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Modulation by Cytokines of Parameters of Astrogliosis *in vitro* and *in vivo*

Trevor Tejada-Bergés

Department of Neurology and Neurosurgery,
McGill University, Montréal
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Abstract

Astrocytes respond to injury of the central nervous system (CNS) in a process termed reactive astrogliosis. While classically considered detrimental to CNS function, the purpose of reactive astrogliosis is now considered by some to promote CNS recovery. The functional role of reactive astrocytes may be clarified if the molecular mediators of astrogliosis can be identified. This thesis explores the role of cytokines in modulating parameters of astrogliosis both *in vitro* and *in vivo*. Studies with astrocytes isolated from uninjured adult mouse brains suggest that these cells have different biological characteristics as compared to neonatal murine astrocytes. On the other hand, IFN-γ promotes DNA synthesis in both fetal and adult human astrocytes and inhibits that of neonatal and adult mouse astrocytes. Collectively, these results suggest that both age- and species-specific differences may determine the response of glial cells to cytokines. In addition, the presence of one cytokine may influence the response of astrocytes to another; the simultaneous administration of TNF-α with more potent cytokine mitogens to adult human astrocytes abrogates the effects of the latter. Finally, experiments on neonatal mice *in vivo* demonstrate their ability to mount an astrogliotic response given an adequate injury stimulus. The presence of cytokines within the lesion site upregulates this reaction.

Résumé

Les astrocytes réagissent aux atteintes au système nerveux central (SNC) par un processus nommé astrogliose réactive. Longtemps considérée comme dommageable au SNC, le rôle de l'astrogliose réactive est désormais considérée comme bénéfique au rétablissement du système nerveux. La fonction de l'astrogliose réactive pourrait être expliquée en identifiant les médiateurs moléculaires de l'astrogliose. La présente thèse explore le rôle que jouent les cytokines dans la modulation de l'astrogliose *in vitro* et *in vivo*. Des étude faites en isolant des astrocytes de cerveaux de souris adultes intacts semblent démontrer que ces cellules ont des caractéristiques biologiques différentes de celles provenant de souris neonatales. Cependant, alors que l'IFN-γ stimule la synthèse de l'ADN par les astrocytes humains d'âge adulte et embryonnaire, il inhibe cette même synthèse par les astrocytes provenant de souris néonatales et adultes. Ces résultats semblent suggérer que la réaction de cellules gliales aux cytokines soit fonction de l'âge et de l'espèce. De plus, la présence d'une cytokine peut influencer la réaction des astrocytes par rapport à une autre cytokine. Par exemple, l'administration simultanée de TNF-α et de mitogènes plus puissants sur des astrocytes humains supprime les effets des mitogènes. Enfin, des expériences sur des souris néonatales *in vivo* démontre leur capacité d'effectuer une réaction astrogliotique suite à une stimulation suffisante. La présence de cytokines dans le champs de la lésion accélère cette réaction.

Preface

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I Dedicate this Thesis

to my family,

a la memoria de mi abuelo, Doctor Antonio Tejada Guzmán,

and to those friends who still put up with me after all these years.

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Thesis Overview

This thesis is divided into six chapters relating the effects of inflammatory cytokines on parameters of reactive astrogliosis on both human and murine astrocytes cultured *in vitro* and on lesioned murine CNS *in vivo*. Chapter 1 gives a brief introduction of reactive astrogliosis and presents evidence implicating cytokines as modulators of this process. The chapters which follow (Chapters 2-5) represent original work exploring further the role of cytokines, either published (Chapter 3: **Glia 6**: 269-280, 1992; Chapter 4: **Brain Research 653**: 297-304, 1994; Chapter 5: **J Neuroscience 14**: 846-856, 1994) or submitted for publication (Chapter 2).

Nearly all astrocyte proliferation studies *in vitro* have used neonatal rodent or calf bovine brain-derived cells. However, numerous reports indicate that astrogliosis is minimal following stab wounds in these perinatal animals, suggesting that astrocytes isolated from these animals may not represent good models for the study of adult astrogliosis. Thus, Chapter 2 outlines a protocol for the isolation of astrocyte-enriched cultures from adult murine CNS parenchyma. Chapter 3 further addresses the question of age-specific versus species-specific differences of astrocytes in their response to cytokines by using fetal and adult human astrocytes as well as fetal and adult murine astrocytes. Given that astrocytes within the CNS are likely to encounter a multitude of cytokines, acting in a co-operative or antagonistic manner, Chapter 4 explores directly the interaction of TNF-α and IFN-γ on proliferation of adult human astrocytes. Both of these cytokines have previously been reported to be mitogens for these cells. Chapter 5 documents the extent of astrogliosis following injury to the neonatal CNS, and assesses directly the extent to which this can be modified by the injection of exogenous cytokines.

Finally, Chapter 6 is a brief summary of the work presented in this thesis, including possible future directions for work in this field. For clarity, it is hoped that the content of this thesis overview serves as a bridge between the six chapters.

Chapter 1

Reactive Astrogliosis: a Role for Cytokines

Reactive astrogliosis refers to the characteristic response of astrocytes to injury of the central nervous system (CNS) whereby these cells become larger, extend more processes, and significantly increase their cytoplasmic content of glial fibrillary acidic protein (GFAP), an astrocyte-specific intermediate filament (Latov *et al.*, 1979; Ludwin, 1985; Janeczko, 1988; Topp *et al.*, 1989). Proliferation of astrocytes is also reported, although the extent to which hyperplasia of astrocytes contributes to the phenomenon of astrogliosis remains controversial (Graeber *et al.*, 1988; Morshead and van der Kooy, 1990; Matsumoto *et al.*, 1992). Increased GFAP immunoreactivity remains a useful and commonly used marker of astrogliosis since cortical astrocytes normally do not stain with antibodies to GFAP, although they contain this intermediate filament (Bignami *et al.*, 1976). Other astrocytic changes involve an increase in the number of mitochondria, Golgi membranes, endoplasmic reticulum of perinuclear cytoplasm, glycogen content and enzyme levels (Nathaniel and Nathaniel, 1981; Malhotra *et al.*, 1990; Landis, 1994).

Reactive astrogliosis is initiated soon after a traumatic insult. [^{3}H]-thymidine incorporation studies in experimental animal models have shown that the process occurs as early as one hour following CNS injury (Latov *et al.*, 1979) while GFAP-positive cortical astrocytes appear within thirty minutes of a cryogenic lesion (Amaducci *et al.*, 1981). By twenty-four hours, a significant number of astrocytes stain positive for GFAP in most lesion models (Stromberg *et al.*, 1986; Whitaker *et al*, 1987; Norton *et al*, 1992). Measurements of GFAP content by quantitative immunoblots in rats subjected to stab wounds show that although no increase over unoperated controls is detected at 3 h, a substantial rise is apparent by 6 h post-lesioning (Hozumi *et al.*, 1990). GFAP mRNA has been demonstrated to increase six-fold up to 6 h following a penetrating brain lesion (Condorelli *et al.*, 1990). In general, with the exception of hepatic encephalopathy, where GFAP content is reportedly reduced (Norenberg, 1986), CNS trauma induces an increase

in GFAP content regardless of the type of initiating insult. *In vitro*, GFAP content of astrocytes can be altered by treatment with dibutyryl cyclic adenosine 3',5'-monophosphate (db cAMP), prostanoids, various hormones and growth factors (Hertz *et al*, 1978; Morrison *et al.*, 1985; Chiu *et al*, 1985). Ultimately, the role of these GFAP changes in terms of CNS function remains unresolved; however, in as much as they represent an acute alteration in the microenvironment surrounding lesions of the CNS, they are of help to researchers attempting to define more clearly the mediators responsible for the astrogliotic reaction. For our purposes, they represent an effective means of objectively assessing astrogliosis and quantitating the effect of cytokines on this process.

Classically, astrocyte proliferation, changes in GFAP content and immunoreactivity, and subsequent development of glial scars have been used as indicators of reactive astrogliosis; however, it is important to note that these may not co-exist within the same model of injury. In the adult rodent facial nerve crush model, where alterations in GFAP content and immunoreactivity are observed, astrocytes apparently do not undergo proliferation while microglia do (Tetzlaff *et al.*, 1988; Graeber *et al.*, 1988). In EAE, while the intensity of GFAP-immunoreactive astrocytes can increase substantially 10-12 days post-inoculation, this is not accompanied in the same time period by a rise in GFAP content (Goldmuntz *et al.*, 1986; Smith *et al.*, 1987; Aquino *et al.*, 1988). Only at later time points is there an associated change in both content and immunoreactivity of GFAP. Several reasons have been postulated, including increased degradation of GFAP into products that stain but are not detected by SDS-PAGE as GFAP, or increased soluble GFAP that is not reflected in the Triton-insoluble cytoskeletal extract used for SDS-PAGE (Condorelli *et al*, 1990; Aquino *et al.*, 1990). A particularly compelling explanation is that previously hidden antigenic epitopes are exposed as a consequence of physical disassembly of GFAP intermediate filaments following injury-induced astrocyte swelling (Eng *et al.*, 1989; Aquino *et al.*, 1990).

It is desirable that a complete description of the changes seen after CNS injury incorporate the gamut of astrocytic responses, namely cell proliferation, changes in GFAP content and immunoreactivity, as well as the formation of a glial scar. Such

determinants are vital to studies which attempt to dissect the causes of astrogliosis post-injury, and, ultimately, its role as a response to CNS trauma. It is possible that each of these attributes is controlled by different mediators.

The glial scar can be composed of a densely interwoven network of astrocytes, fibroblasts, oligodendrocytes and numerous inflammatory cells such as macrophages, microglia and lymphocytes. Multiple layers of abnormal basal lamina may be associated with the scar (reviewed in Reier *et al.*, 1986, 1989). The reconstituted basal lamina is thought to serve as a glia limitans, encapsulating regions of the CNS that become exposed to the non-CNS environment following injury (Berry *et al.*, 1983). Glial scars are especially prominent in injuries where the pial lining of the brain is breached, with consequent migration of meningeal fibroblasts into the wound cavity; indeed, features of a glia limitans become apparent *in vivo* or *in vitro* whenever astrocytes and fibroblasts come into contact (Abnet *et al.*, 1991).

The formation of glial scars is classically considered undesirable: a source of inhibition of axonal growth or regeneration (reviewed in Reier, 1986), a focus of epileptiform activity (Pollen and Trachtenberg, 1970; Brotchi *et al.*, 1979; Willmore *et al.*, 1981) and a hindrance to remyelination (Raine *et al.*, 1970). However, given that the CNS demonstrates inherent compensatory mechanisms to maintain function after a traumatic insults, why do astrocytes become reactive following injury, particularly considering the postulated detrimental effects of astrogliosis? Several functional properties have been ascribed to reactive astrocytes including the removal of myelin and neuronal debris from the injured area, encapsulation of regions of the CNS that are exposed to non-CNS environment following injury, restoration of some degree of physiological homeostasis in the injured CNS and filling of the space vacated by the dead or dying cells. Increasingly, astrocytes have been ascribed neurotrophic properties (David, 1993; Yong, in press).

Tissue culture studies have demonstrated that an astrocytic monolayer enhances the survival and differentiation of neurons (Lindsay, 1979; Noble *et al.*, 1984; Fallon, 1985; Neugebauer *et al.*, 1988; Chuah *et al.*, 1991), most likely via the production of

adhesion molecules implicated in neuronal development and survival (Noble *et al.*, 1985; Smith *et al.*, 1990). The majority of the astrocytes and neurons used in these studies are from neonatal or embryonic CNS. Presumably, these cells have a greater regenerative potential; nonetheless, Lindsay (1979) used astrocytes derived from adult rat brains. *In vivo*, astrocyte-containing implants serve as adequate substrates for axonal growth (Silver and Ogawa, 1983; Smith *et al.*, 1986; Rudge and Silver, 1990).

Astrocytes are capable of producing a variety of neurotrophic growth factors *in vitro* under both basal or stimulated conditions, including nerve growth factor (NGF), fibroblast growth factor (FGF), ciliary neurotrophic factor (CNTF; Gradient *et al.*, 1990; Yoshida and Gage, 1991; Frei *et al.*, 1989; Ferrara *et al.*, 1988; Rudge *et al.*, 1992; Carroll *et al.*, 1993). Furthermore, reactive astrocytes appear capable of producing NGF, FGF and CNTF *in vivo* (Bakhit *et al.*, 1991; Finklestein *et al.*, 1988; Tourbah *et al.*, 1992; Ip *et al.*, 1993).

Stab wound studies have shown that neurotrophic factors are secreted around the lesion locus following an injury. These factors have been extracted from intact tissue adjacent to the injury and absorbable Gelfoam pieces placed in the wound cavity (Nieto-Sampedro *et al.*, 1983; Whittemore *et al.*, 1985; Ip *et al.*, 1993). Largely, astrocytes have been implicated in the production of these substances simply based on the presence of reactive astrocytes in the lesion areas; however, *in situ* studies by Ip *et al.* (1993) confirmed that astrocytes are responsible for the increased CNTF production. It seems reasonable, therefore, that reactive astrocytes might be responsible for the production of these neurotrophic substances.

Ultimately, the key to determining the role of astrocytes during reactive astrogliosis is not solely to account for neurotrophic properties of astrocytes under unstimulated conditions, but to confirm this capacity under conditions which mimic the reactive changes seen in astrocytes. David *et al.* (1990) used unfixed cryostat sections of transected adult rat optic nerve to study the ability of reactive astrocytes in situ to support axonal growth of chick dorsal root ganglion neurons. After 5 days, 30% of the ganglia placed near the lesion demonstrated extensive neurite growth. None placed distal to the

lesion showed such growth, which was comparable to the normal adult rat optic nerve. More recently, Miller *et al.* (1994) demonstrated that the ability of astrocytes to promote neurite growth was enhanced following treatment with dibutyryl cAMP, Interleukin-1β (IL-1β) and macrophage conditioned medium, all substances which induced reactive changes in astrocytes.

This thesis aims to further clarify the role of cytokines as putative modulators of reactive astrogliosis both *in vitro* and *in vivo*. Previous reports from this laboratory using a stab wound plus callosotomy model in adult rats demonstrated that the resultant astrogliosis was most prominent in cortical areas adjacent to the subarachnoid spaces and decreased gradually into the deeper cortical areas, implicating soluble and diffusible factors in triggering the astrocytic response (Moumdjian *et al.*, 1991). In this regard, a number of molecules have been reported to be mitogens for neonatal astrocytes *in vitro* (reviewed in Yong *et al.*, 1989).

In considering possible soluble mediators of astrogliosis, it is important to consider that injury to the CNS of this type causes the recruitment of intrinsic (microglia) and extrinsic (monocytes, lymphocytes, and NK cells) inflammatory cells capable of releasing a variety of cytokines (Goldmuntz *et al.*, 1986; Giulian, 1987; Leong and Ling, 1992; Perry *et al.*, 1993). Giulian *et al.*(1989) showed that after a needle stab to the rat brain, blood-borne monocytes and microglia appear within 5 h. The largest number of mononuclear phagocytes is seen at 2 days after the injury. Anti-inflammatory agents, chloroquine and colchicine, injected into the animals decreased the number of mononuclear phagocytes in the damaged brain, reduced GFAP-immunoreactivity and inhibited neo-vascularization.

Studies measuring cytokine levels in the brain following CNS injury have demonstrated an increase in IL-1, IL-6 and Tumor Necrosis Factor-α (TNF-α; Nieto-Sampedro and Berman, 1987; Nieto-Sampedro and Chandy, 1987; Woodroofe *et al.*, 1991; Taupin *et al.*, 1993). Evidence that these cytokines further promote inflammation of the CNS is supported by studies indicating that a single microinjection of γ-Interferon (γ-IFN), TNF-α or IL-2 into the normal CNS results in the recruitment of inflammatory

cells in the CNS parenchyma (Watts *et al.*, 1989; Simmons and Willenborg, 1990; Sethna and Lampson, 1991).

Studies where IL-1, IL-2 and γ-IFN are directly injected into the adult rodent brain demonstrated an increase in the extent of GFAP immunoreactivity (Giulian and Lachman, 1985; Giulian *et al.*, 1988; Watts *et al.*, 1989; Yong *et al.*, 1991). Intraocular injections of IFN-γ, TNF-α and IL-1 increased the adherence of inflammatory cells to the vascular endothelium and evoked astrogliosis in rabbits (Brosnan *et al.*, 1989). In EAE, infiltration of lymphocytes and monocytes is prominent and coincides with astrogliosis (Smith *et al.*, 1987; Aquino *et al.*, 1988; Cammer *et al.*, 1990; D'Amelio *et al.*, 1990). Finally, previous *in vitro* studies using neonatal rodent, calf bovine and human adult astrocytes have shown that cytokines, including IL-1, IL-6, TNF-α and γ-IFN, activated T-cell supernatants as well as B cell products are capable of inducing glial proliferation (Giulian and Lachman, 1985; Selmaj *et al.*, 1990; Barna *et al.*, 1990; Yong *et al.*, 1991; Benveniste *et al.*, 1989).

In summary, then, the presence of elevated levels of inflammatory cytokines within the injured CNS, and the multiple reports of cytokines exerting effects on astrocytes, provide compelling rationale to explore the role of inflammatory cytokines as mediators of astrocyte reactivity *in vitro* and *in vivo*.

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Chapter 2

Derivation and Characterization of Astrocytes Cultured from Uninjured Adult Mouse Brains

Trevor Tejada-Bergés and Voon Wee Yong

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Abstract

Much of the work aimed at studying the properties of astrocytes involves the use of primary cell cultures. With few exceptions, these preparations are obtained from embryonic or early neonatal cerebra. Recent reports suggest that the properties of astrocytes can vary as a function of their age and maturity; thus, adult brain-derived primary cultures would be better suited for studying the role of astrocytes in the mature CNS. However, it has proven difficult to culture astrocytes from uninjured adult mouse brains employing strategies similar to those used for immature animals. We report here on the derivation and characterization of astrocyte-enriched cultures from uninjured six-month old adult CD1 mice. By immunocytochemistry we show a predominance of astrocytes with few oligodendrocytes or microglial cells. Fibroblasts are a major contaminant only upon long-term culturing of these cells. The adult brain-derived astrocytes exhibit a significantly lower basal rate of proliferation than that of neonatal-derived astrocytes, as assessed by bromodeoxyuridine (BrdU) incorporation.

Introduction

Astrocytes form a large proportion of the cells within the central nervous system (CNS) and play a dynamic role in the functioning of the brain. Thus, they form an integral part of the blood-brain barrier, maintain ionic homeostasis, clear neurotransmitters from the synaptic cleft, secrete trophic factors and may perform immunomodulatory functions. During development, astrocytes serve to guide axons to their proper sites (Rakic, 1981). Upon injury to the CNS, astrocytes undergo hypertrophy and proliferation in a response termed reactive gliosis (reviewed in Lindsay, 1986; Hatten *et al.*, 1991). Although considered detrimental to CNS regeneration (Reier *et al.*, 1983; Reier, 1986), reactive gliosis may actually be an attempt to promote CNS repair (David, 1993; Yong, 1994). Studies point to reactive astrocytes as a source of neurotrophic substances around the site of a lesion (Nieto-Sampedro *et al*, 1983; Whittemore, 1985; Needels, 1986; Nieto-Sampedro *et al.*, 1987; Miller *et al.*, 1994).

Attempts at understanding the role of astrocytes in physiology and pathology have capitalised on the ability to isolate enriched cultures of astrocytes and to examine these under relatively defined conditions. With few exceptions, most studies have utilised embryonic or neonatal tissues. Few investigators have attempted to grow viable astrocyte cultures from the uninjured, mature murine CNS. Whether perinatal cells represent good models of adult brain astrocytes is a subject of debate, since differences between neonatal and adult astrocytes have been documented.

Gliosis is a feature of adult CNS injuries, but occurs to a lesser extent as a result of lesions during embryonic or neonatal life (Berry *et al.*, 1983; Barret *et al.*, 1984), although this notion has recently been contested (Trimmer and Wunderlich, 1990; Hatten *et al.*, 1991). Hatten *et al.* (1991) and others (Bacher & Bunge, 1990; Wang *et al.*, 1990) suggest that the state of differentiation rather than the strict chronological age of an animal is important in determining whether a gliotic response does or does not occur. The implantation of fetal- or neonatal-derived astrocytes into the brain of adult animals at the time of CNS injury decreases the extent of gliosis (Kruger *et al.*, 1986; Smith &

Silver, 1988; Smith & Miller, 1991); on the other hand, astrocytes which have matured *in vitro* do not have this ability (Smith & Silver, 1988; Smith & Miller, 1991). Furthermore, neonatal-derived astrocytes are reportedly capable of promoting the regression of an established glial scar after transplantation into chronic injury sites (Houle & Reier, 1988). The maturation of astrocytes *in vitro* can alter their capacity to enhance neurite extension (Smith *et al.*, 1990). Recently, we observed a differential proliferative response between adult human and neonatal mouse astrocytes to γ-interferon and Interleukin-1 (Yong *et al.*, 1991a). While mitogenic for adult human astrocytes, these cytokines either did not increase DNA synthesis or induced a significant decrease in the proliferation of neonatal mouse astrocytes, suggesting that age may be a determining factor in mediating the proliferative response to extrinsic factors.

In view of the concern that neonatal astrocytes may not be representative of adult-derived astrocytes, we set out to devise a scheme for isolating astrocytes from the unlesioned brain of adult mice. In this communication, we describe a method for obtaining cultures of non-neuronal brain cells enriched for astrocytes from mature animals, and their subsequent characterization using cell-specific antisera. Contrary to previous reports that astrocytes dissociated from normal adult brain grow poorly (Singh & Van Alstyne, 1978; Lindsay *et al.*, 1982), the cells we have isolated show a significant basal level of mitotic activity; albeit lower than that of perinatal-derived astrocytes, supporting our assumption that these adult-derived cells are different from neonatal-derived ones. The generation of these cells allows one to address issues regarding the age-dependent role astrocytes might play in promoting or inhibiting regeneration in the mature CNS, and the possible differences between perinatal- and adult-derived astrocytes in response to extrinsic signals.

Materials and Methods

Tissue Culture of Adult Murine Astrocytes

Animals were obtained from Charles River Canada (Montreal, Quebec). These consisted of retired female CD1 breeders approximately six months old and weighing roughly 30 g each. On average, 10 animals were used for each preparation.

Animals were euthanized via ether anaesthesia, their cerebral lobes removed and washed twice in sterile phosphate-buffered saline (PBS). The tissue was cut into 1-3 mm^3 fragments, rinsed extensively in PBS and incubated with 0.25% trypsin (Gibco) and 100 µg/ml DNAse (Sigma) in PBS for 15 min at 37°C with no agitation. The floating suspension was removed and passed through a 132 µm nylon mesh which served to retain undissociated fragments. Fetal bovine serum (FBS) was added to the filtrate at a final concentration of 1% to inactivate the trypsin. The undissociated tissue was reincubated with 0.125% trypsin and 100 µg/ml DNAse for another 15 min at 37°C. Once again, the dissociated cells were filtered through a 132 µm mesh, while the softened tissue fragments which remained were sieved via two consecutive passes through nylon messes of 210 and 132 µm pore size, respectively. The cells were subsequently centrifuged at 1,500 rpm for 15 min, resuspended in PBS, mixed in a 30% Percoll (Pharmacia) gradient and centrifuged once again at 15,000 rpm for 30 min at 4°C. The viable cells, floating between an upper layer of myelin and debris and a lower layer of red blood cells were removed and diluted in 5 volumes of PBS and collected by centrifugation at 600 rpm for 10 min. Finally, the cells were washed twice in PBS, suspended in feeding medium (approximately 2 x 10^6 cells/ml) and seeded onto poly-L-lysine- (PL) coated (10 µg/ml) 25 cm^2 Falcon flasks (4 ml per flask). Feeding medium consisted of Eagle's Minimum Essential Medium (1X) supplemented with 5% heat-inactivated FBS, 1 mg/ml dextrose and 20 µg/ml gentamicin (all purchased from Gibco).

The following day, all cellular debris was removed and the cultures were flooded with feeding medium. Cells were maintained at 37°C in a humidified incubator (5% CO_2), and the medium was changed 2 to 3 times per week.

After 10 to 14 days *in vitro*, the cells were removed from the flasks using 0.05% trypsin and 20 µg/ml DNAse. FBS was added to inactivate the trypsin and the floating

cells were collected, centrifuged (2 000 rpm for 10 min) and resuspended in feeding medium. The cells were subsequently plated onto PL-coated (10 µg/ml) 9 mm Aclar coverslips at a density of 10^4 cells per coverslip, and used for experiments within 1 to 2 weeks following retrypsinization to minimize the proportion of contaminating fibroblasts.

A proportion of cells remained adherent to the culture flask following the retrypsinization process. These strongly adherent cells were flooded with feeding medium; in some cases, these were retrypsinized (0.075% trypsin) and the cells collected for further characterization after vigorous shaking of the flasks.

Tissue Culture of Neonatal Murine Astrocytes

Neonatal mouse astrocytes were from 1 day old CD1 outbred pups. Whole brain was dissected into 1 mm^3 pieces and incubated with 1% trypsin and 100 µg/ml DNAse in PBS for 10 min at 37°C. FBS at 1% final concentration was added to inactivate the trypsin. Following trituration with a Pasteur pipette, the cell suspension was passed through a 132 mm mesh to retain undissociated fragments. The filtrate was collected, washed and centrifuged twice in feeding medium, and then plated onto uncoated 25 cm^2 Falcon flasks at a density of 1 x 10^6/ml of feeding medium. Cells were left for 7 to 10 days at 37°C in a humidified incubator (5% CO_2) with medium changes every 2 to 3 days. Feeding medium during this period was similar to that of the adult cells, except it contained 10% FBS. The cells were removed 7 to 10 days following the initial dissociation process and plated on coverslips in the same manner as the adult astrocytes. Purity of astrocytes for up to 2 weeks following replating was over 90% as judged by immunofluorescence for glial fibrillary acidic protein (GFAP; not shown).

Indirect Immunofluorescence Labelling

The major cell types in the adult mouse brain preparations were characterised by staining with a variety of antibodies. Antibodies were diluted in a solution of 2% horse serum, 2% HEPES buffer (1mM) and 10% goat serum in Hank's balanced salt solution, arbitrarily termed HHG (all constituents purchased from Gibco). Staining with rabbit

anti-GFAP (1:100; DAKO) and Rh-conjugated sheep anti-fibronectin (FN; 1:100; Serotec) was performed on cells fixed for 15 min at -20°C in 5% glacial acetic acid:95% absolute ethanol (acid:alcohol). Mouse anti-galactocerebroside (GalC; mouse hybridoma supernatant, used neat; Ranscht *et al.*, 1982), rat anti-Mac-1 (1:100; Boehringer-Mannheim) and rat anti-Thy-1 (1:10; gift from Dr. Trevor Owens, McGill University) antibodies were applied to live cultures, which were subsequently fixed in 2% paraformaldehyde for 5 min at 4°C followed by 15 min at -20°C with acid:alcohol. To assess the background level of fluorescence, cells serving as negative controls were incubated in HHG instead of primary antisera.

A FITC-conjugated goat anti-rabbit Ig (1:100; Cappell) was applied to stain for GFAP; however, a Rh-conjugated goat anti-rabbit Ig (1:100; Cappell) was used when double-staining for Thy-1 antigen and GFAP. A Rh-conjugated goat anti-mouse IgG (1:150; Cappell) was used for GalC visualisation. Cells incubated with primary antisera for Mac-1 and Thy-1 were labelled with a biotinylated anti-rat IgG (1:100; Vector) followed by Streptavidin-FITC (1:100; Boehringer-Mannheim). The cells on coverslips were incubated with 16 µl of diluted antisera for 45 min at room temperature in a humid chamber. The coverslips were mounted with Gelvatol on glass slides and viewed using a Leitz fluorescence microscope. Photomicrographs of stained cells were taken on Kodak Tri-X Pan film. To determine the percentage of different cell types present in these cultures, the number of positively labelled cells on half of a coverslip double stained for either GFAP/GalC or Thy-1/FN was divided by the total number of cells within the same region (maximum of 300 cells counted).

Assessing Astrocytic Proliferation

The number of astrocytes undergoing mitosis was visualised using a GFAP-BrdU double immunofluorescence technique. The cells were pulsed for various periods of time with 10 µM BrdU (Sigma), a thymidine analogue incorporated during the S-Phase of mitosis. This method allows precise identification of a proliferative astrocyte. The GFAP-BrdU double immunofluorescence technique is described in detail elsewhere

(Yong and Kim, 1987; Yong *et al.*, 1991a). Briefly, the cells were fixed in 70% EtOH at -20°C for 30 min and rehydrated with PBS. The cells were incubated with a polyclonal rabbit antibody to GFAP (1:100; DAKO) for 45 min at room temperature, followed by a FITC-conjugated goat anti-rabbit Ig (1:100). To denature the cellular DNA and allow the antibody access to any BrdU which may have been incorporated , the cells were treated with 2N HCL for 10 min followed by sodium borate (pH 9,0) for another 10 min. The BrdU was labelled with a monoclonal mouse antibody to BrdU (1:25; Becton-Dickinson) followed by a Rh-conjugated goat anti-mouse Ig secondary antibody (1:150). The coverslips were coded by a colleague and mounted on glass slides with Gelvatol to allow for blind analysis. Using a Leitz fluorescence microscope equipped with FITC and Rh optics, the number of GFAP-BrdU double-positive cells as a percentage of all GFAP' astrocytes was assessed.

Statistical analysis

Treated and untreated responses were analysed using a one-way ANOVA with Duncan's multiple comparisons of all groups. A difference was considered significant if $p < 0.05$.

Results

Primary Cell Cultures

The initial dissociation procedure produced a cell suspension with much cellular debris. On average, 2.5-3 x 10^6 viable (Trypan blue-excluding) cells were recovered initially for each animal used. By 24 hours, most viable cells had attached to the flask, and a change of medium at this time removed the bulk of cellular debris. Upon examination, there was no evidence of viable cells in this suspension which was removed. Cultures became cleaner and free of debris with each subsequent change of medium. After a few days in culture, the cells began to change morphology, producing a mixed population of cells. By two weeks *in vitro*, when the cultures were retrypsinized, the cells

had formed an almost confluent layer. At this time, an average of 2.5 x 10^5 viable cells were recovered per animal used.

A puzzling observation was that freshly dissociated cells did not adhere or grow as well on coverslips as they did in flasks. Initially, attempts were made to grow the cells directly onto PL-coated plastic and/or glass coverslips. Except for a few cultures, these cells did not grow well. However, if the cells were seeded onto PL-coated flasks and allowed to grow for a period of 1 to 2 weeks, the cells adhered to the Aclar coverslips very soon following retrypsinization. Moreover, these cultures were highly enriched for astrocytes. Thus, all subsequent isolations involved initial seeding on flasks to establish the cultures followed by retrypsinization onto coverslips for characterization.

Characterization of Cells

Antisera directed at cell-specific surface antigens or cytoskeletal proteins were used to identify which cells made up the bulk of our cultures. The use of an antibody against the astrocyte-specific marker, GFAP, showed that the majority of the cells in our cultures were of this type (88-96%; Table 1). These GFAP$^+$ cells exhibited a range of morphology (Fig. 1a-c) similar to that observed for adult human astrocytes (Yong *et al.*, 1990), but the majority had large nuclei, cytoplasm-rich pericarya, and extended few thick processes. While the large majority of the cells within our cultures were astrocytes (over 88%), the percentage of these cells varied from preparation to preparation (Table 1).

A small number of cells stained positively for GalC (1-2%; Table 1) demonstrating the relative absence of oligodendroglia in these astrocyte-enriched cultures. The GalC$^+$ cells usually appeared singly (Fig. 2a-c), although groups of cells were observed (Fig. 2d). Many of the GalC$^+$ cells extended sheet-like processes (Fig. 2c) probably in an attempt to form a myelin-like sheath as suggested by others (Vick *et al.*, 1990). As mentioned in the Methods, some cells remained attached after the initial retrypsinization procedure. By using a second treatment of trypsin and vigorous shaking of the flasks, some of these strongly adherent cells were recovered; the number of GalC$^+$

cells in the second recovery was substantially increased (Fig. 2d). Indeed, using a Nikon inverted phase contrast light microscope, cells resembling oligodendrocytes morphologically were seen in the flasks following the initial retrypsinization (Fig. 3a). The strong adherence of the oligodendrocytes to the PL-coated flasks might explain the high purity of our astrocyte cultures.

After 2 to 3 weeks post-retrypsinization, the number of GFAP-negative cells began to increase. These cells exhibited streaky FN staining (Fig. 4a). Thy-1 antigen and FN double staining demonstrated that these cells were also Thy-1-positive (Fig. 4a,b) supporting our assumption that they were fibroblasts (Lindsay *et al.*, 1982). Thy-1 staining was also evident in a large proportion of GFAP$^+$ astrocytes (Fig. 1c,d). This is in disagreement with a report by Lindsay *et al.* (1982), but supports the results of Pruss (1979). As a result, we were unable to enrich for astrocytes using anti-Thy-1-mediated immunocytolysis as described (Lindsay *et al.*, 1982). Eventually, fibroblasts were the only contaminating cell-type present in significantly large numbers (>5%) appearing only after long-term culturing (over 1 month) of these cells.

Monocytes/macrophages were absent from the trypsinized portion of our cultures as assessed by staining with anti-Mac-1 (Fig. 5b). As a positive staining control we used cultures of neonatal mouse astrocytes (Fig.5a). Cells resembling microglia remained adherent to the flasks following retrypsinization (Fig.3b).

Basal Rate of Astrocytic Proliferation

In order to determine their approximate rate of proliferation, we compared the rate of BrdU-uptake of both adult- and neonatal-derived astrocytes *in vitro* in 5% FBS-containing medium. Cells on coverslips were pulsed with 10 mM BrdU for 2, 16, 24, 48 and 96 hours. The cells were subsequently fixed and stained for GFAP and BrdU as outlined in the Methods. Figure 6 illustrates the results of this double immunofluorescence technique. Figure 7 shows the basal rate of BrdU uptake of both adult- and neonatal-derived astrocytes. The rate was variable among adult cell preparations. However, the basal level of BrdU incorporation by the cells derived from

uninjured adult animals was significantly lower than that of neonatal astrocytes for each period of BrdU pulse tested.

Discussion

While methods for obtaining highly enriched cultures of embryonic or neonatal rodent astrocytes have been well described (Manthorpe *et al.*, 1979; McCarthy and de Vellis, 1980; Stieg *et al.*, 1980), a much more limited number of studies have attempted to isolate astrocytes from mature rodent animals. Singh and Van Alstyne (1978) demonstrated that while they were unable to grow astroglia from normal adult rat striatum, they were capable of culturing these cells from striatum 5 days after the induction of reactive gliosis in response to a chemical lesion with kainic acid. In 1982, Lindsay *et al.* observed a somewhat similar phenomenon. They reported that pure astrocyte cultures could be grown from the corpus callosum of adult rats 5 days after a knife lesion, but not from uninjured rat brain. More recently, Norton *et al.* (1988) and Vick *et al.* (1990) reported the ability to derive enriched astrocyte cultures from the adult rat brain; while Vernadakis *et al.*(1984) were able to grow an astrocyte-containing mixed culture from the cerebral hemispheres of aged mice.

In this report, we have described a method for obtaining viable cultures from the brains of uninjured adult mice which are highly enriched for astrocytes (>88% GFAP$^+$). To our knowledge, this is the first description of a protocol for growing astrocyte-enriched preparations from uninjured adult mouse brain. Some GalC$^+$ cells were seen in these cultures, but the majority of these oligodendrocytes remained attached to the flasks following the retrypsinization process. This was also the case for cells of the monocyte lineage as none of the cultured cells in the adult preparations stained for Mac-1; however, cells resembling microglia were evident in the flasks post-trypsinization. It is interesting to note that the adhesive properties of oligodendrocytes and microglia, as compared to those of astrocytes, are reversed in our adult murine cultures from those of neonatal-derived cells where oligodendrocytes and microglia do not attach as strongly (McCarthy

and de Vellis, 1980; Giulian and Baker, 1986). Furthermore, oligodendrocytes from the adult rat or human brain have poor adhesive properties which allow their purification by removal of floating cells following a day of incubation on uncoated flasks (Yong *et al.*, 1991b).

As a result of the added difficulty of culturing astrocytes from mature animals, most *in vitro* studies have capitalized on the ability to culture astrocytes from the brain of perinatal animals. However, perinatal-derived astrocytes may not be a good model system for studying the properties of astrocytes in the adult animal. Young, relatively undifferentiated astrocytes may have different biologic properties from older, more fully differentiated cells. For example, during the development of the CNS, astrocytes have a high mitotic rate which decreases as a function of age (Korr, 1986). However, cells in the adult rodent brain do retain the ability to divide (McCarthy and Leblond, 1988; Schipper and Wang, 1990), often in response to injury (Latov *et al.*, 1979; Miyake *et al.*, 1988) and disease (Smith *et al.*, 1987). Further apparent differences between neonatal and adult cells have been elaborated in the introduction.

Norton and Farooq (1989) have reported on the presence of vimentin$^+$/GFAP$^-$ /GalC$^-$ cells in cultures of mature rat brain which become vimentin$^+$/GFAP$^+$, mimicking the vimentin to GFAP transition which occurs developmentally (Dahl, 1981). They propose that the astrocytes growing in their cultures arise from a population of glial precursor cells present in the adult brain. At present, we are unable to make any conclusions regarding the origin of the GFAP-staining in our cultures. However, progenitor cells have been isolated from the optic nerves of adult rats (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989) and striata of adult mice (Reynolds and Weiss, 1991). The O2A progenitor cells isolated from the optic nerves of rats appear fundamentally distinct from their counterparts in perinatal animals (Wolswijk and Noble, 1989). This further argues for the use of age-matched control preparations *in vitro* if an attempt is to be made at correlating these results with *in vivo* observations. It would be of interest to ascertain whether the enriched astrocyte cultures described here are indeed

mature astrocytes successfully isolated from the adult mouse brain or rather astrocytes maturing from progenitor cells present in adult animals.

In summary, the results reported in this communication demonstrate that it is possible to grow astrocyte-enriched cultures from the brains of mature, uninjured mice. Contrary to earlier reports, these cells are viable, and exhibit a modest basal level of proliferation as measured by BrdU incorporation. The mitotic rate of adult mouse astrocytes is, however, significantly lower than that of perinatal-derived astrocytes supporting our assumption that these cells are distinguishable from the cells routinely isolated from neonatal cerebra. The protocol we have described lays the foundation to address the possible age-dependent neurotrophic properties of astrocytes, and the differences between perinatal- and adult-derived astrocytes in response to extrinsic signals.

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Figure Legends

Figure 1: **Characterization by indirect immunofluorescence of astroglial cells derived from uninjured adult mouse brain.** Cells stained with an antibody to GFAP showed a range of morphology (A-C). A large proportion of the GFAP-positive cells in C stained for Thy-1 (D); however, some cells were Thy-1 negative (arrows). X 1 (XX).

Figure 2: **Galactocerebroside immunofluorescence of adult mouse-derived oligodendrocytes after one (A,B) or two (C,D) retrypsinization procedures.** Most of the cells appeared singly (A-C), although oligodendrocytes were more numerous following a second retrypsinization (D). Many cells extended GalC-positive sheet-like processes (C). X 1 (XX).

Figure 3: **Phase contrast of oligodendrocytes (A) and microglia (B) which remained attached to culture flasks following an initial retrypsinization process (3 days post-retrypsinization).** X 1 200.

Figure 4: **Fibronectin and Thy-1 antigen double immunofluorescence staining of fibroblasts in adult mouse preparations.** These cells appeared in significant numbers only after long-term culturing. X 1 (XX).

Figure 5: **Immunofluorescence staining of adult mouse-derived preparations for monocytes using an anti-Mac-1 antibody.** No positive staining cells were evident (B). Neonatal mouse cultures were used as a positive control (A). X 1 (XX).

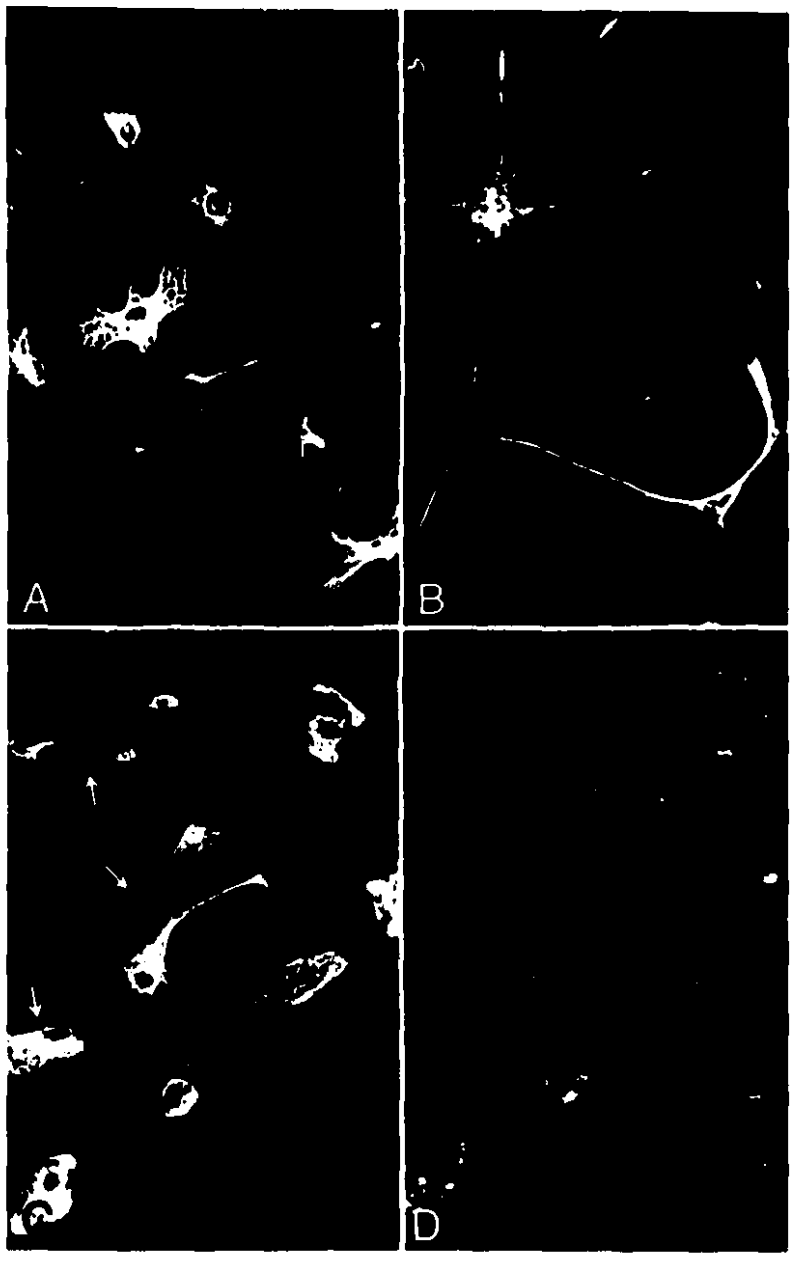
Figure 6: **GFAP-BrdU double immunofluorescence of adult mouse astrocytes.** GFAP is shown in A while the corresponding BrdU labelling is shown in B. Astrocytes that have incorporated BrdU are indicated by arrows. X 1 (XX).

Figure 7: **Adult mouse astrocytes have a significantly lower rate of BrdU incorporation than neonatal mouse astrocytes at all periods of BrdU pulse tested.** Values are mean ± SEM with the number of coverslips analysed shown in parentheses (significant if $p < 0.05$, using 1-way ANOVA with Duncan's multiple comparisons). All results were obtained from coded specimens.

Table 1. Percentages of Major Cell Types Following Retrypsinization of Adult Murine Cultures

	Cell Types		
Culture (DIV)[a]	Astrocytes (% $GFAP^+$)[b]	Oligodendrocytes (% $GalC^+$)[b]	Fibroblasts (% $Thy\text{-}1^+FN^+$)[b]
W95 (16)	95.7 ± 0.8	0.7 ± 0.3	0 ± 0
W310 (20)	90.6 ± 0.9	2.3 ± 0.5	2.4 ± 0.8
W305 (27)	88.4 ± 1.3	1.8 ± 0.8	5.8 ± 0.6

[a] DIV represents days in vitro after initial dissociation procedure.
[b]Mean ± SEM of four coverslips as described in Materials and Methods. Total percentages do not add up to 100% as some cells were unidentifiable.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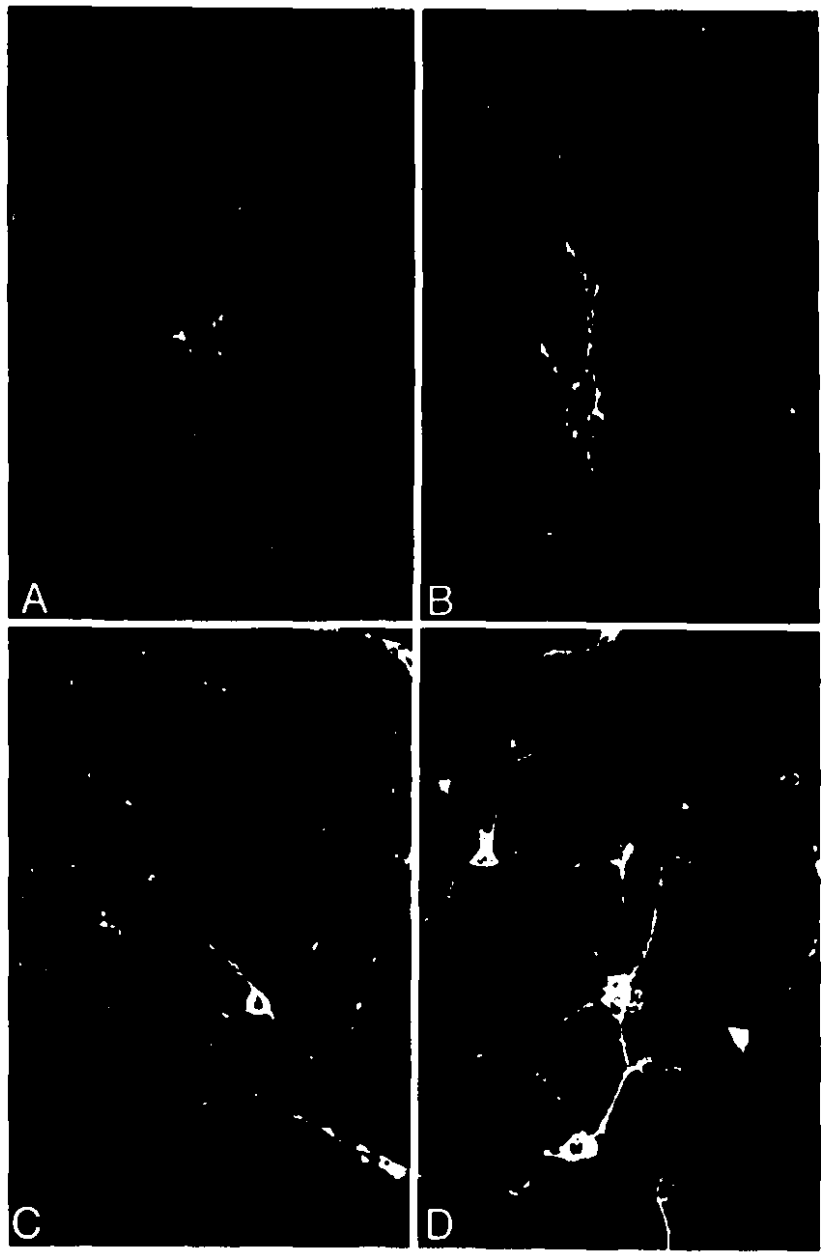
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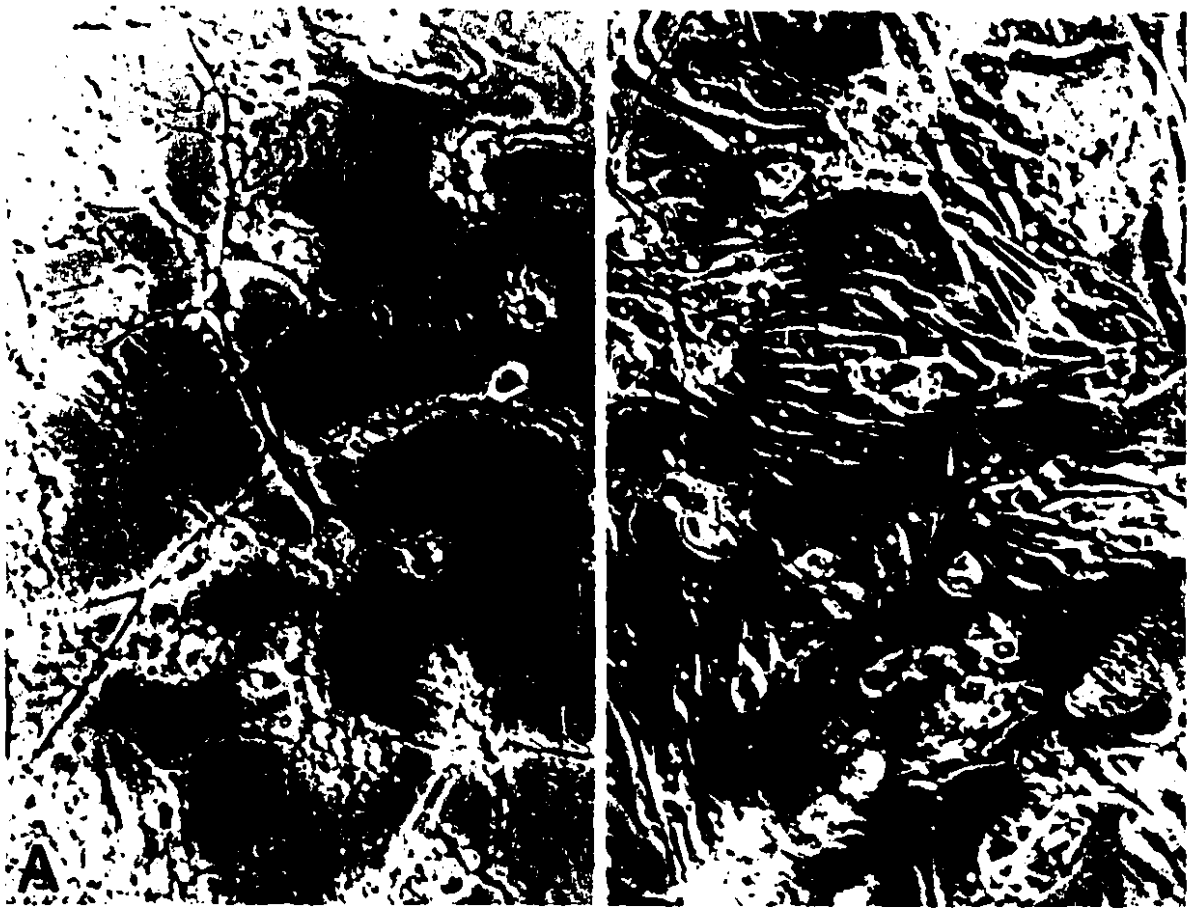
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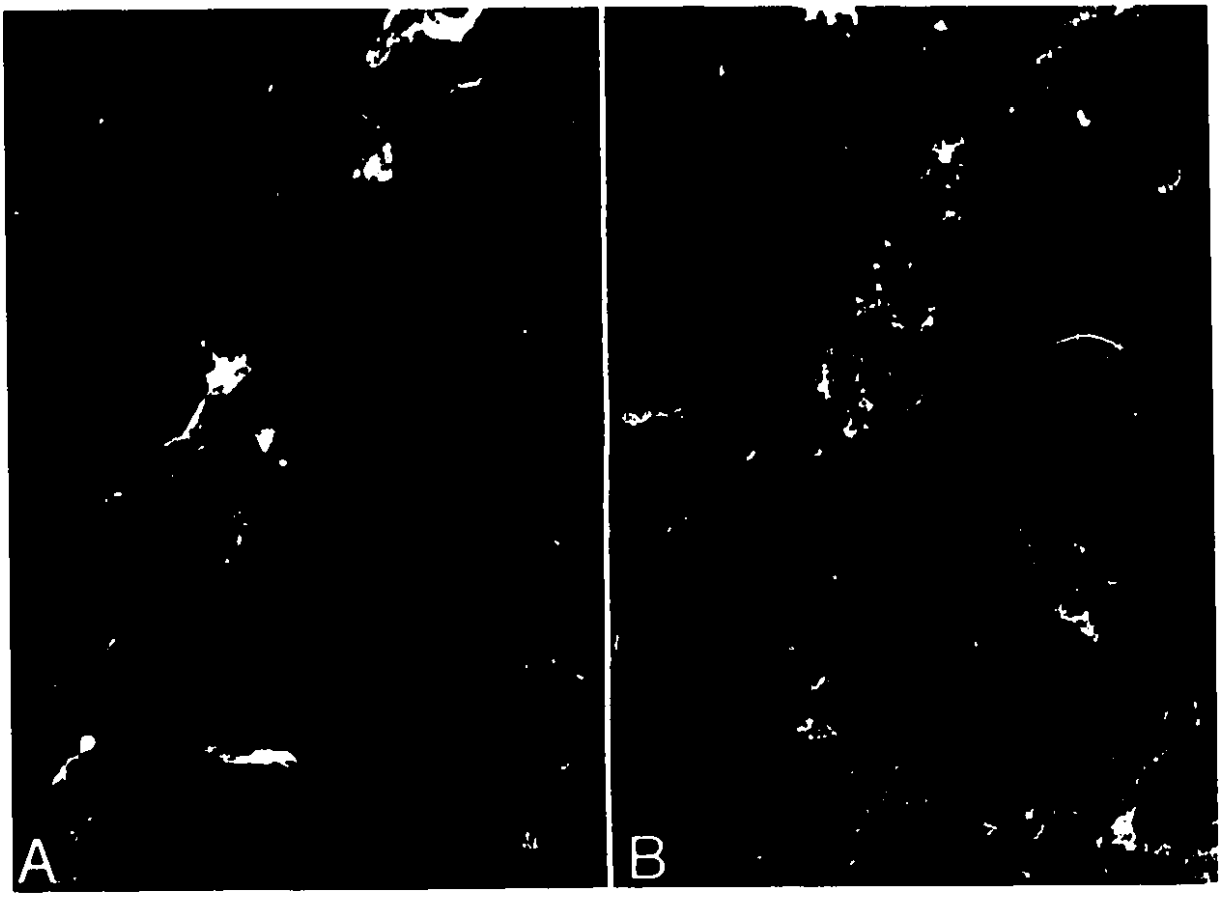
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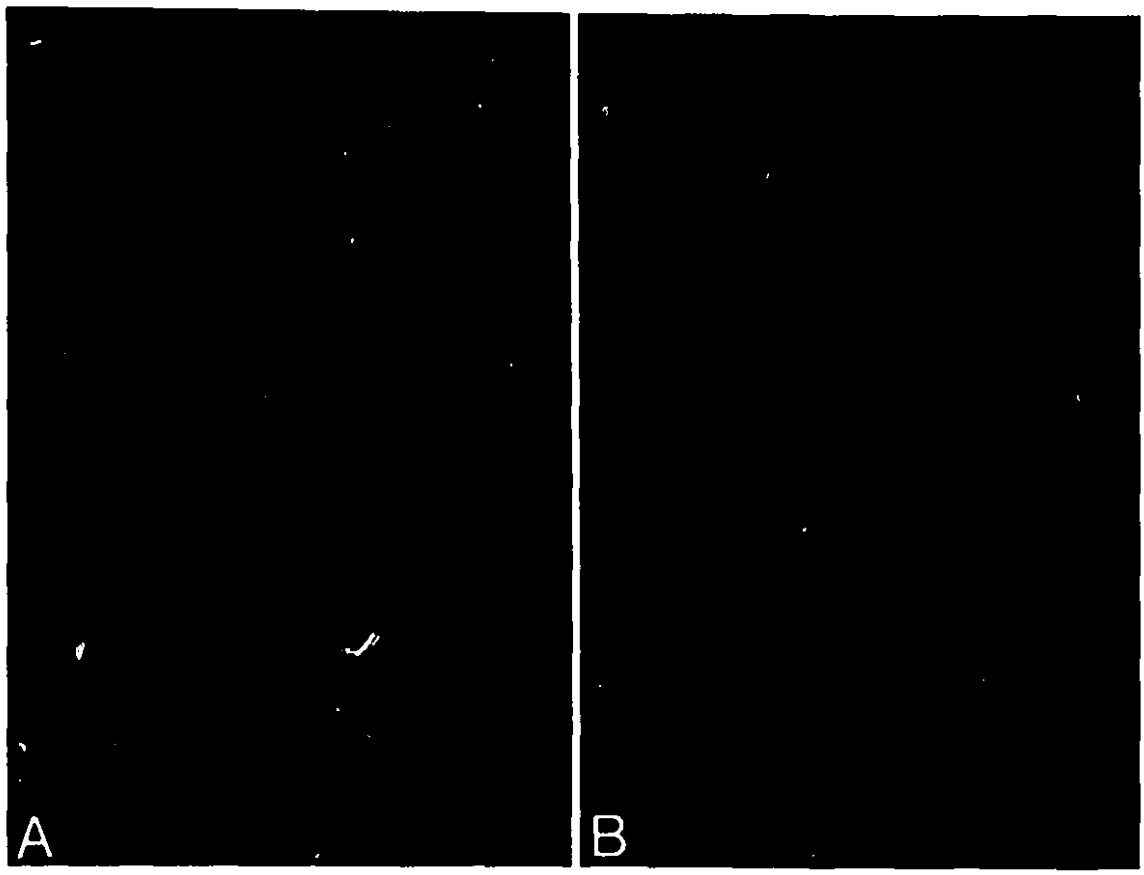
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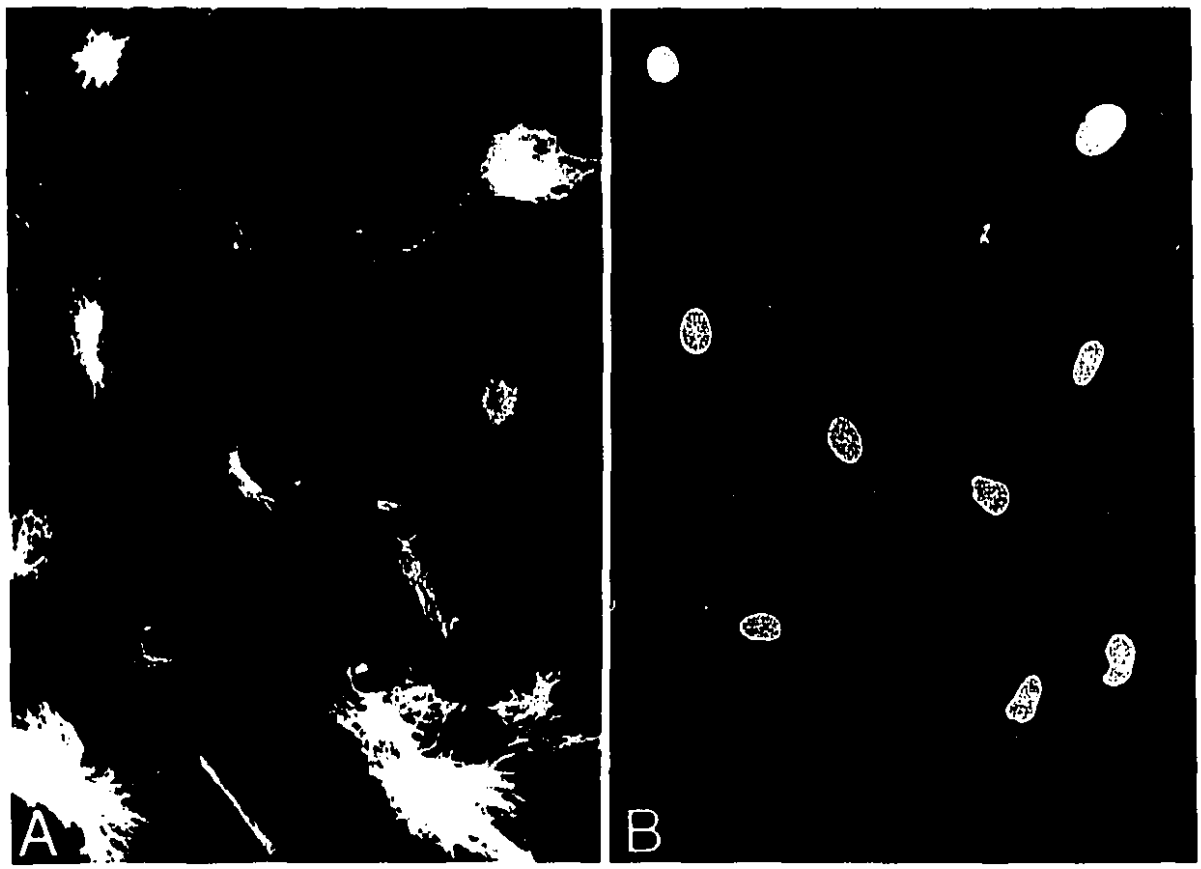
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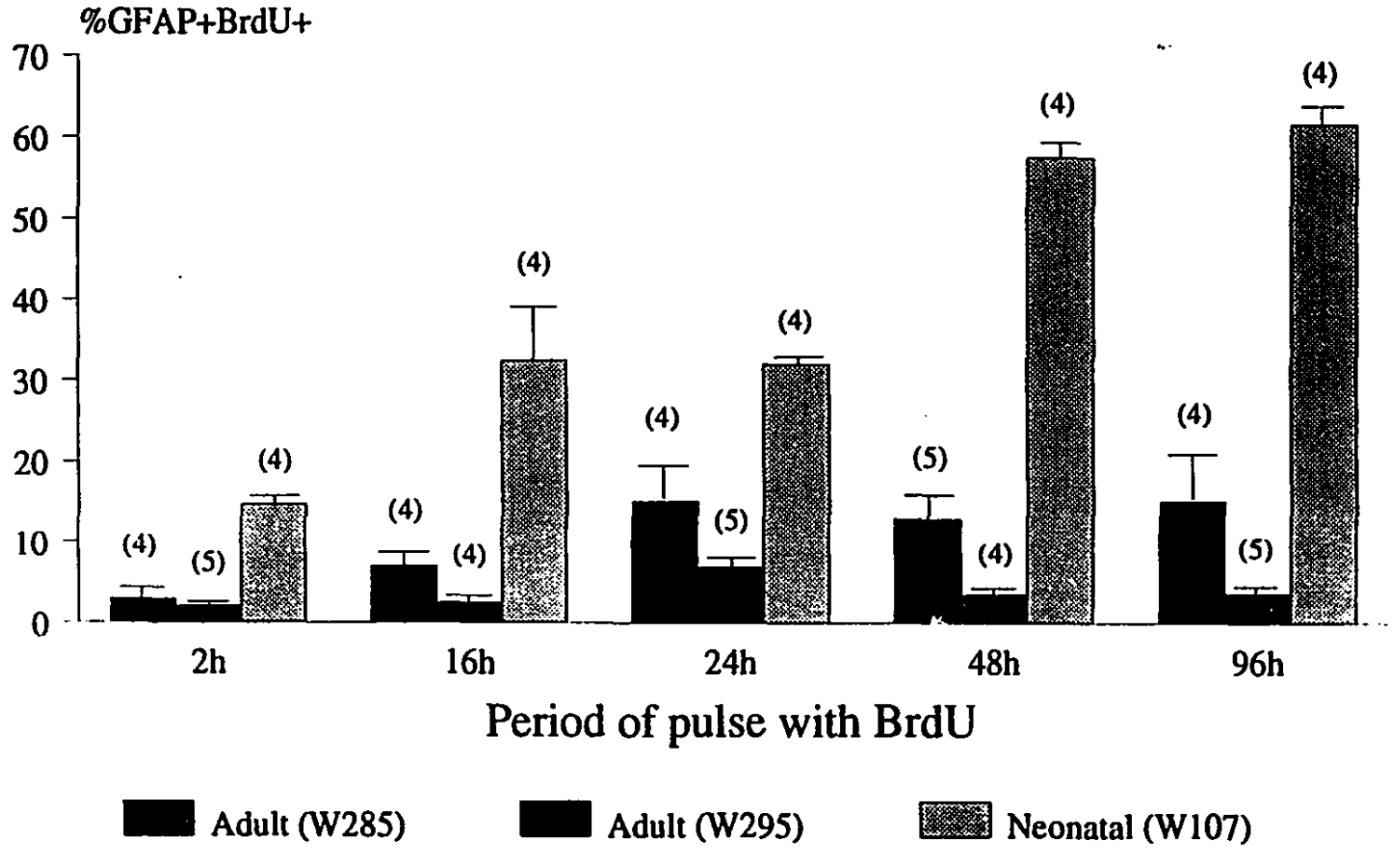
Comparison of BrdU Uptake: Adult and Neonatal Mouse Astrocytes



Chapter 3

Differential Proliferative Response of Human and Mouse Astrocytes to ϒ-Interferon

Voon Wee Yong, Trevor Tejada-Bergés, Cynthia G.Goodyer, Jack P. Antel, and Fiona P. Yong

(Glia 6: 269-280, 1992)

Abstract

We have previously shown that γ-interferon promoted the proliferation of adult human astrocytes isolated from brain biopsy specimens. In contrast, in the present study, astrocytes derived from neonatal mouse brains and treated with recombinant murine γ-interferon responded by a decrease in proliferation (average of 50% at 100 U/ml). The basal rate of proliferation as assessed by bromodeoxyuridine incorporation was markedly increased in neonatal mouse astrocytes when compared to the adult human cells, suggesting that age, and the corresponding metabolic activity of cells, could be important determinants in the mitogenic response of astrocytes to cytokines. However, subsequent examinations of fetal human and adult mouse astrocytes with comparable basal rate of proliferation to neonatal mouse and adult human cells respectively, showed γ-interferon to promote DNA synthesis in fetal human astrocytes while inhibiting that of adult

mouse astrocytes. The results suggest species-specific differences in the proliferative response of human and mouse astrocytes to the cytokine γ-interferon.

Introduction

Reactive gliosis, where astrocytes undergo proliferation and hypertrophy, is a prominent feature following many types of injury to the central nervous system (CNS). Attempts to understand the molecular signals that induce gliosis have included studies aimed at assessing mediator(s) of proliferation of cultured astrocytes. To this end, a number of soluble molecules have been reported to be mitogens for astrocytes *in vitro*. This list includes fibroblast growth factor (FGF), epidermal growth factor (EGF), glial growth factor from the bovine pituitary (GGF-BP), platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), biologically active phorbol esters, and certain vasoactive peptides such as vasoactive intestinal peptide and endothelin (reviewed in Yong *et al.*, 1989).

Attention has also focused on cytokines as inducers of astrocytic proliferation. This has merit in that inflammatory mononuclear cells such as lymphocytes, bloodborne monocytes/macrophages, and intrinsic brain microglia cells are known to be present around lesion sites in the brain, particularly when the blood brain barrier is breached. Thus, supernatants derived from lymphocytes activated *in vitro* can induce proliferation of rodent (Fontana *et al.*, 1980; Merrill *et al.*, 1984) or human (Barna *et al.*, 1985) astrocytes. In addition, specific purified cytokines including interleukin (IL)-1 (Giulian and Lachman, 1985; Giulian *et al.*, 1988; Nieto-Sampedro and Berman, 1987), tumor necrosis factor (TNF-α) (Barna *et al.*, 1990; Selmaj *et al.*, 1990), and IL-6 (Selmaj *et al.*, 1990) are reported mitogens.

With few exceptions, the aforementioned studies utilized astrocytes derived from the neonatal or fetal brain. Gliosis, on the other hand, is a feature of adult CNS injuries and occurs to a lesser extent (if at all) in insults inflicted during embryonic or neonatal life (Sumi and Hager,1968; Bignami and Dahl,1976; Berry *et al.*, 1983; Barrett *et al.*, 1984), although this contention has recently been challenged (Eclancher *et al.*, 1990; Trimmer and Wunderlich, 1990; Hatten *et al.*, 1991). Studies of proliferation of astrocytes from the adult brain may therefore be more relevant to understanding the mediators of gliosis postinjury. Our laboratory has been examining soluble factors that could produce proliferation of adult human astrocytes cultured from brain biopsy specimens. While autopsy derived astrocytes could not be induced to proliferate (Yong *et al.*, 1988), biopsy-derived adult human astrocytes undergo a low basal rate of proliferation *in vitro*. Supernatants collected from activated CD4+ or CD8+ human T-lymphocytes stimulated DNA synthesis as measured by bromodeoxyuridine incorporation (Yong *et al.*, 1991a). The effects of the lymphocyte supernatants could be completely blocked by a neutralising antibody to γ-interferon (γ-IFN), but not by neutralising antibodies to IL-1 or TNF-α. Added alone, recombinant human γ-IFN evoked proliferation of adult human astrocytes.

When adult human astrocytes were treated with EGF, FGF, PDGF, and IGF-1, these being mitogens for embryonic or neonatal rodent and human astrocytes as reported by several groups including our own, no increase in proliferation was observed (Yong *et al.*, 1991a). Such findings suggest that the age of the brain, with concordant changes in the state of maturity and metabolic activity of cells, could be a determining factor in influencing whether or not a proliferative response would occur. In the present manuscript, we have directly compared the mitogenic capability of γ-IFN on adult human as well as neonatal mouse astrocytes. The results show that in contrast to adult human cells, γ-IFN reduced the proliferation of neonatal mouse astrocytes. To further resolve whether this differential response was due to the age of the animals from which the astrocytes were derived, or species differences, astrocytes were cultured from fetal human and adult mouse brains. Subsequent treatment with γ-IFN revealed a proliferative response for

fetal human astrocytes but an inhibition of DNA synthesis for adult mouse cells. These results suggest species differences in the proliferative response of astrocytes to γ-IFN.

Materials and Methods

Cell Culture

Adult human glial cultures were isolated from the temporal cortex of 11 patients (average age 29.1 ± 2.7 years, mean ± SEM, Table 1) undergoing surgical resection to ameliorate intractable epilepsy. Cell dissociation was by a protocol described in detail elsewhere (Yong *et al.*, 1990, 1991b). For the majority of the cultures used, the purity of astrocytes following removal of oligodendrocytes and most microglial cells was about 70%; the remaining 30% consisted mainly of microglial cells which presently cannot be further eliminated. In a minority of cultures, where removal of microglia cells was not actively sought, the proportion of microglia cells was substantially higher (up to 80%); however, we have previously demonstrated that the mitogenic action of γ-IFN did not depend on the relative presence/ absence of microglial cells or their secretory products (Yong *et al.*, 1991a). Plating density on 10 μg/ml poly-lysine (PL)-coated Aclar fluorocarbon 9 mm coverslips was 10^4 cells/coverslip. Feeding medium was Eagle's minimum essential medium supplemented with 5% fetal bovine serum (FBS), 1 mg/ml dextrose, and 20 μg/ml gentamicin.

Neonatal mouse astrocytes were from 1-day-old CD1 outbred pups. Whole brain was dissected into cubes of 1 mm and incubated with 1% trypsin and 100 μg/ml DNAse in phosphate-buffered saline (PBS) for 10 min at 37°C. The strong trypsin solution was removed and the cubes further incubated with 0.1% trypsin and 100 μg/ml DNAse in PBS for 30 min at 37°C. FBS at 1% final concentration was added to inactivate the trypsin. Following trituration with a Pasteur pipette, the cell suspension was passed through a nylon mesh of 130 mm size to retain undissociated fragments. The filtrate was collected, washed and centrifuged twice in feeding medium, and plated into 25 cm^2 Falcon flasks at density of 5 million cells in 5 ml feeding medium. Cells were left for 7 to

10 days at 37°C in a humidified incubator, with medium change every 2 to 3 days. Feeding medium during this period was as described for the human cells, except that the FBS content was 10%. To remove cells from the flasks, 0.05% trypsin was used for 10 min at 37°C. FBS at 1% final concentration was added to inactivate the trypsin and the floating cells were then collected, centrifuged, and resuspended in feeding medium. At this stage, the feeding medium containing 5% FBS described for the human cells was used. Cells were plated onto two different types of coverslips coated with 10 µg/ml PL. In the first, mouse astrocytes were plated onto 9 mm Aclar coverslips at density of 10^4 cells/ coverslip for bromodeoxyuridine studies (see below); this is identical to the conditions for adult human cells. The second utilizes 13 mm glass coverslips at plating density of 25,000 cells/coverslip (for [^{3}H]-thymidine studies). Purity of astrocytes for up to 2 weeks following replating was over 90% as judged by immunofluorescence for glial fibrillary acidic protein (GFAP), an intermediate filament of astrocytes. Mouse astrocytes were used for experiments within 10 days following retrypsinization since the proportion of contaminating fibroblasts increases steadily thereafter.

Fetal human astrocytes were derived from the brains of abortuses of 10 to 18 weeks gestation. These tissues were obtained at the time of therapeutic abortions (by dilatation and curretage) with approval from local Institutional Ethics Review Committee. To obtain viable cells, cubes of approximately 1 to 3 mm were incubated with 0.25% trypsin and 100 µg/ml DNAse for 30 min at 37°C. FBS at 1% final concentration was added and the suspension was triturated with a glass pipet to further break up undissociated fragments. Following filtration through a 130 mm nylon mesh filter, the filtrate was centrifuged at 1,200 rpm for 10 min. The cell pellet was resuspended and washed twice in the 5% FBS feeding medium described. Twenty million cells in 10 ml feeding medium were plated onto 10 µg/ml PL-coated 100 mm dishes. Feeding medium was changed only once every 4 days to allow neurons to die. After 2 weeks, cells were removed from the dishes with 0.05% trypsin and plated onto glass or Aclar coverslips as per the neonatal mouse astrocytes. Medium change was effected every 2 days. Purity of astrocytes following replating was over 90%, which was maintained for at least a month

thereafter. Cells were used for the present experiments within 2 weeks following replating.

Adult mouse astrocytes were from 6 month old CD1 outbreds. The protocol is described in detail in Chapter 2. In brief, 1-3 mm brain cubes were incubated with 0.25% trypsin and 100 µg/ml DNAse for 15 min at 37°C. The floating suspension was removed and passed through a 130 mm nylon mesh. FBS was added to the filtrate to inactivate trypsin. The undissociated tissues were collected from the nylon mesh and were reincubated with 0.125% trypsin and 100 ug/ml DNAse for another 15 min at 37°C. Once again, the dissociated cells were filtered through a 130 mm mesh, while the softened tissue fragments which remained were sieved via two consecutive passes through nylon meshes of 210 and 130 mm pore size, respectively. The cells were subsequently centrifuged in Percoll as per the adult human cells, collected, washed, and seeded onto PL-coated flasks with the 5% FBS feeding medium described above. The following day, all cellular debris were removed and the cultures refed. After 10 to 14 days *in vitro*, the cells were removed from the flasks using 0.05% trypsin, and plated onto PL-coated Aclar coverslips at 10^4 cells per coverslip. These were used for experiments within 1 to 2 weeks following retrypsinization. Purity of astrocytes during this period ranged from 88 to 96%. Longer term cultures had a substantial proportion of contaminating fibroblasts.

Assessment of Proliferation of Astrocytes

Two different methods were employed based largely on the source of astrocytes being analysed: GFAP-bromodeoxyuridine (BrdU, an analog of thymidine) double immunofluorescence protocol and [^{3}H]-thymidine incorporation. The GFAP-BrdU technique which specifically visualizes proliferating astrocytes was used for all the adult human cultures since these contained contaminating microglial cells; it was important to exclude the results of possible microglial proliferation. Adult mouse astrocytes were also examined using GFAP-BrdU immunofluorescence despite the enrichment of astrocytes, since basal [^{3}H]-thymidine incorporated tended to be low. The second method of

[^{3}H]-thymidine incorporation into the S-phase of proliferating cells was used for the majority of neonatal mouse and fetal human astrocyte cultures since the purity of astrocytes was in excess of 90%, and because basal [^{3}H]-thymidine incorporation in control cultures were of a sufficient magnitude to allow reliable detection of inhibitors or promoters of proliferation. This was a much faster assay than GFAP-BrdU immunofluorescence since the latter had to be visually examined and the proportion of GFAP$^+$ cells (astrocytes) with BrdU incorporation tabulated.

The GFAP-BrdU procedure has been described in detail elsewhere (Yong and Kim, 1987; Yong *et al.*, 1988, 1991a). Following staining, coverslips were mounted on glass slides with Gelvatol; at this point, the coverslips were coded to permit for blind analyses. Using a double immunofluorescence microscope, the number of GFAP$^+$ cells was counted. Of these, the number and thus the percentage of astrocytes that have incorporated BrdU was tabulated. Figure 1 depicts astrocytes immunolabelled for both GFAP and BrdU.

To perform [^{3}H]-thymidine uptake and liquid scintillation counting, cells on coverslips were incubated with 1 mCi [^{3}H]-thymidine for 16 h. Each coverslip was then rinsed thoroughly with PBS and placed into a vial containing 5 ml of Cytoscint^R (ICN Chemical, Irvine, CA). The amount of radioactivity was then quantified using a β-counter. We routinely use this method of whole cell counting rather than isolating the DNA because we have determined that the amount of radioactivity trapped in non-DNA compartments after thorough washes with PBS is of a negligible quantity (Couldwell *et al.*, 1990).

In another set of experiments, neonatal mouse astrocytes were treated with test factors (see Results) and then analysed simultaneously using GFAP-BrdU immunofluorescence, [^{3}H]-thymidine incorporation, and counting of cell numbers, to allow comparisons between these methods. For counting of cell numbers, astrocytes were seeded at a density of 200,000 cells in 400 µl feeding medium in 60 mm PL-coated dishes. The next day, 3 ml of feeding medium and appropriate test factors were added. Cells were left for 4 days at 37°C, and removed from their dishes with 0.25% trypsin (30

min). The dishes were washed thoroughly with PBS to maximize cell yield. Following centrifugation at 1,200 rpm, 10 min, cells were suspended in 150 µl of PBS. A 15 µl aliquot was taken for cell counting using a haemocytometer to obtain values for total number of cells per sample.

Treatment of Cells with Test Factors

Unless otherwise described, all cells were treated with test agents for a total period of 4 days. For adult human cultures, 10 mM BrdU was added during the last 48 h because of their very low basal rate of proliferation (see results). For neonatal mouse or fetal human cells, 10 mM BrdU or 1 µCi [^{3}H]-thymidine were administered during the last 16 h because of their higher rate of proliferation than adult human astrocytes (see Results); adult mouse astrocytes were treated with BrdU during the last 24 h of experiment. These time frames were selected based on preliminary experiments to optimize conditions for identifying mitogens or inhibitors of astrocytic proliferation. All experiments were conducted in feeding medium containing 5% FBS unless indicated otherwise; the rate of proliferation of control cultures in this 5% FBS medium is henceforth referred to as basal rate.

Test agents were recombinant human or mouse γ-IFN (both from Genzyme), FGF (Boehringer-Mannheim), and 4β-phorbol-12,13-dibutyrate (PBD), a biologically active phorbol ester. Unless otherwise stated, for γ-IFN, human cells were treated with the recombinant human cytokine while mouse cells were exposed to the murine reagent.

Chromium Release Assay

To assess whether recombinant mouse γ-IFN could be cytotoxic to neonatal mouse astrocytes, cells were seeded at a density of 50,000 cells/well in flat-bottom 96-well plates. Two days later, the cells were incubated with 1 µCi/well of ^{51}Chromium for 18 h. Cells were gently washed twice with 5% FBS-containing feeding medium and allowed to stand for 30 min after which γ-IFN (100 or 500 U/ml final concentration) was added for 6 or 18 h; longer incubation periods were not possible because of the inherent

toxicity of ^{51}Chromium (see Results). Supernatants (A) were then removed and counted in a γ-counter for spontaneous release of ^{51}Chromium. Aliquots of 5 M NaOH were then added to each well for 20 min and supernatants (B) removed and counted for remaining radioactivity. The percentage lysis was calculated by dividing the cpm of supernatant A by the total labelling (A + B). Positive controls for cell lysis were 30% ethanol in normal feeding medium.

Results

Effects of Υ-IFN on Adult Human or Neonatal Mouse Astrocytes

In an earlier report that involved astrocytes from four adult human series, we showed that recombinant human γ-IFN at 10, 100, and 1,000 U/ml enhanced the proliferation of adult human astrocytes (Yong *et al.*, 1991a). We have extended that study and demonstrate here that 100 U/ml of γ-IFN enhanced proliferation in all of 11 different adult human cultures tested; in eight of these, statistical significance was attained (Table 1).

In contrast to the human cells, application of 100 U/ml of recombinant murine γ-IFN to neonatal mouse cultures resulted in an average of 50% inhibition of [^{3}H]-thymidine incorporation; this was evident in all 12 different experiments involving eight series of mouse cultures (Fig. 2). The inhibitory effects of γ-IFN were dose-dependent (Fig. 3) and did not appear to be due to general cell cytotoxicity as assessed using a ^{51}Chromium release assay (Table 2). In a different set of experiments, neonatal mouse cultures were treated with a single dose of 100 U/ml γ-IFN for different time periods ranging from 6 to 96 h; [^{3}H]-thymidine was added during the last 16 h for all time points except for the 6 h mark (6 h [^{3}H]-thymidine pulse). This was to examine whether there was an initial burst of proliferation which was not reflected in the subsequent 4 day harvest. Figure 4 shows that recombinant murine γ-IFN did not at any period increase the proliferation of neonatal mouse astrocytes.

Correspondence in BrdU Incorporation, [^{3}H]-Thymidine Uptake, and Cell Numbers

Since the neonatal mouse astrocyte results were obtained by using [^{3}H]-thymidine incorporation in contrast to the GFAP-BrdU immunofluorescence for adult human cells, it was important to show that the two methods produce comparable results. Neonatal mouse astrocytes were incubated with agents known to affect their proliferation for a total of 4 days, with 10 mM BrdU or 1 mCi [^{3}H]-thymidine added during the last 16 h. These agents included γ-IFN (100 U/ml), FGF (20 ng/ml), or PDB (100 nM). By both methods of assessing proliferation, PDB and FGF were mitogenic while γ-IFN was inhibitory. Results of both assays were virtual mirror images of one another (Fig. 5) and correlation analysis of [^{3}H]-thymidine uptake with the BrdU data gave a correlation value of 0.99 ($P = 0.001$).

A third set of neonatal mouse astrocytes was exposed to the above treatments for 4 days, and counting of cell numbers then performed as described in Methods. The resultant changes in cell numbers in treatment groups compared to controls mirrored data obtained using either the GFAP-BrdU or [^{3}H]-thymidine techniques (Fig. 5). Indeed, correlation analyses of cell numbers with GFAP-BrdU or [^{3}H]-thymidine uptake results gave in both cases a correlation value of 0.99 ($P = 0.001$). These experiments illustrate that the differential response of adult human and neonatal mouse astrocytes to γ-IFN was not due to the techniques used to assess proliferation. Furthermore, the techniques of GFAP-BrdU immunofluorescence and [^{3}H]-thymidine incorporation to assay for astrocytic proliferation are reflective of changes in actual cell numbers.

Basal Rate of Proliferation of Neonatal Mouse and Adult Human Astrocytes

We have compared the rate of BrdU incorporation of neonatal mouse and adult human astrocytes in 5% FBS-containing medium in an attempt to investigate their relative mitotic capability. Cells on coverslips, and plated similarly at 10,000 cells/coverslip, were pulsed with 10 μM BrdU for different periods ranging from 2 to 96 h and subjected to GFAP-BrdU immunohistochemistry. As shown in Figure 6, adult

human astrocytes have a much lower rate of proliferation. Discernible BrdU uptake (0.2% of astrocytes) was detected only after 24 h of BrdU incubation, and this increases to 3% and 12% after 48 and 96 h, respectively. In contrast, a significant proportion of neonatal mouse astrocytes had incorporated BrdU by 2 h (15%); corresponding values for 24, 48, and 96 h were 32%, 57%, and 61%, respectively.

The proliferation rate of adult human astrocytes could be enhanced if the content of FBS in the medium was increased. Thus, for a total BrdU treatment period of 96 h, the % of $GFAP^+$ cells which had incorporated BrdU in 15% FBS medium was 9.3 ± 1.9 (mean ± SEM); in 30% FBS, the value was 13.2 ± 1.8%.

Effects of γ-IFN Treatment on Proliferation of Fetal Human and Adult Mouse Astrocytes

The differential response of adult human and neonatal mouse astrocytes to γ-IFN could be due to at least two factors: the age of the subjects from which the cells were derived (which is reflected in the vast difference in basal rate of BrdU incorporation as shown in Fig. 6), or species differences. These possibilities were addressed by culturing astrocytes from fetal human or 6-month-old adult mouse brains, and exposing the cells to cytokines. Figure 7 demonstrates that a single application of recombinant human γ-IFN over 4 days increased the proliferation of fetal human astrocytes when used at 100 and 1,000 U/ml. The results reflected those for adult human astrocytes with the exception that 10 U/ml was not an effective dose for fetal human cells. Because of this discrepancy, which could have been due to a faster rate of metabolism of γ-IFN by fetal cells, a single dose of γ-IFN for 2 days, or a 4-day treatment period with two applications every second day, were instituted. Under such conditions, γ-IFN significantly promoted proliferation of fetal human astrocytes from 10 to 1,000 U/ml (Fig. 7). In light of the changes in frequency and period of treatment for fetal human cells, neonatal mouse astrocytes were similarly treated with recombinant murine γ-IFN. However, inhibition of proliferation was observed under all paradigms (Fig. 7).

The basal rate of proliferation of fetal human astrocytes is high, and is equivalent to that of neonatal mouse astrocytes (Fig. 6). Thus, despite comparable basal rates of

proliferation, neonatal mouse astrocyte cultures were inhibited by γ-IFN while fetal human cells responded to this cytokine by an increase in DNA synthesis.

Adult mouse astrocytes, as with their neonatal counterparts, responded to recombinant murine γ-IFN with a decrease in the rate of proliferation (Table 3). With regards to the basal rate of proliferation, different series of mouse cultures tended to have varying rates (see Chapter 2); these either approximated those of adult human astrocytes, or were between those of adult human and neonatal mouse/fetal human astrocytes. However, the inhibitory effect of γ-IFN on adult mouse astrocytes was evident in cultures that had comparable low basal rate of proliferation (Experiment 1 of Table 3) to adult human astrocytes (see Table 1), or in cultures with higher basal proliferative rate (Experiments 2 and 3 of Table 3).

Effects of Murine γ-IFN on Human Cells and Vice Versa

The results presented thus far have utilized recombinant mouse γ-IFN on mouse cells, and human γ-IFN on human astrocytes. To address the possibility that the recombinant murine reagent contain inhibitory factors other than γ-IFN, fetal human astrocytes were incubated with 100 U/ml of murine γ-IFN for 4 days. Similarly, neonatal mouse cells were exposed to recombinant human γ-IFN at 100 U/ml. By [^{3}H] -thymidine analyses, there was no change in proliferation of either human (101 ± 5% of controls, n of 11 coverslips) or mouse (103 ± 8% of controls, n of 8) astrocytes under these circumstances. The failure of murine γ-IFN to affect human cells, and vice versa, is in accordance with reports that interaction of γ-IFN with its receptor to elicit a response occurs in a species specific manner (Hemmi *et al.*, 1989; Gray *et al.*, 1989).

Discussion

A major consequence of injuries to the CNS is the hypertrophy and proliferation of astrocytes. As the gliotic process evolves, an end-result can be the formation of a densely interwoven glial scar composed of many different cell types, myelin debris, collagen dense bundles in the extracellular space, and multiple layers of abnormal basal lamina (reviewed in Reier, 1986). The formation of glial scars has classically been considered undesirable and included among the many postulated detriments are inhibition of axonal growth or regeneration (reviewed in Reier, 1986), the genesis of a site of electrical instability and epilepsy (Pollen and Trachtenberg, 1970; Brotchi, 1979), and interference with remyelination (Raine and Bornstein, 1970).

More recent evidence, however, suggests that the process of astrocyte reactivity may actually be an attempt by these cells to promote CNS recovery and regeneration. This concept has evolved from studies indicating that normal astrocytes have neurotrophic properties (reviewed in Manthorpe *et al.*, 1986), that reactive astrocytes around the locus of a lesion may be the source of neurotrophic factors (Nieto-Sampedro *et al.*, 1983, 1987; Whittemore *et al.*, 1985; Needels *et al.*, 1986), and that early postnatal astrocytes embedded on nitrocellulose filters can provide a terrain suitable for axons to traverse the cerebral midline to reform a corpus callosum in previously acallosal perinatal or adult animals (Silver and Ogawa, 1983; Smith *et al.*, 1986). More recently, David *et al.* (1990) demonstrated that the reactive astrocyte-containing proximal portion of a transected adult rat optic nerve could support axonal growth of chick dorsal root ganglion neurons; ganglia placed distal to the lesion site, where no gliosis was evident, showed no such growth but this could be altered if the distal optic nerve was treated with macrophages from the lesioned CNS.

To attempt to resolve the neurotrophic or inhibitory potential of reactive astrocytes, one means would be to identify and regulate mediators of the proliferation of astrocytes. To this end, using cultured astrocytes as targets, several mitogens for astrocytes including cytokine and non-cytokine growth factors have been described. Largely, these studies utilized astrocytes from perinatal animals. At issue is whether the perinatal astrocytes serve as good model systems to understanding the evolution and

properties of the reactive astrocyte in the adult CNS, since gliosis occurs to a lesser extent in injuries inflicted during embryonic or neonatal life (Sumi and Hager, 1968; Bignami and Dahl, 1976; Berry *et al.*, 1983; Barrett *et al.*, 1984).

Using adult human astrocytes as targets, we have shown that γ-IFN is a mitogen (Yong *et al.*, 1991a). We have reconfirmed this observation (Table 1) and further show that in contrast, neonatal mouse astrocytes responded to γ-IFN by a reduction in the rate of proliferation (Figs. 2-4). This differential proliferative response is not due to the age of the brains from which the astrocytes were derived, or to the disparate basal rate of proliferation, since fetal human astrocytes with similar characteristics (age and basal rate of proliferation) to neonatal mouse cells responded to γ-IFN by an increase in proliferation. Furthermore, the different means of assessing proliferation did not affect the outcome of the results since GFAP-BrdU immunofluorescence, [^{3}H]-thymidine incorporation or cell number counting all gave comparable data (Fig.5). Species differences likely is the main factor accounting for the differential response of human and mouse astrocytes to γ-IFN since adult mouse astrocytes responded in a similar manner to their neonatal counterparts (Table 3).

In reviewing the literature, we note that there have been reports of γ-IFN not affecting the rate of proliferation of astrocytes; however, these utilized human cytokine on calf bovine (Selmaj *et al.*, 1990) or neonatal rate (Benveniste *et al.*, 1989). As the results here suggest, the response of cells to recombinant γ-IFN occurs in a species specific manner. This has recently been confirmed by the observation that recombinant murine γ-IFN decreased the proliferation of neonatal mouse astrocytes (Johns *et al.*, 1992).

We have previously shown that application of murine γ-IFN into the adult mouse brain at injury increases the extent of GFAP-immunoreactive astrocytes (Yong *et al.*, 1991a). While the latter may appear at first glance to be at odds with the current adult mouse proliferation data here, the results emphasize the need for carefully distinguishing between an increase in GFAP immunoreactivity and proliferation *in vivo*. Clearly, an

increase in the number of GFAP-positive astrocytes (which may be due to exposure of previously hidden antigenic epitopes as a result of astrocyte hypertrophy) does not necessarily implicate astrocyte proliferation. Indeed, in the facial nerve resection model, where GFAP immunoreactive astrocytes appear, no proliferation of astrocytes has been documented (Graeber *et al.*, 1988). Furthermore, others have found that in the animal disease experimental allergic encephalomyelitis, increases in GFAP immunoreactivity did not correlate with alterations in GFAP content (Smith and Eng, 1987; Aquino *et al.*, 1988). Taken together, these and the current results suggest that the many prominent changes of a reactive astrocyte (increases in GFAP immunoreactivity and content, hypertrophy, and cellular proliferation) are not necessarily correlated. Indeed, the molecular mediators for each of these may be different. It is also possible that the same factor that increases the extent of GFAP immunoreactivity in the brain following an injury may concomitantly inhibit astrocytic proliferation. In conclusion, the response of astrocytes to γ-IFN occurs in a species specific manner. While this cytokine increases the proliferation of human astrocytes, it decreases that of mouse astrocytes. The age of the animals, and the corresponding basal rate of proliferation, were not determining factors. Studies aimed at defining the molecular mediators of astrocytic proliferation or gliosis will have to take into account the possible role of species differences.

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Figure Legends

Figure 1. ***GFAP-BrdU double immunofluorescence of adult (A,B) and fetal (C,D) human astrocytes.*** GFAP is shown in A and C while the corresponding BrdU labelling is featured in B and D. Astrocytes with BrdU incorporation are indicated by arrows in A and C. x 1,200. The morphology of neonatal and adult mouse astrocytes is similar to that shown for fetal human cells; adult human astrocytes (A) tend to be more fibrous in morphology.

Figure 2. ***Recombinant mouse γ-IFN decreases the proliferation rate of neonatal mouse astrocytes in 12 different experiments involving eight series of mouse cultures.*** Treatment period was for 4 days with 1 μCi [^{3}H]-thymidine added during the last 16 h. Each point is the mean ± SEM of four coverslips of cells. Student's t test was used to analyze statistical significance from untreated sister culture controls: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$,

Figure 3. ***Recombinant mouse γ-IFN decreases the proliferation of neonatal mouse astrocytes.*** Data from one representative experiment are shown, and each bar is the mean ± SEM of four coverslips, except for 500 U/ml where n was 7. *$P < 0.05$, using one-way ANOVA with Duncan's multiple comparisons.

Figure 4. ***Time course response of proliferation of neonatal mouse astrocytes to 100 U/ml of γ-IFN.*** Samples were harvested for liquid scintillation counting at the time points indicated. No initial burst in proliferation was observed. Results from one representative experiment are shown as mean ± SEM of four coverslips. *Significantly different from controls, $P < 0.05$ (one-way ANOVA with Duncan's multiple comparisons).

Figure 5. ***Results of proliferation of neonatal mouse astrocytes using either [^{3}H]-thymidine incorporation GFAP-BrdU double immunofluorescence or counting of cell numbers show good correspondence.*** Values are mean ± SEM, with number of samples shown in parentheses. All treated groups are statistically significant from controls (ANOVA, $P < 0.05$). Correlation analyses of results from all methods give r values of 0.99, with $P = 0.001$.

Figure 6. ***Neonatal mouse and fetal human astrocytes have comparable basal rates of proliferation which are much higher than that for adult human astrocytes.*** Values are mean ± SEM of four coverslips per culture, from one representative experiment. All results were obtained from coded specimens.

Figure 7. ***Υ-IFN increases the proliferation of fetal human astrocytes while decreasing that of neonatal mouse astrocytes.*** This was evident whether a 2- or 4-day treatment period was used, or when γ-IFN was applied twice over a 4-day period. Values ± SEM of [^{3}H]-thymidine uptake are expressed as % of controls, with the number of samples for fetal human cells indicated in parentheses. For neonatal mouse astrocytes, eight coverslips were used per data point. Results are pooled from seven experiments. *$P < 0.05$ using one-way ANOVA with Duncan's multiple comparisons.

Table 1. Effects of 100 U/ml recombinant human gamma-interferon on different adult human astrocyte cultures[a]

Culture Series	Age of Donor (years)	% GFAP-BrdU double positive cells	
		Controls	Gamma-Interferon
W16	17	5.7 ± 1.1 (6)	15.4 ± 5.7 (3)*
W32	49	2.8 ± 1.1 (6)	28.9 ± 7.6 (3)**
W57	36	0.7 ± 0.5 (4)	4.6 ± 0.9 (4)**
W66	27	1.1 ± 0.4 (6)	3.9 ± 1.5 (6)
W92	20	3.6 ± 0.8 (4)	9.9 ± 1.5 (4)**
W93	21	1.7 ± 0.7 (4)	3.3 ± 1.4 (4)
W94	26	4.4 ± 1.5 (4)	6.3 ± 0.6 (4)
W98	27	13.1 ± 0.9 (4)	39.3 ± 6.1 (4)***
W137	35	0.0 ± 0.0 (4)	11.6 ± 2.4 (4)***
W160	30	0.0 ± 0.0 (4)	11.7 ± 3.1 (4)**
W178	32	0.5 ± 0.3 (4)	9.7 ± 2.8 (4)**

[a] Values of the % of proliferating astrocytes are given as mean ± SEM, with the number of coverslips tested shown in parentheses. On each coverslip, an average of 127 ± 5 GFAP+ astrocytes were counted. Cultures were treated over a 4-day period. Significantly different from respective controls using Student's t test: *P < 0.05, **P < 0.01, ***P < 0.001.

Table 2. Gamma-interferon is not cytotoxic to neonatal mouse astrocytes: ^{51} Chromium release assays after 6 and 18 h treatments

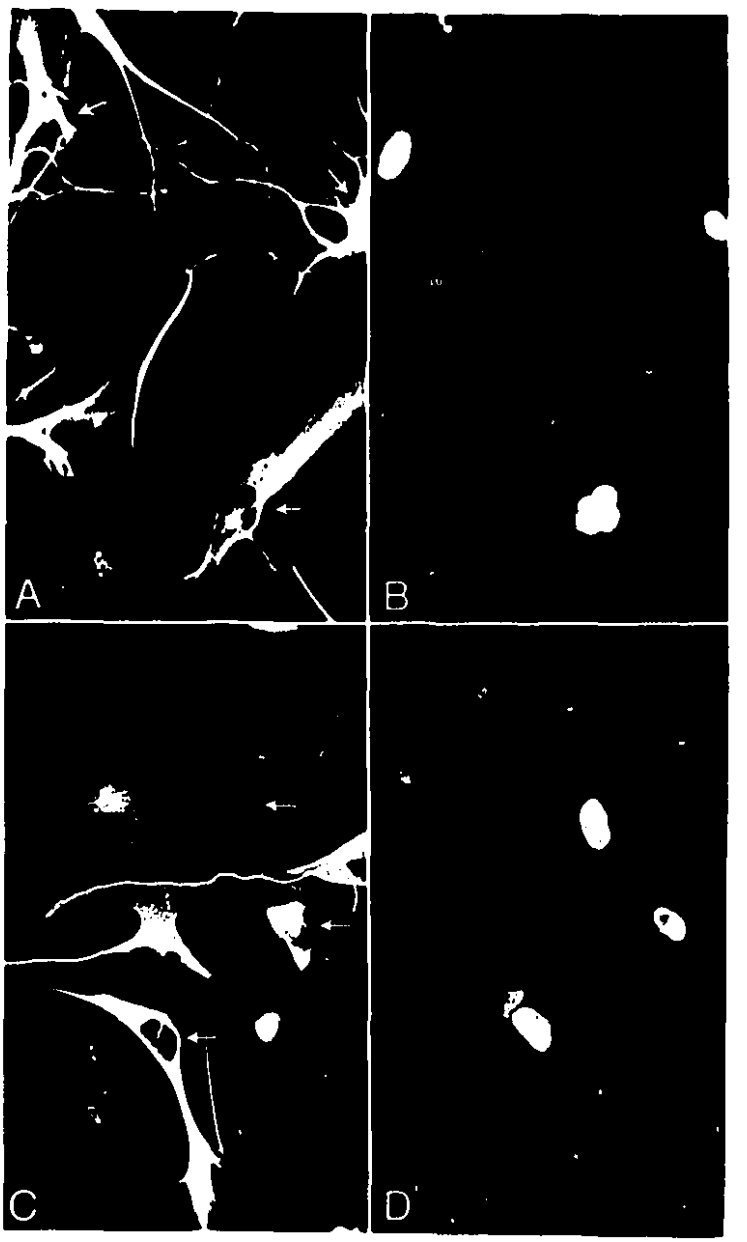
Agent	^{51}Chromium release (average % lysis) 6h	18 h
Normal Feeding Medium	19.6 ± 0.5 (24)	37.7 17 1.3 (24)
Positive Controls (30% EtOH)	83.1 ± 0.8 (11)*	84.4 ± 1.0 (12)*
Gamma-interferon 100 U/ml	18.7 ± 1.4 (10)	40.3 ± 2.3 (8)
Gamma-interferon 500 U/ml	22.6 ± 1.1 (7)	40.0 ± 2.0 (8)

[a] Values are mean ± SEM, with the number of samples shown in parentheses. ^{51} Chromium itself can lyse cells as determined by teh increase in normal control values over time.
*Significantly different from normal feeding medium controls, $P < 0.05$ (one-way ANOVA ith Duncan's multiple comparisons).

Table 3. Response of adult mouse astrocytes to gamma-interferon[a]

Cytokine	Experiment Number	% GFAP-BrdU double positive cells		% of Control
		Control	Cytokine	
γ-IFN 10 U/ml	1	8.2 ± 2.2 (5)	1.8 ± 0.9 (5)*	22.0
	2	29.2 ± 4.0 (5)	18.9 ± 5.5 (3)	64.7
γ-IFN 100 U/ml	1	8.2 ± 2.2 (5)	3.7 ± 1.7 (4)	45.1
	2	29.2 ± 4.0 (5)	9.6 ± 1.9 (4)*	32.9
	3	26.6 ± 0.6 (4)	13.8 ± 2.2 (4)*	51.9
γ-IFN 1000 U/ml	2	29.2 ± 4.0 (5)	9.6 ± 0.9 (4)*	32.9
	3	26.6 ± 0.6 (4)	11.0 ± 2.9 (4)*	41.4

[a]Values are mean ± SEM with the number of coverslips shown in parentheses. On each coverslip, an average of 165 ± 4 GFAP+ cells were counted. Results are from three experiments (Experiments 1 to 3) where cells were treated for 4 days with cytokines. In experiment 2, four other coverslips were incubated with 100 U/ml of γ-IFN for 2 days; the decrease in proliferation was apparent by this point (15.0 ± 2.7% of double positive cells, $P < 0.05$ from controls, representing a 48 % reduction). All results were analysed by one-way ANOVA with Duncan's multiple comparisons because of multiple treatment paradigms for each experiment. *$P < 0.05$.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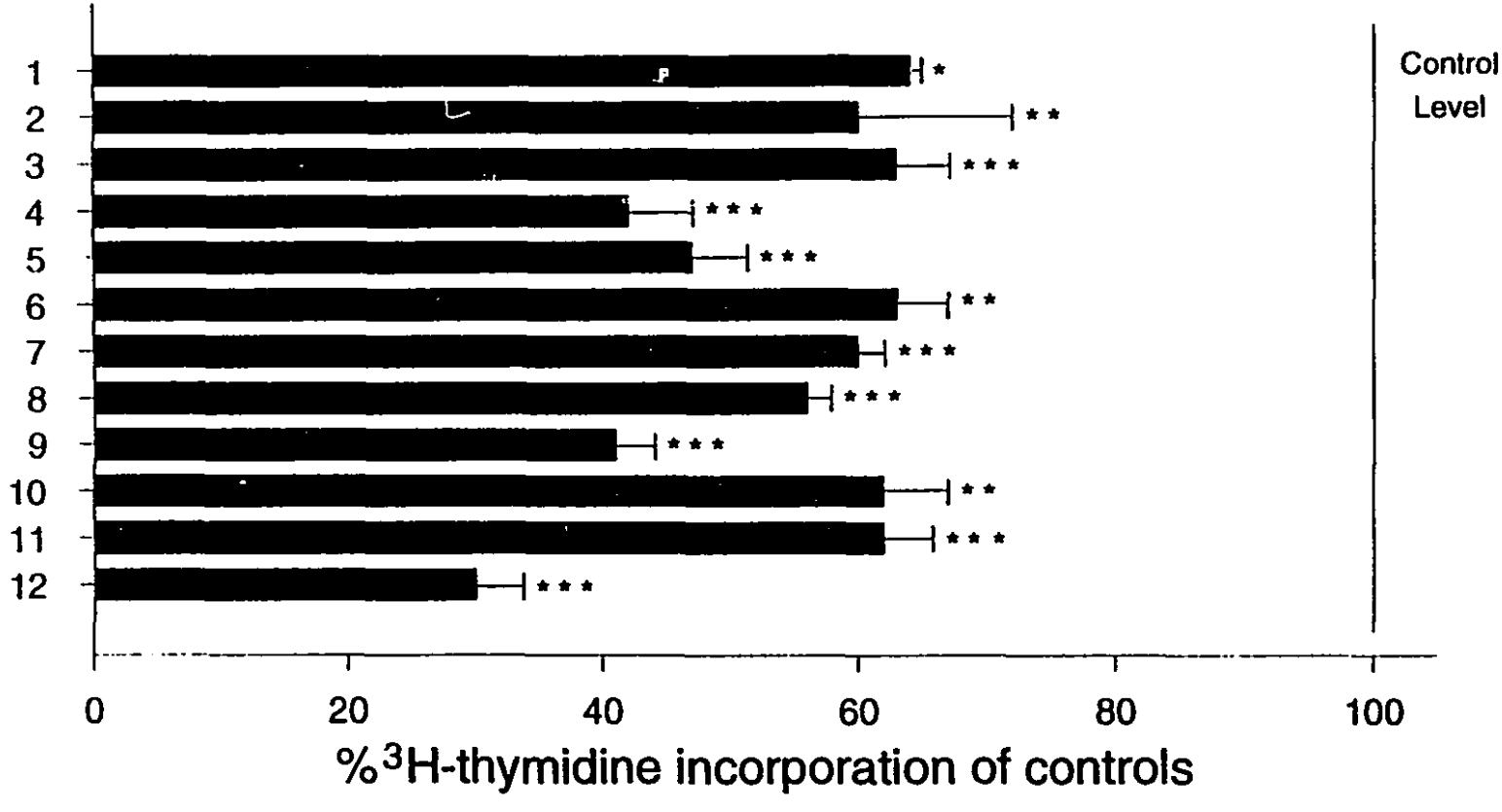
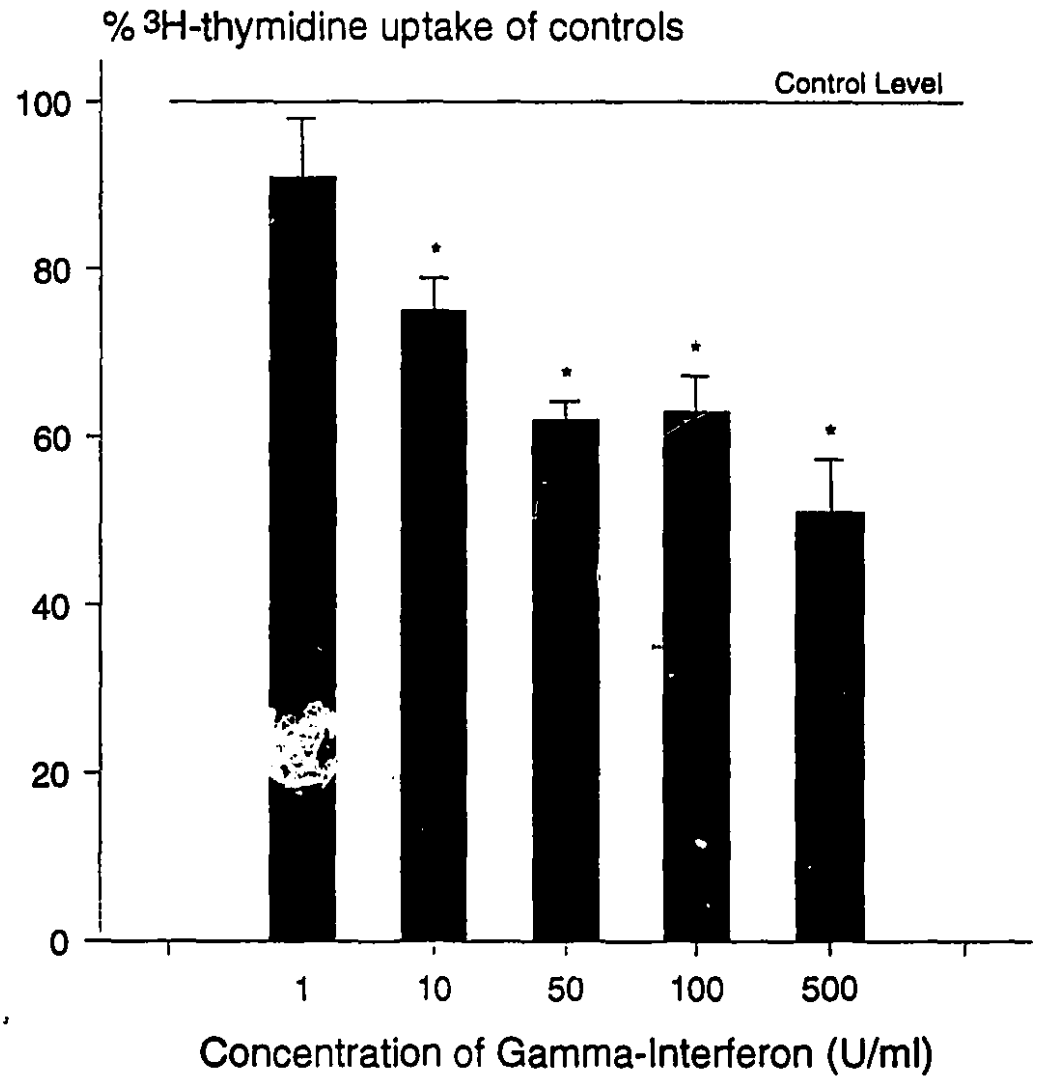
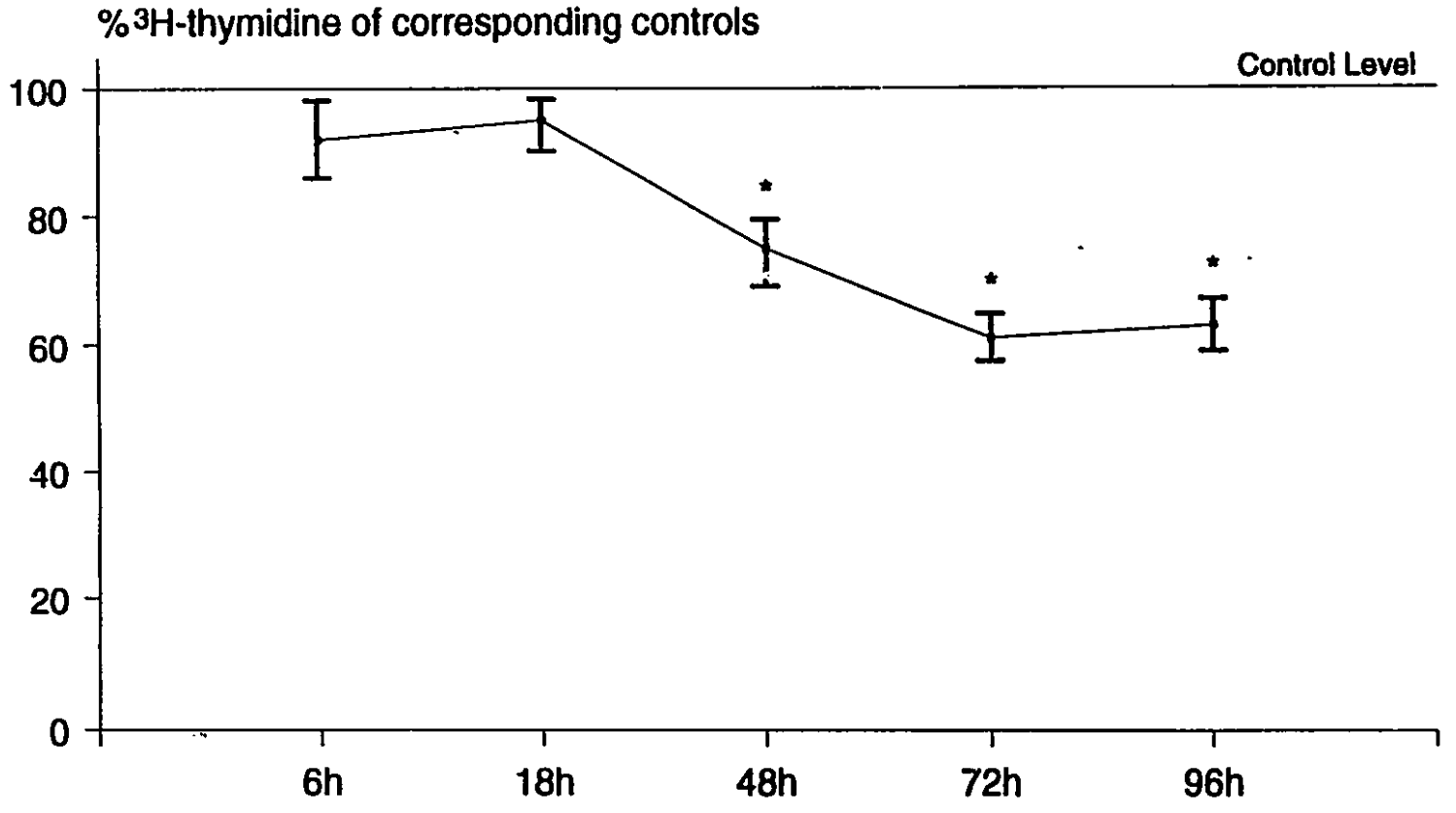
Recombinant Mouse Gamma-Interferon (100U/ml) Decreases Proliferation Of Neonatal Mouse Astrocytes.



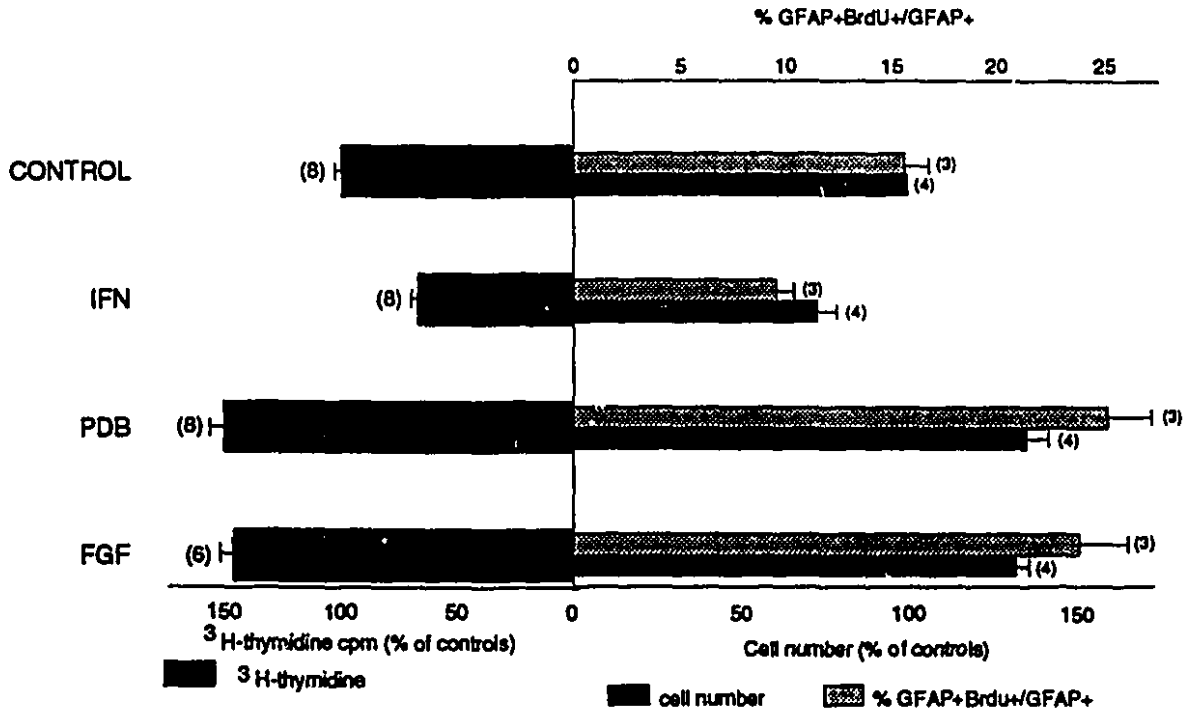
Dose-Dependent Reduction In Proliferation Of Neonatal Mouse Astrocytes By Gamma-Interferon



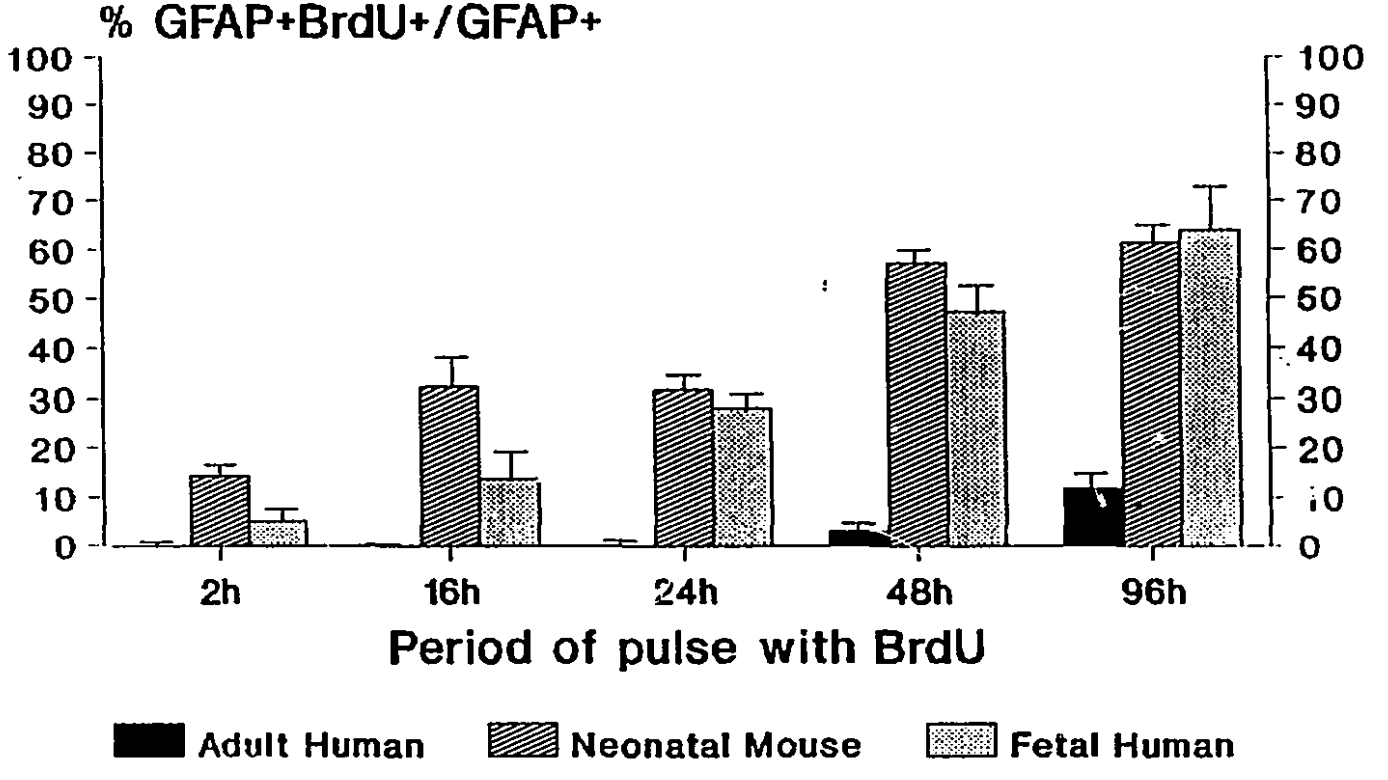
Time Course Response Of Proliferation Of Neonatal Mouse Astrocytes To Gamma Interferon (100U/ml)



Correspondence In Proliferation Of Neonatal Mouse Astrocytes As Assessed By ^{3}H-thymidine incorporation, GFAP-BrdU immunofluorescence, and cell numbers

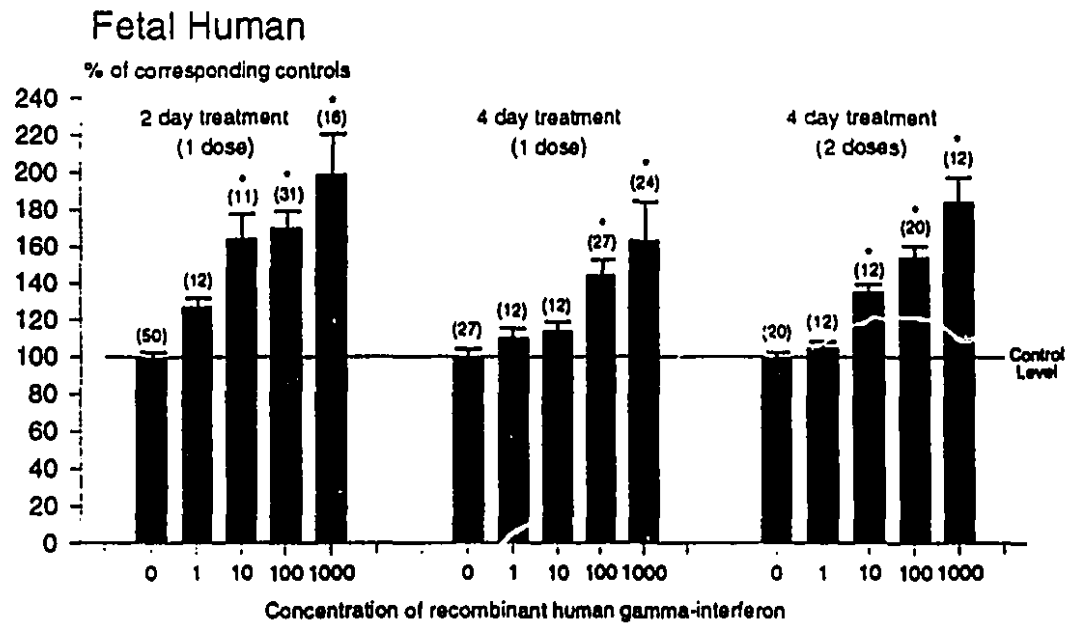


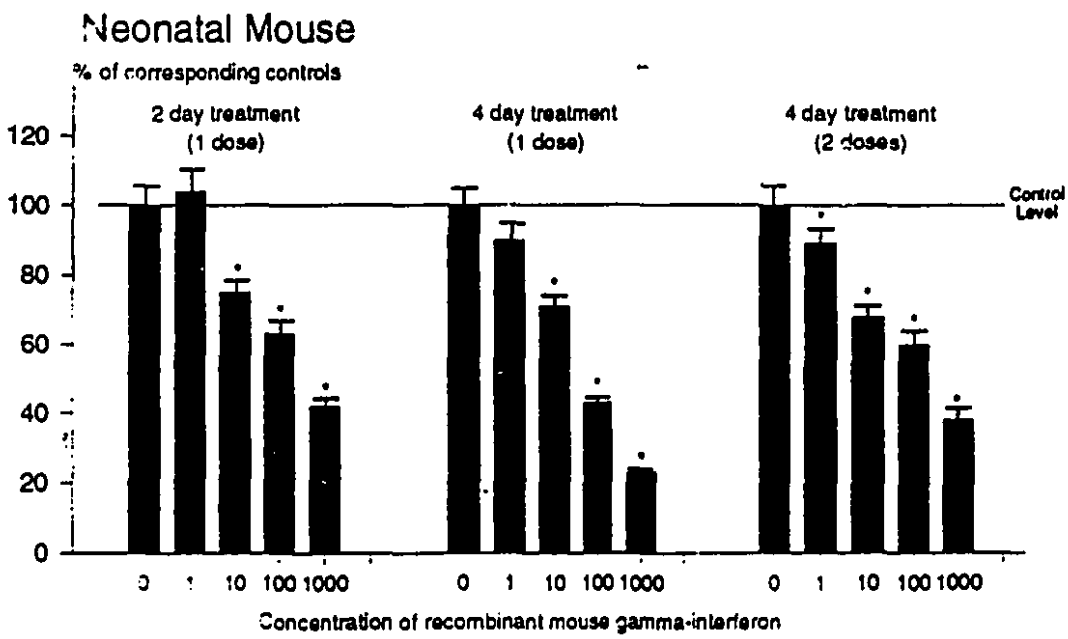
Comparison Of BrdU Uptake: Adult And Fetal Human, And Neonatal Mouse Astrocytes



Values are mean + SEM, of 4 coverslips per culture. All results were obtained from coded specimens.

Effects Of γ-IFN On Proliferation Of Fetal Human Or Neonatal Mouse Astrocytes As A Function Of Period And Frequency Of Treatment





Chapter 4

The Astrocyte Mitogen, Tumor Necrosis Factor-α , Inhibits the Proliferative Effect of More Potent Adult Human Astrocyte Mitogens, γ-Interferon and Activated T-Lymphocyte Supernatants

Trevor Tejada-Bergés and Voon Wee Yong

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Abstract

The proliferative response of adult human astrocytes to tumor necrosis factor-a (TNF-α) was examined. Applied alone, TNF-α was dependent on the content of serum in the feeding medium, being mitogenic only in conditions of over 15% serum in medium. In accordance with previous results, supernatants from activated human CD8⁺ T-lymphocytes (CD8 SN) and recombinant human interferon-γ (IFN-γ) enhanced proliferation of adult human astrocytes in 5% serum-containing medium. Simultaneous administration of TNF-α (10-1000 units), however, abrogated the mitogenic effects of either CD8 SN or IFN-γ ; the inhibitory effect of TNF-α was lost if applied 2 days following IFN-γ treatment. These studies show that while TNF-α is an astrocyte mitogen under selected conditions, it inhibits proliferation induced by other mitogens. In this manner, TNF-α may be important in regulating the proliferative response of astrocytes during reactive astrogliosis *in vivo*.

Introduction

Following trauma to the central nervous system (CNS), proliferation of astrocytes occurs as part of a process termed reactive astrogliosis (Cavanagh, 1970; Janeczko, 1988; Latov *et al.*, 1979; Miyake *et al.*, 1988; Topp *et al.*, 1989). The extent to which astrocyte hyperplasia contributes to the overall astroglial reactivity post-injury remains controversial (Graeber et al, 1988; Matsumoto *et al.*, 1992; Morshead and van der Kooy, 1990) and under study. Furthermore, the precise molecular signals which initiate and/or regulate the astrocytic response *in vivo* are not completely understood. A number of soluble molecules are reported mitogens for astrocytes *in vitro*. These include fibroblast growth factor, epidermal growth factor, glial growth factor from the bovine pituitary, platelet-derived growth factor, insulin-like growth factor-1, as well as biologically active phorbol esters (reviewed in Yong *et al.*, 1989).

Inflammatory mononuclear cells such as lymphocytes, blood-derived macrophages and intrinsic brain microglia are featured in many pathologic conditions of the brain, such as inflammatory brain diseases (Cuzner *et al.*, 1988; Perry *et al.*, 1993; Smith and Eng, 1987; Traugott *et al.*, 1983) and trauma resulting from stab wounds to the CNS (Giulian *et al.*, 1989; Sethna and Lampson, 1991; Tsuchihashi *et al.*, 1981). These inflammatory cells release cytokines and likely account for the measured increase in levels of interleukin (IL)-1, IL-2, IL-6 and TNF-α following brain trauma (Nieto-Sampedro and Berman, 1987; Nieto-Sampedro and Chandy, 1987; Taupin *et al.*, 1993; Woodroofe *et al.*, 1991). The cytokines secreted by the infiltrating and/or resident inflammatory cells may mediate features of astrogliosis. This notion is supported by studies where the administration of interleukin (IL)-1 (Giulian and Lachman, 1985; Giulian *et al.*, 1988) and IL-2 (Watts *et al.*, 1989) into the adult rodent brain increases the extent of GFAP immunoreactivity, a convenient marker of astroglial reactivity. Intraocular injections of IFN-γ, TNF-α and IL-1 evoke astrogliosis in rabbits (Brosnan *et al.*, 1989). In a recent study, we demonstrated that a single application of 20 units of cytokines (IFN-γ, IL-1, IL-6, TNF-α and M-CSF) into a cortical stab wound in neonatal

mice, which normally do not exhibit astrogliosis in contrast to similar injury in adults, converted minimal astroglial reactivity to extensive astrogliosis (Balasingam *et al.*, 1994).

In vitro, in keeping with the hypothesis that cytokines may regulate features of astrocyte reactivity, supernatants derived from activated T- or B-lymphocytes induced proliferation of cultured neonatal rodent astrocytes (Benveniste *et al.*, 1989; Fontana *et al.*, 1980; Merrill *et al.*, 1984). We have previously reported that supernatants from activated human T-lymphocytes (CD8 SN) are potent mitogens for adult human astrocytes as assessed by incorporation of bromodeoxyuridine (BrdU; Yong *et al.*, 1991). Purified cytokines such as IL-1 (Giulian and Lachman, 1985; Hunter *et al.*, 1993; Oh *et al.*, 1993), IL-6 (Selmaj *et al.*, 1990) and TNF-α (Oh *et al.*, 1993; Selmaj *et al.*, 1990) can enhance the proliferation of neonatal rodent or calf bovine astrocytes; TNF-α has been shown to promote proliferation of adult human astrocyte (Barna *et al.*, 1990). Finally, our laboratory has reported that recombinant human IFN-γ and IL-1 are mitogenic for adult human astrocytes *in vitro* (Yong *et al.*, 1991; Yong *et al.*, 1992).

While the use of purified cytokines on astrocytes in isolation has defined the capability of certain cytokines to mediate changes to the astrocyte, the brain is likely to encounter a multitude of cytokines during inflammation or trauma to the CNS. Potentially, these cytokines can act in a cooperative or antagonistic manner. Thus, studies where cytokines are applied in combination may more closely approximate the situation encountered *in vivo*. In this regard, TNF-α and IFN-γ are known to synergize in a variety of systems including the lysis of tumor cells (Williamson *et al.*, 1983) and expression of Class II major histocompatibility complex (MHC) antigens on rodent astrocytes (Benveniste *et al.*, 1989; Vidovic *et al.*, 1990). Sugarman *et al.* (1985) reported that, depending on the cell line tested, concomitant treatment with TNF-α and IFN-γ could either inhibit, enhance or minimally alter their growth.

In the present manuscript, we have examined the response of adult human astrocytes to TNF-α added either in isolation, or in combination with other cytokines.

We report that while TNF-α was a mitogen for adult human astrocytes under selected conditions, it inhibited the proliferative effects of more potent astrocyte mitogens, namely CD8 SN and IFN-γ. IL-1 did not display this antagonistic effect of TNF-α. As microglia (Frei *et al.*, 1988) and astrocytes (Robbins *et al.*, 1987; Lieberman *et al.*, 1989; Chung and Benveniste, 1990) are both capable of secreting TNF-α, and astrocytes express high-affinity TNF-α receptors (Benveniste *et al.*, 1989), TNF-α may act in both a para- and autocrine fashion to modulate the proliferative response of astrocytes. As such, these findings may be of relevance to the proliferative response of astrocytes evidenced *in vivo*.

Materials and Methods

Reagents

Recombinant human (rh) IFN-γ was from Boehringer Mannheim (Mannheim) while rhTNF-α and rhIL-1α,β were purchased from Genzyme (Boston, MA). Supernatants collected from $CD8^+$ human T-lymphocytes of over 95% purity (CD8 SN) and activated with an OKT3 antibody and IL-2 were obtained as described previously (Yong *et al.*, 1991). Bromodeoxyuridine (BrdU) was from Sigma (St. Louis, MO). The culture medium components (Eagle's minimum essential medium, fetal bovine serum, dextrose and gentamicin) were all from Gibco (Grand Island, NY). A rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP) was obtained from Dako Corporation (Westchester, PA), while a mouse monoclonal antibody to BrdU was bought from Becton-Dickinson (San José, CA). The secondary antibodies used were affinity-purified goat anti-rabbit Ig conjugated to fluorescein-isothiocyanate (FITC) and goat anti-mouse Ig conjugated to rhodamine (Rh), both of which were purchased from Cappell (Lexington, MA).

Primary glial cell cultures

Human astrocytes were isolated from the brain biopsy specimens of 8 patients (average age: 30 years, ranging from 24 to 35 years) undergoing surgery for the treatment of intractable epilepsy. None of the patients demonstrated signs of progressive neurologic, neoplastic or systemic diseases. Cell dissociation was by a protocol described in detail elsewhere (Yong and Antel, 1992). Briefly, the tissue was incubated with 0.25% trypsin (Gibco) and 20 µg/ml DNAse (Boeringer Mannheim) for approximately one hour at 37°C. The dissociated cells were sieved through a nylon mesh of 132 mm pore size which served to retain undissociated tissue fragments. The undissociated tissue was gently forced through the mesh. The filtrate was centrifuged in a 30% Percoll (Pharmacia, LKB Biotechnology Uppsala) gradient at 15,000 rpm for 30 minutes. The viable cell layer was collected, washed, resuspended in feeding medium and seeded onto uncoated Falcon tissue culture flasks (25 cm^2) at a density of 2 million/ml. All non-adherent cells (largely oligodendrocytes) were removed the following day for other studies. The remaining adherent cells, consisting primarily of astrocytes and microglia, were allowed to develop morphologically for approximately 1 to 2 weeks. During this period, the less-adherent astrocytes tended to stratify themselves on top of the more adherent microglial cells and could be shaken off (160 rpm, 5 hours at room temperature) and seeded onto 9 mm poly-l-lysine-coated (10 µg/ml) Aclar coverslips at a density of 10^4 cells/coverslip. Purity of adult human astrocytes (GFAP-positive cells) was 70%, and microglial cells (Leu-M5 immunoreactivity) were the major contaminating cell type which could not be eliminated completely (Yong and Antel, 1992). Contaminating fibroblasts only appeared in large numbers after approximately one month *in vitro*.

Unless otherwise specified, the feeding medium consists of Eagle's minimum essential medium supplemented with 5% fetal bovine serum (FBS), 1 mg/ml dextrose and 20 µg/ml gentamicin.

Assessment of astrocytic proliferation

The number of astrocytes in the S-phase of the cell cycle was visualized using a GFAP-BrdU double immunofluorescence technique, which allowed precise identification of proliferating astrocytes. The cells were pulsed for 48 hours with 10 mM BrdU (Sigma), a thymidine analog incorporated during the S-phase of mitosis. The GFAP-BrdU double immunofluorescence technique is described in detail elsewhere (Yong and Kim, 1987). Briefly, cells were fixed in 70% ethanol at -20°C for 30 minutes. The cells were rehydrated with phosphate-buffered saline (PBS), and incubated with a polyclonal rabbit antibody to GFAP (1:100) for 45 minutes. The secondary antibody was a goat anti-rabbit Ig conjugated to FITC (1:100; Cappell), and was added for 45 minutes. To denature the cellular DNA and allow the antibody access to any BrdU which may have been incorporated, the cells were treated with 2N HCl for 10 minutes followed by sodium borate (pH 9.0) for another 10 minutes. The BrdU was visualized by a monoclonal mouse antibody to BrdU (1:10) and a Rh-conjugated goat anti-mouse Ig (1:150, Cappel) secondary antibody. Coverslips were mounted on glass slides with Gelvatol and coded to allow for blind analysis. Using a Leitz fluorescence microscope equipped with FITC and Rh optics, the number of GFAP-BrdU double-positive cells as a percentage of all GFAP-positive astrocytes was assessed. Figure 1 illustrates the results of this double-immunofluorescence technique. We have documented that the results obtained with this GFAP-BrdU double immunofluorescence technique correspond well with changes in actual cell numbers (Yong *et al.*, 1992).

Treatment of cells with reagents

Preliminary experiments had indicated that a 4-day incubation period with test factors, with BrdU applied during the last 2 days, was optimal to observe a proliferative response of adult human astrocytes in 5% FBS medium; this regimen likely reflects the low basal rate of proliferation of adult human astrocytes in culture. The period of BrdU pulse could be considerably shortened (e.g. 2 or 16 hours) had the mitotically active fetal human or neonatal mouse astrocytes been utilized (Yong *et al.*, 1992). Thus, all adult human cultures were treated with a single application of the test factors for a period of 4

days, and 10 mM BrdU was added to the medium during the last 48 hours. CD8 SN was applied at a ratio of 1:1 with 5% FBS-containing medium.

Statistical analysis

Treated and untreated responses were analyzed using a one-way ANOVA with Duncan's multiple comparisons of all groups since all experiments involved numerous groups (see Fig. 2 to 5). Differences were considered significant if $p < 0.05$.

Results

The proliferative response of astrocytes to TNF-α is dependent on the content of serum in the feeding medium

Initial experiments in 5% FBS-containing medium showed that 100 U/ml of TNF-α did not enhance proliferation of adult human astrocytes. Subsequent examination revealed that this was dependent on the serum content in the culture medium. Figure 2 illustrates that increasing the serum content in the feeding medium raised the rate of BrdU incorporation in control cultures. Addition of 100 U/ml TNF-α potentiated this response in sister cultures. While the increase in proliferation of adult human astrocytes induced by TNF-α did not attain statistical significance in conditions of 0 to 5% FBS when compared to their corresponding controls, it was statistically significant when cells were grown in 15 and 30% FBS-containing medium.

Effect of TNF-α on adult human astrocytes in the presence of CD8 SN or IFN-γ

In contrast to TNF-α, the more potent mitogens CD8 SN and IFN-γ could enhance proliferation of adult human astrocytes in 5% FBS-containing medium (Fig. 3 and 4), in accordance with our previous observations (Yong *et al.*, 1991). In correspondence with previous findings (Yong *et al.*, 1991; Yong *et al.*, 1992), the basal rate of proliferation of adult human astrocytes tended to be very low and this is indicated by the less than 2% of control astrocytes with BrdU incorporation.

Co-incubation of TNF-α (at 10, 100 or 1000 U/ml) with CD8 SN in 5% FBS-containing medium decreased the proliferative effect of CD8 SN on astrocytes in 4 out of 5 adult human glial cultures (Fig. 3). In the fifth preparation (Series W90), 58% of astrocytes incorporated BrdU in response to CD8 SN. In this series, TNF-α (10, 100 and 1000 U/ml) was incapable of exerting its inhibitory effect.

Similarly, simultaneous treatment of astrocyte cultures with 100 U/ml of both IFN-γ and TNF-α in 5% FBS-containing medium resulted in an inhibition of the IFN-γ-induced proliferative response (Figure 4). The IFN-γ -induced proliferation was further utilized for kinetic analyses to determine when the inhibitory action of TNF-α could manifest. As noted in Figure 5, TNF-α inhibited the mitogenic effect of IFN-γ if added at the same time or at 1 day following IFN-γ application. However, if TNF-α was administered at 2 or 3 days following IFN-γ, the proliferative response of adult human astrocytes to IFN-γ was not antagonized by TNF-α (Fig. 5).

Effect of IL-1α,β on adult human astrocytes in the presence of IFN-γ

In order to test the relative specificity of action of TNF-α , IFN-γ was added in conjunction with another reported mitogen of cultured adult human astrocytes, IL-1 (61). Co-incubation of astrocytes with 100 U/ml each of IFN-γ and IL-1 in 5% FBS-containing medium resulted in no statistically significant inhibition of the IFN-γ-induced mitogenic activity, although an inhibitory trend was evident (Fig. 4).

Discussion

Proliferation of astrocytes during reactive astrogliosis is one of the parameters by which this characteristic glial response to CNS trauma has been described (Cavanagh, 1970; Latov *et al.*, 1979; Janeczko, 1988; Miyake *et al.*, 1988; Topp *et al.*, 1989). If the astroglial response continues unabated, a dense glial scar composed of many different cell types, myelin debris, collagen bundles in the extracellular space and multiple layers

of abnormal basal ganglia may form (for review see Reier, 1986). Astrogliosis has traditionally been considered detrimental to CNS regeneration (Raine and Bornstein, 1970; Reier, 1986) and as a possible source of electrical instability leading to epileptiform activity (Brotchi, 1979; Pollen and Trachtenberg, 1970). More recently, however, reactive astrocytes have been reported to have neurotrophic functions, and that these cells may be responding to injury in an attempt to promote CNS recovery (Silver and Ogawa, 1983; Kesslak *et al.*, 1986; Needels *et al.*, 1986; Smith *et al.*, 1986; Ip *et al.*, 1993; Yong, in press). Clearly, determining the signals which regulate astrogliosis is of importance to understanding the evolution and functional consequence of reactive astrogliosis.

As part of our ongoing research into assessing the role of cytokines as mediators of astrogliosis, we examined the response of mixed populations of cultured adult human glial cells to TNF-α in combination with other mitogens, specifically CD8 SN and IFN-γ. Our results of TNF-α being a mitogen for adult human astrocytes is in partial agreement with other reports of TNF-α promoting the proliferation of bovine, rodent and human astrocytes (Barna *et al.*, 1990; Selmaj *et al.*, 1990; Oh *et al.*, 1993) in that in our hands, the mitogenic effect of TNF-α is serum-concentration-dependent (Fig. 2). In 5% FBS-containing medium, a culture condition in which TNF-α was not mitogenic, TNF-α abrogated the proliferative response of CD8 SN and IFN-γ (Fig. 3 and 4). The inhibitory effect of TNF-α was time dependent in that efficacy was lost if administered 2 days following IFN-γ (Fig. 5). Presumably, by this period, astrocytes had already been committed by IFN-γ to enter the S-phase of the cell cycle. Partial specificity of the inhibitory effect of TNF-α is supported by the results that IL-1 could not inhibit the proliferative effect of IFN-γ. We are unable to specify what factor within the CD8 SN the TNF-α reacted with. Our laboratory has previously reported that a neutralising antibody to IFN-γ mitigated the proliferation evoked by CD8 SN (Yong *et al.*, 1991), and it is possible that TNF-α reacted with IFN-γ contained within the lymphocyte supernatant.

The paradigm supported by our data, that is, one mitogenic cytokine modulating the cellular response to another stimulator of DNA synthesis, is analogous to the results obtained upon treatment of astrocytes with epidermal growth factor (EGF) and fibroblast growth factor (FGF). While EGF was a stronger mitogen for neonatal rodent astrocytes than FGF, FGF pretreatment of cells inhibited the ability of EGF to stimulate DNA synthesis (Huff and Schreier, 1990). Interestingly, concomitant treatment of the neonatal rodent astrocytes with both factors failed to inhibit the rate of proliferation (Huff and Schreier, 1990).

The mechanism by which TNF-α inhibits astrocyte cell division remains unclear, although it is evident that this cytokine can promote the proliferation of some cell types {astrocytes as cited above, an astrocytoma line (Lachman *et al.*, 1987), and fibroblasts (Vilcek *et al.*, 1986)} while mediating cytostatic or cytotoxic responses on others {some tumor cell lines (Williamson *et al.*, 1983; Sugarman *et al.*, 1985)}. The total number of TNF-α receptors have been reported to increase upon treatment with IFN-γ (Aggarwal *et al.*, 1985; Benveniste *et al.*, 1989), and it is conceivable that the number of TNF-α receptors on adult human astrocytes in the co-presence of TNF-α and IFN-γ increased. Thus, one would have expected a potentiation rather than a decrease in astrocytic proliferation. While puzzling initially, our observations correspond with a report by Aggarwal *et al.* (1986) that while exposure of a human cervical carcinoma cell line to concanavalin A caused a 2-fold increase in receptors for TNF-α, these cells were protected by the lectin from the synergistic cytotoxic effects of TNF-α and IFN-γ. Sugarman *et al.* (1985) established that the diverse proliferative responses of three cell lines they studied in response to TNF-α did not reflect variations of number of binding sites. It appears that while the increased biologic consequences of TNF-α may be explained in part by an augmentation in TNF-α receptor expression, such a phenomenon is not sufficient to resolve fully the effects of TNF-α on responsive cells.

It should be noted that the purity of adult human astrocytes used in the present study is 70% at best, with microglia cells comprising the rest. Previous attempts to

further enrich for astrocytes by antibody-dependent complement-mediated lysis of microglia, or the use of leucine methyl ester, had failed to eliminate the resistant microglia cells (Yong and Antel, 1992). Thus, it is conceivable that the effects of TNF-α may be indirect, i.e. by microglial mediation. Although our previous study had indicated that the proliferative response of adult human astrocytes to IFN-γ was not dependent on the microglial content in culture (Yong *et al.*, 1991), this may not be the case for TNF-α. Nonetheless, when and if the opportunity presents itself, it would be preferable to use both astrocyte-purified and mixed glial (astrocytes with microglia) cultures for analyses of the TNF-α response.

Cooperation or antagonism between cytokines has been demonstrated in a number of studies. IFN-γ and TNF-α synergized to maximize expression of Class II MHC molecules on neonatal rodent (Benveniste *et al.*, 1989) and human astrocytes (Arenzana-Seisdedos *et al.*, 1988), as well as on human islet cells (Pujol-Borrell *et al.*, 1987); transforming growth factor-βs prevented the induction by TNF-α and IFN-γ of Class II MHC antigen expression on astrocytes (Schluesener, 1990). Synergy between cytokines has also been reported to enhance lysis of tumor cells (Williamson *et al.*, 1983) and to inhibit or enhance proliferation of normal and transformed cells *in vitro* (Lee *et al.*, 1984; Sugarman *et al.*, 1985). The cell type could also influence the synergy or antagonism of cytokines; while TNF-α increased the IFN-γ-induced Class II MHC expression on astrocytes, TNF-α decreased that induced by IFN-γ on endothelial cells (Tanaka and McCarron, 1990).

In conclusion, while TNF-α in isolation can be a mitogen for adult human astrocytes, it inhibits the proliferative effect of more potent mitogens, namely CD 8 SN and IFN-γ. Studies of astrogliosis *in vivo*, where the brain is likely to encounter a mixture of cytokines, need to take into account the potential interaction between cytokines.

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Figure Legends

Figure 1. **The technique of GFAP-BrdU double immunofluorescence to detect proliferating adult human astrocytes.** GFAP immunoreactivity is displayed in A and the corresponding BrdU labelling is shown in B. Astrocytes that have incorporated BrdU are shown by arrows in A. (X 800).

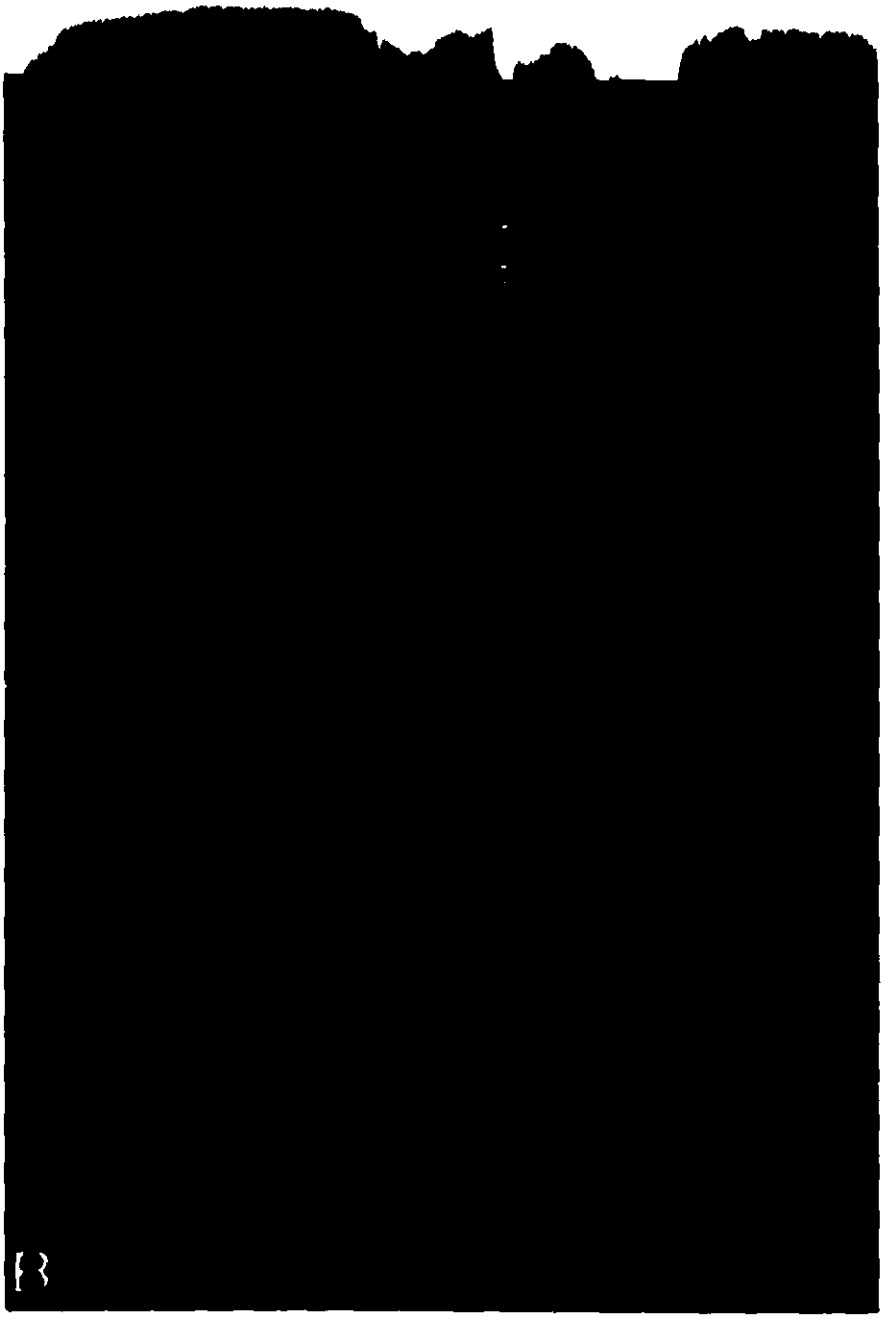
Figure 2. **The mitogenic effect of TNF-α on adult human astrocytes is dependent on the concentration of FBS in the feeding medium.** Values are mean ± S.E.M. of 4 coverslips, where on each coverslip an average of 149 ± 9 cells was evaluated blind. *P<0.05 compared to corresponding controls using 1-way ANOVA with Duncan's multiple comparisons.

Figure 3. **The mitogenic effect of CD8 SN on adult human astrocytes is prevented by the co-administration of TNF-α.** Results from 4 series of adult human astrocytes are shown. Each histogram is the mean ± S.E.M. of 4 coverslips of cells where on each coverslip, 152 ± 7 GFAP$^+$ astrocytes were evaluated blind. In a fifth preparation,where CD8 SN caused 58% of astrocytes to incorporate BrdU, TNF-α did not decrease the mitogenic effect of CD8 SN (results not shown). *P<0.05 compared to CD8 SN alone using 1-way ANOVA with Duncan's multiple comparisons.

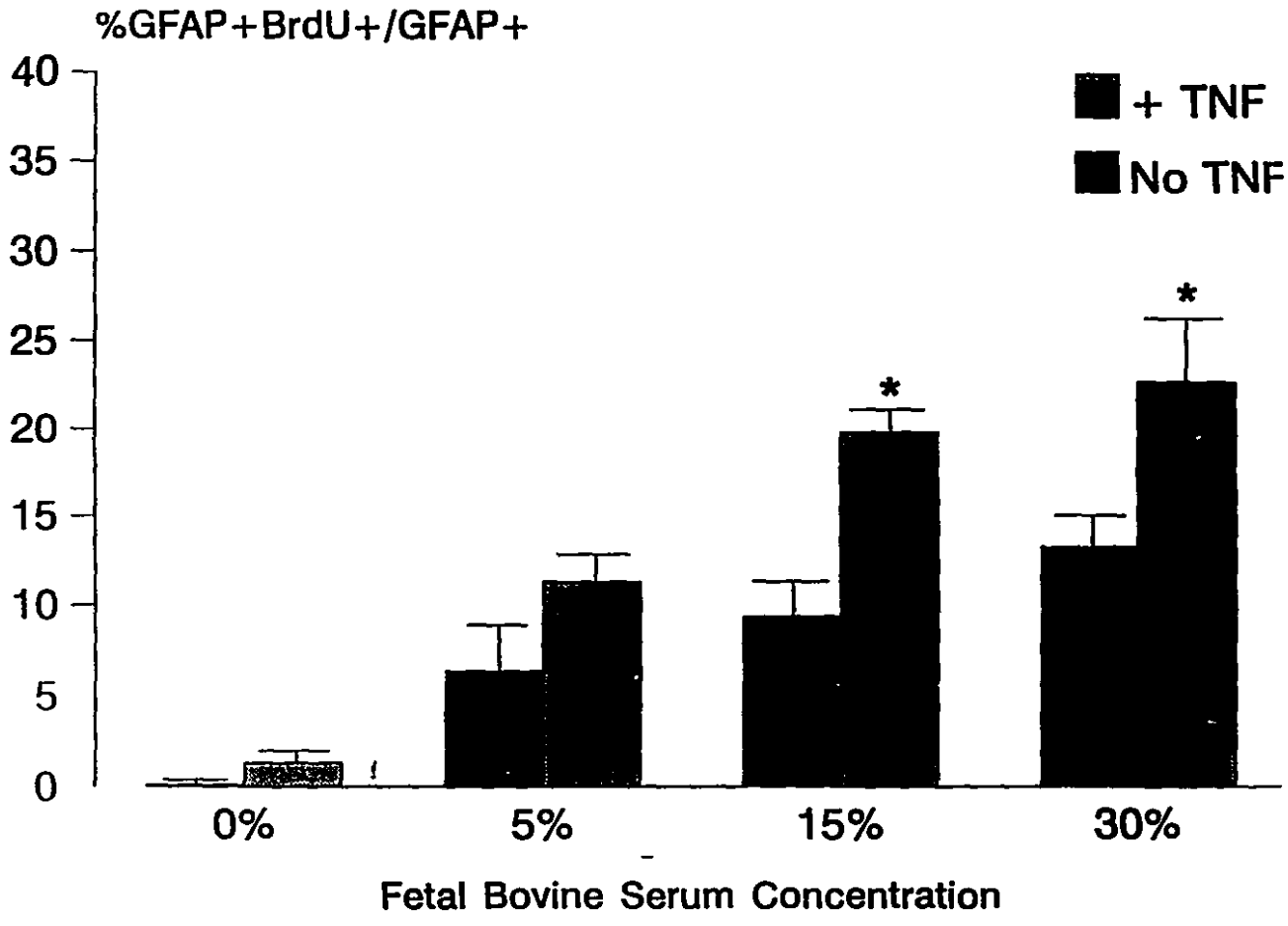
Figure 4. **The proliferative effect of γ-IFN on adult human astrocytes is antagonized by TNF-α but not by IL-1α,β.** Values are the mean ± S.E.M. of 4 coverslips where 168 ± 3 GFAP$^+$ astrocytes per coverslip were evaluated blind. *P<0.05 compared to γ-IFN alone using 1-way ANOVA with Duncan's multiple comparisons.

Figure 5. **The inhibitory action of TNF-α is lost if added 2 days after IFN-γ treatment.** Cells were incubated with 100 U/ml of IFN-γ or TNF-α alone, or were

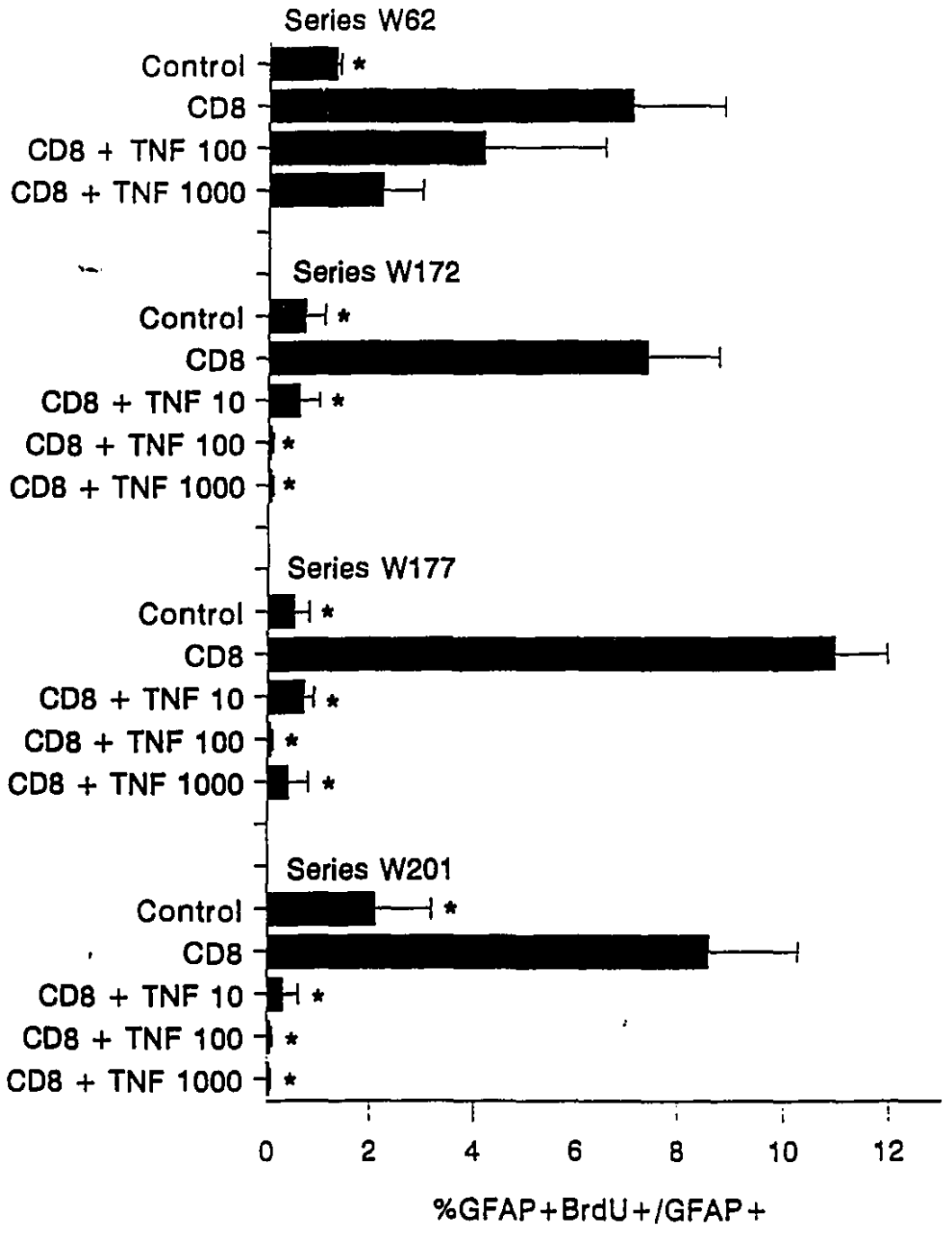
exposed to 100 U/ml of both cytokines. In the latter, TNF-α was added either at the same time as IFN-γ, or at 1, 2 or 3 days following IFN-γ. Values are mean ± S.E.M. from blinded analyses, and statistical significance was assessed by 1-way ANOVA with Duncan's multiple comparisons. *$P<0.05$ compared to IFN-γ alone. $P<0.05$ compared to controls.



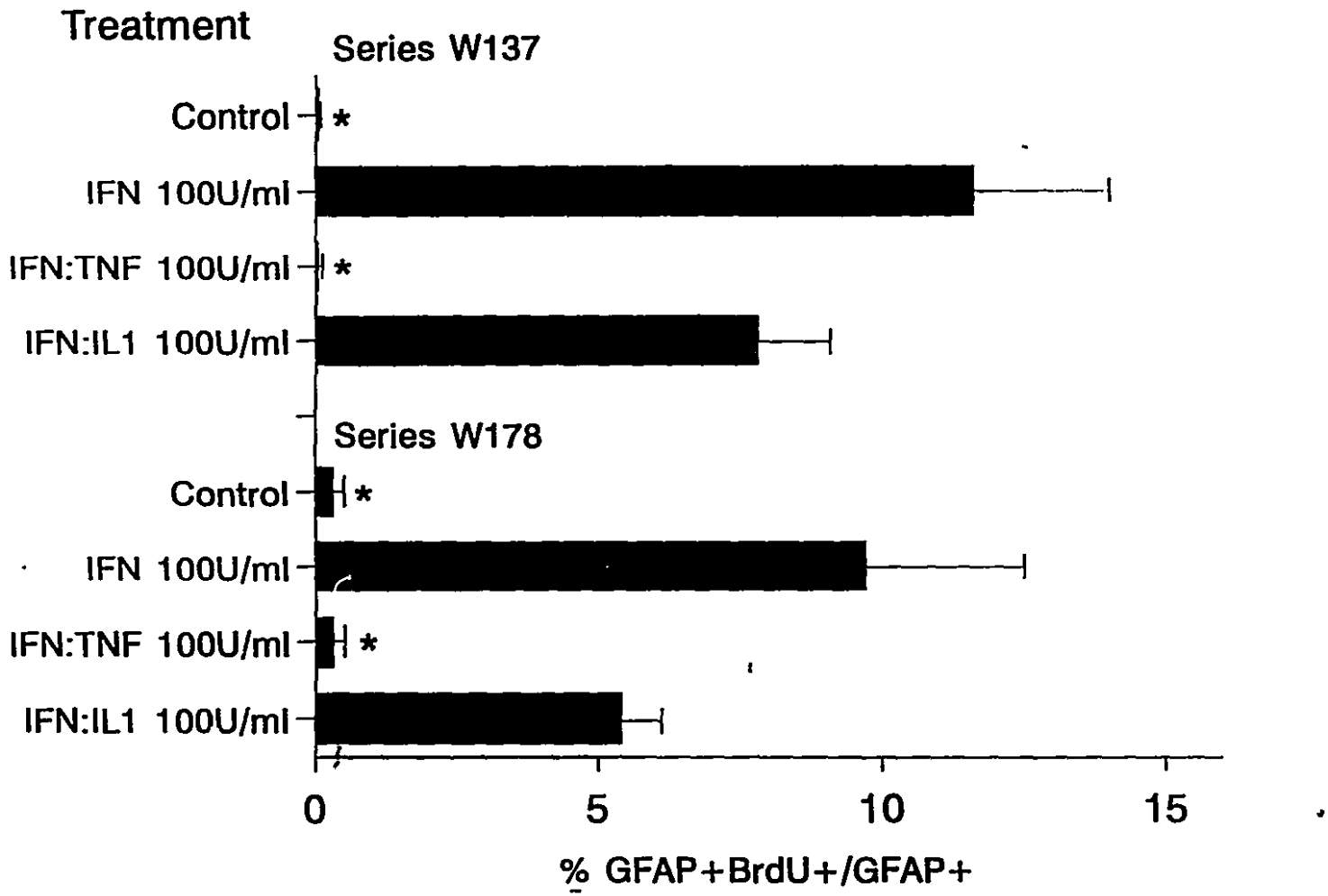
Effect of TNF-α on Proliferation of Adult Human Astrocytes in Medium Containing Different Amounts of Serum



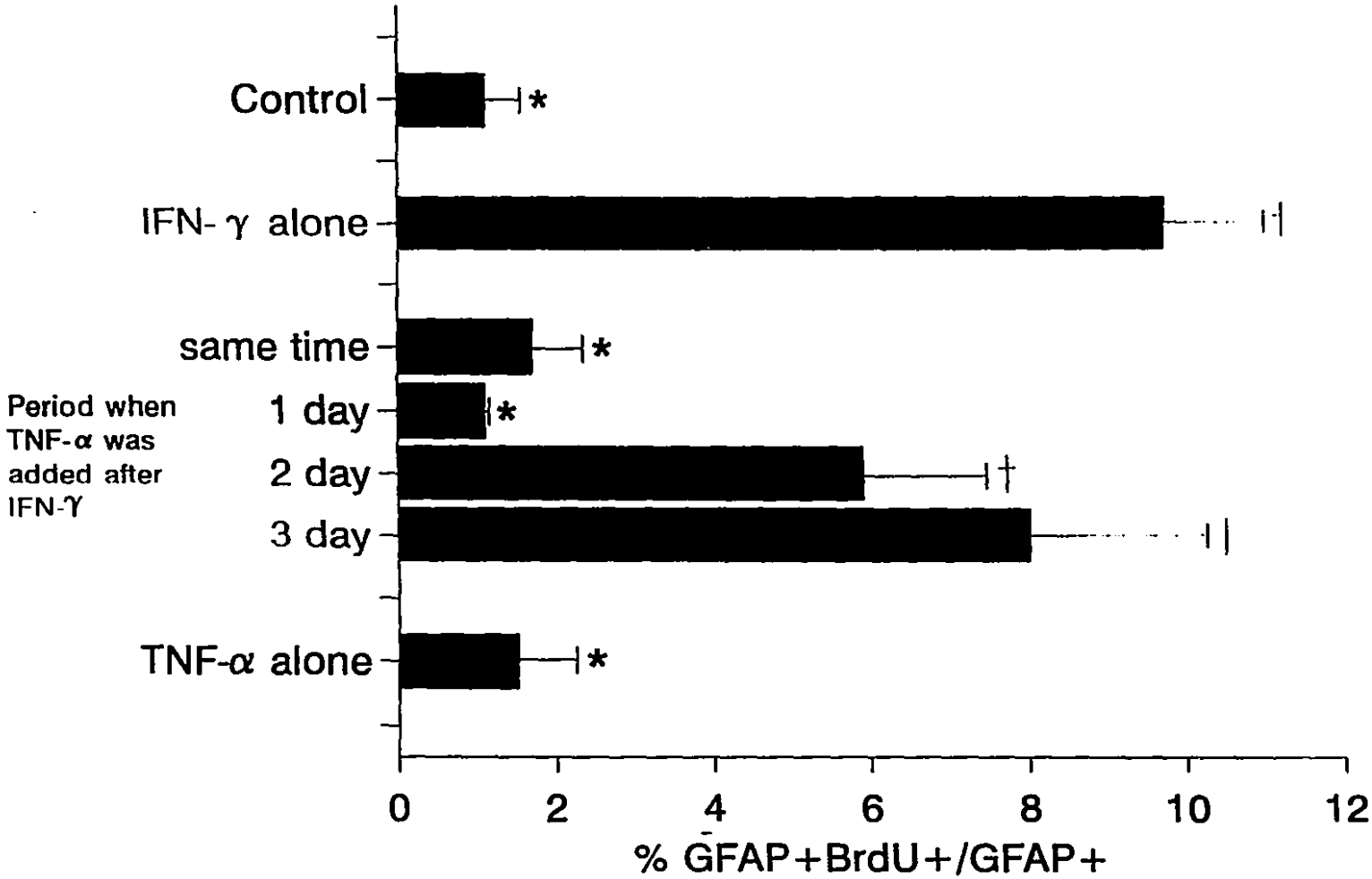
TNF-α Decreases the Mitogenic Effect of CD8 SN on Human Astrocytes in Vitro



Modulation of the Proliferative Effect of IFN-γ by TNF-α and IL-1 on Cultured Human Astrocytes



Temporal Analysis Of The Inhibition By TNF-α Of The IFN-γ -induced Proliferation Of Human Astrocytes



Chapter 5

Reactive Astrogliosis in the Neonatal Mouse Brain and Its Modulation by Cytokines

Vijayabalan Balasingam, Trevor Tejada-Bergés, Erin Wright, Radka Bouckova, and Voon Wee Yong

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Abstract

Reactive astrogliosis is a characteristic response of astrocytes to inflammation and trauma of the adult CNS. To assess the hypothesis that cytokines from inflammatory mononuclear cells that accumulate around lesion sites have a role in modulating astrogliosis, this study sought to take advantage of the neonatal system in which astrogliosis is reported to be minimal following injury and in which the immune system is relatively immature compared to adult animals. A nitrocellulose membrane implant into the cortex of postnatal day 3 mice resulted in a tremendous astrogliotic response 4 d later, as measured by glial fibrillary acidic protein (GFAP) immunoreactivity and GFAP content. In contrast, a neonatal stab wound produced limited astroglial response when compared to the adult stab wound. Utilizing the neonatal stab wound model, cytokines were microinjected into the wound site at the time of injury. All cytokines tested (γ-IFN, IL-1, IL-2, IL-6, TNF-α, and M-CSF) resulted in a significantly increased astrogliosis. The specificity of the cytokine response was demonstrated by the inability of human γ-IFN, but not mouse γ-IFN, in enhancing neonatal mouse astrogliosis, in accordance with reports

that the interaction of γ-IFN with its receptor occurs in a species-specific manner. We conclude that neonatal astrocytes can become reactive if an adequate injury stimulus is presented, and that the release of immunoregulatory cytokines by cells around lesion sites may be a mechanism that contributes to the production of gliosis.

Introduction

Reactive astrogliosis, where astrocytes undergo hypertrophy and/ or proliferation in addition to other histological and enzymatic changes, is a prominent aftermath following trauma and inflammation to the CNS (Latov *et al.*, 1979; Smith *et al.*, 1983; Mathewson and Berry, 1985; Maxwell *et al.*, 1990a,b). A longterm result of the astrocytic reaction can be the formation of a glial scar at the lesion site (Reier *et al.*, 1983; Liuzzi and Lasek, 1987), which, via yet poorly understood mechanisms, may inhibit axonal regeneration or remyelination.

Injury to the CNS also involves the recruitment of both endogenous and exogenous inflammatory mononuclear cells, particularly when the blood-brain barrier is breached (Kitamura *et al.*, 1972; Tsuchihashi *et al.*, 1981; Giulian, 1987; Giulian *et al.*, 1989; Morshead and van der Kooy, 1990; Milligan *et al.*, 1991; Woodroofe *et al.*, 1991; Taupin *et al.*, 1993). The cytokines released by the inflammatory mononuclear cells may have a role in modulating astrogliosis. This notion is supported by studies where the administration of interleukin- 1 (IL-1) (Giulian *et al.*, 1988), interleukin-2 (IL-2) (Watts *et al.*, 1989), and interferon-γ (Yong *et al.*, 1991a) into the adult rodent brain increases the extent of glial fibrillary acidic protein immunoreactivity (GFAP-IR). Intraocular injections of γ-IFN, tumor necrosis factor-α (TNF-α), and IL-1 have also been reported to evoke gliosis in rabbits (Brosnan *et al.*, 1989). In addition, *in vitro* evidence for the

proliferation of neonatal rat or calf bovine astrocytes in response to IL-1, IL-6, and TNF-α (Giulian and Lachman, 1985; Nieto-Sampedro and Berman, 1987; Selmaj *et al.*, 1990), and human astrocytic cell lines or primary human astrocytes to TNF-α and γ-IFN (Barna *et al.*, 1 990; Yong *et al.*, 1991 a, 1992) have given further credence to the direct or indirect role of cytokines in promoting astroglial reactivity.

While the presentation of astrogliosis is common to injuries occurring in the adult CNS, injuries inflicted during embryonic or neonatal periods have been observed to produce minimal astrogliosis, if any at all, in cortical stab wounds (Sumi and Hager, 1968; Bignami and Dahl, 1976; Berry *et al.*, 1983; Maxwell *et al.*, 1990b) and spinal cord lesions (Osterberg and Wattenberg, 1963; Gearhart *et al.*, 1979; Bernstein *et al.*, 1981; Barrett at al., 1984), although this contention has been challenged (Roessmann and Gambetti, 1986; Moore *et al.*, 1987; Trimmer and Wunderlich, 1990). Reasons postulated for the lack of astrogliosis in neonatal CNS injuries have included the relative immaturity and plasticity of neonatal astrocytes and neurons, and the lack of myelin in neonatal animals. Since the immune system in neonatal animals is relatively immature compared to adults (Hobbs, 1969; Abo *et al.*, 1983; Lu and Unanue, 1985; De Paoli *et al.*, 1988; Hannet *et al.*, 1992), the consequent lack of cytokine production to evoke astrogliosis may constitute a probable cause of the lack of astrogliosis following neonatal CNS injuries. To explore this postulate, the aim of the present study was to inflict damage to the neonatal brain, to document the resultant extent of astrogliosis, and to determine whether this extent could be increased by exogenously administered cytokines.

Initial studies using the implantation of a piece of nitrocellulose membrane (NC) into the cerebral cortex of postnatal day 3 (P3) mouse resulted in extensive GFAP-IR and increased GFAP content when measured 4 d post-injury. In contrast, a neonatal stab wound resulted in minimal astrogliosis, in accordance with the multitude of reports that have documented minimal astrogliosis in neonatal animals following a stab injury (Sumi and Hager, 1968; Bignami and Dahl, 1976; Berry *et al.*, 1983; Maxwell *et al.*, 1990b) compared to an adult stab wound (Cavanagh, 1970; Mathewson and Berry, 1985; Hozumi *et al.*, 1990; Maxwell *et al.*, 1990a).

Using the neonatal stab wound model, with its minimal astrogliosis, a bolus dose of cytokines (20 U in 2 μl) was administered to the stab cavity immediately following the injury. We demonstrate that while controls had minimal astrogliosis 4 d after, cytokine-treated animals had extensive astrogliosis. All cytokines tested [γ-IFN, IL-1, IL-2, IL-6, TNF-α, and human macrophage colony-stimulating factor (M-CSF)] provided for enhanced astrogliosis as determined by GFAP-IR. The species specificity of the cytokine effect was demonstrated by the inability of human γ-IFN to evoke a gliotic response in accordance with reports that the interaction of γ-IFN with its receptor occurs in a species-specific manner (Gray *et al.*, 1989; Hemmi *et al.*, 1989; Rubio and de Felipe, 1991; Plata-Salaman, 1992). The results suggest that the occurrence of astrogliosis in neonatal animals is dependent on the type of injury inflicted, and that the release of immunoregulatory cytokines by cells around lesion sites could be a mechanism that contributes to the production of astrogliosis.

Materials and Methods

Creation of brain injury in neonatal mouse

Postnatal day 3 CD 1 mouse pups (of either sex from standard-sized litters) obtained from a commercial source (Charles River Canada, Montreal, Canada) were anaesthetized with inhalational methoxyflurane. An incision was made in the skin overlying the skull, and an iris scissors was used to make a 1 mm cut in the skull. Three different types of injuries were then inflicted in groups of animals. For NC stab injury, a dry 1 mm^2 piece of nitrocellulose membrane (Schleicher and Schuell, Keene, NH) previously boiled in three changes of water to remove surfactant (Rudge *et al.*, 1989) was inserted into the parietal cortex perpendicular to the surface and removed immediately. For NC implant injury, animals were treated in the same manner but the membrane was left in place for the entire duration of the experiment (4 d). For scissors stab animals, an iris scissors was used to make a 1-mm-deep cut to the parietal cortex. The incision was

closed using Krazy Glue and the pups were kept under a heat lamp for 1 hr before being returned to their nursing mothers.

Creation of scissors stab injury in adult mouse brains

Female CD1 retired breeders (Charles River Canada, Montreal, Canada) were anaesthetized with an intraperitoneal injection of chloral hydrate (150 mg/kg) and immobilized in a stereotaxic frame. A midline incision was made and a unilateral circular (2-mm-diameter) craniectomy was performed over the left hemisphere by using a dental drill. The scissors stab injury was inflicted as described for neonates and the animals were kept under a heat lamp for 1 hr post-surgery.

Administration of cytokines to neonatal animals *in vivo*. Immediately following the scissors stab injury, a 22 gauge Hamilton microinjector (Hamilton Company, Reno, NV) attached to a stereotaxis instrument was used to deposit 2 µl of recombinant cytokine solution (10 U/µl) directly to the wound site. Injection rate was 1 ml/min. The skin incision was closed with Krazy Glue as above. The following recombinant cytokines suspended in 0.2% BSA were utilized: murine γ-IFN, human γ-IFN, human IL-1 (α,β), human IL-2, human IL-6, human tumor necrosis factor-α (TNF-α) and human macrophage colony-stimulating factor (M-CSF). These were chosen to reflect cytokines predominantly released by T-lymphocytes (γ-IFN, IL-2, and M-CSF) or microglia/ macrophages (IL-1, IL-6, TNF-α) that are likely to be present at lesion sites, although astroglia have the potential to make some of these cytokines under selective conditions (Wesselingh *et al.*, 1990). Except for γ-IFN, all the human cytokines used are described to be effective on murine cells by the manufacturers (Genzyme, Cambridge, MA; United Biotechnology Inc., Lake Placid, NY).

Qualitative and quantitative assessment of GFAP-IR in situ

All animals were killed by CO_2 asphyxiation 4 d following surgery; this time point was chosen to reflect our findings (Moumdjian *et al.*, 1991; Yong *et al.*, 199 la) and those of others (Norton *et al.*, 1992) that the extent of GFAP-IR is likely to be extensive

at this juncture, at least in adult stab injuries. Brain was removed and quick-frozen in isopentane on dry ice. Ten micrometer coronal sections were obtained on gelatin-coated slides and subjected to immunofluorescence for GFAP (glial fibrillary acidic protein), a cytoplasmic intermediate filament specific for astrocytes (Eng, 1985). In brief, sections were air dried for 10 min and fixed in 70% ethanol for 20 min. After washing with phosphate-buffered saline (PBS), each section was treated for 2 hr with 3% ovalbumin (Sigma) as a blocking step prior to incubation with a rabbit anti-GFAP polyclonal antibody (1:100; Dako Corp.) for 6 hr at room temperature. Following a brief rinse with PBS, a goat anti-rabbit immunoglobulin conjugated to FITC (1:75; Jackson) was introduced for 1 hr. Negative control for immunohistochemistry was replacement of the primary antibody with the diluting medium for antibody, HHG (1 mM HEPES buffer, 2% horse serum, 10% goat serum in Hanks' balanced salt solution). This was followed by a brief rinse in PBS and a final water rinse before mounting with Gelvatol. Slides were coded so that the qualitative assessment of GFAP-IR could be performed blind. Examination was restricted to the cortical regions, since astrocytes in these areas, unlike those in the external glia limitans and corpus callosum, are normally not GFAP-IR although containing this intermediate-filament protein (Bignami and Dahl, 1974). The area of the cortex containing GFAP-IR astrocytes was qualitatively tabulated from + to + + + + in ascending order of cortical area covered by GFAP-IR astrocytes.

Quantitative assessment of GFAP-IR for the different injury models was performed using a confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany) following immunolabelling for GFAP. Only the dorsal cortex ipsilateral to the lesion site and its corresponding contralateral area were scanned for the quantitative assessment. The scanner was mounted on a Leica Fluovert FS Microscope and optical excitation was achieved on the 488 nm line of an argon laser. The emitted fluorescent light was directed through a band-pass filter peaking at 535 ± 7 nm before transmission to the photomultiplier. Samples were scanned with a 2.5x, 0.08 NA objective in order to encompass the cortical ipsilateral hemisphere within the laser's image acquisition domain. The image was reconstructed from the averages of 64 passes per raster line (scan -64), in

an attempt to obtain high signal-to-noise ratio. The same area of cortex (at lesion site) was examined in all animals, the criteria being the density of GFAP-IR cells and the degree of spread of reactivity as one moved away from the wound site. Areas with GFAP-IR were traced out on each section to encompass only regions contributing to a cumulative immunofluorescence intensity with a standard deviation of 30. This criterion was adopted to achieve uniform conservative estimates on the extent of astrogliosis in all groups except the scissors stab and NC stab animals. For the latter samples the relatively small GFAP-IR cortical areas necessitated scanning using a 10x, 0.30 NA objective in order to facilitate image acquisition. Images were also obtained (at scan -8) with a 40x, 1.3 NA oil immersion objective for all groups, as a means of verifying the presence of GFAP-IR astrocytes in all samples scanned with a lower-powered objective.

Quantification of GFAP content

GFAP protein extraction and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out via a modified protocol as previously described (Hozumi *et al.*, 1990). Cortical lesion site was resected 4 d following injury and quick-frozen on dry ice. In addition, areas corresponding to the lesion site on the contralateral side as well as areas ipsilaterally anterior to the lesion site were also resected. Pooled resected cortical tissue from uninjured normal animals served as controls. Each sample (approximately 10 mg) was homogenized in 50 vol of 100 mM phosphate buffer (pH 7.4) containing 8 M urea at 4°C. The homogenate was boiled for 5 min on a heat block at 100°C and then analyzed for total protein content with a protein assay kit (Bio-Rad) that utilized bovine serum albumin as a standard. Six milligrams of protein extract were further diluted in sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1-0.05% bromophenol blue) and boiled for 5 min at 100°C. Samples (in 20 µl vol) were electrophoresed on a 10% SDS-PAGE for 45 min at 200 V constant voltage, with different quantities of purified bovine GFAP (Boehringer-Mannheim) as standards.

After electrophoresis, the samples were transblotted to a piece of Immobilon P membrane (Millipore) at 100 V constant voltage for 1 hr at 4°C. The membrane was

incubated in 5% skim milk in PBS (Blotto) followed by anti-GFAP antibody solution (Dako; diluted 1:1000 in Blotto) for 2 hr at 4°C. The membrane was rinsed in PBS and incubated in ^{125}I-Protein A (2 μCi diluted in 20 ml of Blotto) for 1 hr. GFAP protein content was quantified on a phosphor-imager (Molecular Dynamics) using IMAGE QUANT software. For each membrane, the concentration of GFAP in each sample was determined by comparison to a standard curve constructed from the integrated volumes occupied by the different purified bovine GFAP standards. The correlation coefficient for the standard curves was always 0.98 or better. To allow for comparisons between different membranes, and because several initial Western blots on the same samples showed good reproducibility, GFAP content of test samples was expressed as a ratio of GFAP content from the pooled normal cortices on the same SDS-PAGE. Representative autoradiograms of Western blots have been included (see Figs. 4, 6).

Assessment of in vitro astrocyte proliferation

The procedure for the culture of neonatal astrocytes from postnatal day 1 CD1 pups, and assessment of proliferation have been described in detail elsewhere (Yong, 1992). In all experiments, cells were treated once with test agents (see Table 3) and maintained for 4 d; 1 μCi of [^{3}H]-thymidine was administered during the last 16 hr of the experiment.

Results

Astrogliosis can occur after neonatal injury

We confined our inspection of astrogliosis to the cortical regions since the cortex of normal animals show no GFAP-IR astrocytes (Fig. 1) although containing this intermediate filament protein; in contrast, normal brains demonstrate GFAP staining of the corpus callosum and the glia limitans (Bignami and Dahl,1974). Initial experiments using a piece of NC inserted into the cortex of neonatal mouse for 4 d (NC implant) evoked extensive astrogliosis as determined by the area of GFAP-IR (Figs. 1, 3). To

reconcile this observation with the multitude of reports that have documented minimal astrogliosis in neonatal animals following a CNS stab injury (Osterberg and Wattenberg, 1963; Sumi and Hager, 1968; Bignami and Dahl, 1976; Gearhart *et al.*, 1979; Bernstein *et al.*, 1981; Berry *et al.*, 1983; Barrett at al., 1984; Maxwell *et al.*, 1990b), we performed a scissors stab injury to the cortex in P3 mice. Four days later, GFAP-IR was minimal (Fig. 2); in contrast, a similar scissors stab injury to the adult mouse brain (Fig. 2) resulted in extensive gliosis with a spatial distribution similar to that described by Mathewson and Berry (1985). Furthermore, in neonatal animals stabbed with a piece of NC membrane that was then removed (NC stab), GFAP-IR was also minimal (Fig. 2), suggesting that the increase in the GFAP-IR observed in neonatal NC implant group was likely a factor of the duration of the NC implant *in vivo*.

We quantified the area of the cortex containing GFAP-IR astrocytes in the different injury models. Figure 3 confirms the extensive increase in GFAP-IR in the neonatal NC implant group, and the minimal astrogliosis following scissors stab or NC stab to the P3 pups. Similar quantification for adult scissors stab injury indicates that the extent of gliosis in neonatal NC implant animals was even higher than that following adult scissors stab injury (Fig. 3).

Protein extracts from the resected areas circumscribing the lesion sites were electrophoresed on SDS-PAGE (Fig.4). GFAP content (expressed as micrograms GFAP/mg total protein) at the lesion site was significantly increased in the neonatal NC implant injury model over both NC stab, scissors stab, and normal animals to correlate with the observed increase in GFAP-IR (Table 1).

In a previous report (Moumdjian *et al.*, 1991), we demonstrated that following a large stab wound to the adult rat brain, the extent of astroglial reactivity was extensive and involved also the contralateral hemisphere. In the present study with neonatal mice, no contralateral astrogliosis was documented for any of the injuries performed, including the NC implant group, where ipsilateral astrogliosis was extensive (Figs. 1, 2). Furthermore, when GFAP protein content was quantified from tissue some distance from the lesion site (corresponding contralateral area or ipsilateral areas at least 4 mm from the

lesion), no changes could be documented from controls, even in the NC implant group (data not shown). Thus, while NC implant in neonatal mouse brains resulted in an increase in GFAP-IR (Fig. 1) and GFAP protein content (Fig. 4), this was focal and remained confined to the area immediately circumscribing the lesion.

The above findings of the increased GFAP-IR and GFAP content in the neonatal NC implant group, but not in the NC stab or scissors stab animals (Table 1), suggest that the occurrence of astrogliosis in the neonatal brain is clearly feasible and is dependent on the type of injury inflicted.

Cytokines can enhance neonatal astrogliosis

Qualitative analysis

To assess the contribution of the immune system in producing astrogliosis, we augmented the neonatal's immature immune system with the administration of cytokines. The scissors stab-injured animals with its minimal astrogliosis now demonstrate enhanced GFAP-IR to most cytokines. As shown in Table 2, recombinant mouse γ-IFN, IL-1, IL-2, IL-6, TNF-α, and M-CSF elicited increased GFAP-IR when compared to vehicle (0.2% BSA)-treated controls. In contrast, human γ-IFN did not evoke astrogliosis over that of vehicle-treated controls, in accordance with reports that the interaction of γ-IFN with its receptor to elicit a response occurs in a species-specific manner (Gray *et al.*, 1989; Hemmi *et al.*, 1989; Rubio and de Felipe,1991; Plata-Salaman,1992). Vehicle (0.2% BSA)treated controls displayed moderate astrogliosis, compared to scissors stab-injured animals with minimal astrogliosis (Table 2).

Quantitative analysis

Our choice of cytokine (rmγ-IFN) for quantification of extent of astrogliosis was determined by our long-standing interest in γ-IFN and its effects on glia (Yong *et al.*, 199 la,b, 1992). The extent of astrogliosis was measured by the density of GFAP-IR cells and the degree of spread of reactivity as one moved away from the wound site (Fig. 5).

Figure 3 shows that the administration of 0.2% BSA as a vehicle following a scissors stab injury increases the extent of GFAP-IR (mean area of $180 \pm 27 \times 10^3$ mm^2) compared to the scissors stab injury alone (mean area of $30 \pm 2 \times 10^3$ mm^2). Comparisons between rmγ-IFN and its 0.2% BSA vehicle shows that the deposition of 20 U of rmγ-IFN (mean area of $634 \pm 54 \times 10^3$ μm^2) further increased the extent of astrogliosis by 3.5-fold ($p < 0.01$, Student's t test).

Measurements of GFAP content (Fig. 6) of 10 mg samples circumscribing the cytokine-injected scissors stab site show that when expressed as a ratio of normal brains, GFAP contents for BSA and rmγ-IFN groups are, respectively, 2.0 ± 0.4 (n of 8 samples) and 1.9 ± 0.3 (n of 9 samples). Thus, while the GFAP content in tissue circumscribing the injected scissors stab site was increased over that of normal brains, rmγ-IFN did not elevate GFAP content when compared to its BSA vehicle control. Hence GFAP content (with no change) does not appear to reflect astrogliosis on the basis of GFAP-IR (3.5-fold increase) following rmγ-IFN treatment (Fig. 3).

Proliferative response of neonatal astrocytes to cytokines

We further addressed the contribution of cytokines toward proliferation of astrocytes, a frequent finding of astrogliosis (Cavanagh, 1970; Latov *et al.*, 1979; Janeczko, 1988, 1991; Takamiya *et al.*, 1988; Topp *et al.*, 1989), by testing for their *in vitro* mitogenic capabilities. [^{3}H]-thymidine measurements revealed that only rmγ-IFN and recombinant human IL-1 (rhIL1) could alter proliferation in an antimitotic fashion (Table 3). Epidermal growth factor, a non-cytokine growth factor that served as a positive control to indicate viability and responsiveness of neonatal murine astrocytes to a defined mitogen, increased the proliferation of neonatal murine astrocytes. Thus, the ability of a cytokine to alter the proliferation of neonatal astrocytes *in vitro* does not predict its capability in enhancing GFAP-IR *in vivo*.

Discussion

The occurrence of astrogliosis following injury and inflammation to the adult CNS is a stereotypical occurrence recognized by increased GFAP-IR, a long-standing neuropathological hallmark (Latov *et al.*, 1979; Smith *et al.*, 1983; Mathewson and Berry, 1985; Aquino *et al.*, 1988; Takamiya *et al.*, 1988; Hozumi *et al.*, 1990; Maxwell *et al.*, 1990a; Moumdjian *et al.*, 1991). These studies have highlighted a number of salient features associated with astrogliosis: astrocytic hypertrophy is more prominent and precedes astrocytic hyperplasia, astrocytic hypertrophy can occur in the contralateral cortex or other sites remote from the lesion site, and an increase in GFAP-IR is not necessarily paralleled by an increase in GFAP content. The reactive changes to astrocytes at the site of injury could be accounted for by the evolution of a number of factors related to the injury itself, including neuronal necrosis, mechanical changes in the tissue, ionic changes (Mathewson and Berry, 1985), or perhaps the disruption of the blood-brain barrier with consequent migration of inflammatory cells with the potential of cytokine production.

In contrast, the majority of studies have shown that injury to neonatal CNS elicits minimal astrogliosis, if any (Osterberg and Wattenberg, 1963; Sumi and Hager, 1968; Bignami and Dahl, 1976; Gearhart *et al.*, 1979; Bernstein *et al.*, 1981; Berry *et al.*, 1983; Barrett at al., 1984; Maxwell *et al.*, 1990b), although this contention has been challenged (Roessmann and Gambetti,1986; Moore *et al.*, 1987; Trimmer and Wunderlich, 1990). Previous studies have implicated the accumulation of inflammatory mononuclear cells and their cytokines at lesion sites of the CNS in modulating the reactive astrocytic changes (Kitamura *et al.*, 1972; Tsuchihashi *et al.*, 1981; Giulian *et al.*, 1987, 1989; Morshead and van der Kooy, 1990, Milligan *et al.*, 1991; Woodroofe *et al.*, 1991; Yong *et al.*, 1991a; Taupin *et al.*, 1993). Given the relative immaturity of the immune system in neonates compared to adults (Hobbs, 1969; Abo *et al.*, 1983; Lu and Unanue, 1985; De Paoli *et al.*, 1988; Hannet *et al.*, 1992), we postulated that the immaturity of the immune system in neonates may contribute to the lack of neonatal astrogliosis.

It was first necessary to document that the neonatal CNS had limited astrogliosis following injury. This was first initiated by implanting a piece of NC membrane into the P3 mouse brain for 4 d. What was observed, however, was a tremendous GFAP-IR (Fig. 1). In contrast, an iris scissors stab wound to the neonatal brain (Fig. 2), similar to the stab models used by others with reports of minimal astrogliosis (Sumi and Hager, 1968; Bignami and Dahl, 1976; Berry *et al.*, 1983; Maxwell *et al.*, 1990b), evoked little GFAP-IR and a small increase in GFAP content (Table 1). The cause of the NC implant in evoking astrogliosis in neonatal animals is probably due to its continued presence *in vivo*, since in the NC stab animals (injury using an NC membrane, which was then removed immediately), minimal astrogliosis was observed 4 d later (Fig. 2). It is probable that the continued presence of the NC implant against a background of a maturing immune system provides a sufficient stimulus to evoke an immune cascade; the release of adequate cytokines at the lesion site may then contribute to the extensive astrogliosis observed (Fig. 3). Whatever the explanation, the conclusion is that reactive astrogliosis as measured by GFAP-IR and GFAP content (Fig. 4) can occur in the neonatal brain, and that it is dependent on the type of injury inflicted (Table 1).

In the NC implant model, astrogliosis was characterized by both an increased synthesis of GFAP intermediate filaments and hypertrophy of the astrocytic cytoplasmic processes. The functional role for the increase in this intermediate filament is not known. Smith *et al.* (1986) have reported that reactive astrocytes could migrate on to an NC implant within 24-48 hr post-implantation in neonatal animals and that these astrocytes formed a terrain that facilitated axonal extension and regeneration. These neonatal astrocytes also appeared to lack the expression of putative growth-inhibitory molecules such as chondroitin-6-sulfate proteoglycan and cytotactin that were present in adult astrocytes (McKeon *et al.*,1991). These findings suggest that neonatal reactive astrocytes may have potential regenerative properties.

To test the hypothesis implicating cytokines as contributors toward astrogliosis, we chose to utilize the neonatal stab model with its inherent minimal gliotic response. A single microinjection of cytokines (rmγ-IFN, rhIL-1, rhIL-2, rhIL-6, rhTNF-α, and

rhM-CSF, all of which are described to be effective in mouse cells by the manufacturer) into the cerebral cortex of the neonatal mouse produced an astrogliotic response (Table 2), similar to that seen in adult stab wound models by GFAP immunoreactivity (Fig. 2) (Mathewson and Berry, 1985; Moumdjian *et al.*, 1991; Yong *et al.*, 1991a). The specificity of the cytokine effect was demonstrated by the inability of rhγ-IFN to evoke an astrogliotic response beyond that of vehicle-treated controls (Table 2), an observation that is in accordance with reports indicating a species-specific interaction between γ-IFN and its receptor (Gray *et al.*, 1989; Hemmi *et al.*, 1989; Rubio and de Felipe, 1991; Plata-Salaman, 1992). The finding that a single administration of cytokines can induce significant astrogliosis in the neonatal stab model with its inherent minimal astrogliosis is consistent with the postulate that the lack of astrogliosis following neonatal injury is related to an immature immune system. This immature immune system would then be partially reconstituted by cytokine administration.

The quantification of the extent of astrogliosis evoked by rmγ-IFN ($634 \pm 54 \times 10^3$ mm^2) revealed a 3.5-fold increase over that of vehicle-treated controls ($180 \pm 27 \times 10^3$ mm^2) as determined by GFAP-IR (Figs. 3 & 5). However, the analyses of GFAP content from tissue circumscribing the injection sites for rmγ-IFN and vehicle-treated controls did not differ but it was higher than unoperated normal controls (Fig. 6). Thus, GFAP content did not reflect the extent of astrogliosis on the basis of GFAP-IR following rmγ-IFN treatment. A similar type of occurrence has been documented in animals with experimental autoimmune encephalomyelitis (EAE), where GFAP content of the spinal cord did not differ from controls at 13-18 d post-inoculation (dpi), a period when intense GFAP-IR was observed in the EAE groups (Smith *et al.*, 1983; Goldmuntz *et al.*, 1986); correlation of GFAP-IR with GFAP content was observed at later periods (35-65 dpi) (Aquino *et al.*, 1988). The most likely interpretation for the non-correspondence between GFAP-IR and GFAP content may be that as astrocytes swell and GFAP filament dissociate there is an increased availability of antigenic epitopes to antibodies for GFAP (Aquino *et al.*, 1988; Eng *et al.*, 1989). This phenomenon seen in EAE for the initial

increase in GFAP-IR before eventual increase in GFAP content appears similar to that observed in our neonatal rmγ-IFN scissors stab model.

Why do all cytokines tested induce astrogliosis? While it is possible that all these cytokines have direct effects on astrocytes, an indirect phenomenon through a possible common pathway is also likely. This route may conceivably be by the recruitment of inflammatory mononuclear cells, including a final effector cell and its cytokine(s), to the lesion site. This possibility is supported by the report of Brosnan *et al.* (1989), who described the occurrence of astrogliosis and increased adherence of inflammatory cells to the vasculature after intraocular injection of γ-IFN, TNF-α, and IL-1. In addition, Watts *et al.* (1989) have demonstrated the disruption of the blood-brain barrier and the recruitment of inflammatory cells to the intracerebral injection site of IL-2. Furthermore, Simmons and Willenbourg (1990) have described the occurrence of a widespread inflammatory response to a single microinjection of γ-IFN or TNF-α in the lumbosacral cord. Finally, Sethna and Lampson (1991) have observed a that a single intracerebral injection of γ-IFN resulted in the recruitment of many types of inflammatory cells to the brain. Our laboratory is currently testing the direct and indirect role of cytokines in mediating gliosis *in vivo*. We are examining the role of γ-IFN as a final common mediator, given the identification of a specific receptor for mouse γ-IFN on neonatal mouse astrocytes (Rubio and de Felipe, 1991), and given the potent effects of γ-IFN on astrocytes in mixed or purified cultures *in vitro* (Yong *et al.*, 1991 a,b, 1992).

An important question now arises: which cells are responsible for the production of cytokines? Conceivably, infiltrating mononuclear phagocytes (macrophages) and other cells of the immune system (e.g., T-lymphocytes and NK cells) are potentially involved. Cells intrinsic to the CNS could also be potential sources of cytokines. In this regard, microglia, astrocytes, and even neurons have been suggested to synthesize cytokines under selective conditions (Giulian *et al.*, 1987; Wesselingh *et al.*, 1990; Logan *et al.*, 1992; Tchelingerian *et al.*, 1993). The nature of the *in vivo* cellular elements contributing to cytokine(s) production following injury remains to be elucidated.

Our investigations demonstrate that the ability of a cytokine to alter the proliferation of neonatal astrocytes *in vitro* does not predict its capability in enhancing GFAP-IR *in vivo*. While all cytokines tested *in vivo* increased GFAP-IR (Table 2), our *in vitro* studies demonstrated an antimitotic effect by rmγ-IFN (Yong *et al.*, 1992) and IL-1, without any significant effects by the other cytokines (Table 3). It is also worth noting that while others have found IL-1, IL-6, and TNF-α to be mitogenic for neonatal rat and calf bovine astrocytes (Giulian and Lachman, 1985; Nieto-Sampedro and Berman, 1987; Selmaj *et al.*, 1990), these cytokines were not mitogenic for neonatal mouse astrocytes. This apparent discrepancy may be due to species differences, since we have demonstrated that while rmγ- was inhibitory for proliferation of neonatal or adult mouse astrocytes, rhγ-IFN was a mitogen for fetal and adult human astrocytes (Yong *et al.*, 1992).

In conclusion, astrogliosis can occur in the neonatal brain if a sufficient stimulus (NC implant) is present. For the neonatal scissors stab wound model with its inherent minimal astrogliosis, a single administration of cytokines induces extensive astrogliosis. These results implicate immunoregulatory cytokines as important contributing factors to the production of astrogliosis following an injury to the CNS.

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Figure Legends

Figure 1. **Implant of NC membrane into P3 mouse pups for 4 d increases the extent of GFAP-IR at the implant site (C).** In C, the cortical area occupied by GFAP-IR astrocytes is encompassed within the traced outline. In normal neonates (A and B, representing the ipsilateral and contralateral hemispheres)7 or in the contralateral hemisphere of NC implant pups (D), GFAP-IR was detected only in the corpus callosum (top of each frame) and glia limitans (bottom of each frame). Images in A-D were acquired by confocal laser scanning microscopy (described in detail in Materials and Methods) using a 2.5x objective. E is a higher magnification of the traced area in C, acquired using a 40x objective, to denote the morphology and reactive nature of the astrocytes.

Figure 2. **A scissors stab (A, B) or an NC stab (C, D) to P3 mouse pups results in minimal astrogliosis when compared to NC implant in neonates (Fig. 1) or scissors stab to adult mice (E, F).** Because of the small GFAP-IR cortical areas, neonatal brain sections in A-D were scanned using a 10x objective (in contrast to 2.5x for NC implant in Fig. 1) to facilitate image acquisition. For visual comparisons, the images for adult scissors stab injury (E, F) are 10x objective acquisitions. A, C, and E are hemispheres ipsilateral to the lesion site, while the corresponding contralateral hemispheres are depicted in B, D, and F.

Figure 3. **Quantitative comparisons of the cortical area covered by GFAP-IR astrocytes in the different injury paradigms in mouse pups.** Injury was inflicted on P3, and animals were killed 4 d after. Adult scissors stab animals have been included for reference. Each data point represents the image acquired from a single brain section. Two brain sections per animal, taken from the lesion site in all cases, from seven animals per group, were analyzed. Scissors stab (mean GFAP-IR area of $30 \pm 2 \times 10^3$ mm^2) or NC

stab (mean area of 53 ± 5 X 10^3 mm^2) to neonates elicited little astrogliosis when compared to neonate NC implant (mean area of 1016 ± 37 X 10^3 mm^2) or adult scissors stab injuries (496 ± 22 X 10^3 mm^2). The introduction of 2 ml of 0.2% BSA to the neonatal scissors stab wound site resulted in increased GFAP-IR (mean area of 180 ± 27 X 10^3 mm^2) compared to scissors stab alone, while 20 U of γ-IFN in 0.2% BSA enhanced astroglial reactivity even further (mean area of 634 ± 54 X 10^3 mm^2).

Figure 4. **GFAP content of tissues (approximately 10 mg wet weight) circumscribing the lesion site in the different injury paradigms in neonatal animals.** GFAP content was read off a standard curve generated by different amounts of purified GFAP (10-100 ng). The mouse GFAP bands ran at a slightly different molecular weight than the GFAP standards (51 kDa) probably because the latter were of bovine extract. GFAP content of normal mouse cortex was 0.3 μg/gm total protein, which corresponds to normal values reported by Goodlett *et al.* (1993).

Figure 5. **Increased GFAP-IR in mouse pups treated with rmγ-IFN following a scissors stab wound (C, D) compared to vehicle (0.2% BSA) treatment (A, B).** A and C are at the lesion site while B and D are hemispheres contralateral to the lesion. Quantification of the area of GFAP-IR in each group is displayed in Figure 3; on average, rmγ-IFN increased GFAP-IR by 3.5-fold over its BSA vehicle control.

Figure 6. **Despite the increase in GFAP-IR elicited by γ-IFN compared to 0.2% BSA vehicle, content of GFAP did not differ between the two groups.**

Table 1. Astrogliosis in neonatal NC implant injury

Injury	Area of GFAP-IR ($\times 10^3 \mu m^2$)	GFAP content (ratio of normal)
Neonatal scissors stab	30 ± 2 (14)	1.3 ± 0.1 (8)
Neonatal NC stab	53 ± 5	1.6 ± 0.2 (6)
Neonatal NC implant	1016 ± 37 (14)*	3.0 ± 0.3 (13)*
Adult Scissors Stab	496 ± 22 (14)	Not done

Values are mean ± SEM, with the number of samples shown in parentheses. Two brain sections per animal were scanned to give the area of GFAP-IR. For measurements of GFAP content, a 10 mg piece of tissue circumscribing the lesion site was used per animal. *$P < 0.05$ compared to all the other groups, using one-way ANOVA with Duncan's multiple comparisons.

Table 2. Cytokines qualitatively increase the extent of GFAP-IR following neonatal scissors stab injury

Treatment	Number of mice	Extent of GFAP-IR
Scissors stab alone	2	+
0.2 % BSA	4	++
Human γ-IFN	4	++
Mouse γ-IFN	6	++++
IL-1α,β	6	++++
IL-2	4	++++
IL-6	6	++++
TNF-α	4	++++
M-CSF	4	++++

Brain sections were analyzed blind and the extent of GFAP-IR in the ipsilateral cortex abulated from a scale of + (minimal) to ++++ (extensive). On average, four brain sections er animal were analyzed blind.

Table 3. Proliferative response of neonatal mouse astrocytes to cytokines in vitro

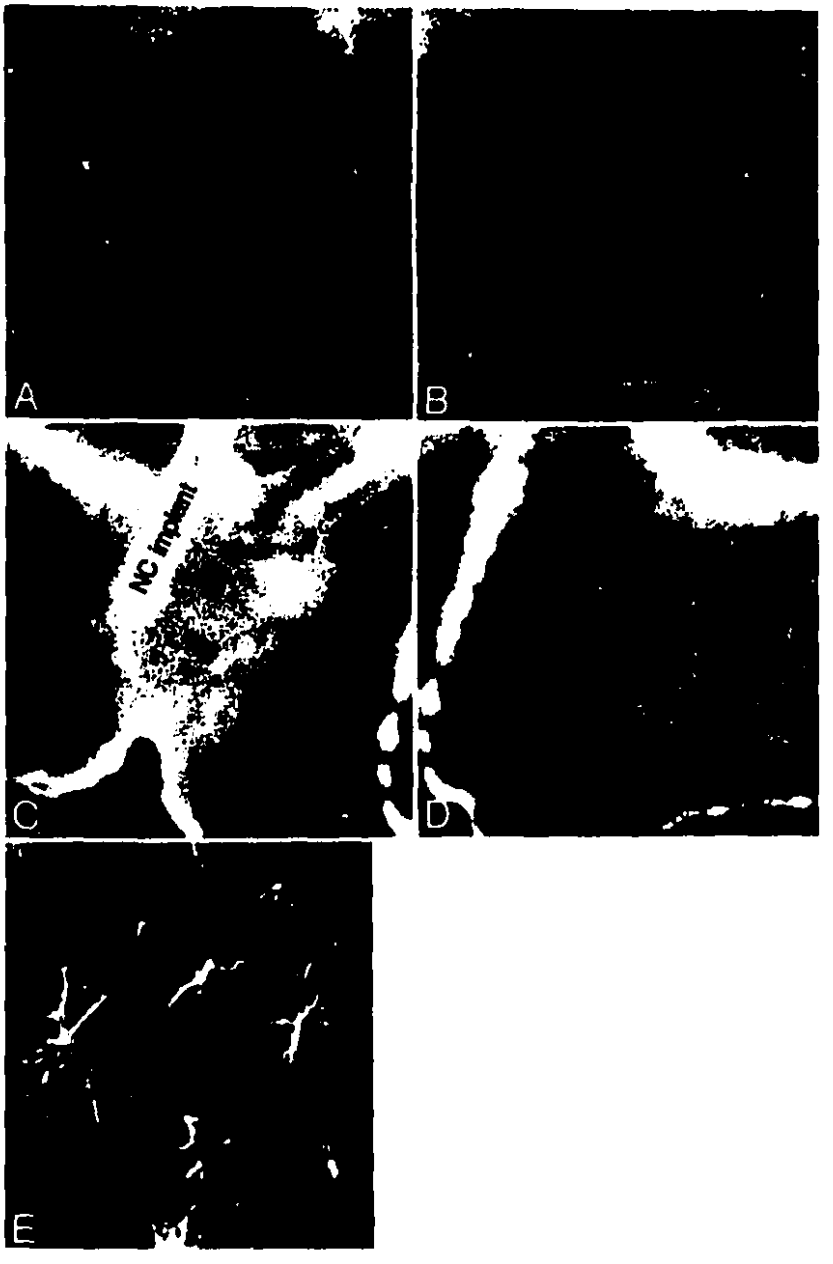
Treatment	Concentration	^{3}H-thymidine (% of controls)
Control	-	100 ± 2 (129)
BSA	0.2%	102 ± 3 (15)
Mouse γ-IFN[a]	100 U/ml	49.8 ± 3 (18)*
Human γ-IFN[a]	100 U/ml	98.8 ± 4 (12)
IL-1α,β	1 U/ml	85.8 ± 5 (7)
	10 U/ml	71.0 ± 3 (15)*
	100 U/ml	78.4 ± 4 (23)*
	500 U/ml	78.0 ± 4 (12)*
IL-2	10 U/ml	97.3 ± 4 (7)
	100 U/ml	123 ± 4 (16)
IL-6	10 U/ml	111 ± 5 (12)
	100 U/ml	101 ± 6 (12)
	500 U/ml	94.1 ± 6 (11)
TNF-α	1 U/ml	117 ± 7 (12)
	10 U/ml	99.6 ± 6 (15)
	100 U/ml	90.0 ± 5 (15)
	500 U/ml	82.2 ± 6 (14)
M-CSF	5 CFU/ml	98.9 ± 5 (12)
	10 CFU/ml	96.9 ± 4 (11)
	50 CFU/ml	111 ± 3 (12)
EGF[b]	5 ng/ml	323 ± 34 (12)*

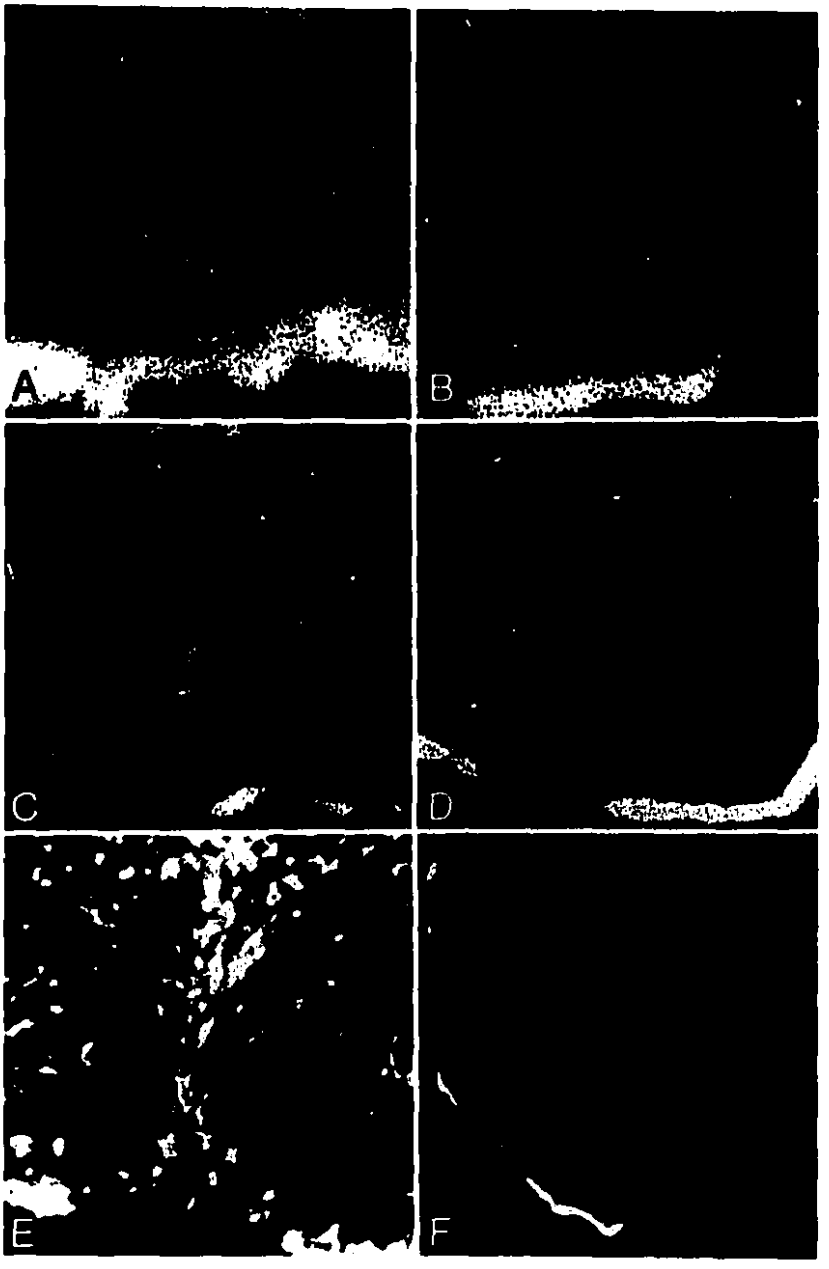
Results have been compiled from 16 different experiments involving 12 mouse culture series. In all experiments, cells were treated for 4 days with agents: 1μCi of ^{3}H-thymidine was administered during the last 16 hours of experiment. Values are mean ± SEM with number of coverslips analyzed shown in parentheses.

[a]Confirms published results (Yong et al., 1992) that mouse but no human γ-IFN produces decrease in proliferation rate at 10, 100, and 1000 U/ml.

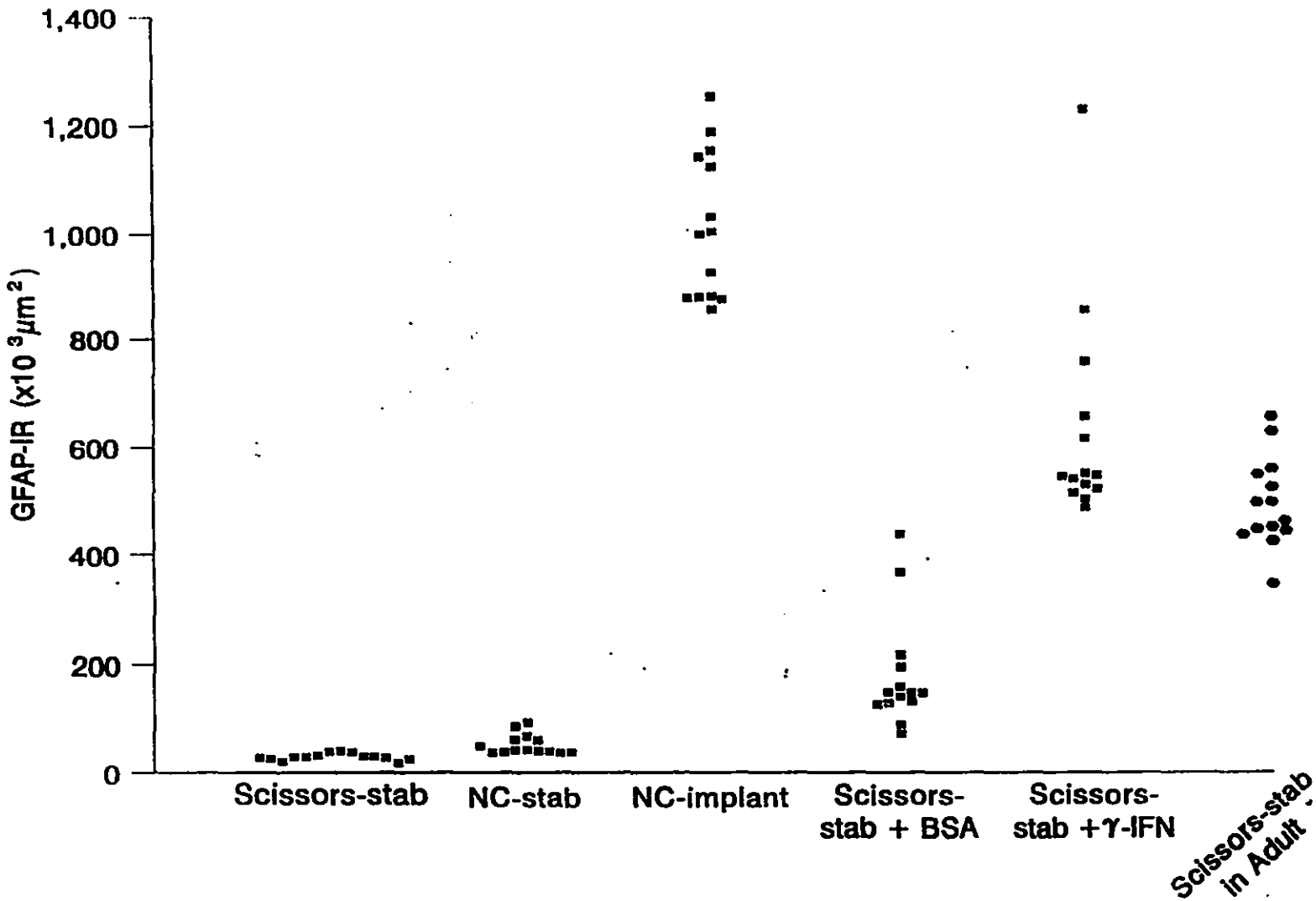
[b] This non-cytokine growth factor was used as a positive mitogenic control.

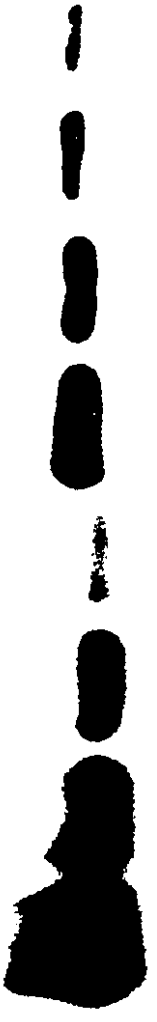
*$P < 0.05$ compared to controls (one-way ANOVA with Duncan's multiple comparisons).


A
B
NC implant
C
D
E


A
B
C
D
E
F

Extent Of GFAP-IR In Neonatal Mouse Brain In Response To Different Injuries





Normal

Scissors-stab

NC-stab

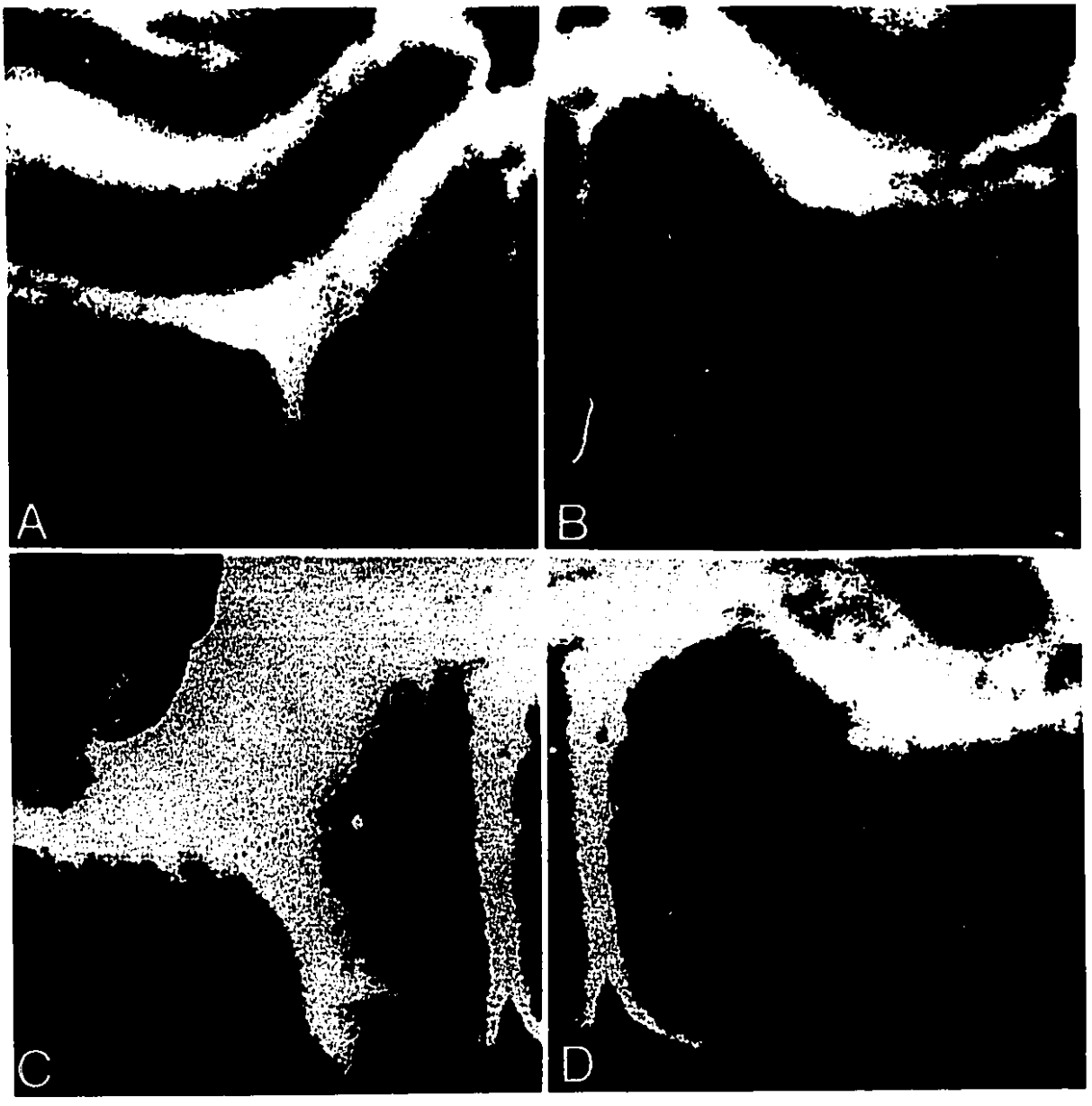
NC-implant

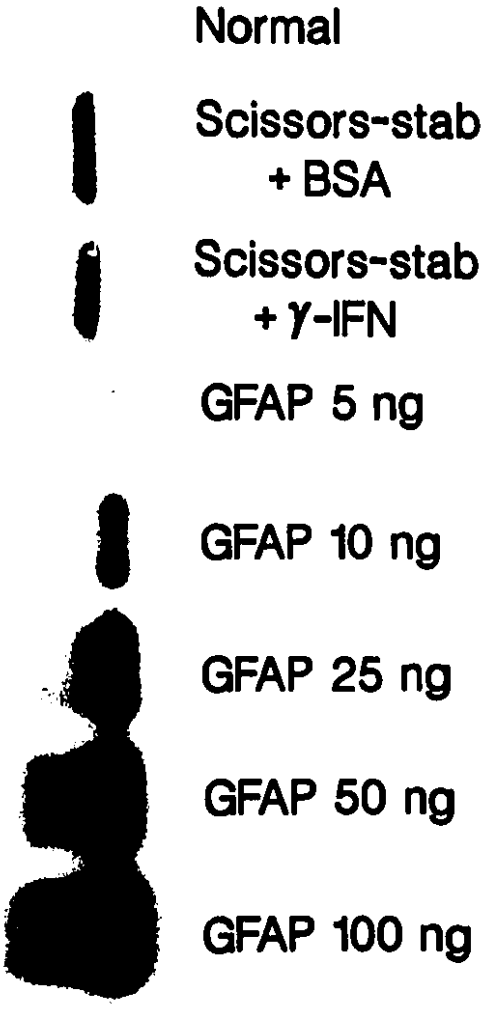
GFAP 10 ng

GFAP 25 ng

GFAP 50 ng

GFAP 100 ng


A
B
C
D


Normal
Scissors-stab
+ BSA
Scissors-stab
+ γ-IFN
GFAP 5 ng
GFAP 10 ng
GFAP 25 ng
GFAP 50 ng
GFAP 100 ng

Chapter 6

Conclusion and Future Directions

This thesis has focused on cytokines as mediators of astrogliosis both *in vitro* and following CNS trauma *in vivo*. The conclusions contained herein further implicate cytokines in this process and present one with a number of questions which can be further addressed. First of all, which cytokine or cytokines are key modulators of astroglisis and, furthermore, which inflammatory cells are responsible for their production?

As of yet, it remains unclear whether one cytokine is key or whether a multitude of cytokines are responsible for astrocytic reactivity. To this end, receptors for IFN-γ (Rubio and de Felipe, 1991) and IL-1 (Ban *et al.*, 1993) have been localized on astrocytes, suggesting that these molecules may be important and direct mediators of the astrocytic response to inflammation. Our results so far suggest that a number of cytokines are responsible. Whether they exert their influence directly or indirectly via a final common molecule remains unclear. It is possible that different parameters of the gliotic reaction are controlled separately, or to a greater extent, by different cytokines and that the combination of cytokines that one might expect *in vivo* results in a multiplicity of effects, which is more than is evident when each of these is tested in isolation.

In terms of cellular sources for these cytokines, a number of studies have demonstrated the presence of macrophages, microglia, NK cells and T lymphocytes at the site of injury (Giulian, 1987; Giulian et al, 1989; Perry *et al.*, 1993). Further studies need to quantitate the presence of specific types of inflammatory cells in experimental paradigms where astrogliosis is evident. Furthermore, the potential role of neurons and astrocytes as producers of cytokines cannot be overlooked. Reports have indicated that neurons can produce IL-1, IL-2, IL-3, M-CSF, TNF-α and a IFN-γ-like molecule. Astrocytes, on the other hand, have long been shown capable of producing IL-1, IL-3, IL-

6, M-CSF, IFN-γ, TNFα and TGFβ$_1$ under both basal and stimulated conditions (reviewed in Yong, in press). Thus, the potential for a complex network of para- and autocrine signals mediating astrogliosis is in place.

Finally, as mentioned previously, the functional role of reactive gliosis is unclear, however, evidence is emerging that reactive astrocytes have neurotrophic properties. It is becoming increasingly clear that cytokines play a central role in the response of the CNS to injury. On the one hand, they induce a variety of astrocytic changes such as cellular proliferation, upregulation of cytoplasmic proteins and induction of the neurotrophic properties of astrocytes, including the production of growth factors, expression of extracellular matrix and cell adhesion molecules. As well, cytokines themselves are capable of directly enhancing the differentiation and survival of neurons *in vitro*. The combined presence of cytokines, reactive astrocytes and neurotrophic factors increases the likelihood of interactions within the CNS microenvironment. A key question which follows is whether modulation of reactive astrogliosis is possible by varying the inflammatory response to CNS trauma and what effect this might have on the putative neurotrophic role of astrogliosis.

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