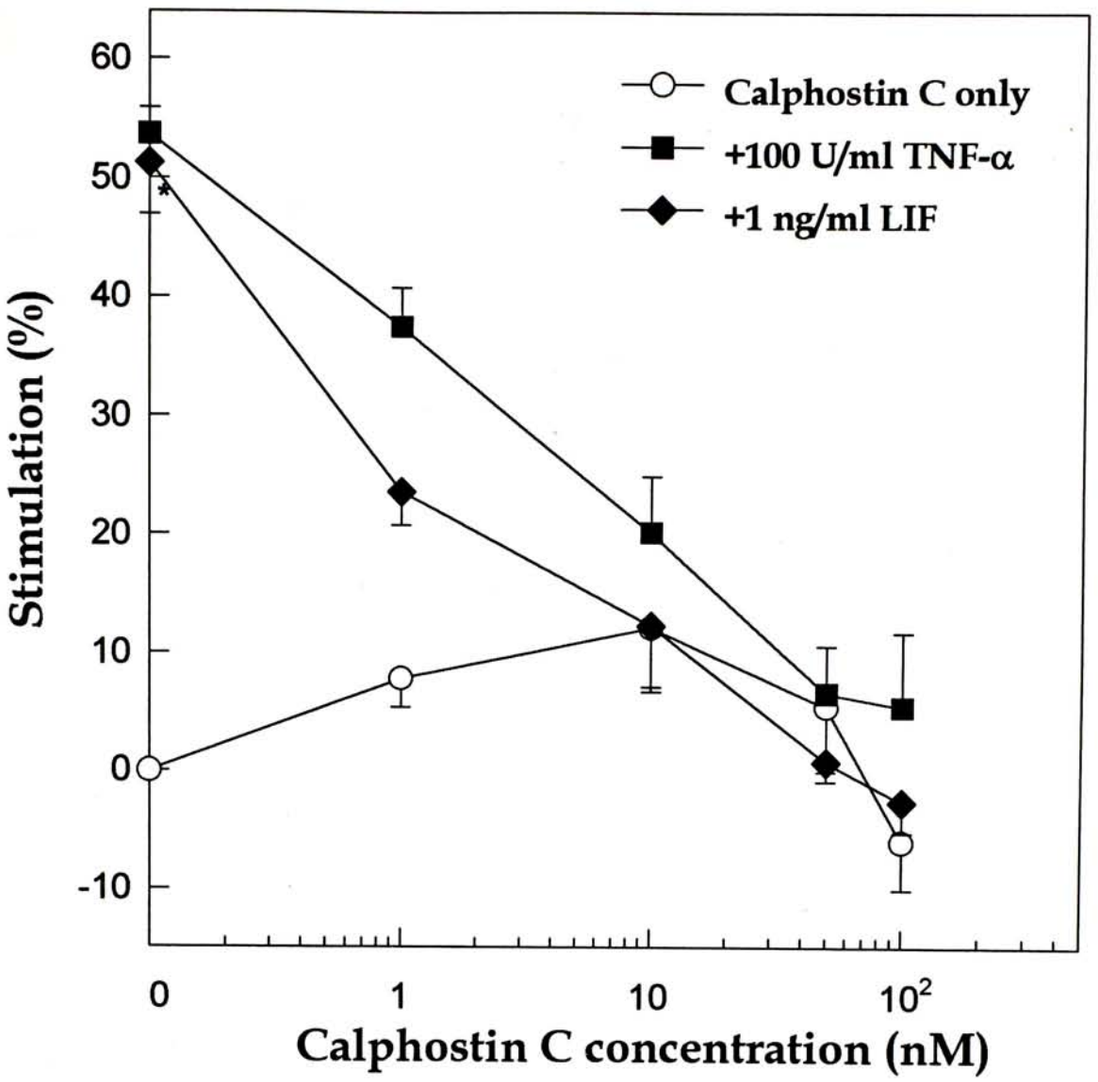


Fig. 3.12 Effects of various concentrations of calphostin C on TNF-α or LIF-induced proliferation in C6 cells. Cells were cultured for 48 hours with or without TNF-α (100 U/ml) or LIF (1 ng/ml) in the presence of indicated concentrations of calphostin C. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: TNF-α ≈ 4.3 nM, LIF ≈ 1 nM.

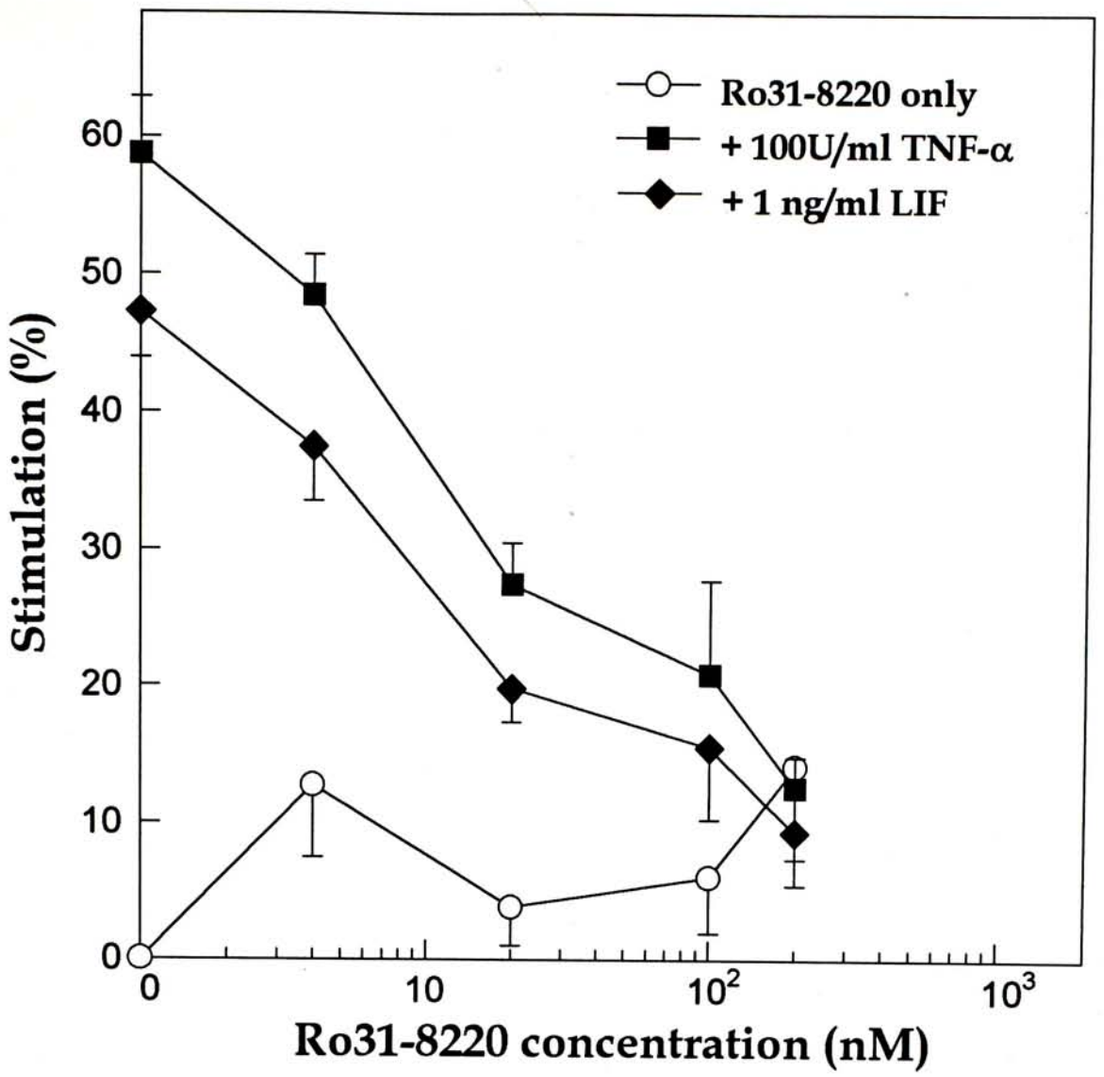


Fig. 3.13 Effects of various concentrations of Ro31-8220 on TNF-α or LIF-induced proliferation of C6 cells. C6 cells were cultured for 48 hours with or without TNF-α (100 U/ml) or LIF (1 ng/ml) in the presence of indicated concentrations of Ro31-8220. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: TNF-α ≈ 15 nM, LIF ≈ 10 nM.

concentrations of staurosporine (0.5 nM) or calphostin C (100 nM) where a slight inhibitory effect was observed (Fig. 3.11 and 3.12).

Staurosporine at a low concentration (0.125 nM) inhibited the proliferation induced by the optimal dose of TNF-α (100 U/ml), or LIF (1 ng/ml) significantly, and complete inhibition of the proliferative action induced by either TNF-α or LIF was observed with 0.5 nM of this inhibitor (Fig. 3.11). The IC_{50} values for TNF-α and LIF were estimated to be ~0.3 nM and ~0.34 nM of staurosporine, respectively.

Calphostin C, which is a potent PKC inhibitor, reduced the proliferation induced by TNF-α or LIF in a concentration-dependent manner (Fig. 3.12); and complete or almost complete inhibition was observed with 50 nM of calphostin C. The IC_{50} values for TNF-α and LIF were estimated to be ~4.3 nM and ~1 nM, respectively.

To ensure that the proliferative effect of TNF-α and LIF was mediated through PKC, the effect of Ro31-8220 was tested. Similar to calphostin C and staurosporine, Ro31-8220 also reduced the proliferative effect of TNF-α and LIF in a concentration-dependent manner (Fig. 3.13) The IC_{50} values for TNF-α and LIF were estimated to be ~15 nM and ~10 nM, respectively.

Comparing the inhibitory effects of the three PKC inhibitors examined, staurosporine, calphostin C and Ro31-8220 (Fig. 3.11 -- 3.13), it was clear that the inhibitory effect of these three inhibitors was in the order of staurosporine >> calphostin C > Ro31-8220.

Results described above showed that the PKC activators stimulated proliferation in C6 cells (Fig. 3.10), and the proliferative effect of TNF-α and LIF was blocked by PKC inhibitors (Figs. 3.11-3.13). In order to demonstrate that PKC was indeed involved in the proliferation of C6 cells, cells were treated with optimal dose of PMA or PDA in the presence of different concentrations of one of the three PKC inhibitors for 48 hours before [^{3}H]-thymidine incorporation was measured. Data showed that DNA synthesis in C6 cells induced by either PMA or PDA was significantly inhibited by staurosporine (Fig. 3.14), calphostin C (Fig. 3.15) or Ro31-8220 (Fig. 3.16) in a dose-dependent manner. Moreover, the effect of PMA appeared to be more sensitive to staurosporine and Ro31-8220 (Figs. 3.14 and 3.16), while that of PDA was more sensitive to the action of calphostin C (Fig. 3.15). Like the inhibitory effects of these PKC inhibitors on cytokine-induced proliferation, staurosporine was the most potent inhibitor of PKC activator-induced proliferation in C6 cells, and complete inhibition was observed with 0.25 nM of staurosporine (Fig. 3.14). Whatever on cytokine- or PKC activator-induced proliferation, the effect order of these three inhibitors was parallel to that of their

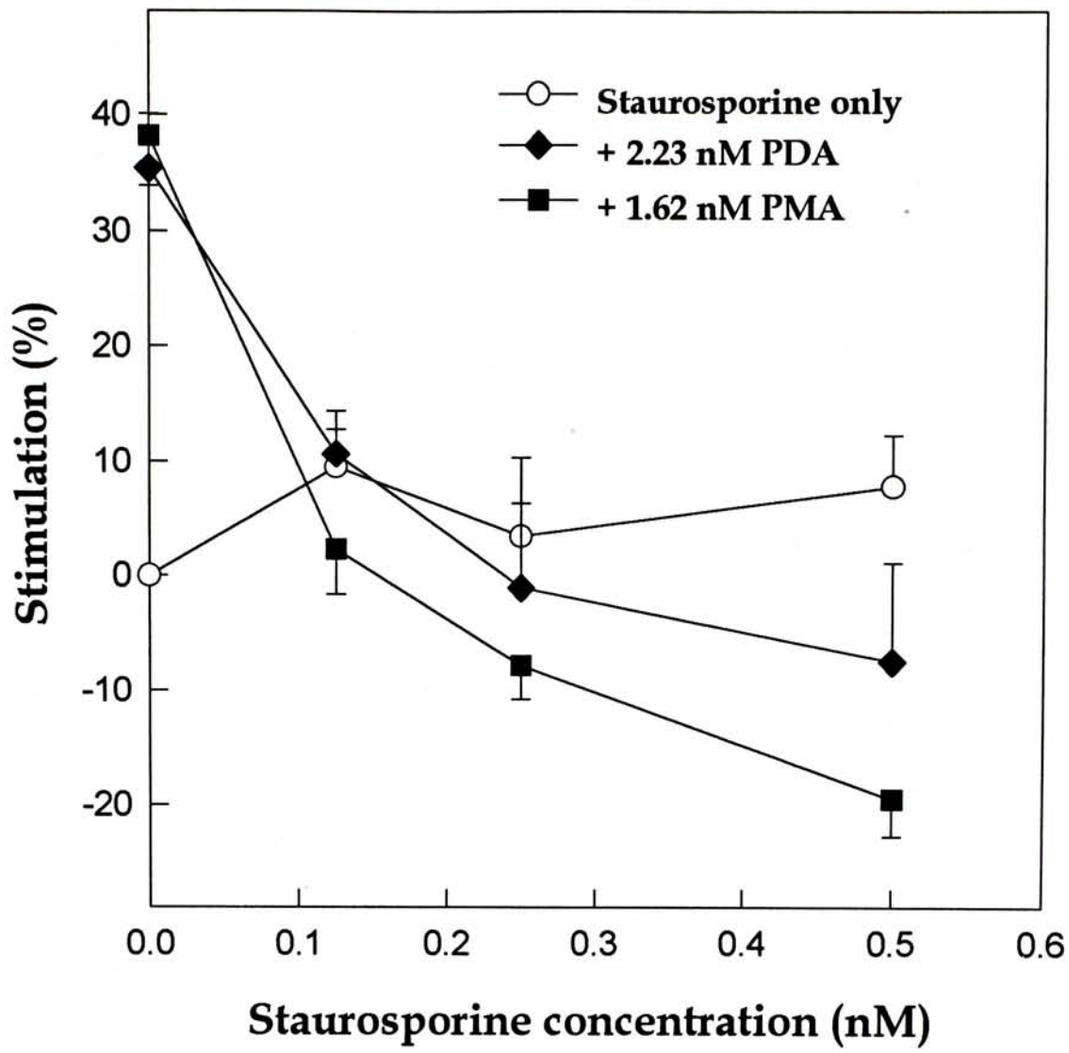


Fig. 3.14 Effects of various concentrations of staurosporine on PMA- or PDA-induced C6 cell proliferation. C6 cells were cultured for 48 hours with various concentrations of staurosporine, in the presence or absence of 1 ng/ml PMA or PDA. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. Estimated IC_{50}: PMA ≈ 0.067 nM, PDA ≈ 0.09 nM.

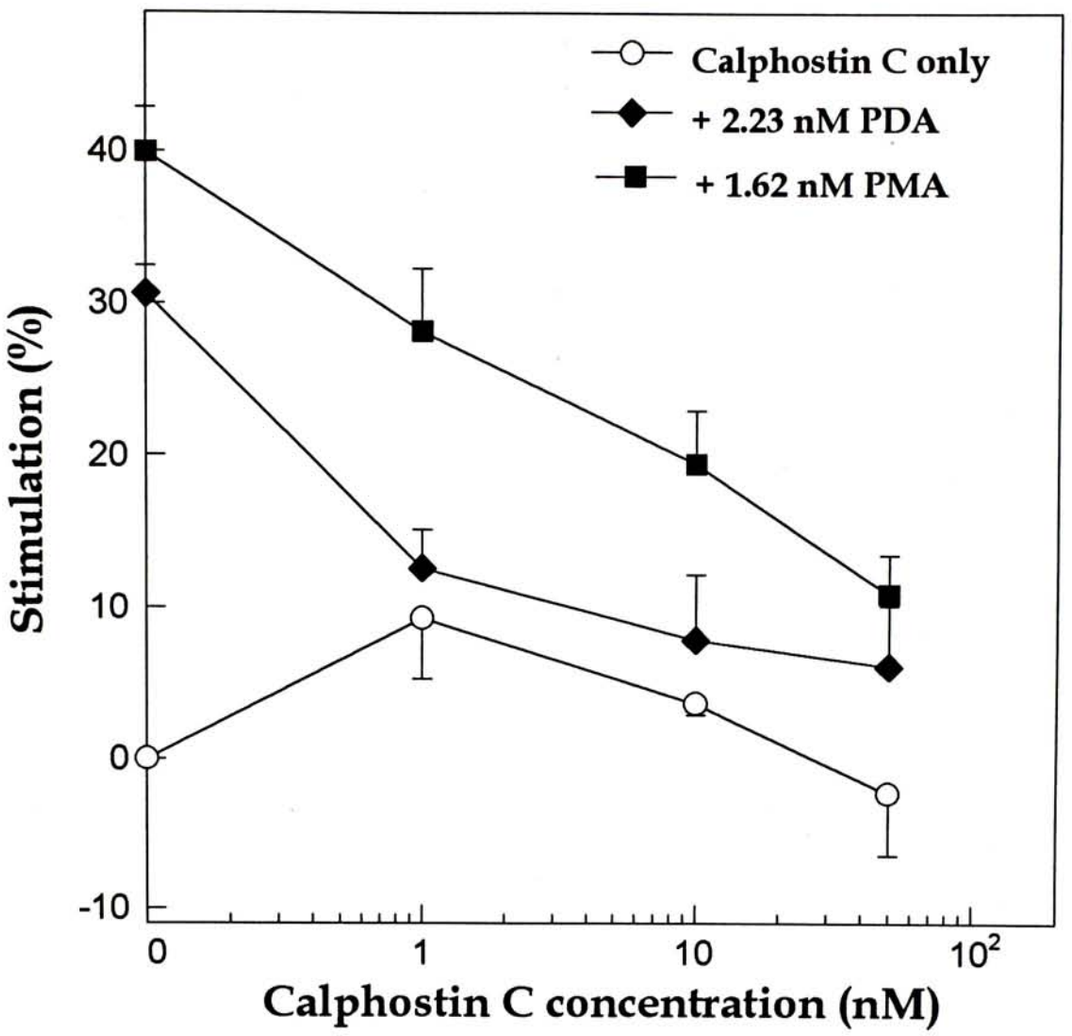


Fig. 3.15 Effects of various concentrations of calphostin C on PMA- or PDA-induced C6 cell proliferation in C6 cells. Cells were cultured for 48 hours with various concentrations of calphostin C, in the presence or absence of 1 ng/ml PMA or PDA. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: PMA ≈ 9 nM, PDA ≈ 0.3 nM.

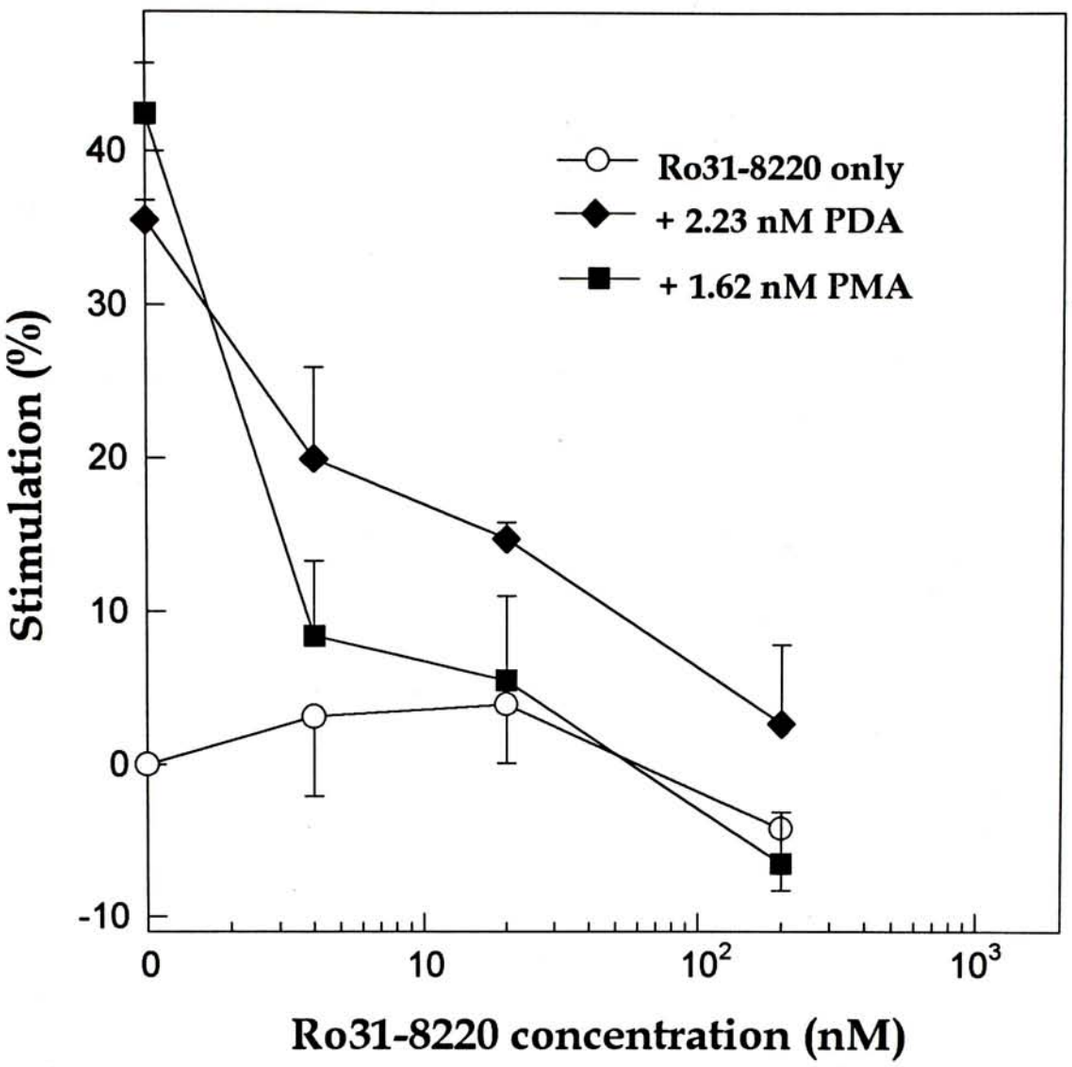


Fig. 3.16 Effects of various concentrations of Ro31-8220 on PMA- or PDA-induced C6 cell proliferation in C6 cells. Cells were cultured for 48 hours with various concentrations of Ro31-8220, in the presence or absence of 1 ng/ml PMA or PDA. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: PMA ≈ 1.5 nM, PDA ≈ 7 nM.

inhibition on PKC activity (Couldwell *et al.*, 1994; Davis *et al.*, 1992; Keller and Niggli, 1993; Kobayashi *et al.*, 1989).

3.3.2 The Involvement of Tyrosine Kinase in the Cytokine-induced C6 Cell Proliferation

Tyrosine kinase, a common second messenger, was reported to be involved in both cytokine and LPS-induced nitric oxide synthase expression in C6 cells (Feinstein *et al.*, 1994a). It is of interest to examine whether tyrosine kinase activity also plays a role in cytokine-induced C6 cell proliferation. Therefore, the effects of two selective tyrosine kinase inhibitors, herbimycin A and tyrphostin, were investigated in this study. The latter has been reported to be highly specific for the enzyme tyrosine kinase (Bianchi *et al.*, 1995; Feinstein *et al.*, 1994a). Figures 3.17 and 3.18 showed the inhibitory effects of herbimycin A and tyrphostin on TNF-α- and LIF-induced C6 cell proliferation. Neither inhibitor alone had significant effect on the incorporation of [^{3}H]-thymidine, while herbimycin A, at 25 nM, and tyrphostin, at around 1 μM, completely blocked the stimulation induced by these two cytokines (Figs. 3.17 and 3.18). This observation also suggested that herbimycin A was more potent than tyrphostin, as the latter required μM concentrations in order to block the cytokine-induced proliferation completely. This result was in agreement with that herbimycin A blocks tyrosine kinase more

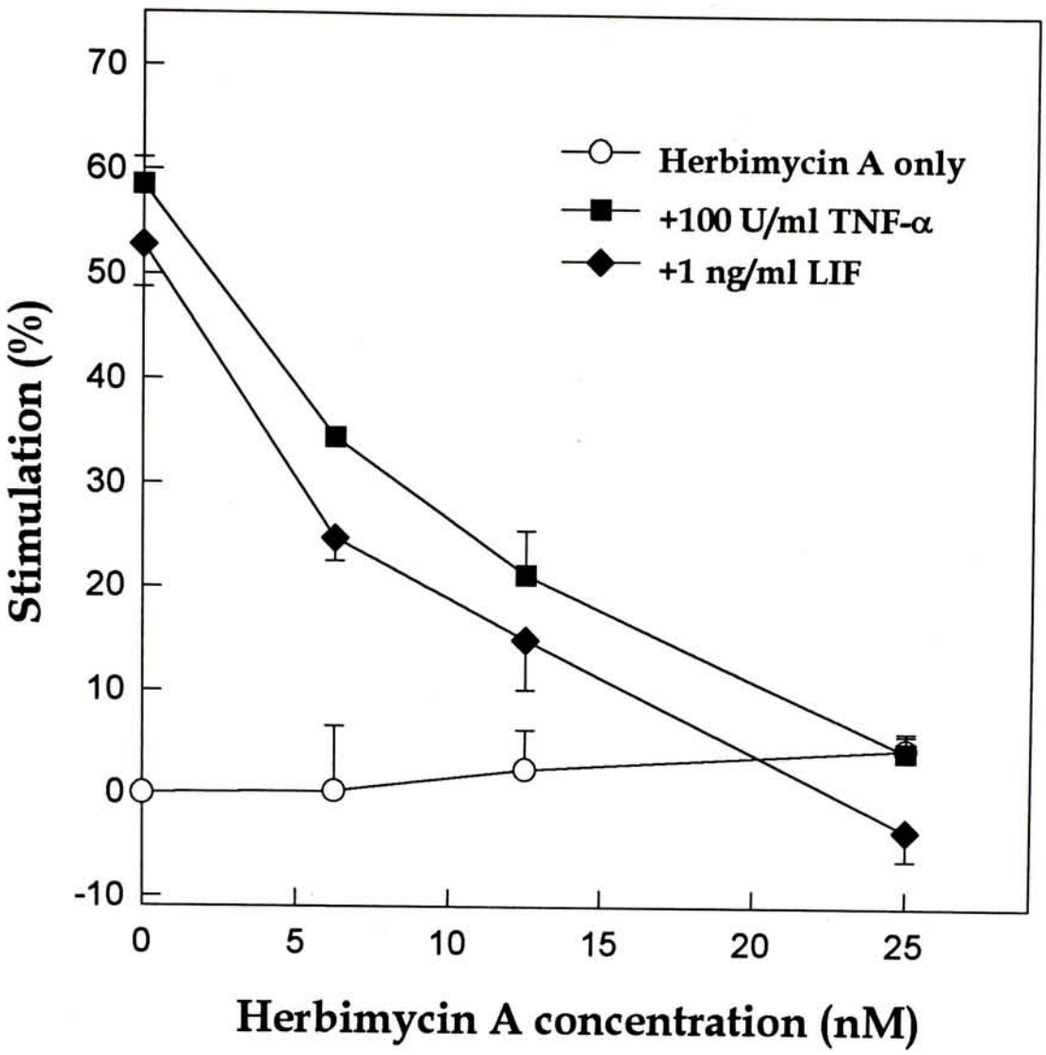


Fig. 3.17 Effects of various concentrations of herbimycin A on TNF-α or LIF-induced proliferation. C6 cells were cultured for 48 hours with or without TNF-α (100 U/ml) or LIF (1 ng/ml) in the presence of indicated concentrations of herbimycin A. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. Estimated IC_{50}: TNF-α ≈ 8.2 nM, LIF ≈ 5.8 nM.

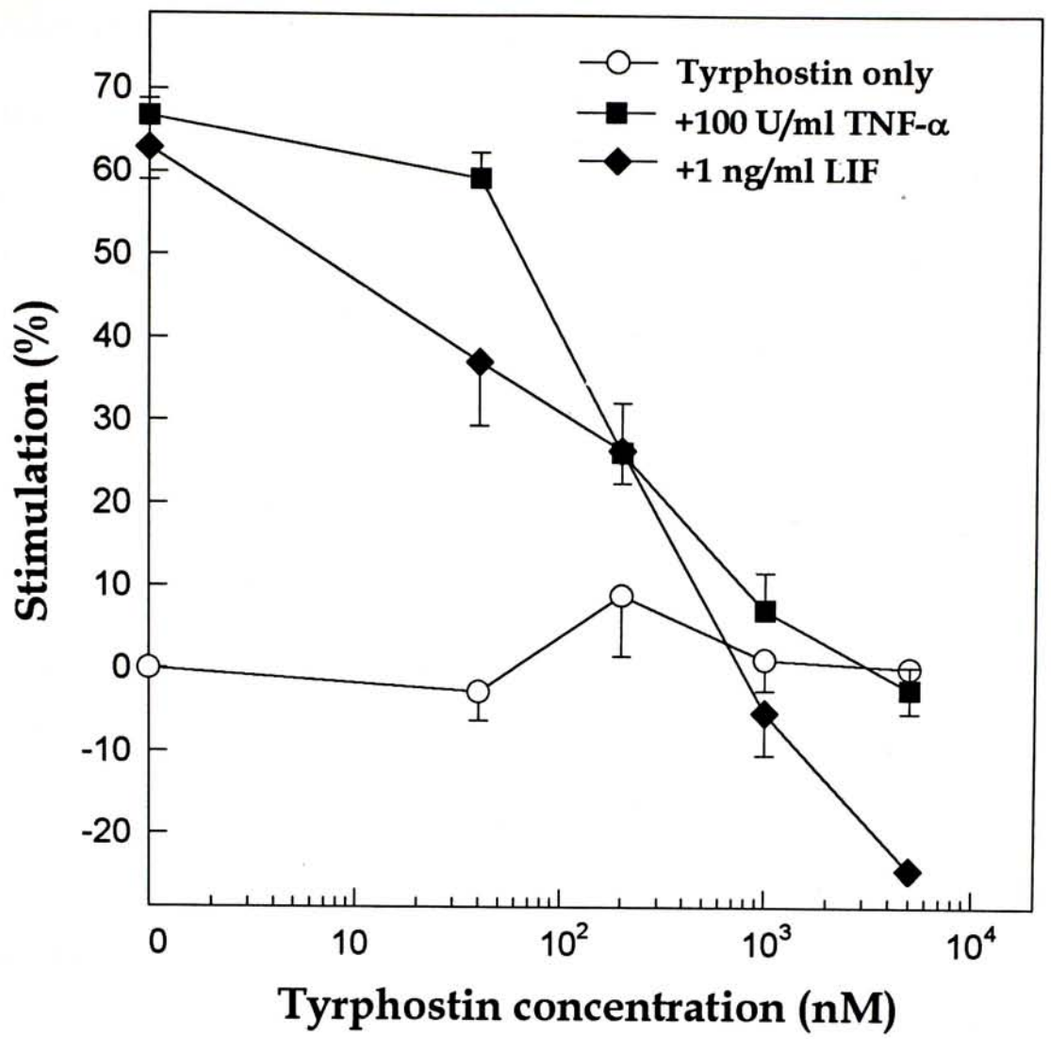


Fig. 3.18 Effects of various concentrations of tyrphostin on TNF-α or LIF-induced proliferation. C6 cells were cultured for 48 hours with or without 100 U/ml TNF-α or 1 ng/ml LIF in the presence of indicated concentrations of tyrphostin. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: TNF-α ≈ 0.14 μM, LIF ≈ 0.12 μM.

effectively than tyrphostin (Migita *et al.*, 1994; Gazit *et al.*, 1989). In the presence of 25 nM of herbimycin A or 5 μM of tyrphostin, almost no proliferative effect could be observed (Figs. 3.17 and 3.18). Another point to be noted was that the action of LIF appeared to be slightly more sensitive to these two tyrosine kinase inhibitors which would indicate that the LIF-induced proliferation is mediated, at least in part, via tyrosine kinase.

In order to investigate whether there is any relationship between tyrosine kinase and protein kinase C activity on the proliferation of C6 cells, the cells were treated with a PKC activator in combination with a tyrosine kinase inhibitor for 48 hours, and cell proliferation was measured by the method as described in Section 2.6. From Figures 3.19 and 3.20, the stimulatory effect of PMA and PDA was blocked by either herbimycin A or tyrphostin. Moreover, herbimycin A was found to be more effective than tyrphostin (Compare Figs. 3.19 and 3.20), which was cooperated with their inhibitory effects on tyrosine kinase activity (Gazit *et al.*, 1989; Migita *et al.*, 1994). The stimulatory action of PDA was more sensitive to these two tyrosine kinase inhibitors. Like before (Figs. 3.17 and 3.18), neither herbimycin A nor tyrphostin alone showed any significant proliferative effect on C6 glioma cells.

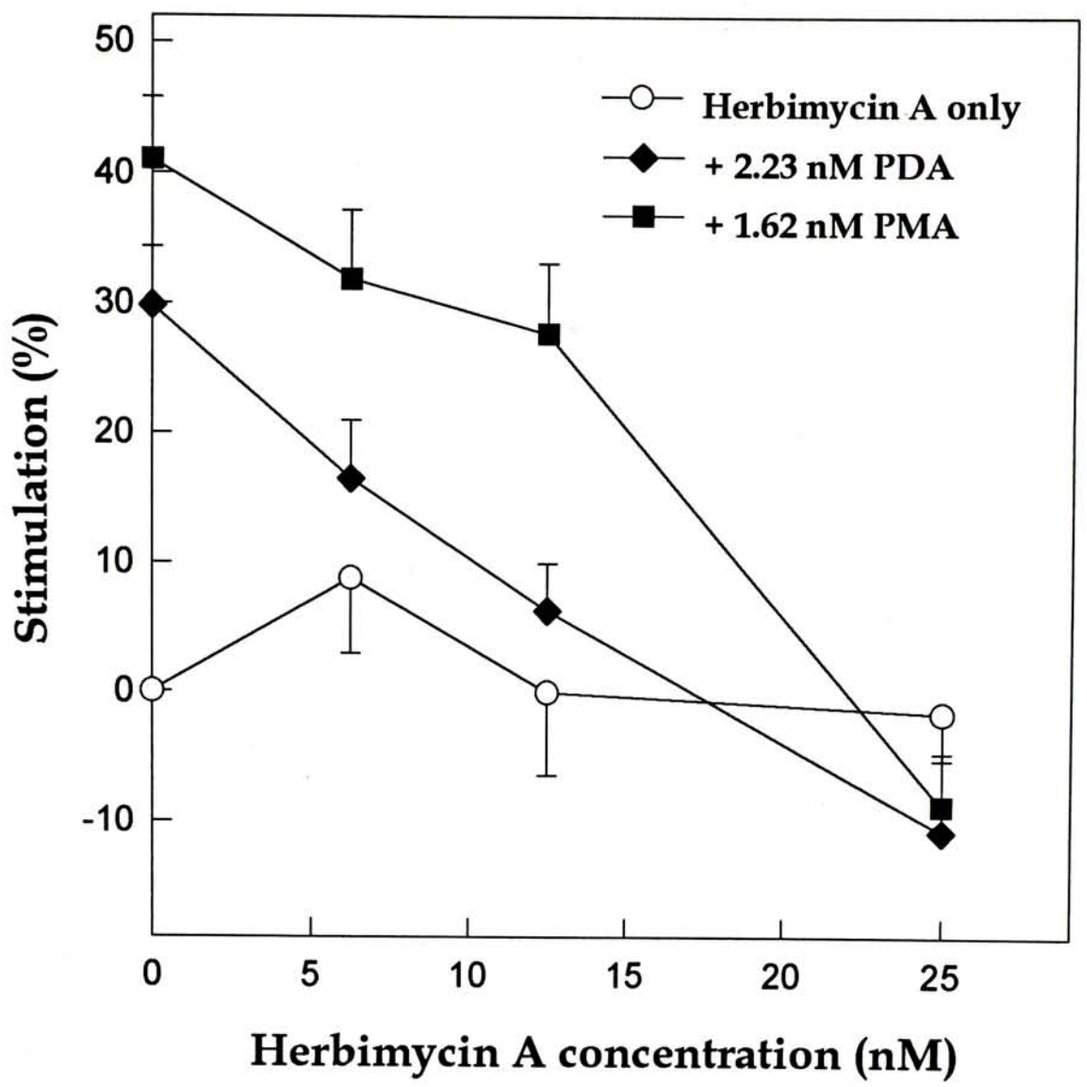


Fig. 3.19 Effects of various concentrations of herbimycin A on PMA- or PDA-induced C6 cell proliferation. C6 cells were cultured for 48 hours with various concentrations of herbimycin A, in the presence or absence of 1 ng/ml PMA or PDA. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. Estimated IC_{50}: PMA ≈ 15 nM, PDA ≈ 7 nM.

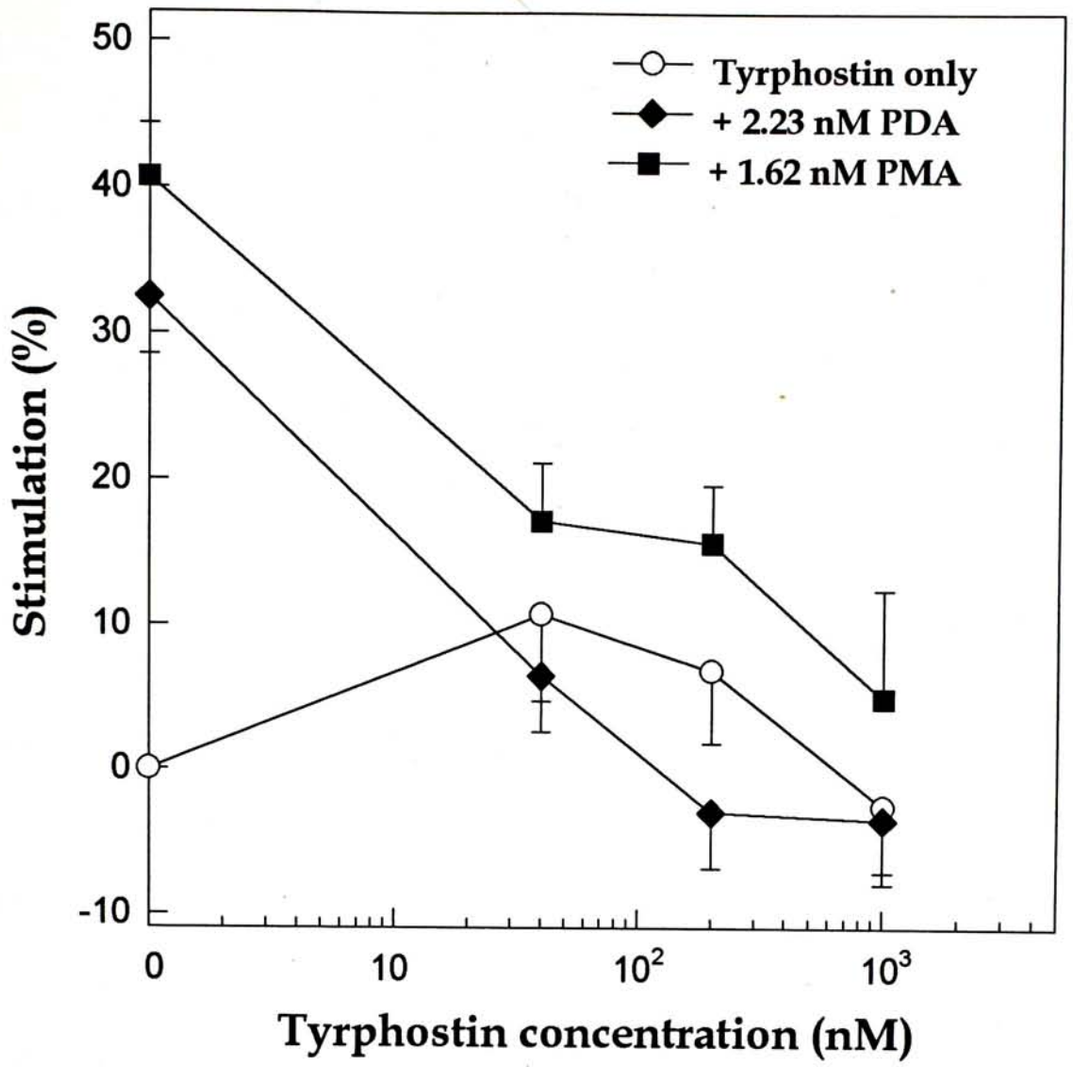


Fig. 3.20 Effects of various concentrations of tyrphostin on PMA- or PDA-induced C6 cell proliferation. C6 cells were cultured for 48 hours with various concentrations of tyrphostin, in the presence or absence of 1 ng/ml PMA or PDA. DNA synthesis was estimated as described before (Section 2.4). Values shown are mean ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: PMA ≈ 36 nM, PDA ≈ 26 nM.

3.3.3 The Involvement of Calcium Ions in Cytokine-induced C6 Cell Proliferation

As Ca^{2+} has been shown to mediate the action of a number of neurotransmitters and biologically active peptides (Frazer *et al.*, 1990; Meyer & Miller, 1990), the actions of A23187, a calcium ionophore, and three different calcium channel blockers, namely $LaCl_3$, verampil and nifedipine on cytokine-induced proliferation were tested.

Figure 3.21 showed the effect of A23187 on cytokine-induced C6 cell proliferation. A23187 alone was able to increase the proliferation of C6 cells, and maximum stimulation was observed with 20.7 nM. This stimulation was further enhanced by TNF-α, IL-1α, IL-1β and LIF. Among the cytokines studied, maximum stimulation (72 % increase in [^{3}H]-thymidine incorporation) was observed with 10 U/ml of TNF-α plus 20.7 nM A23187. In the presence of 83 nM of A23187, with or without cytokine, the proliferative effect decreased, and this may be due to the cytotoxic effect of this calcium ionophore at higher concentrations.

Since the increase in intracellular calcium ion concentration by A23187 stimulated proliferation in C6 cell, the effects of some Ca^{2+} channel blockers were then examined. The three Ca^{2+} channel blockers--$LaCl_3$, verampil and nifedipine, themselves did not affect [^{3}H]-thymidine incorporation significantly (Figs. 3.22-3.24); however,

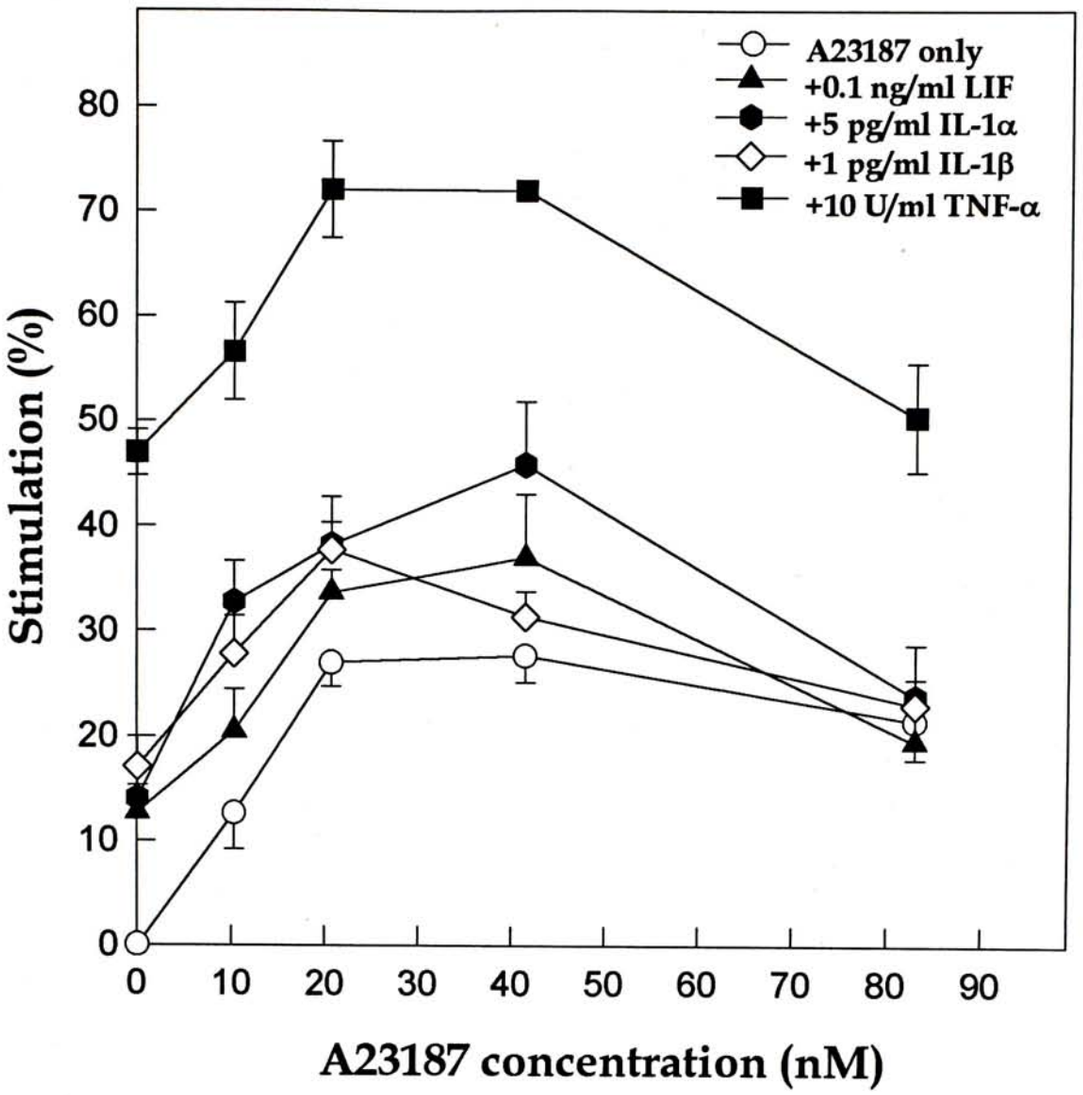


Fig. 3.21 Effects of A23187 and cytokine on C6 cell proliferation. C6 cells were cultured for 48 hours in control medium or in the presence of a cytokine plus various concentrations of A23187. DNA synthesis was estimated as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells.

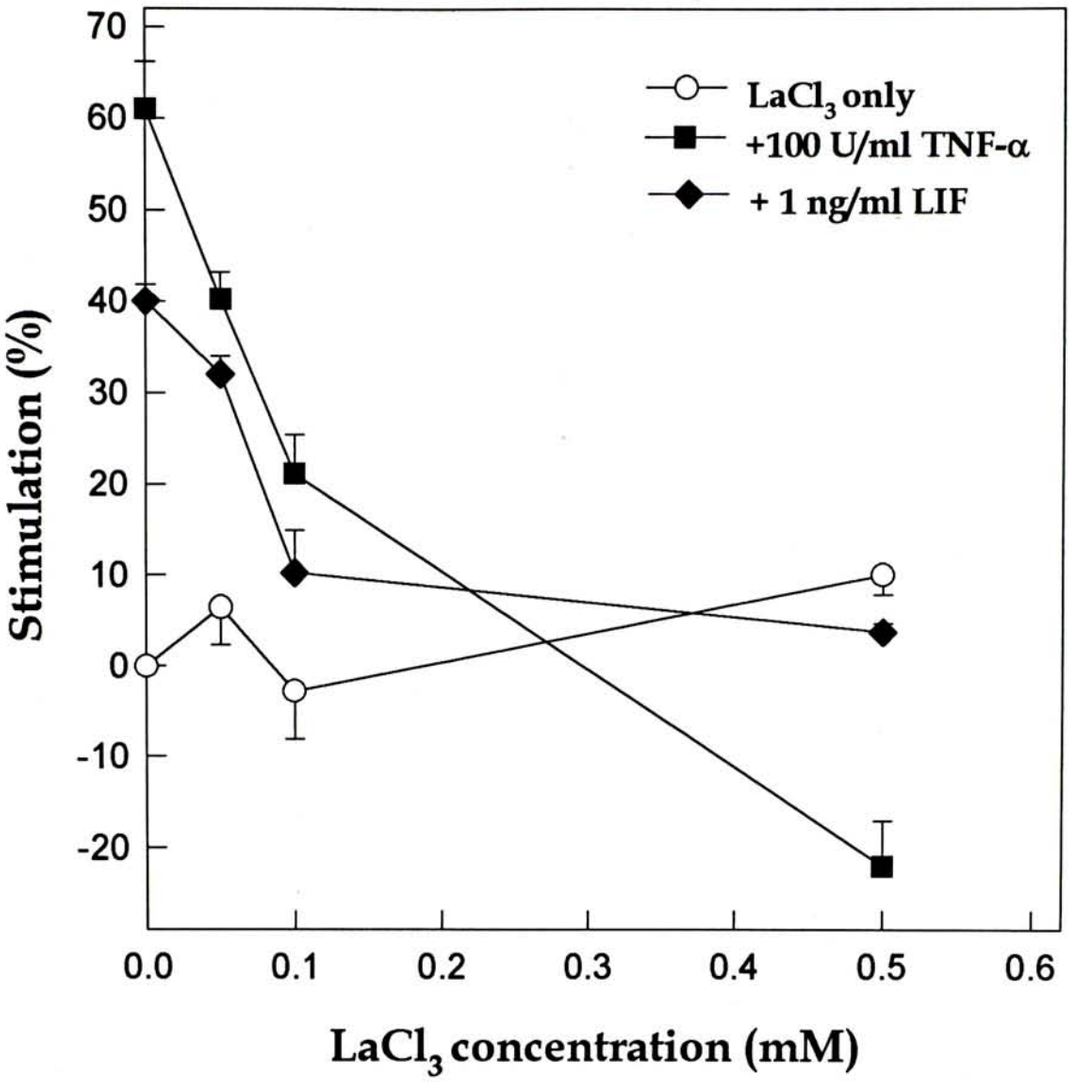


Fig. 3.22 Effect of $LaCl_3$ on cytokine-induced proliferation in C6 cells. Cells were cultured for 48 hours with or without TNF-α (100 U/ml) or LIF (1 ng/ml) and in the presence of various concentrations of $LaCl_3$. [^{3}H]-thymidine incorporation was estimated as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells. Estimated IC_{50}: TNF-α ≈ 0.06 mM, LIF ≈ 0.11 mM.

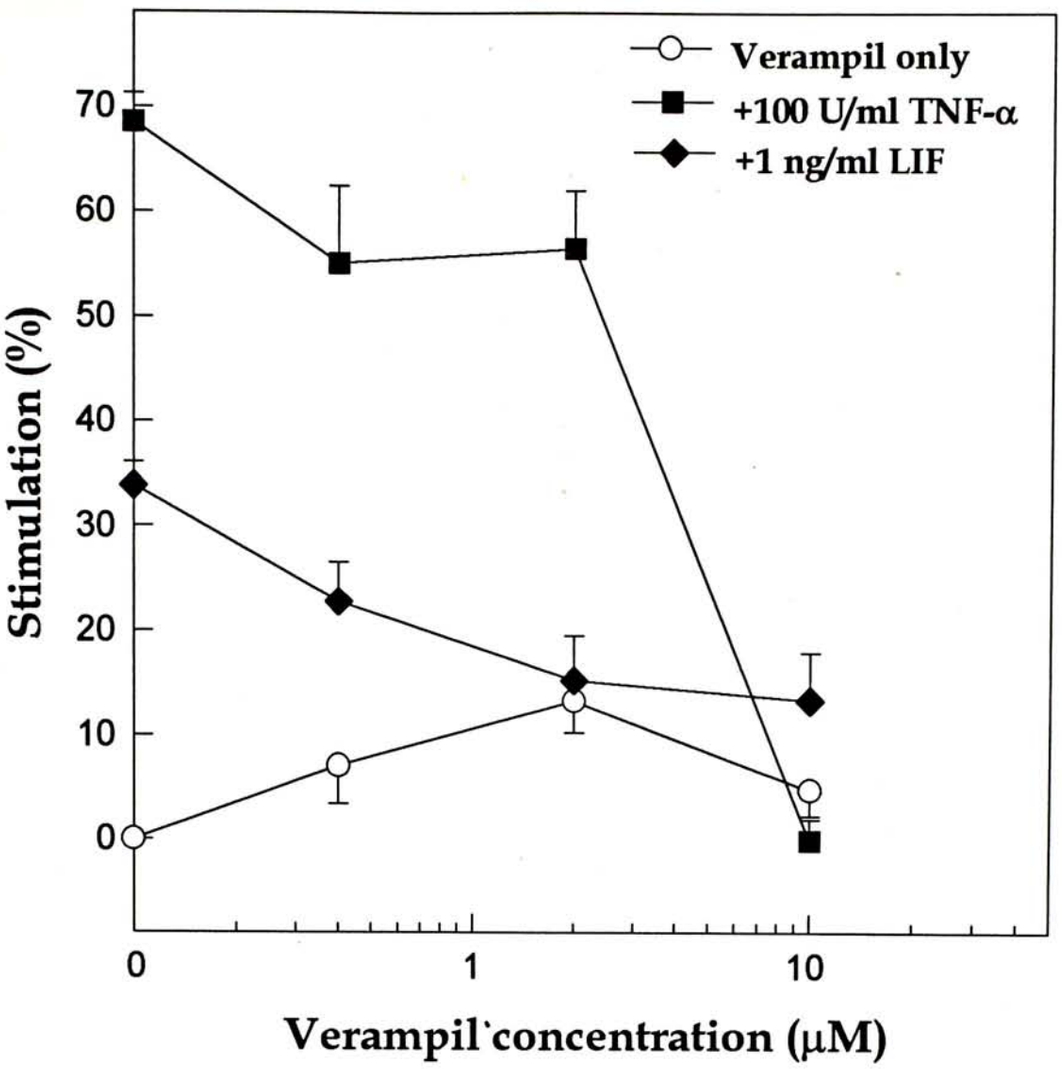


Fig. 3.23 Effect of verampil on cytokine-induced proliferation in C6 cells. Cells were cultured for 48 hours with or without TNF-α (100 U/ml) or LIF (1 ng/ml) and in the presence of various concentrations of verampil. [^{3}H]-thymidine incorporation was estimated as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: TNF-α ≈ 1.25 μM, LIF ≈ 5.25 μM.

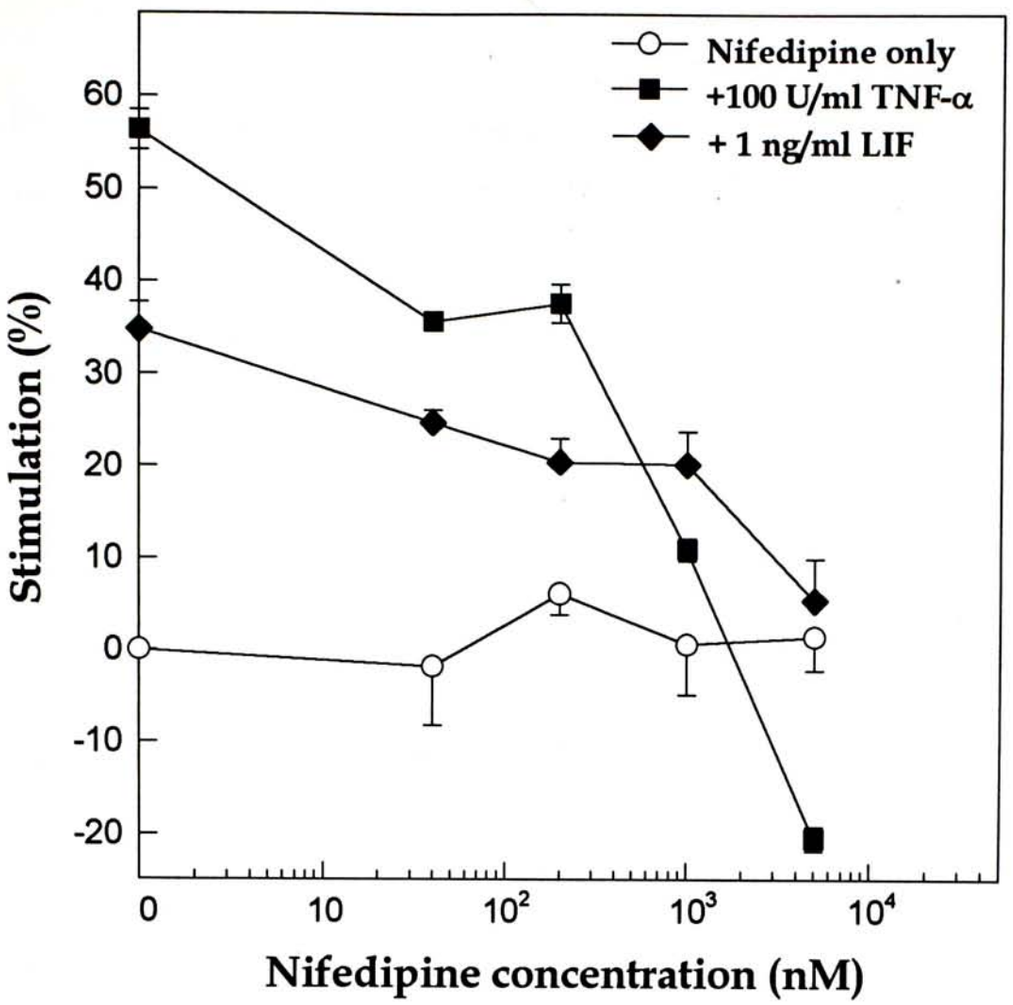


Fig. 3.24 Effect of nifedipine on cytokine-induced proliferation in C6 cells. Cells were cultured for 48 hours with or without TNF-α (100 U/ml) or LIF (1 ng/ml) and in the presence of various concentrations of nifedipine. [^{3}H]-thymidine incorporation was estimated as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: TNF-α ≈ 0.4 μM, LIF ≈ 1.8 μM.

they effectively reduced the TNF-α– and LIF-induced C6 cell proliferation in a dose-dependent manner (Figs. 3.22-3.24). TNF-α and LIF were chosen as the former produced the highest while the latter the least proliferative effect in the presence of A23187 (Fig. 3.21), although the maximum degree of inhibition varies with the compounds added. From the Figures 3.22, 3.23 and 3.24, it was clear that the stimulatory effect of TNF-α was more sensitive to these three channel blockers, and complete or almost complete inhibition was observed with the $LaCl_3$ (0.3 mM), verampil (10 μM) and nifedipine (5 μM). On the other hand, the stimulatory effect of LIF was less sensitive to these calcium channel blockers (Figs 3.22-3.24). To further prove that calcium ions are involved in cytokine-induced proliferation, the effect of EGTA, a potent calcium chelator, was studied. Figure 3.25 showed that EGTA, at several concentrations, had no significant effect on [^{3}H]-thymidine incorporation in C6 cells. However, the cytokine-induced proliferation was significantly inhibited by this calcium chelator. In agreement with the results obtained with calcium channel blockers, the TNF-α-induced proliferative effect was more sensitive to EGTA (Fig. 3.25).

3.3.4 The Involvement of Cyclic Nucleotides in Cytokine-induced C6 Cell Proliferation

Since the accumulation of cGMP in C6 cells can be regulated by cytokines (Simmons & Murphy, 1993; Vigne *et al.*, 1993), the effects of

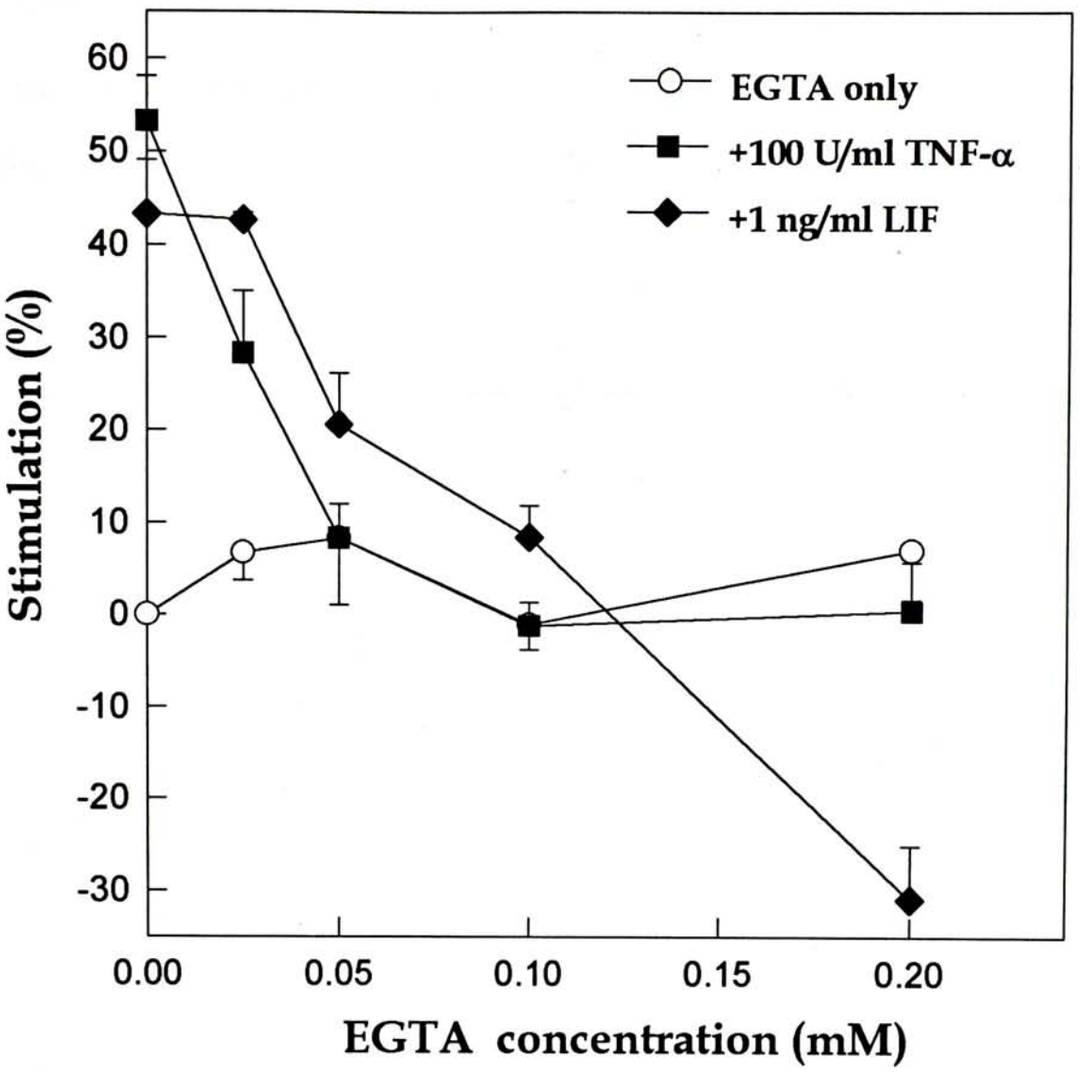


Fig. 3.25 Effect of EGTA on cytokine-induced proliferation in C6 cells. Cells were cultured for 48 hours with or without TNF-α (100 U/ml) or LIF (1 ng/ml) and in the presence of various concentrations of EGTA. [^{3}H]-thymidine incorporation was estimated as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells. Estimated IC_{50}: TNF-α ≈ 26 μM, LIF ≈ 48 μM.

dbcAMP and/or dbcGMP on C6 cell proliferation were investigated. Moreover, it is of particular interest to examine whether these two cyclic nucleotides mediate the proliferative effect of TNF-α or LPS on C6 cells.

Figure 3.26 showed that both dbcAMP and dbcGMP can increase C6 cell proliferation and maximum stimulation was observed with 1 mM of either cyclic nucleotide. The maximum stimulation observed with dbcAMP was 29 % which was slightly lower than that of dbcGMP (38 %). At 100 μM of dbcAMP, the incorporation of [^{3}H]-thymidine was significantly inhibited, while significant stimulation was observed with the same concentration of dbcGMP. The reason for such difference is unclear.

3.3.5 The Involvement of Nitric Oxide in Cytokine-induced C6 Cell proliferation

Figure 3.26 has clearly shown that cyclic nucleotides, especially cGMP, may be involved in the signalling pathway of cytokine-induced proliferation in C6 cells. Since cyclic GMP production is related to nitric oxide biosynthesis in C6 cells (Demerle-Pallardy *et al.*, 1993; Simmons & Murphy, 1993; Vigne *et al.*, 1993), it is of interest to study the effects of sodium nitroprusside (SNP), an exogenous NO donor, and N^G-methyl-L-arginine (NMA) and N^W-nitro-L-arginine methyl ester (NAME), both being nitric oxide synthase (NOS)

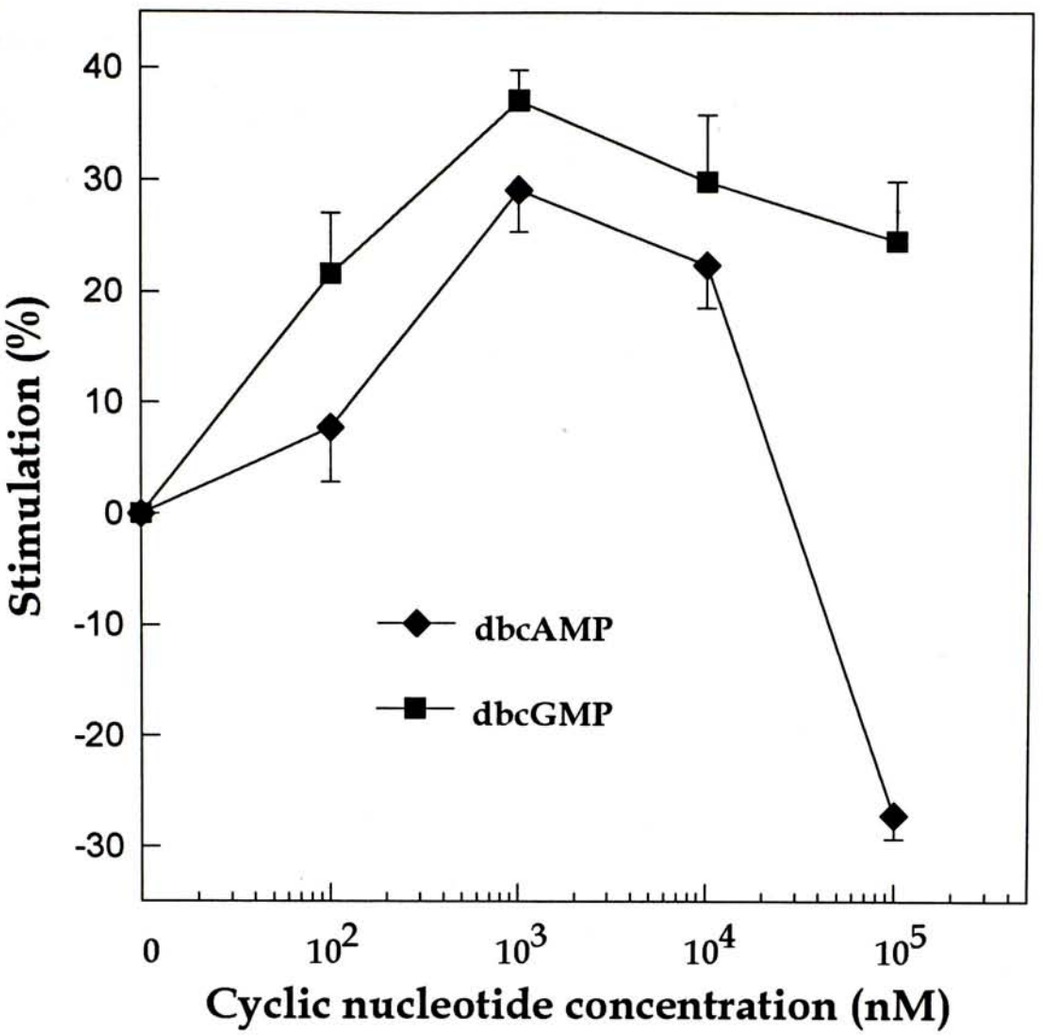


Fig. 3.26 The effect of cyclic nucleotides on C6 cell proliferation. C6 cells were cultured for 48 hours in medium or in the presence of indicated concentrations of dbcGMP or dbcAMP. DNA synthesis was estimated as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale.

inhibitors, on C6 cell proliferation. Contrary to expectation, SNP at concentrations of 10^{-7} - 10^{-4} M had no effect on [^{3}H]-thymidine incorporation, while higher concentrations were inhibitory (Fig. 3.27). On the other hand, dose-dependent inhibition of TNF-α– and LIF-induced proliferation by both NOS inhibitors--NMA and NAME was observed (Figs. 3.28 and 3.29). The proliferative effects of TNF-α and LIF were decreased to 15 % and 5 % respectively by 400 μM of NMA, and to -3 % and 1 % respectively by 500 μM of NAME. However, at these concentrations NMA or NAME alone exerted only a slight effect (< 10 % inhibition or stimulation) on C6 cell proliferation (Figs. 3.28-3.29).

To assess the effects of various cytokines on NOS activity in C6 cells, nitrite accumulation in the cell culture media was determined as described by Section 2.8.2. When C6 glioma cells were incubated with various cytokines, including 500 U/ml TNF-α, 5 ng/ml LIF and 1 ng/ml IL-1α or β, for three days, the increases in nitrite concentration were found to be 41 %, 38 % , 50 % and 51 % in the presence of TNF-α, LIF, IL-1α and IL-1β, respectively (Figure 3.30) when compared to that of the control cells.

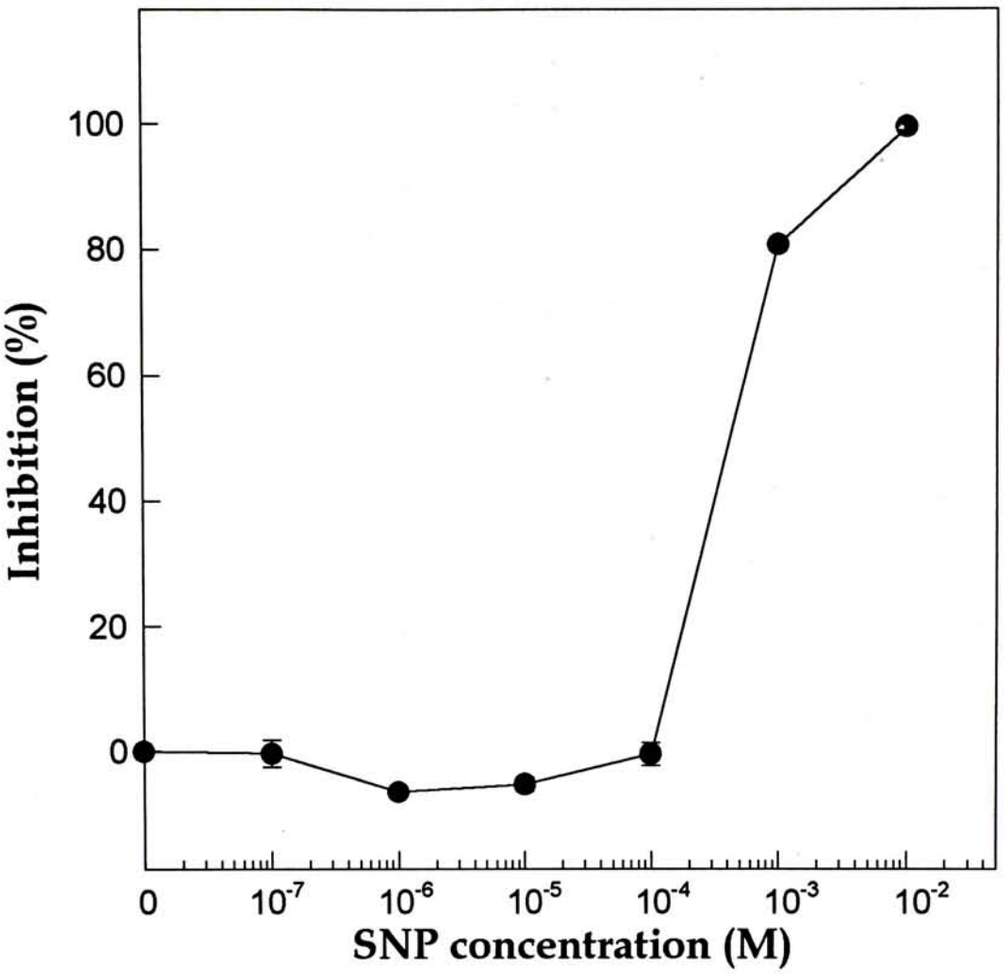


Fig. 3.27 The effect of SNP on C6 cell proliferation. C6 cells were cultured for 48 hours in presence or absence of various concentrations of SNP. [^{3}H]-thymidine incorporation was estimated as described before and % Inhibition = 100 × ($CPM_{control}$ - CPM_{sample})/$CPM_{control}$ (Section 2.4). The amount of [^{3}H]-thymidine incorporation was 60023 ± 889.84 cpm/well. Values shown are means ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale.

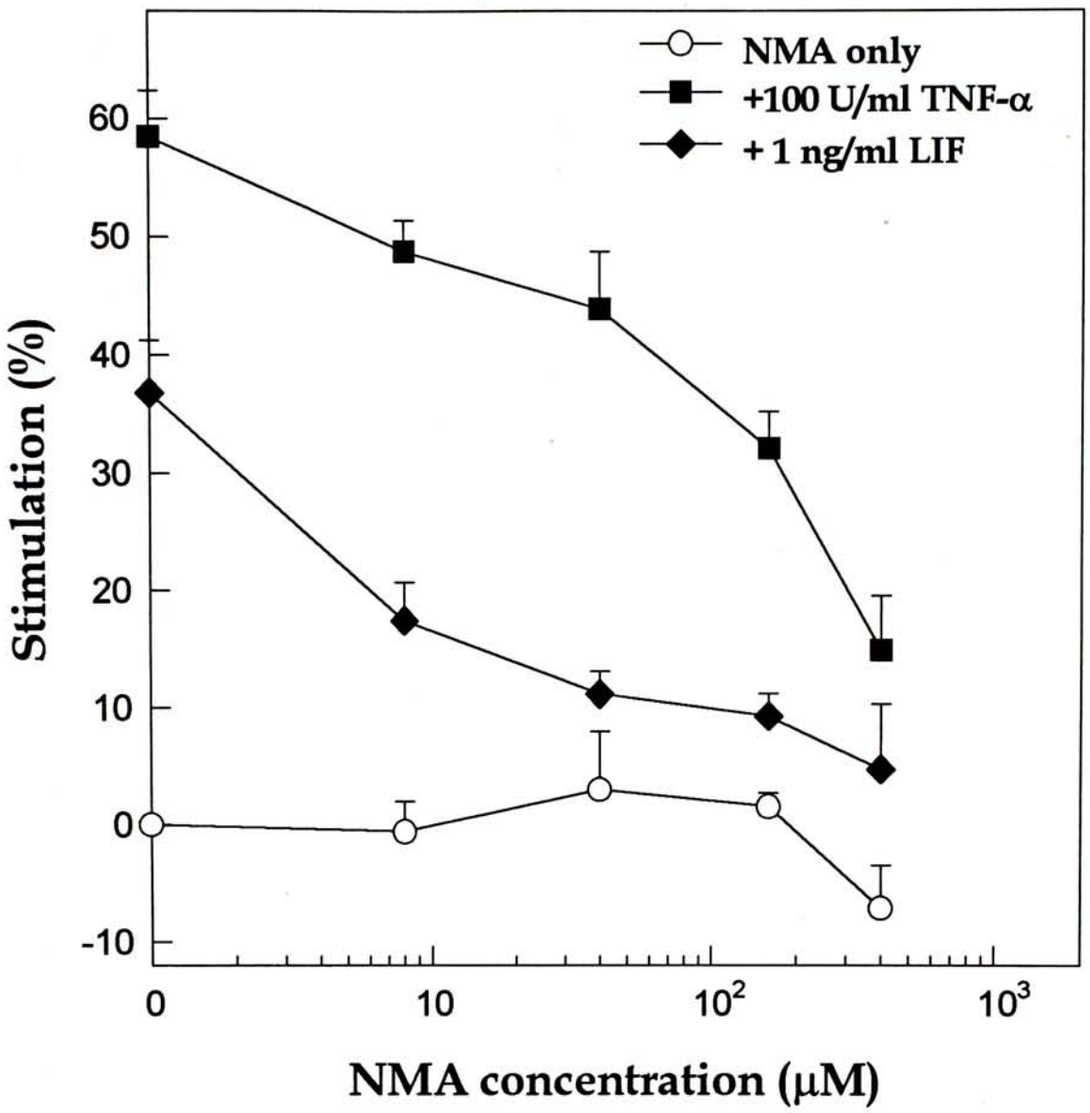


Fig. 3.28 Effect of NMA on cytokine-induced C6 cell proliferation. C6 cells were cultured for 48 hours in the presence or absence of the TNF-α (100 U/ml) or LIF (1 ng/ml), with or without NMA. [^{3}H]-thymidine incorporation was determined as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: TNF-α ≈ 208 μM, LIF ≈ 8 μM.

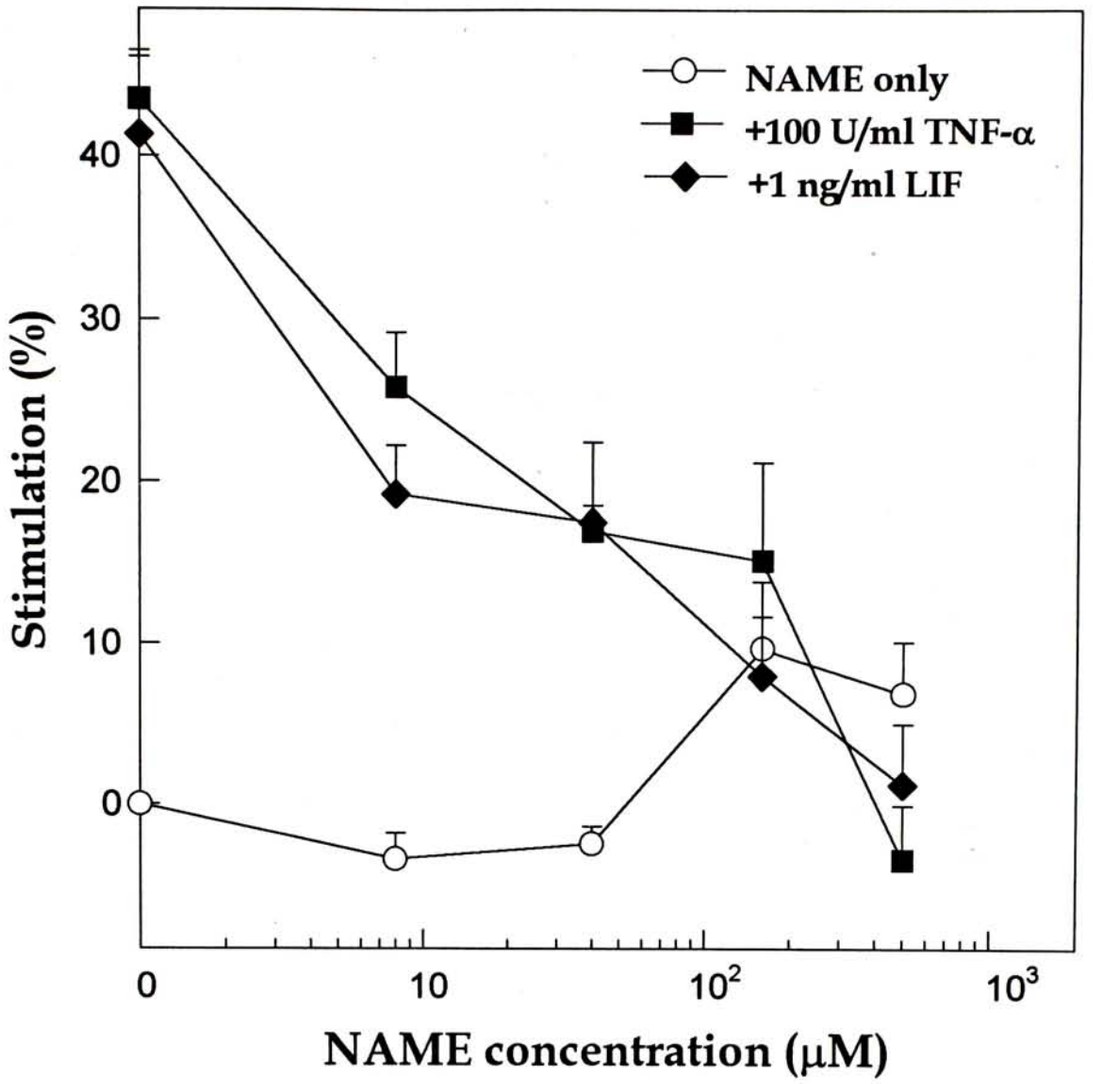


Fig. 3.29 Effect of NAME on cytokine-induced C6 cell proliferation. C6 cells were cultured for 48 hours in the presence or absence of the TNF-α (100 U/ml) or LIF (1 ng/ml), with or without NAME. [^{3}H]-thymidine incorporation was determined as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale. Estimated IC_{50}: TNF-α ≈ 15 μM, LIF ≈ 7 μM.

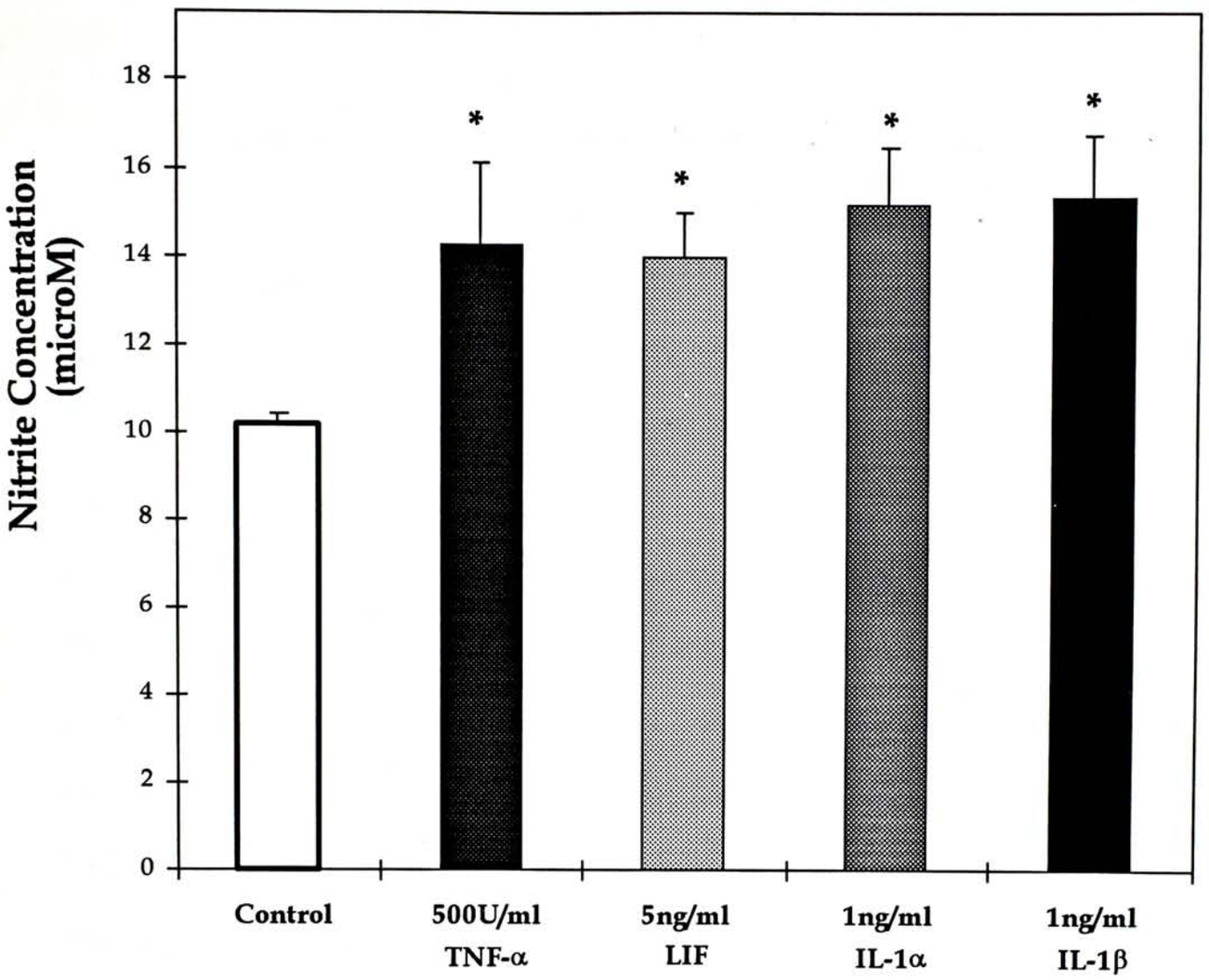


Fig. 3.30 Effects of various cytokines on nitrite production in C6 cells. C6 cells were incubated with the indicated cytokine for three days, and the nitrite formed in the culture media was determined using the Griess reagent as described in Section 2.8.2. Values shown are means ± standard error of quadruplicate wells. * $P < 0.05$ compared with control by Student's *t* test.

3.3.6 The Involvement of β-Adrenergic Receptor in Cytokine-induced C6 Cell Proliferation

Since astrocytes are known to express β-adrenergic receptors on their surfaces (Mantyh *et al.*, 1995), the effects of isoproterenol, a β-adrenergic agonist, and propranolol, an antagonist of β-adrenergic receptor, on the proliferation of C6 cells were also investigated. It was found that the addition of 1 μM of isoproterenol alone caused a 30 % increase in [^{3}H]-thymidine incorporation in C6 cells (Fig. 3.31). On the other hand, propranolol at concentrations varying from 0.4 to 25 μM had no significant effect on the [^{3}H]-thymidine incorporation (Fig. 3.31). The stimulatory effect of isoproterenol was not affected by concentrations of ≤ 10 μM of propranolol, but greatly reduced to below the control level with 25 μM of propranolol. Interestingly enough, 25 μM propranolol also greatly reduced the stimulatory effect of TNF-α-induced [^{3}H]-thymidine incorporation in C6 cells (Fig. 3.32). The mechanism involved, however, is unclear at present. Further experiments are needed to confirm the involvement of β-adrenergic receptor in cytokine-induced C6 cell proliferation.

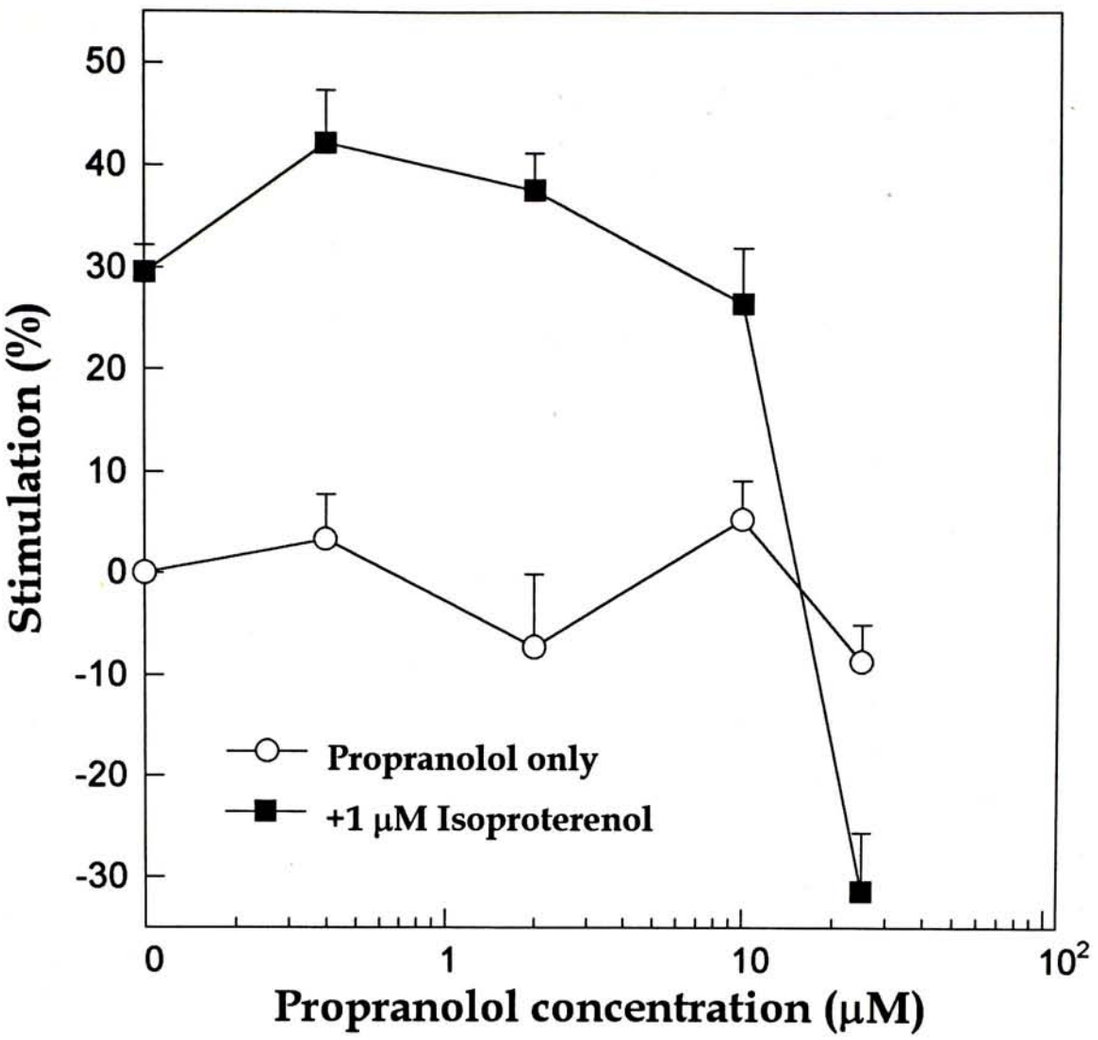


Fig. 3.31 Effects of isoproterenol and propranolol on C6 cell proliferation. C6 cells were cultured for 48 hours in presence or absence of 1 μM isoproterenol, plus various concentrations of propranolol. [^{3}H]-thymidine incorporation was estimated as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale.

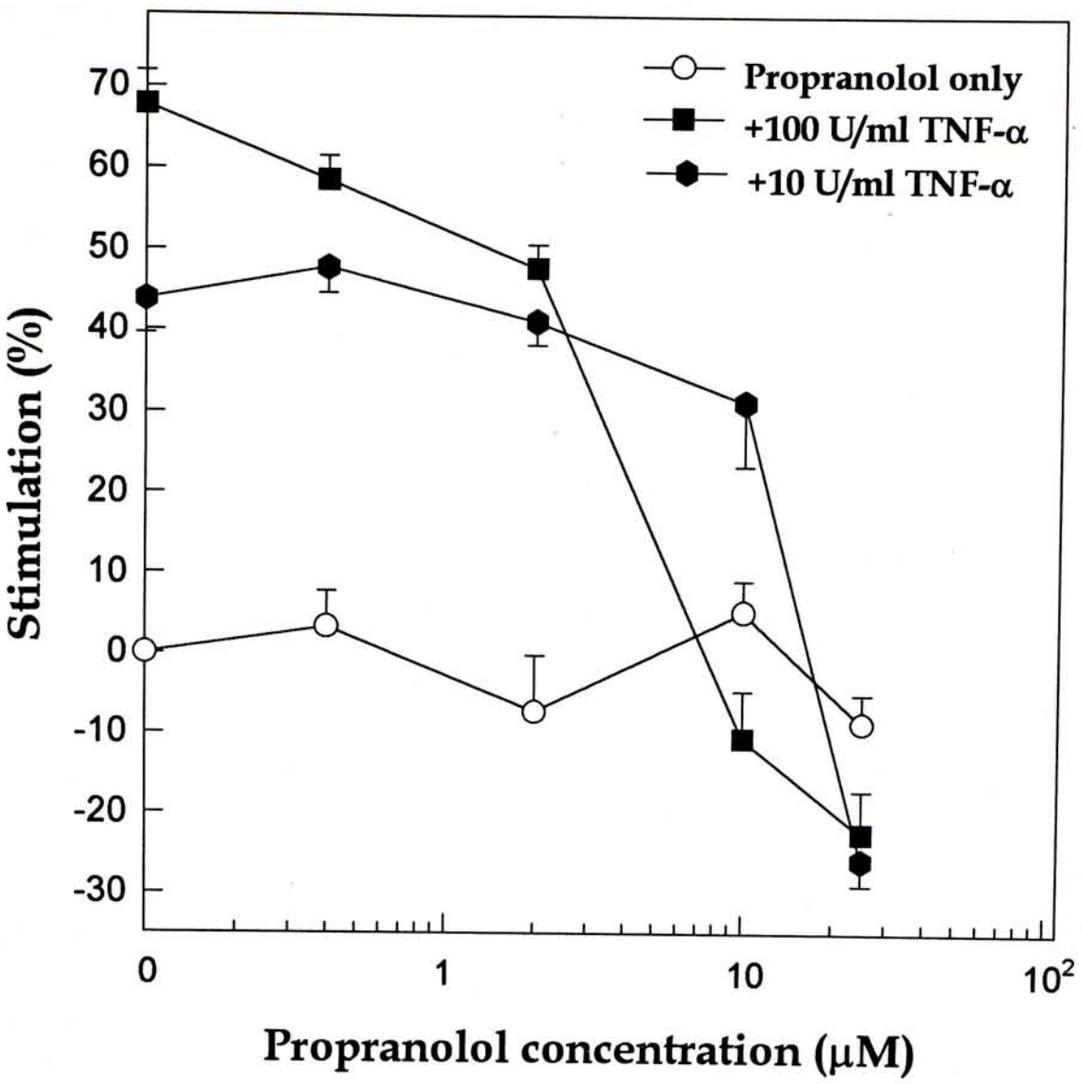


Fig. 3.32 The inhibition of TNF-α-induced C6 cell proliferation by propranolol. C6 cells were cultured for 48 hours in medium or in the presence of 10 or 100 U/ml TNF-α, plus various concentrations of propranolol. DNA synthesis was estimated as described before (Section 2.4). Values shown are means ± standard error of quadruplicate wells. The X-axis was represented in a logarithmic scale.

Chapter 4 DISCUSSION AND CONCLUSIONS

Results of the present study indicated that TNF-α, IL-1α, IL-1β and LIF stimulate [^{3}H]-thymidine incorporation in C6 cells, in a dose- and time-dependent manner. Not only was DNA synthesis increased but the growth of the C6 cells was also markedly enhanced by these cytokines. In addition, the present work demonstrated that LPS also stimulates proliferation of C6 cells (Fig. 3.5). LPS is known to be a potent cytokine inducer (Lee *et al.*, 1993b), thus its effect may be via the cytokine(s) produced. These findings are in agreement with reports that TNF-α and IL-1 stimulate proliferation of astrocytes, both *in vivo* and *in vitro* (Giulian & Lachman, 1985; Selmaj *et al.*, 1990), as well as in rat C6 glioma cells (Munoz-Fernandez *et al.*, 1991; Munoz-Fernandez & Fresno, 1993). The morphological studies showed that neither TNF-α nor LIF caused any significant changes in the cytokine-treated cells (Figs. 3.7-3.8). The observation with TNF-α is in agreement with that reported by Munoz-Fernandez *et al.* (1991). C6 cells are known to express both the astrocytic and oligodendritic phenotypes (Brodie & Vernadakis, 1991; Parker *et al.*, 1980; Vernadakis *et al.*, 1991). Our studies with GFAP antibody showed that over 90 % (Figs. 3.9) of the untreated C6 cell or C6 cells exposed to TNF-α for 48 hours were GFAP positive. Since GFAP is a marker protein for astrocytes (Eng *et al.*, 1971), the results suggest that the

cells used in the present study were mainly astrocytic. This finding may explain why TNF-α was mitogenic for astrocytes but caused damages to myelin and oligodendrocytes *in vitro* (Selmaj & Raine, 1988, Selmaj *et al.*, 1990). The proliferative effect observed suggest that cytokines, including TNF-α, LIF, IL-1α and IL-1β, could play an important role during nerve tissue regeneration by stimulating the proliferation of glial cells as an autocrine growth factor and inducing differentiation of neurons as a neurotrophic factor. Since glial cells play an important role in guiding developing neurons (Rakic, 1971a and b), it is possible that cytokines may influence neuron development.

Primary and murine astrocytes can secrete IL-6 in response to a variety of stimuli, including virus, IL-1, TNF-α, IFN-γ, LPS and calcium ionophore (Benveniste *et al.*, 1990; Frei *et al.*, 1989; Lieberman *et al.*, 1989; Norris *et al.*, 1994). Astrocytoma cells are known to express specific high-affinity receptors for IL-6 (Taga *et al.*, 1987), and that this cytokine has a mitogenic effect on astrocytes (Selmaj *et al.*, 1990). It is possible that the proliferative effect of TNF-α and other cytokines tested is mediated by the IL-6 produced upon stimulation with these cytokines. However, this possibility seems rather unlikely as no proliferative effect of IL-6 on C6 cells was observed. This notion is further supported by the observation that IL-6 was less potent that TNF-α and IFN-γ in requiring much higher doses of up to 1,000 U/ml to reach maximum stimulation of thymidine incorporation in glial

cells (Munoz-Fernandez & Fresno, 1993). Receptors for LIF have not been reported on C6 cells, thus it is not known if this cytokine exerted its action by binding to its, or related, receptors. In this connection, it is interesting to note that the LIF receptor has a significant sequence homology to the β subunit of IL-6 receptor (Gearing *et al.*, 1991). As TNF-α also induces the secretion of granulocyte-colony stimulating factor and granulocyte-macrophage colony stimulating factors in astrocytes (Malipiero *et al.*, 1990), the possible role of these two colony-stimulating factors in mediating the proliferative effect of TNF-α and other cytokines on C6 cells cannot be excluded from the present study and is currently under investigation in our laboratory.

The data showed that LIF was inhibitory to C6 cell proliferation at 10 ng/ml, while lower concentrations of LIF were found to be stimulatory (Fig. 3.2). This differential effect of cytokine on glial cell proliferation had been reported in studies with IL-4, which affected cell proliferation in a similar fashion (Brodie & Goldreich, 1994). The reason for the stimulatory and inhibitory effects of these cytokines in C6 cells is unclear at present. In view of the fact that multiple receptors of different affinities for various cytokines exist in other cells (Miyajima *et al.*, 1992), the biphasic effects of these cytokines may be due to the possibility that different cytokine concentrations affect interactions between the cytokine with its receptors of different affinities. This possibility is supported by binding studies which

showed the existence of high and low-affinity IL-4 receptors and the presence of both 55 kD and 75 kD TNF receptors in certain blood cells (Barna *et al.*, 1992; Foxwell *et al.*, 1989).

In the present study, it was found that the proliferative effects of TNF-α and LIF required a relatively short exposure time to these cytokines. It seems reasonable to speculate that receptors for these two cytokines already exist on the cell surface. More, since the proliferative effects of TNF-α and IL-1α or β were additive rather than synergistic (data not shown), it is possible that these cytokines caused proliferation by affecting a (some) common signalling pathway(s) in C6 glioma cells. This thought seems more likely than the action of different cytokines on common receptors as selective receptors for various cytokines had been demonstrated (Barna *et al.*, 1992; Benveniste *et al.*, 1990; Gearing *et al.*, 1991; and Selmaj *et al.*, 1990).

Our study demonstrated that both TNF-α- and LIF-induced proliferation in C6 cells involve protein kinase C (PKC) (Figs. 3.10-3.16), Ca^{2+} (Figs. 3.21-3.25) and tyrosine kinase (Figs. 3.17-3.20). The involvement of PKC mediating the action of TNF-α was demonstrated by observations that potent PKC inhibitors, Ro31-8220, staurosporine and calphostin C (Gescher, 1992), inhibited both the TNF-α- and LIF-stimulated proliferation in C6 cells (Fig. 3.11-3.13); whereas PKC activators, PMA and PDA, induced proliferation (Fig.

3.10). In addition, the proliferative effects of PMA and PDA were also blocked by the three PKC inhibitors tested (Figs. 3.14-3.16). This finding is supported by the recent reports that TNF-α and IL-1β activated PKC in cultured rat astrocytes (Norris *et al.*, 1994), and that astrocyte proliferation is regulated by PKC (Bhat, 1989; Honegger, 1986; Sawada *et al.*, 1993). However, our finding is different from that of Munoz-Fernandez & Fresno (1993), who reported that the proliferative effect of TNF-α and IFN-γ in C6 cells was unaffected by H7, the only PKC inhibitor tested in their study. The lack of effect of H7 in their report may be due to the different potencies of various PKC inhibitors on different PKC isozymes (Gescher, 1992). In fact, we have found that Ro31-8220, a cogener of staurosporine, reduced the PMA inhibitory action on C-type natriuetic peptide-induced cGMP formation in C6 cells, while H7 was ineffective (Tsang, D. *et al.*, unpublished observation).

Since some PKC isoforms are calcium-dependent (Berridge, 1993), the effects of A23187, a calcium ionophore, as well as some calcium channel blockers on cytokine-induced C6 cell proliferation were investigated. A23187 alone stimulated C6 cell proliferation (Fig. 3.21); and in the presence of a sub-optimal dose of cytokines, such as 10 U/ml of TNF-α, 0.1 ng/ml of LIF, 5 pg/ml of IL-1α and 1 pg/ml of IL-1β, A23187 further enhanced the proliferation induced by these cytokines. The involvement of calcium in mediating the proliferative effects of TNF-α and LIF is further supported by observations that

blockers of calcium influx--$LaCl_3$, verampil and nifedipine, inhibited the proliferative effects of these two cytokines in a concentration-dependent manner (Figs. 3.22-3.24). In addition, EGTA, a Ca^{2+} chelator, also has similar inhibitory action on the cytokine-induced C6 cell proliferation (Fig. 3.25). The finding that Ca^{2+} is involved in the proliferative action of cytokines agrees with reports that cytosolic calcium is a key messenger in controlling many cellular processes, including gene transcription and programmed cell death (Nicotera *et al.*, 1992; Petersen *et al.*, 1994). The mechanism by which Ca^{2+} stimulated proliferation in C6 cells is unclear at present. As certain neurotransmitters, e.g., glutamate, are known to exert their effects by affecting ion channels and/or transporters (Frazer *et al.*, 1990; Meyer & Miller, 1990), it is conceivable that TNF-α may affect Ca^{2+} fluxes in C6 cells. This reasoning is supported by our findings that the cytokine-induced proliferative effect was inhibited by some selective calcium channel blockers, $LaCl_3$, verampil and nifedipine (Figs. 3.22-3.24). Another possibility is due to the intrinsic channel-formation property of TNF-α. It has been shown that TNF-α can insert into the hydrocarbon core of phospholipid bilayers (Baldwin *et al.*, 1988). Moreover, TNF-α has been reported to form pH-dependent, voltage-dependent, ion-permeable channels in planar lipid bilayer membranes and increase the sodium permeability of human U937 histiocytic lymphoma cells (Kagan *et al.*, 1992). As A23187 is known to activate PKC, and that some PKC isoforms are calcium-dependent (Berridge, 1993), thus, it is quite possible that the action of Ca^{2+} is

mediated by activating PKC. However, this idea requires further testing with C6 cells. In view of the fact that the products produced upon the hydrolysis of phosphatidyl inositol 4, 5 bisphosphate by phospholipase C can activate PKC and mobilize calcium, the present findings do not allow us to discern whether calcium mobilization or activation of PKC is the primary signal transducing pathway mediating the proliferative effect of the cytokines studied. As A23187 has been shown to enhance phospholipase A_2 activity in pulmonary arterial smooth cells (Chakraborti *et al.*, 1991), and that IL-1β induced phospholipase A_2 activity in C6 cells, the involvement of phospholipase A_2 in mediating the proliferative effects of cytokines in C6 cells is certainly worthy of serious consideration. This study is currently under close examination in our laboratory.

Since tyrosine kinase is a well known second messenger in cytokines function (Stahl & Yancopoulos, 1993), its involvement in cell proliferation was also examined in this project. It was found that C6 cell proliferation induced by TNF-α and LIF was sensitive to the presence of the selective tyrosine kinase inhibitors, herbimycin A and tyrphostin (Bianchi *et al.*, 1995; Feinstein *et al.*, 1994a). The mechanism whereby TNF-α- and LIF-induced cell proliferation in C6 cells remains to be elucidated. In this connection it is interesting to note that the activity of nitric oxide synthase could be enhanced by LPS, or the combinations of IFN-γ, IL-1β and TNF-α, and this activation was mediated by the tyrosine kinase pathway(Feinstein *et al.*, 1994a and

b). In the present study, it was found that the two tyrosine kinase inhibitors were able to block the proliferative effect of PMA and PDA. This would suggest that in the cytokine-induced proliferation signal cascade the tyrosine kinase activation may be located down-stream to that of PKC activation.

As mentioned above, Feinstein *et al.* (1994a and b) reported that tyrosine kinase was involved in the expression of nitric oxide synthase in glial cells. Moreover, it was observed that high concentrations of N^G-monomethyl-L-arginine, an active nitric oxide synthase (NOS) inhibitor, but not N^G-monomethyl-D-arginine, a stereoisomer without NOS inhibitory activity, reduced the cytokine-induced growth of C6 cells (Munoz-Fernandez & Fresno, 1993). These observations suggest that nitric oxide may be involved in cytokine-induced proliferation of C6 cells. The present study demonstrated that the nitric oxide synthase inhibitors, N^G-methyl-L-arginine and N^W-nitro-L-arginine methyl ester (Knowles & Moncada, 1994), reduced the proliferative effect of TNF-α or LIF on C6 cells (Figs. 3.28 and 3.29), while neither inhibitors alone affected the [^{3}H]-thymidine incorporation of the cells. However, the participation of nitridergic pathway in mediating cytokine-induced proliferation in C6 cells seems to be less important as it was recently reported that LPS alone was not a sufficient inducer, but rather the presence of cytokines was needed to stimulate NOS expression (Feinstein *et al.*, 1994b). Moreover, the addition of TNF-α or IFN-γ alone, but without LPS,

had no effect on NOS activity in C6 cells (Feinstein *et al.*, 1994b). Furthermore, Simmons and Murphy (1993) found that in C6 cells LPS and cytokine increased the production of cGMP, one of the primary products related to the signal transduction of nitric oxide (Synder & Bredt, 1992) and this stimulatory action had a rigid dependency on the presence of L-arginine. Since no extra L-arginine (except the small amount present in the culture medium) was added in our experiments, this would suggest that the nitridergic pathway is less important in mediating the cytokine-induced proliferation in C6 cells. Even in the presence of added L-arginine, neither TNF-α alone nor a combination of IFN-γ and IL-1β caused any detectable increase in cGMP production in C6 cells until 4 hours after the addition of cytokines (Simmons and Murphy, 1993). In our study, however, significant proliferative effect of TNF-α was observed after 60 minutes exposure to TNF-α (Fig. 3.4) Moreover, the exogenous nitric oxide donor, sodium nitroprusside at non-cytotoxic concentrations, did not show any proliferative effect on C6 glioma cells (Fig. 3.27). Though the nitric oxide formation was induced by cytokines including TNF-α, LIF, IL-1α and IL-1β (Fig. 3.30), the increase was much lower than the reported data of Munoz-Fernandez & Fresno (1993). The reason for the discrepancy is unclear, but may be due to the higher concentrations of cytokines and longer time of incubation used in their proliferation assays. In short, it seems reasonable to conclude that the nitridergic pathway plays a necessary but less

important role compared to other transducing pathways, in mediating the proliferative effect of cytokines in C6 cells.

Recently, it was reported that cGMP production in glial cells was induced and regulated by cytokines (Simmons & Murphy, 1992 and 1993; Vigne *et al.*, 1993). These authors found that the cytokine-induced cGMP production had a rigid dependency on L-arginine, and was NO-dependent. However, we were unable to show the increase in cGMP production by TNF-α in several attempts (data not shown). The reason may be due to the lack of extra addition of L-arginine in our studies. On the other hand, in agreement with the reports of Simmons & Murphy (1992 and 1993), we found that cGMP as well as cAMP increased [^{3}H]-thymidine incorporation in C6 cells (Fig. 3.26). The mechanism through which cGMP increased [^{3}H]-thymidine incorporation in C6 cells remains unclear. It is possible that the action of cGMP may involve changes in calcium fluxes and phospholipases as it had been shown that cGMP could regulate calcium fluxes and induce the formation of inositol phosphates in astroglia (Furukawa *et al.*, 1986; Hirata *et al.*, 1990).

In summary, the present study showed that a number of cytokines, including TNF-α, LIF, IL-1α and IL-1β, stimulated [^{3}H]-thymidine incorporation in C6 glioma cells. The reason why so many cytokines can stimulate C6 cell proliferation is unclear, it may be that these cytokines after binding to their respective receptors trigger the

same signal transducers in C6 cells, and this would explain the redundant biological effects of cytokines (Meager, 1990). The proliferative effect of these cytokines on C6 cells suggests that cytokines produced by glial cells could play an important role in the nervous system in response to trauma and inflammation. Similar conclusions have been reached by other investigators in their studies with cultured astrocytes (Chung & Benveniste, 1990; Eddleston & Mucke, 1993; Kasahara *et al.* 1990). In addition, astrocytes are known to synthesize and secrete NGF and other neurotrophic factors which play an important role in the survival, growth and differentiation of neurons in the CNS (Furukawa *et al.*, 1986). Recent studies indicate that NGF synthesis can be regulated by a variety of growth factors and cytokines (Yoshida & Gage, 1992), and can be induced in reactive astrocytes during brain injury (Goedert *et al.*, 1986). Our finding that various proinflammatory cytokines can stimulate C6 cell proliferation indicates that these and possibly other cytokines may provide neurotrophic effect in the CNS during instances of injury and inflammatory events. In addition to these pathological implications, increasing evidence supports the ideas that cytokines coordinate migration and proliferation of glial cells, as well as regulate neuronal network formation during normal development of the nervous system (Merrill, 1992).

References

Aderem, A.A. (1993) How cytokines signal messages within cells. *J. Infect. Disease* **167**:S2-7

Aderka, D., Le, J. and Vilcek, J. (1989) IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *J. Immunol.* **143**:3517-3523.

Aggarwal, B.B. and Vilcek, J. (1992) Tumor necrosis factor: structure, function and mechanism of action. Marcel Dekker, New York, p89-98.

Akiyama, H. and McGeer, P.L. (1990) Brain microglia constitutively express beta-2-integrins. *J. Neuroimmunol.* **30**:81-93.

Akiyama, T., Ishida, J., Nakagawa, S., Ogaware, H., Watanable, S., Itoh, N., Shibuy, M. and Fukami, Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J.Biol. Chem.* **262**:5592-5595.

Alheim, K., Andersson, C., Tingsborg, S., Ziolkowska, M., Schultzberg, M. and Bartfai, T. (1991) Interleukin-1 expression is inducible by nerve growth factor in PC12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. (USA)* **88**:9302-9306.

Arai, K., Lee, F. Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. (1990) Cytokines: Coordinators of immune and inflammatory responses. *Annu. Rev. Biochem.* **59**:783-836.

Baldwin, R.L., Chang, M.P., Bramhall, J., Graves, S., Bonavida, B., Wisnieski, B.J. (1988) Capacity of tumor necrosis factor to bind and penetrate membranes is pH-dependent. *J. Immunol.* **141**:2352-2357.

Bandtlow, C.E., Meyer, M., Lindholm, D., Spranger, M., Heumann, N.R. and Thoenen, H. (1990) Regional and cellular codistribution of interleukin 1β and nerve growth factor mRNA in the adult rat brain:

possible relationship to the regulation of nerve growth factor synthesis. *J. Cell. Biol.* **111**:1701-1711.

Barna, B.P., Barnett, G.H., Jacobs, B.S. and Estes, M.L. (1992) Divergent responses of human astrocytoma and non-neoplastic astrocytes to tumor necrosis factor alpha involve the 55 kDa tumor necrosis factor receptor. *J. Neuroimmunol.* **43**:185-190.

Bartfai, T. and Schultzberg, M. (1993) Cytokines in neuronal cell types. *Neurochem. Int.* **22**:435-444.

Bell, R.M. and Burn, D.J. (1991) Lipid activation of protein kinase C. *J. Biol. Chem.* **266**:4661-4664.

Benda, P., Lightbody, J., Sato, G., Levine, L. and Sweet, W. (1968) Differentiated rat glial cell strain in tissue culture. *Science* **161**:370-371.

Benveniste, E.N. (1992a) Inflammatory cytokines within the central nervous system: sources, function and mechanism of action. *Am. J. Physiol.* **263**:C1-C6.

Benveniste, E.N. (1992b) Cytokines: Influence on glial cell gene expression and function. In: *"Chem. Immunol. Vol 52, Blalock, J.E. (ed.)"* Karger Pub. Ltd., Basel, p106-153.

Benveniste, E.N., Sparacio, S.M. and Bathea, J.R. (1989) Tumor necrosis factor-α enhances interferon-γ mediated class II antigen expression on astrocytes. *J. Neuroimmunol.* **25**:209-219.

Benveniste, E.N., Sparacio, S.M., Norris, J.G., Grenett, H.E. and Fuller, G.M. (1990) Induction and regulation of interleukin-6 gene expression in astrocytes. *J. Neuroimmunol.* **30**:201-212.

Benveniste, E.N., Vidovic, M., Panek, R.B., Norris, J.G., Reddy, A.T. and Benos, D.J. (1991) Interferon-γ-induced astrocytes class II major histocompatibility complex gene expression is associated with both protein kinase C activation and Na^+ entry. *J. Biol. Chem.* **266**:18119-18126.

Berkenbosch, F., van Oers, J., Delrey, A., Tilders, F. and Besedovsky, H. (1987) Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1. *Science* **238**:524-526.

Berridge, M.J. (1993) Inositol trisphosphate and calcium signalling. *Nature* **361**:315-325.

Bethea, J.R., Chung, I.Y., Sparacio, S.M., Gillespie, G.Y. and Benveniste, E.N. (1991) Interleukin-1 beta induction tumor necrosis factor-alpha gene expression in human astroglioma cells. *J. Neuroimmunol.* **36**:179-191.

Bethea, J.R., Gillespie, G.Y., Chung, I.Y. and Benveniste, E.N. (1990) Tumor necrosis factor production and receptor expression by human astrocytoma cell line. *J. Neuroimmunol.* **30**:1-13.

Beutler, B. and Cerami, A. (1989) The biology of cachectin/TNF-α primary mediator of the host response. *Annu. Rev. Immunol.* **7**:625-655.

Beutler, B. (1992) Tumor necrosis factors: the molecules and their emerging role in medicine, Raven Press, New York, p178-185.

Bhat, N.R. (1989) Role of protein kinase C in glial cell proliferation. *J. Neurosci. Res.* **22**:20-27.

Bianchi, L., Arcangeli, A, Bartolini, P., Mugnal, G., Wanke, E. and Olivotto, M. (1995) An inward rectifier K^+ current modulated in neuroblastoma cells the tyrosine phosphorylation of the pp125 FAK and associated proteins: role in neuritogenesis. *Biochem. Biophys. Res. Commun.* **210**:823-829.

Blalock, J.E. (1994) The immune system. Our sixth sense. *The Immunologist* **2**:8-15.

Booss, J., Esiri, M.M., Tourtelotte, W.W. and Mason, D.Y. (1983) Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. *J. Neurol. Sci.* **62**:219-232.

Brett, J., Gerlach, H., Nawroth, P., Steinberg, S., Godman, G. and Stern, D. (1989) Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. *J. Exp. Med.* **169**:1977-1991.

Brodie, C. and Goldreich, N. (1994) Interleukin-4 modulates the proliferation and differentiation of glial cells. *J. Neuroimmunol.* **55**:91-97.

Brodie, C. and Vernadakis, A. (1991) Muscle derived factors induce proliferation and astrocytic expression in C6 glial cells. *Glial* **4**:269-275.

Burstein, S.A., Mei, R.-L., Henthorn, J., Friese, P. and Turner, K. (1992) Leukemia inhibitory factor and interleukin-11 promote maturation of murine and human megakaryocytes *in vitro*. *J. Cell. Physiol.* **153**:305-312.

Cammer, W., Tansey, F., Abramovitz, M., Ishigaki, S. and Listowsky, I. (1989) Differential localization of glutathione-S-transferase Yp and Yb subunits in oligodendrocytes and astrocytes of rat brain. *J. Neurochem.* **52**:876-883.

Cataldi, A., Miscia, S, Lisio, R., Rana, R. and Cocco, L. (1990) Transient shift of diacylglycerol and inositol lipids induced by interferon in Daudi cells. Evidence for a different pattern between nuclei and intact cells. *FEBS Lett.* **269**:465-468.

Chakraborti, S., Micheal, J.R. and Patra, S.K. (1991) Protein kinase C dependent and independent activation of phospholipase A_2 under calcium ionophore (A23187) exposure in rabbit pulmonary arterial smooth muscle cells. *FEBS Lett.* **285**:104-107.

Charnay, Y., Paulin, C., Chayvialle, J.-A. and Dubois, P.M. (1983) Distribution of substance P like immunoreactivity in the spinal cord and dorsal root ganglia of the human fetus and infant. *Neuroscience* **10**:41-55.

Cheema, S.S., Richards, L., Murphy, M. and Bartlett, P.F. (1994) Leukemia inhibitory factor prevents the death of axotomised sensory

neurons in the dorsal root ganglia of the neonatal rat. *J. Neurosci. Res.* **37**:213-218.

Chung, I.Y. and Benveniste, E.N. (1990) Tumor necrosis factor-alpha production by astrocytes: Induction by lipopolysaccharide, interferon-gamma and interleukin-1. *J. Immunol.* **144**:2999-3007.

Clemens, M.J. (1991) Introduction to cytokines. In: *"Cytokines"*. Bios Scientific Publisher Chapter 1, Oxford, p1-18.

Conquet, F. and Brulet, P. (1990) Development expression of myeloid leukemia inhibitory factor gene in preimplantation blastocysts and in extraembryonic tissue of mouse embryos. *Mol. Cell. Biol.* **10**:3801-3805.

Couldwell, W.T., Antel, J.P. and Young, V.W. (1992) Protein kinase C activity correlates with the growth rate of malignant gliomas: Part II. effects of glioma mitogens and modulators of protein kinase C. *Neurosurgery* **31**:717-724.

Couldwell, W.T., Hinton, D.R., He, S., Chen, T.C., Sebat, I., Weiss, M.H. and Law, R.E. (1994) Protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines. *FEBS Lett.* **345**:43-46.

Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U. and Ullrich, A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. *Science* **233**:859-866.

Davis, P.D., Elliott, L.H., Harris, W., Hill, C.H., Hurst, S.A., Keech, E., Kumar, M.K., Lawton, G., Nixon, J. S. and Wilkinson, S.E. (1992) Inhibitors of protein kinase C. 2. substituted bisindolylmaleimides with improved potency and selectivity. *J. Med. Chem.* **35**:994-1001.

de Giovine, F.S. and Duff, G.W. (1990) Interleukin-1: the first interleukin. *Immunol. Today* **11**:13-14.

de Vellis, J., Schjeide, O.A., Clemente, C.D. (1967) Protein synthesis and enzymatic patterns in the developing brain following head X-irradiation of newborn rats. *J. Neurochem.* **14**:499-511.

Demaine, A.G. (1989) The molecular biology of autoimmune disease. *Immunol. Today* **10**:357-361.

Demerle-Pallardy, C., Lonchampt, M.-O., Chabrier, P.-E. (1993) Nitric oxide synthase induction in glial cells: effect on neuronal survival. *Life Sci.* **52**:1883-1890.

Dermietzel, R. (1974) Junctions in the central nervous system of the cat. I. Membrane fusion in central myelin. *Cell Tissue Res.* **149**:121-135.

Dinarello, C.A., Cannon J.G., Wolf, S.M., Bernheim, H.A., Beutler, B., Cerami, A., Figari, I.S., Palladino, M.A. Jr. and O'Connor, J.V. (1986) Tumor necrosis factor is an endogenous pyrogen and induces production of interleukin-1. *J. Exp. Med.* **163**:433-1450.

Drummond, G.E., Iyer, N.T., Keith, J. (1962) Hydrolysis of ribonucleoside 2',3'-cyclic phosphates by a diesterase from brain. *J. Biol. Chem.* **237**:3535-3539.

Dustin, M.L., Rothlein, T., Bhan, A.K., Dinarello, C.A. and Springer, T.A. (1986) Induction by IL-1 and interferon-γ: Tissue distribution, biochemistry, and function of natural adherence molecule (ICAM-1). *J. Immunol.* **137**:1270-1274.

Eddleston, M. and Mucke, L. (1993) Molecular profile of reactive astrocytes -- implications for their role in neurologic disease. *Neurosci.* **54**:15-36.

Eiseman, E. and Bolen, J.B. (1990) Src-related tyrosine kinases as signaling components in hematopoietic cells. *Cancer Cells* **2**:303-310.

Eng, L.F., Venderhaeghen, J.J., Bignami, A. and Gerstl, B. (1971) An acidic protein isolated from fibrous astrocytes. *Brain Res.* **28**:351-354.

Escary, J.L., Perreau, J., Dumenil, D., Enzine, S. and Brulet, P. (1993) Leukemia inhibitory factor is necessary for maintenance of hematopoietic stem cells and thymocyte stimulation. *Nature* **363**:361-364.

Fan, G. and Katz, D.M. (1993) Non-neuronal cells inhibit catecholaminergic differentiation of primary sensory neurons: role of leukemia inhibitory factor. *Development* **118**:83-93.

Farrar, W.L., Kilian, P.L., Ruff, M.R., Hill, J.M. and Pert, C.B. (1987) Visualization of interleukin 1 receptors in brain. *J. Immunol.* **139**:459-463.

Feinstein, D.L., Galea, E., Cermak, J., Chugh, P., Lyandvert, L. and Reis, D.J. (1994a) Nitric oxide synthase expression in glial cells: suppression by tyrosine kinase inhibitors. *J. Neurochem.* **62**:811-814.

Feinstein, D.L., Galea, E., Roberts, S., Berquist, H., Wang, H. and Reis, D.J. (1994b) Induction of nitric oxide synthase in rat C6 glioma cells. *J. Neurochem.* **62**:315-321.

Fierz, W., Endler, B., Reske, K., Wekerle, H. and Frontana, A. (1985) Astrocytes as antigen presenting cells. I. Induction of Ia antigen expression on astrocytes by T cells via immune interferon and its effect on antigen presentation. *J. Immunol.* **134**:3785-3793.

Fontana, A., Fierz, W. and Wekerle, H. (1984) Astrocytes present myelin basic protein to encephalitogenic T-cell lines. *Nature* **307**:273-276.

Fontana, A., Kristensen, F., Dubs, R., Gemsa, D. and Weber, E. (1982) Production of prostaglandin E and an interleukin-1-like factor by cultured astrocytes and C6 glioma cells. *J. Immunol.* **129**:2413-2419.

Foxwell, B.M.J., Woerly, G. and Ryffel, B. (1989) Identification of interleukin-4 receptor-associated proteins and expression of both high- and low-affinity binding on human lymphoid cells. *Eur. J. Immunol.* **19**:1637-1643.

Frazer, A., Maayani, S. and Wolfe, B.B. (1990) Subtypes of receptors for serotonin. *Annu. Rev. Pharmacol. Toxicol.* **30**:307-348.

Frei, K., Malipiero, U.V., Leist, T.P., Zinkernagel, R.M., Schwab, M.E. and Fontana, A. (1989) On the cellular source and function of interleukin-6 produced in the central nervous system in viral diseases. *Eur. J. Immunol.* **19**:689-694.

Frei, K, Siepl, C., Groscurth, P., Bodmer, S., Schwerdel, C. and Fontana, A. (1987) Antigen presentation and tumour cytotoxicity by interferon-γ-related microglial cells. Eur. *J. Immunol.* **17**:1271-1278.

Freshney, R.I. (1987) The Transformed Phenotype. In: "*Culture of animal cells: A manual of basic technique (2nd ed.)*", Alan R.Liss Inc., Chapter 15, New York, p197-206.

Frohman, E.M., Frohman, T.C., Duatin, M.L., Veyuvegula, B., Choi, B., Gupta, A., van den Noort, S. and Gupta, S. (1989a) The induction of intercellular adhesion molecule 1 (ICAM-1) expression on human fetal astrocytes by interferon-γ, tumour necrosis factor-α, lymphotoxin, and interleukin-1: relevance to intracerebral antigen presentation. *J. Neuroimmunol.* **23**:117-124.

Frohman, E.M., van den Noort, S. and Gupta, S. (1989b) Astrocytes and intracerebral immune responses. *J. Clin. Immunol.* **9**:1-9.

Furukawa, K.I., Ohshima, N., Tawada-Iwata, Y. and Shigekawa M. (1991) Cyclic GMP stimulates Na^+/Ca^{2+} exchange in vascular smooth muscle cells in primary culture. *J. Biol. Chem.* **266**:12337-12341.

Furukawa, S., Furukawa, Y., Satoyoshi, E. and Hayashi, K. (1986) Synthesis/secretion of nerve growth factor by mouse astroglial cells in culture. *Biochem. Biophys. Res. Commun.* **136**:57-63.

Galea, E., Feinstein, D.L. and Reis, D. J. (1992) Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures. *Proc. Natl. Acad. Sci. (USA)* **89**:10945-10949.

Gazit, A., Yaish, P., Gilon, C. and Levitzki, A. (1989) Tyrphostins I: synthesis and biological activity of protein tyrosine kinase inhibitors. *J. Med. Chem.* **32**:2344-2352.

Gearing, D.P., Thut, C.J., vanden Bos, T., Gimpel, S.D., Delaney, P.B., King, J., Price, V., Cosman, D. and Beckmann, M.P. (1991) Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp 130. *EMBO J.* **10**:2839-2848.

Gescher, A. (1992) Towards selective pharmacological modulation of protein kinase C -- opportunities for the development of novel antineoplastic agents. *Brit. J. Cancer* **66**:10-19.

Giulian, D (1984) Peptides from the regenerating central nervous system of the goldfish stimulate glia. *Proc. Natl. Acad. Sci. (USA)* **81**:3567-3571.

Giulian D. and Baker, T.J. (1985) Peptides released by ameboid microglia regulate astroglial proliferation. *J. Cell Biol.* **104**:2411-2418.

Giulian, D. and Baker, T.J. (1986) Characterization of ameboid microglia isolated from developing mammalian brain. *J.Neurosci.* **6**:2163-2178.

Giulian, D., Baker, T.J., Shih, L.-C.N. and Lachman, L. (1986) Interleukin-1 of the central nervous system is produced by ameboid microglia. *J. Exp. Med.* **164**:594-604.

Giulian, D. and Lachman, L.B. (1985) Interleukin-1 stimulation of astroglial proliferation after brain injury. *Science* **228**:497-499.

Giulian, D., Woodward, J., Young, D.G., Krebs, J.F. and Lachman, L.B. (1988a) Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. *J. Neurosci.* **8**:2485-2490.

Giulian, D., Young, D.G., Woodward, J., Brown, D.C. and Lachman, L.B. (1988b) Interleukin-1 is an astroglial growth factor in developing brain. *J. Neurosci.* **8**:709-714.

Goedert, M., Fine, A., Hunt, S.P. and Ulrich, A. (1986) Nerve growth factor mRNA in peripheral and central rat tissue and in the human central nervous system: Lesion effects in the rat brain and levels in Alzheimer's disease. *Mol. Brain Res.* **1**:85-92.

Gordon S. and Hirsch, S. (1981) Differentiation antigens and macrophage heterogeneity. *Adv. Exp. Med. Biol.* **155**:391-400.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Analysis of nitrate, nitrite and [^{15}N]-nitrate in biological fluids. *Anal. Biochem.* **126**:131-138.

Gschwendt, M., Kittstein, W. and Marks, F. (1991) Protein kinase C activation by phorbol esters: do cysteine-rich regions and pseudosubstrate motifs play a role? *Trends Biochem. Sci.* **16**:167-169.

Haour, F.B., Ban, E.M., Milon, G.M., Baran, D. and Fillion, G.M. (1990) Brain interleukin-1 receptors: characterization and modulation after lipopolysaccharide injection. *Prog. Neuroendocrinimmunol.* **3**:196-204.

Hartung, H.P., Jung S., Stoll, G., Zilasek, J., Schmidt, B., Archelos, J. and Toyka K. (1992) Inflammatory mediators in demyelinating disorders of the CNS and PNS. *J. Neuroimmunol.* **40**:197-210.

Hauser, S.L., Bhan, A.K., Gilles, F.H., Hoban, C.J., Reinherz, E.L., Schlossman, S.F. and Weiner, H.L. (1983) Immunohistochemical staining of human brain with monoclonal antibodies that identify lymphocytes, monocytes and the Ia antigen. *J. Neuroimmunol.* **5**:197-205.

Hendry, I.A., Murphy, M., Hilton, D.J., Nicola, N.A. and Bartlett, P.F. (1992) Binding and retrograde transport of leukemia inhibitory factor by the sensory nervous system. *J. Neurosci.* **12**:3427-3434.

Heuschling, P. (1995) Nitric oxide modulated γ-interferon-induced MHC class II antigen expression on rat astrocytes. *J. Neuroimmunol.* **57**:63-69.

Hilton, D.L. (1992) LIF: lots of interesting function. *Trends Biochem. Sci.* **17**:72-6.

Hirata, M., Kohse, K.P., Chang, C.H., Ikebe, T and Murad, F. (1990) Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells. *J. Biol. Chem.* **265**:1268-1273.

Hirayama, M., Yokochi, T., Shimokata, K., Lida, M. and Fujuki, N. (1986) Induction of human leukocyte antigen-A, B, C and DR on cultured human oligodendrocytes and astrocytes by human γ-interferon. *Neurosci. Lett.* **72**:369-374.

Holen, I., Stromhang, P.E., Gordon, P.B., Fengsrud, M., Berg, T.O., Seglen, P.O. (1995) Inhibition of autophagy and multiple steps in asialoglycoprotein endocytosis by inhibitors of tyrosine protein kinase (tyrphostins). *J. Biol. Chem.* **270**:12823-12831.

Honegger, P. (1986) Protein kinase C-activating tumor promoters enhance the differentiation of astrocytes in aggregating fetal brain cell cultures. *J. Neurochem.* **46**:1561-1566.

Hosoda, K., Feussner, G.K. and Rydelek-Fitzgerald, L. (1994) Agonist and cyclic AMP-mediated regulation of β_1-adrenergic receptor mRNA and gene transcription in rat C6 glioma cells. *J. Neurochem.* **63**:1635-1645.

Hu, D.E.and Fan, T.P. (1995) Protein kinase C inhibitor calphostin C prevents cytokine-induced angiogenesis in the rat. *Inflammation* **19**:39-54.

Hug, H.and Sarre, T.F. (1993) Protein Kinase C isoenzymes: divergence in signal transduction. *Biochem. J.* **291**:329-343.

Jonakait, G.M. (1993) Neural-immune interactions in sympathetic ganglia. *Trends Neurosci.* **16**:419-423.

Jonakait, G.M. and Schotland, S. (1990) Conditioned medium from activated splenocytes increases substance P in sympathetic ganglia. *J. Neurosci. Res.* **26**:24-30.

Kagan, B.L., Baldroin, R.L., Mounoz, D. and Wisnieski, B.J. (1992) Formation of ion-permeable channels by tumor necrosis factor-α. *Science* **255**:1427-1430.

Kasahara, T., Yagisama, H., Yamashita, K., Yamaguchi, Y and Akigama, Y. (1990) IL-1 induces proliferation and IL-6 mRNA expression in a human astrocytoma cell line: Positive and negative modulation by cholera toxin and cAMP. *Biochem. Biophys. Res. Commun.* **167**:1242-1248.

Kempski, O., Staub, F., Jansen, M., Schodel, M. and Baethmann, A. (1992) Swelling of C6 glioma cells and astrocytes from glutamate, high K^+ concentrations or acidosis. *Prog. Brain Res.* **94**:69-75.

Keller, H.U. and Niggli, V. (1993) The PKC-inhibitor Ro31-8220 selectively suppresses PMA- and diacylglycerol-induced fluid pinocytosis and actin polymerization in PMNs. *Biochem. Biophys. Res. Commun.* **194**:1111-1116.

Kilbourn R. G. and Belloni I. (1990) Endothelial cell production of nitric oxides in response to interferon in combination with tumor necrosis factor, interleukin-1, or endotoxin. *J. Natl. Cancer Inst.* **82**:772-776.

Kimball, E.S., Persico, F.J. and Vaugholc, J.L. (1988) Substance P, neurokinin A, and neurokinin B induce generation of IL-1 like activity in P388D1 cells. *J. Immunol.* **141**:3564-3569.

Kishimoto, T. (1989) The biology of interleukin-6. *Blood* **74**:1-10.

Kishimoto, T., Taga, T. and Akira, S. (1994) Cytokine signal transduction. *Cell* **76**:253-262.

Knowles, R.G. and Moncada, S. (1994) Nitric oxide synthases in mammals. *Biochem. J.* **298**:249-258.

Kobayashi, E., Nakano, H., Morimoto, M. and Tamaoki, T. (1989) Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specfic inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **159**:548-553.

Kurzrock, R., Estrov, Z., Wetzler, M., Gutterman, J.U. and Talpaz, M. (1991) LIF: not just a leukemia inhibitory factor. *Endocrine Rev.* **12**:208-217.

Lachman, L.B., Brown, D.C. and Dinarello, C.A. (1987) Growth promoting effect of recombinant interleukin-1 and tumour necrosis factor for a human astrocytoma cell line. *J. Immunol.* **138**:2913-2916.

Le, J.M. and Vilcek, J. (1989) Biology of disease: Interleukin-6: A multifunctional cytokine regulating immune reactions and the acute phase protein response. *Lab. Invest.* **61**:588-602.

Lee, M.H. and Bell, R.M. (1991) Mechanism of protein kinase C activation by phosphatidylinositol 4, 5-bisphosphate. *Biochemistry* **30**:1041-1049.

Lee, S.C., Dickson, D.W., Liu, W. and Brosnan, C.F. (1993a) Induction of nitric oxide synthase activity in human astrocytes by interleukin-1β and interferon-γ. *J. Neuroimmunol.* **46**:19-24.

Lee, S.C., Liu W., Dickson, D.W., Brown C.F. and Berman, J.W. (1993b) Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. *J. Immunol* **150**:2659-2667.

Lees, M.B. and Brostoff, S.W. (1984) Myelin. In: *"Proteins of myelin"*, (2nd ed., Morell ed.), p197-217.

Lieberman, A.P., Pitha, P.M., Shin, H.S. and Shin, M.L. (1989) Production of tumor necrosis factor-alpha and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. *Proc. Natl. Acad. Sci. (USA)* **86**:6348-6352.

Linnekin, D. and Farrer, W.L. (1990) Signal transduction of human interleukin-3 and granulocyte-macrophage colony-stimulating factor through serine and tyrosine phosphorylation. *Biochem. J.* **271**:317-324.

Linskey, M.E. and Gilbert, M.R. (1995) Glial differentiation: A review with implications for new directions in neuro-oncology. *Neurosurgery* **36**:1-22.

Lotz, M., Vaughan, J.H. and Carson, D.A. (1988) Effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science* **241**:1218-1221.

Male, D.K., Pryce, G. and Hughes, C.C.W. (1987) Antigen presentation in brain: MHC induction on brain endothelium and astrocytes compared. *Immunology* **60**:453-459.

Malipiero, U.V., Frei K and Fontana, A. (1990) Production of hemopoietic colony-stimulating factor by astrocytes. *J. Immunol.* **144**:3816-3821.

Mangoura, D., Sakellaridis, N., Jones, J. and Vernadakis, A. (1989) Early and late passages of C6 glial cell growth : Similarities with primary glial cells in culture. *Neurochem. Res.* **14**:941-947.

Mannoji, H., Yeger, H. and Becker, L.E. (1986) A specific histochemical marker (lectin *Rincinus communis* agglutinin-I) for normal human microglia and application to routine histopathology. *Acta Neuropathol.* **71**:341-343.

Mantyh, P.W., Rogers, S.D., Allen, C.J., Catton, M.D., Ghilardi, J.R., Levin, L.A., Maggio, J.E. and Vigna, S.R. (1995) Beta 2-adrenergic receptors are expressed by glia *in vivo* in the normal and injured central nervous system in the rat, rabbit, and human. *J. Neurosci.* **15**:152-164.

Martin, S.B. and Tracey, K.J. (1992) Tumour necrosis factor α (TNF) in neuroimmunology. *Adv. Neuroimmunol.* **2**:125-138.

Massa, P.T., Dorries, R. and ter Meulen, V. (1986) Viral particles induce Ia antigen expression on astrocytes. *Nature* **320**:543-546.

Massa, P.T. and ter Meulen, V. (1987) Analysis of Ia induction on Lewis rat astrocytes *in vitro* by virus particles and bacterial adjuvant. *J. Neuroimmunol.* **13**:257-271.

Massa, P.T., Schmipl, A., Wecker, E. and ter Meulen, V. (1987) Tumor necrosis factor amplifies virus-mediated Ia induction on astrocytes. *Proc. Natl. Acad. Sci. (USA)*, **84**:7274-7245.

McCarthy, K.D. and Salm, A. K. (1991) Pharmacologically distinct subsets of astroglia can be identified by their calcium response to neuroligands. *Neuroscience* **41**:325-333.

Meager, A. (1990) The cytokine concept. In: "*Cytokines*", Open University Press, Chapter 1, Milton Keynes, p1-4.

Merrill, J.E. (1987) Macroglia: neural cells responsive to lymphokines and growth factors. *Immunol. Today* **8**:146-150.

Merrill, J.E. (1992) Tumor necrosis factor alpha, interleukin 1 and related cytokines in brain development: normal and pathological. *Dev. Neurosci.* **14**:1-10.

Merrill, J.E. and Chen, I.S.Y. (1991) HIV-1, macrophages, glial cells, and cytokines in AIDS nervous system disease. *FASEB* **5**:2391-2397.

Messens, J. and Slegers, H. (1992) Synthesis of glial fibrillary acidic protein in rat glioma in chemically defined medium: Cyclic AMP-dependent transcriptional and translational regulation. *J. Neurochem.* **58**:2071-2080.

Metcalf, D. (1991) The leukemia inhibitory factor (LIF). *Int. J. Cell Cloning* **9**:95-108.

Meyer, M.L. and Miller, R.L. (1990) Excitatory amino acid receptors, second messengers and regulation of intracellular Ca^{2+} in mammalian neurons. *Trends Pharmacol. Neurosci.* **11**:254-260.

Migita, K., Eguchi, K., Kawabe, Y., Mizokami, A., Tsukada, T. and Nagataki, S. (1994) Prevention of anti-CD3 monoclonal antibody-induced thymic apoptosis by protein tyrosine kinase inhibitors. *J. Immunol.* **153**:3457-3465.

Miyajima, A., Kitamura, T., Harada, N., Yokota, T. and Arai, K.-I. (1992) Cytokine receptors and signal transduction. *Annu. Rev. Immunol.* **10**:295-331.

Morganti-Kossmann, M.C., Kossmann, T and Wahl, S.M. (1992) Cytokines and neuropathology. *Trends Pharmacol. Sci.* **13**:286-291.

Morrison, R.S., Saneto, R.P. and de Vellis (1982) Developmental expression of rat brain mitogens for cultured astrocytes. *J. Neurosci. Res.* **8**:435-442.

Munoz-Fernandez, M.A., Armas-Portela, R., Diaz-Nido, J., Alonso, J.L., Fresno, M. and Avila, J. (1991) Differential effects of tumor necrosis factor on the growth and differentiation of neuroblastoma and glioma cells. *Exp. Cell Res.* **194**:161-164.

Munoz-Fernandez, M.A. and Fresno, M. (1993) Involvement of nitric oxide on the cytokine induced growth of glial cell. *Biochem. Biophys. Res. Commun.* **194**:319-325.

Murphy, M., Reid, K., Brown, M.A. and Bartlett, P.F. (1993) Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. *Development* **117**:1173-1182.

Murphy, M., Reid, K., Hilton, D.J. and Bartlett, P.F. (1991) Generation of sensory neurons is stimulated by leukemia inhibitory factor. *Proc. Natl. Acad. Sci. (USA)* **88**:3498-3501.

Murray, R., Lee, F. and Chiu, C.-P. (1990) The genes for leukemia inhibitory factor and interleukin-6 are expressed in mouse blastocyst prior to the onset of hemopoiesis. *Mol. Cell. Biol.* **10**:4953-4956.

Naitoh, Y., Fukata, J. and Tominaga, T. (1988) Interleukin-6 stimulates the secretion of adrenocorticotropic hormone in conscious, freely-moving rats. *Biochem. Biophys. Res. Commun.* **155**:1459-1463.

Naparstek, Y., Cohen, I.R., Fuks, Z. and Vlodavsky, I. (1984) Activated T lymphocytes produce a matrix-degrading heparin sulphate endoglycosidase. *Nature* **310**:241-244.

Nathan, C. (1992) Nitric oxide as secretory product of mammalian cells. *FASEB J.* **6**:3051-3064.

Navia, B.A., Jordan, B.D. and Price, R.W. (1989) The AIDS dementia complex. *Annu. Neurol.* **19**:517-524.

Nawa, H., Yamamori, T., Le, T. and Patterson, P.H. (1990) Generation of neuronal diversity: analogies and homologies with hematopoiesis. *Cold Spring Harbor Symp. Quant. Biol.* **55**:247-253.

Nicola, N.A. (1994) An introduction to the cytokines. In: *"Guidebook to cytokines and their receptors"*. Oxford University Press. p1-8.

Nicotera, P., Bellomo, G. and Orrenius, S. (1992) Calcium-mediated mechanisms in chemically induced cell death. *Annu. Rev. Pharmacol. Toxicol.* **32**:449-470.

Noble,M. and Murray, K. (1984) Purified astrocytes promote the *in vitro* division of bipotential glial progenitor cell. *EMBO J.* **3**:2243-2247.

Nomura, H., Shiosaka, S., Inagaki, S., Ishimoto, I., Senba, E., Sakanaka, S., Takatsuki, K., Matsuzaki, T., Kubota, Y., Saito, H., Takase, S., Kogure, K. and Tohyama, M. (1982) Distribution of substance P like immunoreactivity in the lower brainstem of the human fetus: An immunohistochemical study. *Brain Res.* **252**:315-325.

Norenberg, M.D. and Martinez-Hernandez, A. (1979) Fine structural localization of glutamine synthetase in astrocytes of rat brain. *Brain Res.* **161**:303-310.

Norris, J.G., Tang, L.-P., Aparacio, S.M. and Benveniste, E.N. (1994) Signal transduction pathways mediating astrocyte IL-6 induction by IL-1β and tumor necrosis factor-α. *J. Immunol.* **152**:841-850.

Ozaki, M., Morii, H., Qvist, R. and Watanabe Y. (1994) Interleukin-1β induces cytosolic phospholipase A_2 gene in rat C6 glioma cell line. *Biochem. Biophys. Res. Commun.* **205**:12-17.

Parker, K.K., Norenberg, M.D. and Vernadakis, A. (1980) "transdifferentiation" of C6 glial cells in culture. *Science* **208**:179-181.

Patterson, P. H. (1994) Leukemia inhibitory factory, a cytokine at the interface between neurobiology and immunology. *Proc. Natl. Acad. Sci. (USA)* **91**:7833-7835.

Patterson, P.H. and Chun, L.L. (1974) The influence of non-neuronal cells on catecholamine and acetylcholine synthesis and accumulation in cultures of dissociated sympathetic neurons. *Proc. Natl. Acad. Sci. (USA)* **71**:3607-3610.

Patterson, P.H. and Nawa, H. (1993) Neuronal differentiation factor/cytokines and synaptic plasticity. *Cell* **72**:123-137.

Perry, V.H. and Gordon, S. (1988) Macrophages and microglia in the nervous system. *Trends Neurosci.* **11**:273-277.

Perry, V.H., Hume, D.A. and Gordon, S. (1985) Immunohistochemical localization of macrophages and microglia in adult and developing mouse brain. *Neuroscience* **15**:313-326.

Peters, A., Palay, S.L., Webster, H.de F. (1976) The fine structure of the nervous system. In: *"The neurons and supporting cells"*. Saunders, Philadelphia, p242-244.

Petersen, O.H., Petersen, C.C.H. and Kasai, H. (1994) Calcium and hormone action. *Annu. Rev. Physiol.* **56**:297-319.

Pfeffer, L.M., Eisenkraft, B.L., Reich, N.C., Improta, T., Baxter, G., Daniel-Issakani, S. and Strulovici, B. (1991) Transmembrane signaling by interferon alpha involves diacylglycerol production and activation of the epsilon isoform of protein kinase C in Daudi cells. *Proc. Natl. Acad. Sci. (USA)* **88**:7988-7992.

Pfeffer, L.M., Strulovici, B. and Saltiel, A.R. (1990) Interferon-alpha selectively activates the beta isoform of protein kinase C through phosphatidylcholine hydrolysis. *Proc. Natl. Acad. Sci. (USA)* **87**:6537-6541.

Pimentel, E. (1994). Interleukins and cytokines In: *"Handbook of Growth Factors, Vol II Hematopoietic growth factors and cytokines"*. CRC Press, Chapter 2, p35-261.

Pober, J.S., Gimbrone, M.A., Cotran, R.S., Reiss, C.S., Burakoff, S.J., Fiers, W., Rothlein, R. and Springer, T.A. (1983) Ia expression by vascular endothelium is inducible by activated T-cells and by human γ-interferon. *J. Exp. Med.* **157**:1339-1353.

Pohlman, T.H., Stanness, K.A., Beatty, P.G., Ochs, H.D. and Harlan, J.M. (1986) An endothelial cell surface factor(s) induced *in vitro* by lipopolysaccharide, interleukin-1, and tumor necrosis factor-alpha increases neutrophil adherence by a cd18-dependent mechanism. *J.Immunol.* **136**:4548-4553.

Prineas, J.W. (1979) Multiple sclerosis. Presence of lymphatic capillaries and lymphoid tissue in the brain and spinal cord. *Science* **203**:1123-1125.

Prineas, J.W. and Wright, R.G. (1978) Macrophages, lymphocytes and plasma cells in the perivascular compartment in chronic multiple sclerosis. *Lab. Invest.* **38**:409-421.

Prober, J.S., Gimbrose, M.A., Lapierre, L.A., Mendrick, D.L., Fiers, W., Rothlein, R. and Springer, T.A. (1986) Overlapping patterns of activation of human endothelial cells by interleukin-1, tumor necrosis factor, and immune interferon. *J. Immunol.* **137**:1893-1896.

Pulver, M., Carrel, S., Mach, J.P. and de Tribolet, N. (1987) Cultured human fetal astrocytes can be induced by interferon-γ to express HLA-DR. *J. Neuroimmunol.* **14**:123-133.

Raff, M.C., Lillien, L.E., Richardson, W.D., Burne, J.F. and Noble M. (1988) Platelet-derived growth factor from astrocytes drived the clock that times oligodendrocyte development in culture. *Nature* **333**:562-565.

Raff, M.C., Miller R.H. and Noble, M. (1983) A glial progenitor cell that develops into an astrocyte or an oligodendrocyte depending on the culture medium. *Nature* **303**:390-396.

Raff, M.C., Mirsky, R., Fields, K.L., Lisak, R.P., Dorfman, S.H., Silberberg, D.H., Gergson, N.A., Leibowitz, S. and Kennedy, M.C. (1978) Galactocerebroside is a specific cell-surface antigenic marker for oligodrendrocytes in the culture. *Nature* **274**:813-815.

Rakic, P. (1971a) Guidance of neurons migrating to the fetal monkey neocortex. *Brain Res.* **33**:471-476.

Rakic, P. (1971b) Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in Macacus Rhesus. *J. Comp. Neurol.* **141**:283-312.

Revel, J.P., Napolitano, L. and Fawcett, D.W. (1960) Identification of glycogen in electron micrographs on thin tissue sections. *J. Biophys. Biochem. Cytol.* **8**:575-589.

Rigi, M., Mori, L., de Libero, G., Sironi, M., Biondi, A., Mantovani, A., Donini, S.D. and Ricciardi-Castagnoli, P. (1989) Monokine production by microglial cell clones. *Eur. J. Immunol.* **19**:1443-1448.

Robbins, D.S., Shirazi, Y., Drysdale, B.E., Leiberman, A., Shin, H.S. and Shin, M.L. (1987) Production of cytotoxic factor for oligodendrocytes by stimulated astrocytes. *J. Immunol.* **139**:2593-2597.

Roszman, T.L. and Brooks, W.H. (1992) Signaling pathways of the neuroendocrine-immune network. In: *"Neuroimmunoendocrinology"* (2nd rev ed., Chem. Immunol., Blalock, J.E.(ed.)), Karger, Ba sel, Vol 52, p170-190.

Satoh, T, Nakamura, S., Taga, T., Matsuda, T., Hirano, T. and Kaziro, Y. (1988) Induction of neuronal differentiation in PC12 cells by B-cell stimulatory factor 2/interleukin-6. *Mol. Cell. Biol.* **8**:3546-3549.

Sawada, M., Suzumura, A., Ohno, K. and Marunouchi, T. (1993) Regulation of astrocyte proliferation by prostaglandin-E2 and the alpha subtype of protein kinase C. *Brain Res.* **613**:67-73.

Selmaj, K.W., Farooq, M., Norton, W.T., Raine, C.S. and Brosnan, C.F. (1990) Proliferation of astrocytes *in vitro* in response to cytokines. A primary role for tumour necrosis factor. *J. Immunol.* **144**:129-135.

Selmaj, K.W. and Raine, C.S. (1988) Tumor necrosis factor mediates myelin and oligodendrocyte damage *in vitro*. *Annu. Neurol.* **23**:339-346.

Sendtner, M., Kreutzberg, G. W. and Thoenen, H. (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature* **345**:440-441.

Shadiack, A.M., Hart, R.P., Carlson, C.D. and Jonakait, G.M. (1993) Interleukin-1 induces substance P in sympathetic ganglia through the induction of leukemia inhibitory factor (LIF). *J. Neurosci.* **13**:2601-2609.

Silvennoinen, O., Witthuhn, B., Quelle, F.W., Cleveland, J.L., Yi, T. and Ihle, J.N. (1993) Structure of the JAK2 protein tyrosine kinase and

its role in IL-3 signal transduction. *Proc. Natl. Acad. Sci. (USA)* **90**:8429-8433.

Simmons, M.L. and Murphy, S. (1992) Induction of nitric oxide synthase in glial cells. *J. Neurochem.* **59**:897-905.

Simmons, M.L. and Murphy, S. (1993) Cytokines regulate L-arginine-dependent cyclic GMP production in rat glial cells. *Eur. J. Neurosci.* **5**:825-832.

Skias, D.D., Kim, D., Reder, A.T., Antel, J.P., Lancki, D.W., Fitch, F.W. (1985) Susceptibility of astrocytes to class I MHC antigen-specific cytotoxicity. *J. Immunol.* **138**:3254-3258.

Small, P.K., Riddle, P. and Noble, M. (1987) Evidence for migration of oligodendrocyte-type-2 astrocyte progenitor cells into the developing rat optic nerve. *Nature* **328**:155-157.

Southern, C., Schulster, D., and Green, I.C. (1990) Inhibition of insulin secretion by interleukin-1β and tumour necrosis factor-α via an L-arginine-dependent nitric oxide generating mechanism. *FEBS Lett.* **276**:42-44.

Spriggs, D.R., Deutsch, S., and Kufe, D.W. (1992) Genomic structure, induction, and production of TNF-α. In *"Tumor necrosis factor: structure, function and mechanism of action"*, (Aggarwal, B. B. and Vilcek, J. eds.), Marcel Dekker, New York. p3-34.

Stahl, N. and Yancopoulos, G.D. (1993) The alphas, betas, and kinases of cytokine receptor complexes. *Cell* **74**:587-590.

Suckling, A.J., Kirby, J.A. and Rumsby, M.G. (1983) Characterization by acid α-napthyl acetate esterase staining of the spinal cord cellular infiltrate in the acute and relapse phases of chronic relapsing experimental allergic encephalomyelits. *Prog. Brain Res.* **59**:317-322.

Sun, Y., Rao, M, Zigmand, R.E. and Landis, S.C. (1994) Regulation of vasoactive intestinal peptide expression in sympathetic neurons in culture and after axotomy: the role of cholinergic differentiation factor/leukemia inhibitory factor. *J. Neurobiol.* **25**:415-430.

Suzumura, A., Mezitis, S.G.E., Gonatas, N.K. and Silberberg, D.H. (1987) MHC antigen expression on bulk isolated macrophage-microglia from newborn mouse brain: induction of Ia antigen expression by γ-interferon. *J. Neuroimmunol.* **15**:263-278.

Suzumura, A., Silberberg, D.H. and Lisak, R.P. (1986) The expression of MHC antigens on oligodendrocytes: Induction of polymorphic H-2 expression by lymphokines. *J. Neuroimmunol.* **11**:179-190.

Synder, S.H. and Bredt, D.S. (1992) Nitric oxide as a neuronal messenger. *Trends Pharmacol. Sci.* **12**:125-128.

Taga, T., Kawanishi, Y., Hardy, R.R., Hirano, T., Kirhimoto, T. (1987) Receptors for B cell stimulatory factor 2. Quantitation, specificity, distribution, and regulation of their expression. *J. Exp. Med.* **166**:967-981.

Taga, T. and Kishimoto, T. (1992) Cytokine receptors and signal transduction. *FASEB J.* **6**:3387-3396.

Takao, T., Tracey, D.E., Mitchell, W.M. and DeSouza, E.B. (1990) Interleukin-1 receptors in mouse brain: characterization and neuronal location. *Endocrinology* **127**:3070-3078.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**:1397-1402.

Traugott, U., Reinherz, E.L. and Raine, C.S. (1983) Multiple sclerosis: Distribution of T-cells, T-cell subsets and Ia-positive macrophages in lesions of different ages. *J. Neuroimmunol.* **4**:201-221.

Tweardy, D., Mott, P. and Glazer, E. (1990) Monokine modulation of human astroglial cell production of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. I. Effects of IL-1α and IL-1β. *J. Immunol.* **144**:2233-2241.

Ullrich, A. and Schlessinger, J. (1990) Cloning of p13 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* **65**:83-90.

van Snick, J.V. (1990) Interleukin-6: an overview. *Annu. Rev. Immunol.* **8**:253-278.

Vernadakis, A., Kentroti, S., Brodie, C., Mangoura, D. and Sakellaridis, N. (1991) C-6 glioma cells of early passage have progenitor properties in culture. In: "*Plasticity and Regeneration of the Nervous System*", (Private, A., Timiras, P.S., Giacobini, E. and Vernadakis, A. eds),. Plenum Press, New York, p131-197.

Vernadakis, A., Lee, K.' Kentroti, S. and Brodie, C. (1992) Role of astrocytes in aging: late passage primary mouse brain astrocytes and C6 glial cells as models. *Prog. Brain Res.* **94**:391-409.

Vigne, P., Damais, C. and Frelin, C. (1993) IL1 and TNFα induce cGMP formation in C6 astrocytoma cells via the nitridergic pathway. *Brain Res.* **606**:332-334.

Villiger, P.M., Geng, Y. and Lotz, M. (1993) Induction of cytokine expression by leukemia inhibitory factor. *J. Clin. Invest.* **91**:1575-1581.

Virchow, R. (1860) Cellular pathology as based upon physiological and pathological histology (Edited and translated by Chance, F.) Churchill, London, p21-30.

Waniewski, R.A. and Martin, D. L. (1986) Exogenous glutamate is metabolized to glutamine and exported by rat primary astrocyte cultures. *J. Neurochem.* **47**:304-313.

Weiss, A. and Littman D.R. (1994) Signal transduction by lymphocyte antigen receptors. *Cell* **76**:263-274.

Westermark, B, Ponten, J and Hugosson, R. (1973) Determinants for the establishment of permanent tissue culture lines from human gliomas. *Acta Path. Microbiol. Scand., Sect. A.* **81**:791-805.

Wolswijk, G. and Noble, M. (1989) Identification an adult-specific glial progenitor cell. *Development* **105**:387-400.

Wong, G.H.W., Bartlett, P.F., Clark-Lewis, I., Battye, F. and Schrade, J.W. (1984) Inducible expression of H-2 and Ia antigens on brain cells. *Nature* **310**:688-691.

Yamaguchi, M., Matsuzaki, N., Hirota K., Miyake, A. and Tanizawa, O. (1990) Interleukin-6 possibly induced by interleukin-1β in the pituitary gland stimulates the release of gonadotropins and prolactin. *Acta Endrocrinol.* **122**:201-205.

Yasukawa, K., Hirano, T., Watanable, Y., Muratani, K., Matsuda, T., Nakai, S. and Kishimoto, T. (1987) Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6). *EMBO J.* **6**:2939-2945.

Yoshida, K. and Gage, F.H. (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibriblast growth factor and other cytokines. *Brain Res.* **569**:14-25.

Zidovetzki, R. and Lester, D.S. (1992) The mechanism of activation of protein kinase C: a biophysical perspective. *Biochem. Biophys. Acta* **1134**:261-272.