Cytokines and Astroglial Reactivity

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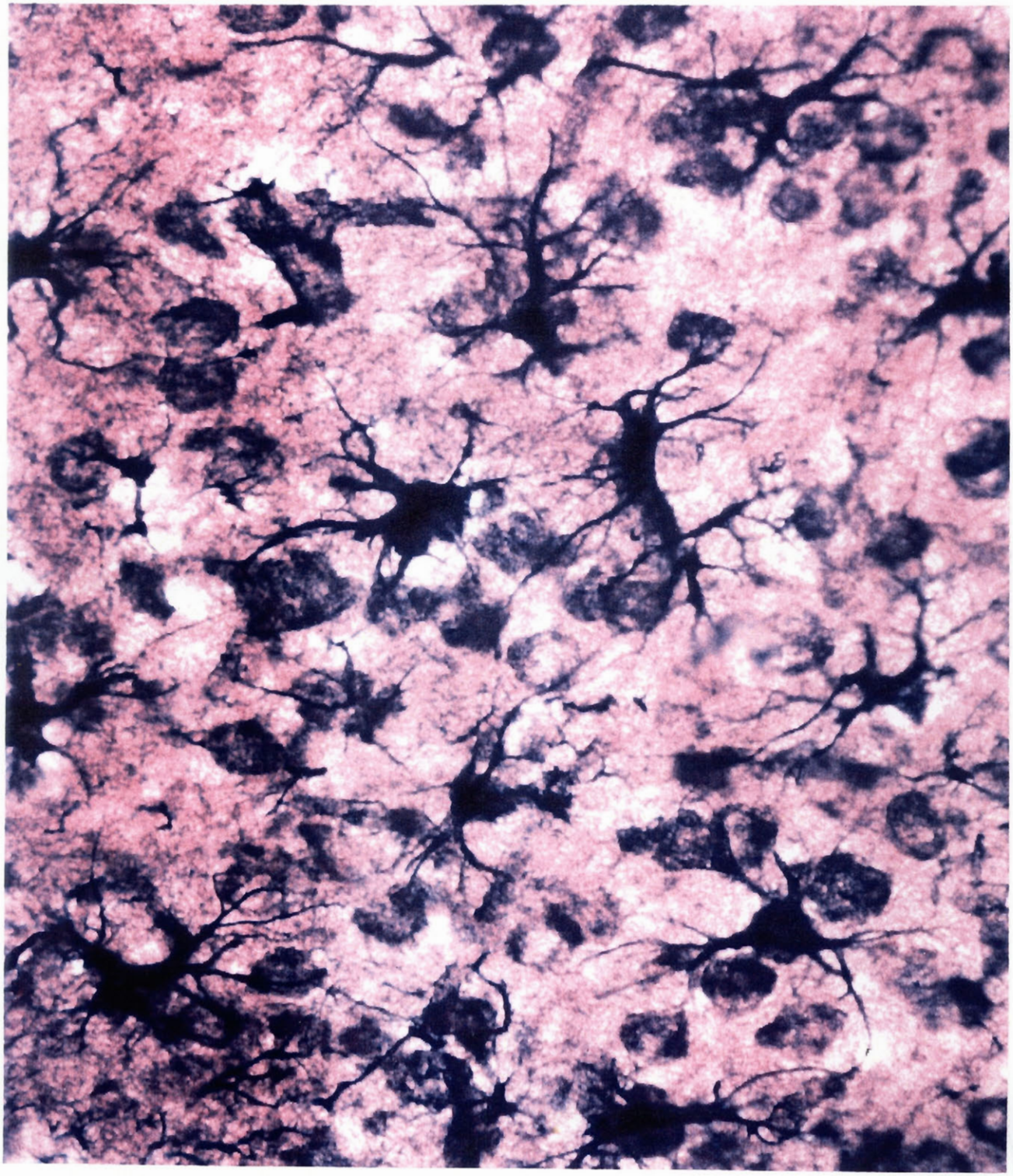

Reactive Astrocytes in the Adult Cortex

I know that, whatsoever God doeth, it shall be for ever:
nothing can be put to it, nor any thing taken from it:
and God doeth it, that men should fear before him.

Ecclesiastes 3:14
King James Version

To my wife

ACKNOWLEDGEMENTS

La nature se dévoilant devant la science

(Nature Reveals Herself to Science)

My first encounter with the Montreal Neurological Institute (MNI) was in the fall of 1989, shortly after I had registered with the Faculty of Medicine as a MD/PhD student. As I was interested in the world of neuroscience research, I made the student's pilgrimage to the institute to register as a graduate student in the Department of Neurology and Neurosurgery. Upon entry into the main lobby of the MNI, my first encounter was with "a beautiful, young woman with head tilted to one side and a demure expression on her

face. Both arms raised while they were parting the upper part of her robe revealing her lovely naked breast''. I was to later learn that La Nature was a custom-made white marble copy of the original in the Louvre in Paris.

On looking around the room, I noticed a painting of a ram's head in the centre of the ceiling with an inscription in Greek, presumably written by Galen which claims: "I have seen a wounded brain heal". This was surrounded by paintings of neurons within 3 of the 6 layers of the cerebral cortex.

Those first solemn moments were to be in later years the inspiration to tackle the technically challenging task ahead of me. In later years as I sat in the lobby late at night, I regained my vision to return the next day in the tranquillity and mystery that lay in these paintings. If there is one single place in the MNI, I would like to remember, it would be this dimly and uniquely decorated lobby created by Dr. Wilder Graves Penfield.

The dominant influence of my intellectual curiosity and playful enthusiasm for science with the ability to recognize the need for diligent perseverance was my friend, professor and research mentor Ira Greenbaum PhD. He was a professor in the Department of Biology, from whom I took my first challenging undergraduate biology course in Chordate Anatomy. He made it possible for me the engineering student (double majoring in electrical and bioengineering) to work in his karyology laboratory. He transformed my work into a game of investigation and enquiry. Entrenched with passionate zeal he showed me the beauty of chromosomal architecture. He taught me skills in tissue culture, to animal handling and designing experiments. It is to this gentleman that I attribute my success to as he saw beyond me an invested his time in me preparing me for the MNI. Although his labours have not been fruitful in his own sphere of interest, it came to bear fruit at the MNI.

To my supervisor and mentor, Voon Wee Yong Ph.D,, I wish to express my indebtedness and gratitude for having given of his time and talents unselfishly by spending long hours and a great deal of patience in developing my research acumen. I am particularly indebted to his fine example as a scholar and a gentleman both in the laboratory and domestic aspects of his world. Finally, of his many contributions to my research development, his single most important contribution to me is best described by the following statement:

Dans les champs de l'observation le hazard ne favourise que les esprits préparés

(In the fields of observation, chance favours only the mind that is prepared)

Louis Pasteur

Address at the University of Lille

I would also like to express my gratitude towards Jack P Antel MD who invited me to join his Neuroimmunology group and who was personally responsible for introducing me to my supervisor. Gratitude also goes to Allan Tenenhouse MD Ph.D or Dr. T. as he is affectionately known to all Mudphudders (MD/PhD students) for his interest in my welfare during the course of my MD/PhD years with his support and gentle threats. Finally, I wish to mention Alain Beaudet MD Ph.D and his French Connection in the person of Andre Morin Ph.D (a post-doctoral fellow from Lyons, France) for their optimism in my experimental endeavours by inculcating in me Pablo Picasso's philosophy:

Je ne cherche pas, je trouve.

(I search not, I find)

I am greatly appreciative for the moral support, love and encouragement my mother has given me through the years. Her guidance and wisdom has been instrumental in giving my life both drive and purpose.

Finally, and most importantly, my thanks goes to my wife, Pierrette, who has been patient and understanding when she did not have to be and strong and resourceful when she did.

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ABSTRACT/RÉSUMÉ

ABSTRACT

Astroglial reactivity is a prominent universal event following trauma and inflammation to the adult central nervous system. In contrast, injury inflicted during early postnatal life produces minimal astroglial reactivity. This divergent response is dictated by the nature of the complex cascade of cellular events that follow anisomorphic injuries. In the adult animal, an inflammatory phase which involves the activation of intrinsic resident microglia and the infiltration of extrinsic blood-borne monocytes occurs prior to astroglial reactivity. The central hypothesis of this thesis therefore states that immunoregulatory cytokines from these intrinsic and extrinsic inflammatory cells contribute towards astroglial reactivity. The hypothesis was first tested by designing a neonatal injury paradigm that would facilitate the occurrence of astroglial reactivity via an immune mediated process. This was accomplished by converting the minimal astroglial response of a neonatal stab-wound to that of extensive astroglial reactivity via the exogenous administration of cytokines. Secondly, the chronological evolution of astroglial reactivity in the neonatal animal was examined, compared and contrasted to that in the adult; the nature of the neonatal chronic injury paradigm as a sufficient injury stimulus to evoke astroglial reactivity was found to correlate with the increasing presence of microglia/macrophages at the lesion site. As a final test to the hypothesis implicating cytokines in modulating astrogliosis, experiments were conducted to attempt to attenuate astroglial reactivity in adult animals following traumatic injury. Interleukin-10, a cytokine synthesis inhibitory factor, attenuated astroglial reactivity by suppressing the microglial/macrophage population at the lesion site. These experimental observations, taken together, strongly implicate the importance of immune mediated mechanisms in astroglial reactivity.

RÉSUMÉ

La réactivité des astrocytes est un phénomène universel important consécutif à un traumatisme et à une inflammation du système nerveux central de l'adulte. Au contraire de l'adulte, une lésion induite durant la période post-natale précoce produit une réactivité minime des astrocytes. La différence de réponse est due à la nature de la cascade complexe des évènements cellulaires résultant des lésions anisomorphes. Chez l'animal adulte la réactivité des astrocytes est précédée par une phase inflammatoire qui entraine l'activation de la microglie intrinsèque locale et l'infiltration des monocytes extrinsèques d'origine sanguine. En conséquence, l'hypothèse principale de cette thèse est que les cytokines immunorégulatrices provenant des cellules inflammatoires endogènes et exogènes contribuent à la réactivité des astrocytes. Dans un premier temps nous avons mis cette hypothèse à l'épreuve en créant un paradigme de lésion néonatale qui encourage la réactivité des astrocytes via un processus impliquant un médiateur immunitaire. Ceci a été obtenu en transformant la réaction minimale des astrocytes induite par une incision en une réaction substantielle par l'ajout de cytokines exogènes. Dans un deuxiéme temps, nous avons procédé à une étude chronologique de l'évolution de la réactivité des astrocytes chez l'animal nouveau né comparé à l'adulte. La lésion néonatale chronique a été suffisante pour stimuler une réaction des astrocytes corrélée à l'augmentation du nombre de microglie/macrophage constatée au niveau immunohistochimique et ultrastructural. Enfin, afin de confirmer l'hypothèse du rôle des cytokines comme modulateurs de l'astroglyose, des expériences ont été faites pour diminuer la réactivité post-traumatique des astrocytes chez l'animal adulte. L'interleukine-10, un facteur inhibiteur de la synthèse des cytokines, a diminué la réaction des astrocytes en supprimant le nombre de microglie/macrophage autour de la lésion. Considérées conjointement, ces observations expérimentales suggèrent fortement l'importance du rôle des médiateurs immunitaires dans la réactivité des astrocytes.

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CHAPTER 6: General Discussion

CHAPTER 7: Future Perspectives

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

As stated in the "Guidelines Concerning Thesis Preparation", Faculty of Graduate Studies and Research, McGill University:

Elements in the thesis that are to be considered as ***contributions to original knowledge*** *must be clearly indicated in the preface or by a separate statement at the begining or at the end of the thesis. This requirement is* ***mandatory for Ph.D. thesis.***

CHAPTER 2

1. The neonatal CNS is capable of manifesting extensive astroglial reactivity if an adequate injury stimulus is (chronic nitrocellulose (NC)-implant) is provided.

2. The inherent minimal astroglila reactivity following a neonatal stab-wound injury can be converted to extensive astroglial reactivity by the single application of cytokines.

3. The ability of a cytokine to alter proliferation of *in vitro* astrocytes does not appear to predict its ability to enhance astroglial reactivity *in vivo*.

CHAPTER 3

4. The nature of the neonatal chronic (NC-implant) injury as an adequate stimulus to evoke astroglial reactivity was correlated to the increasing presence of reactive microglia/macrophages at the immunohistochemical and ultrastructural level.

5. The acute NC-Stab injury displayed minimal astroglial reactivity and reactive microglia/macrophage presence, but had a rapid and significant increase in mRNA and content for glial fibrillary acidic protein. This observation suggest an accleration of astroglial development following injury in neonatal animals.

6. The extensive astroglial reactivity elicited by either the neonatal chronic (NC-implant) or adult injuries resolved with time to leave only lesion bordering reactive astrocytes which form a glial scar. The transient or reversible astroglial reactivity occurs only in regions remote from the lesion site which are devoid of neuronal death.

CHAPTER 4

7. Interleukin (IL)-10 attenuated astroglial reactivity in a dose response fashion as documented by a decrease in the number and the hypertrophic state of these reactive astrocytes.

8. *In vitro* astrocyte experiments suggest that IL-10 does not directly interact with astrocytes to attenuate an astroglial response.

9. The mechanism of interleukin-10 activity in decreasing astroglial reactivity appears to involve the suppression of microglia/macrophage activation. In this regard, one avenue is via the suppresion of TNF-α mRNA.

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MANUSCRIPTS AND AUTHORSHIP

As stated in the "Guidelines Concerning Thesis Preparation", Faculty of Graduate Studies and Research, McGill University:

*"Candidates have the option, **subject to the approval of their Department**, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.*

*If this option is chosen, **connecting text, providing, logical bridges between the different papers, are mandatory.***

*The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. **The thesis must include, as separate chapters or sections:** (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this reviews appropriate, and (5) a final overall conclusion and/or summary.*

Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e.g. in appendices) to allow a clear and a precise judgement to be made of the importance and originality of the research reported in the thesis.

*In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent;** supervisors must attest to the accuracy of such claims at the Ph.D. oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."*

CONTRIBUTIONS OF THE AUTHORS ON CO-AUTHORED PAPERS

As supervisor of my Ph.D. training, Voon Wee Yong provided guidance and is the senior author on all manuscripts. Except for the following individuals listed below, I conducted all the experiments of this thesis.

CHAPTER 2

Reactive Astrogliosis in the Neonatal Mouse Brain and its modulation by Cytokines.
Balasingam V, Tejada-Berges T, Wright E, Bouckova R, Yong VW
(*J Neurosci 14:846-856, 1994*)

Tejada-Berges T and Wright E: In earlier studies Mr. Tejada-Berges, a Masters student, and Mr. Wright, a summer student, assisted in performing preliminary injury studies in neonatal animals. The creation of the injury paradigm was taught to these gentlemen by myself, as I was responsible for the design of these trauma models.

Bouckova R: As a technician in the VW Yong's laboratory, Ms. Bouckova provided technical support in performing protein gel electrophoresis.

CHAPTER 3

Neonatal Astroglial Reactivity: Discordance of GFAP mRNA and protein content with immunoreactivity, and its dependence on macrophage presence.
Balasingam V, Dickson K, Brade A, Yong VW (Submitted)

Dickson K: As a technician in the VW Yong's laboratory, Ms. Dickson provided technical support in performing protein gel electrophoresis.

Brade A: As a summer student in VW Yong' laboratory, Mr. Brade performed the initial Northern analysis of the different neonatal injury paradigms.

CHAPTER 4

Attenuation of Astroglial Reactivity by Interleukin-10

Balasingam V, Yong VW (Submitted)

Co-authored with supervisor.

★★★

PRESENTATIONS

PRESENTATIONS

ORAL

Balasingam V (1995) Attenuation of astroglial reactivity by IL-10. Neurosurgical Rounds. Barrow Neurological Institute, Phoenix, Arizona, USA, October 19, 1995.

Balasingam V (1995) Astroglial reactivity & its modulation by microglia/macrophage - derived cytokines. Proceedings of the 37TH Annual Fellows' Day. Montreal Neurological Institute, Montreal, Canada, June 9, 1995.

Balasingam V (1995) Cytokines and Astroglial Reactivity. Noon Research Seminars. Montreal Neurological Institute, Montreal, Canada, March 14, 1995.

Balasingam V (1993) Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines. Proceedings of the 35TH Annual Fellows' Day, Montreal Neurological Institute. Montreal, Canada, June 11, 1993

POSTER

Balasingam V, Collier B, Voon VW (1995) Attenuation of Astroglial Reactivity by IL-10. (1995) Society for Neuroscience, 25th Annual Meeting. Nov 11-16, 1995, San Diego, USA.

Balasingam V, Voon VW (1995) Astroglial Reactivity and its modulation by Cytokines. Canadian Society for Clinical Investigation/Medical Research Council Special Joint Program, Sept 13-17, 1995, Montreal, Canada.

Balasingam V., Turley J, Yong VW (1993) Cytokines, inflammatory cells and astrogliosis. Society for Neuroscience, 23rd Annual Meeting. November 7-12, 1993, Washington, D.C., USA, Vol. 19.

Balasingam V, Yong VW (1993) Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines. Network of Centres of Excellence in Neural Regeneration and Recovery, 1993 General Meeting. May 27-30, 1993, Montreal, Canada.

Yong VW, Tejada-Berges T, Wright E, and Balasingam V (1992) The characteristics of reactive astrocytosis can be differentially modulated by cytokines. Society for Neuroscience, 22nd Annual Meeting. Anaheim, California, USA, Vol. 18.

Yong VW, Balasingam V (1992) The properties of reactive astrogliosis can be differentially modulated by cytokines. Network of Centres of Excellence in Neural Regeneration and Recovery, 1992 General Meeting. June 11-13, 1992, Toronto, Canada.

Balasingam V, Yong VW (1991) Mediation of neurotrophic properties of reactive astrocytes by the immune system. 6TH Annual Aspen MD-PhD Student Conference, Jul 19-21, 1991, Aspen, Colorado, USA.

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CHAPTER 1

GENERAL INTRODUCTION

PREFACE

The main objective of this general introduction is to provide historical information with a review of the literature to develop a hypothesis implicating the role of cytokines in astroglial reactivity. The first part, entitled historical perspective, provides a brief historical overview on the identification of neuroglia and their participation following trauma to the brain. The second part, entitled present perspective, focuses on a literature review of microglia/macrophages and astroglia in anisomorphic injuries. The third part, entitled cytokines and astroglial reactivity, highlights available evidence in the literature that implicate a role for cytokines in the process of astroglial reactivity. Finally, the fourth part of this chapter addresses the objectives of the thesis.

GENERAL INTRODUCTION

Wounds to the brain and spinal cord do not heal in a similar manner to that seen elsewhere in the body where there is regeneration of the parenchyma. The consequences of traumatic injury to neuronal elements of the central nervous system (CNS) have long been recognized as irreversible from the onset of mechanical insult. A series of complex reactions by non-neuronal cells of the CNS also predominates following CNS injury. These events following trauma have been interpreted differently through the years. This progressive comprehension will be illustrated from a brief historical preview beginning with the identification of non-neuronal cells. Recent evidence is then put forth to suggest a role for immunoregulatory inflammatory peptides from both the blood-borne and intrinsic CNS mononuclear phagocytes in contributing to the evolution of a brain scar. The central hypothesis of this thesis is that these immunoregulatory peptides or *cytokines* play an important role in modulating the specific neuroglial response described as astroglial reactivity following CNS trauma.

Historical Perspective - *Neuroglia* [1]

Definition of Neuroglia

The original concept of neuroglia was introduced by Virchow (1856) as a broad classification for the non-neuronal cellular elements of the CNS. Neuroglia were considered to be a connective tissue element within which nerves are embedded. Customarily, Virchow's paper in 1846 is cited as the first report of neuroglia, a credit bestowed on him by Weigert (1895). Strictly speaking this is not correct. Historians report that the concept of neuroglia came to Virchow in the late 1840's and 1850's as he applied

1. For a comprehensive historical overview of neuroglia (written in english), the reader is referred to Robertson (1897), Penfield (1932), Rio-Hortega (1932), Glees (1955), Malhotra et al., (1990) and Jacobson (1991).

Schwann's cell theory of 1839 to the brain. At that time, the substance of the brain and other organs were illustrated as "*tissues*" woven fabrics or pieces of cloth, as first proposed in the lectures of Xavier Bichat, (given in post-revolutionary France in the 1790's), but published after his death at the age of 32 in 1801. Bichat first suggested that *tissues* are where the functions of life and the dysfunction of disease occur; Virchow inferred from this to claim that cells are where life and disease occur.

In 1846, Virchow reported only the existence of a type of connective tissue, *Bindegewebe*, underneath the single-cell layer of the ependyma which line the ventricles. In 1856, when Virchow republished his 1846 paper, he added a new long footnote stating that " the ependyma is no isolated layer in the narrow sense of the word, but only the surface layer of the *Zwischen-Bindesubstanz* of the brain matter" (Young, 1991). This binding substance or *Bindesubstanz* "forms in the brain, in the spinal cord, and in the higher sensory nerves a sort of *Kitt* in which the nervous elements are embedded" (Somjen, 1988). This German word *Kitt* literally meant putty or glue. Since the brain's connective substance or *Kitt* appeared different from that of other organs, Virchow coined the term *Nervenkitt* (Virchow 1856, 1858). He had no idea of the function of *nervenkitt* and suspected a contributory role in pathologic processes: "This very interstitial tissue of the brain and spinal marrow is one of the most frequent seats of morbid change, as for example of fatty degeneration" (1858 as translated in Young, 1991). Virchow (1858) also proposed the mesodermal origin for these cells, an idea he borrowed from Remak (1852).

Golgi (1870) was the first to provide clear descriptions of non-neuronal cells and used the term *neuroglia*, which is the greek translation of *nervenkitt* or nerve glue used by Virchow, and referred to them as "*stroma connectiva*". In 1873, Golgi developed the potassium dichromate-silver technique which facilitated metallic impregnation of both neurons and glia - starting the modern era of glial cell biology. This technique provided the first morphological definition of neuroglial cells as a distinct class from neurons. Golgi's mercuric chloride method of 1879 and later the rapid method of 1887 were

contributory to the accelerated progress in neurocytology in the 1890's.

Identification of Astroglia

One of the first advances was the recognition of different types of neuroglia. Astrocytes were named by Lenhossék (1891) who recognized them as a separate subclass of stellate-looking cells, although their presence was recognized by Golgi in 1873. Astrocytes were later subdivided into two types by Koelliker (1893) and Andriezen (1893): Protoplasmic astrocytes by their absence of fibres in the cytoplasm and their predominant presence in the grey matter; Fibrous astrocytes by the presence of fibres in the cytoplasm and their predominant location within white matter.

Lenhossék (1893) then went on to show the histogenesis of astrocytes, starting with the detachment of radially oriented spongioblast or glial precursors followed by their migration into the brain parenchyma where they continued to divide to give rise to astrocytes. The transformation of radial glia into astrocytes was confirmed by Ramón y Cajal (1896) in the chick embryo spinal cord. These preliminary findings were re-discovered in the era of modern glial cell biology (Schmechel and Rakic, 1979a,b; Levitt and Rakic, 1980; Choi, 1981; Hajos and Basco, 1984; Benjelloun-Touini et al., 1985; Federoff, 1986; Munoz-Garcia and Ludwin 1986a,b; Hirano and Goldman, 1988). Lenhossék and Ramón y Cajal also showed that radial glial cells are the first to differentiate and their peripheral expansions persist in the spinal cord to form the glia limitans exterior, all of which have been confirmed with modern techniques (Rakic, 1972, 1981; Choi 1981; Liuzzi and Miller 1987).

The next developmental milestone in glial cell biology was achieved by Ramón y Cajal (1913) with his gold chloride-sublimate method for staining astrocytes. This staining technique revealed the soma and processes of astrocytes with great clarity, as a unit independent of syncytial connections. Using this technique Ramón y Cajal was able

to refute: 1) the presence of either a glial or neuronal syncytium connectivity of cytoplasm as proposed by Held (1903); and 2) the mesoblastic origin of protoplasmic and fibrous astrocytes proposed by Andriezen (1893). Ramón y Cajal maintained the names of these two types of astrocytes but insisted on an ectodermal origin for both. He described the protoplasmic astrocytes in the grey matter to have frequent branching and wide expansions, and the fibrous astrocytes of the white matter to have fewer branches, which are straight and long. He ascribed to astrocytes the function of serving neurons in a nutritive and insulating capacity. In addition, he proposed that astrocytes may have the capacity to make and break synaptic contacts by the contraction of their processes.

The Third Element

Neurons and neuroglia were the two major categories of cells in the CNS until Ramón y Cajal introduced the concept of the "tercer elemento" or the "third element". Cells of this "tercer elemento", the adendritic or apolar cells, were thought by him to be of mesodermal origin. Rio-Hortega (1919-1921) made the next great stride when he stained the "third element" with another specific stain for neuroglia, silver carbonate. Rio-Hortega showed that Ramón y Cajal's third element consisted of two types of cells: oligodendrocytes which were the incompletely stained cells that were devoid of processes and a new CNS cell type that he called microglia. These adult microglia when stained with silver carbonate revealed a ramified morphology. He proposed that the resident microglia were of mesodermal origin and contributed to phagocytosis in pathophysiology. It is to the microglia of the CNS that Rio-Hortega (1919a) would ascribe the term "tercer elemento" or the third element because of their different origin and function.

Identification of Microglia

Rio-Hortega (1921) demonstrated that young microglia first appeared in the embryonic CNS after the development of blood vessels. These young microglia were amoeboid in shape and also stained selectively with the silver carbonate technique. He noticed that these amoeboid microglia arrived into the brain via three gateways located at sites where the pia matter was closely related to white matter: (1) the tela choroidea (formed by the invagination of the pia matter) in the third ventricle, (2) the tela choroidea of the fourth ventricle, and (3) beneath the pia matter of the cerebral peduncles. This observation was further correlated by the presence of young amoeboid microglia only within the white matter with none in the grey. Their presence increased in numbers rapidly up to postnatal day 4 in rabbits and rodents. This was followed by a sharp decline in numbers that was seen simultaneously with an increase in the number of adult looking ramified microglia within grey matter by postnatal day 15. Penfield (1928) describes the developmental sequence of microglia reported by Rio-Hortega as follows:" (1) tuberous (containing vacuoles), (2) amoeboid or pseudopodic (containing protoplasmic inclusions), (3) ramified (as seen in normal adult)". On assuming the adult ramified phenotype, it was thought that these cells were in a resting phase and that they acted as sentinels until the occurrence of an insult to the CNS; at such time they would revert to their embryonic form and participate in phagocytosis.

Rio-Hortega also recognized that these young amoeboid microglia present during early postnatal development were the same cells described by Virchow (1867) as *Kornchenzellen*. These cells confined to the white matter were busy phagocytosing under normal conditions. The phagocytic activity of these cells under normal physiological developmental conditions was an observation that these authors were unable to resolve as they associated phagocytosis with pathophysiology.

Identification of Oligodendroglia

Oligodendroglia were actually first identified by Robertson (1899, 1900) with his platinum staining. He named them mesoglia since he believed these cells to be of mesodermal origin but he was unable to ascribe function to them. Rio-Hortega later independently rediscovered these cells by his more reliable staining technique and proposed a ectodermal origin. He named them oligodendrocytes primarily for the shorter and fewer processes recognized in these cells in comparison to astrocytes. These were further subdivided into the intrafasicular oligodendroglia of the white matter and the perineuronal satellite oligodendroglia of the grey matter. From their anatomical position and late postnatal appearance, Rio-Hortega (1922) proposed that oligodendroglia were involved with myelination of the CNS. Penfield (1924a) reaffirmed this notion.

Summary of Neuroglia

The introduction of the Spanish metallic impregnation techniques by Ramón y Cajal (1913, 1916) and Rio-Hortega (1919-1921) contributed significantly to the historical fund of knowledge on neuroglia. These microscopists identified two categories of neuroglial cells, macroglia (second element) and microglia (third element). The macroglia comprise the astrocytes and the oligodendrocytes which are both of ectodermal origin, as are the neurons (first element). In contrast, microglia were considered to be of mesodermal origin.

Neuroglial response to CNS pathophysiology was recognized and reported early on as being degenerative, hyperplastic or even phagocytic in both acute and chronic conditions. However, it was not until the advent of the Spanish metallic impregnation techniques that the specific responding neuroglial cells following injury could be identified.

Historical Perspective - *Microglia in trauma*

Transition of Microglia

Rio-Hortega, who was credited with identifying microglia, was the first to demonstrate the transition of resting ramified microglia into their activated form following traumatic cerebral puncture wounds to the cat. He reported that within 12 hours following injury, the cytoplasm of these activated microglia cells became swollen and elongated with the replacement of their ramified processes with short stubby processes. This was followed by the presence of amoeboid or *compound granular corpuscles* after 24 hours at the lesion site. Rio-Hortega, from these observations, proposed that compound granular corpuscles or Gitterzellen seen in many areas of cerebral destruction and pathology involving phagocytosis arose from resting ramified microglia. Later Rio-Hortega and Jiménez de Asua (1921) recognized the similarity of microglia to tissue macrophages seen elsewhere in the body by Metchnikoff [2] with respect to phagocytic activity. This notion was further strengthened by Metz and Spatz (1924) who demonstrated that the phagocytosis of iron pigment by CNS parenchymal and perivascular microglia was similar to the activity of macrophages seen elsewhere in the body.

Sources of Amoeboid Microglia

Penfield (1924b), who had worked with Rio-Hortega, also observed the presence of numerous similar granulo-adipose cells or Gitterzellen in stab wounds to the dog cortex after 6 days. He assumed these phagocytes to be amoeboid microglia as described by Rio-Hortega. Reconfirmation of this rapid transformation of resting microglia into their activated amoeboid forms was also independently demonstrated by Alberca (1926). Although Rio-Hortega and Penfield were proponents of the notion that compound granular

2. Elie Metchnikoff initiated the study of phagocytosis and coined the term macrophage.

corpuscles or amoeboid microglia were of CNS origin, others like Cone (1925) suggested that amoeboid microglia were of leukocyte origin from the blood. Cone's suggestion was based on observations of CNS infections that went on to pathology. This concept of the double origin of phagocytosing compound granular corpuscles or amoeboid microglia was not a new one as it was originally proposed by Alzheimer (1910). The origin of phagocytic cells in the CNS have long been an item of contention and would be carried into the modern era.

However, these early investigators recognized the similarities between microglia and non-CNS tissue macrophages with regards to histological staining and phagocytic activity at destructive foci. In an attempt to resolve these similarities, Penfield (1925) suggested that microglia be included as part of Aschoff's (1924) reticulo-endothelial system and this notion was also shared by Rezza (1925) and Jiminéz de Azúa (1927). The term reticulo-endothelial system was used by Aschoff to include all groups of cells capable of clearing particles and dyes by phagocytosis in organs such as the liver, lung, bone-marrow, and spleen. Rio-Hortega (1932) was in agreement and later acknowledged that microglia were the reticuloendothelial cells of the CNS.

Historical Perspective - *Astroglia in trauma*

Astrogliosis

Weigert (1895) showed that the overgrowth of neuroglia was invariably a response to CNS injury. This overgrowth of neuroglia or gliosis was believed by him to replace the disappearance of nervous tissue. In contrast, Alzheimer (1910) showed that the disappearance of neural tissue was not necessary for the occurrence of gliosis as evidenced in non-traumatic pathology. Da Fano (1906) in studying experimental brain wounds also noted the presence of an increasing neuroglial population and their interlaced network of fibres at the lesion site. With the advent of the gold chloride sublimate stain

for astrocytes, Ramón y Cajal (1913) and Rio-Hortega (1916) demonstrated that the earlier neuroglial reports of multiplication and hypertrophy of processes to form what Nissl had called *Gliarasen* at the lesion site was of astroglial origin. The Spanish investigators were therefore instrumental in demonstrating that the gliosis described by earlier investigators was actually one mounted by astroglia. This led to the synonymous use of gliosis with astrogliosis even to the present day.

Chronological study of Astrogliosis

Rio-Hortega and Penfield (1927) must be credited for conducting the first systematic chronological study of neuroglia reactivity following trauma to the CNS. They observed that microglia and astroglia were the two cell types that responded reactively to injury. In chronological sequence, Rio-Hortega and Penfield (1927), described that the initial response was mounted by microglia as it transformed from its resting phase to its active phagocytic state for the purposes of clearing degenerating debris. Secondly, this was followed by the swelling and "clasmatodendrosis" or acute degeneration of injured astrocytes located within the wound. Later, by the third day post-injury, surrounding astrocytes underwent amitotic division or proliferation and subsequent hypertrophy with fibrous changes to the cytoplasm. This occurred regardless of whether these astroglial cells were originally protoplasmic or fibrous in origin. Thirdly, compound granular corpuscles or Gitterzellen disappeared from the wound when the products of degeneration had been cleared. This was then followed by the reappearance of ramified microglia in the scar. Finally, they reported that neurons and oligodendrocytes did not enter into the reactive phase of scar formation. However, when axons were damaged, limited regeneration of these axons took place as reported by Ramón y Cajal (1928). Linnel (1929) later confirmed the finding that injured oligodendrocytes underwent degeneration rather than enter the hyperplastic reaction of scar formation. The observations of Rio-Hortega and Penfield (1927) led them to propose the notion that astrocytes serve as "delicate indicators of the action of noxious influences upon the CNS".

Penfield and Buckley (1928) extended the study of astrogliosis by comparing wounds where injured brain tissue was either left in place or completely removed in a clean fashion. They observed that the retention of damaged cerebral tissue initiates vigorous phagocytosis by compound granular corpuscles or Gitterzellen with a resultant greater extent of gliosis. On the other hand, removal of the injured cerebral tissue reduced the total number of compound granular corpuscles and decreased the extent of astrogliosis.

Anisomorphic and Isomorphic Gliosis

Astrogliosis elicited by traumatic injury result in the overgrowth of astroglia with an orientation directed radially towards wound margins (Rio-Hortega and Penfiled, 1927; Penfield and Buckley, 1928). Since this arrangement of reactive astroglia is not in keeping with the original morphological architecture, it is termed *anisomorphic gliosis*. In contrast, astrogliosis recognized in non-traumatic CNS pathology (e.g. chronic inflammatory disease like general paralysis, encephalitis, and toxic diseases that produce rapid neuroglial degeneration) appears to be different. Although astroglial reactivity in these types of insults display hyperplasia (as determined by increased numbers of astrocytes) and hypertrophy, the astroglial processes appear to be directed in a manner respecting the morphological architecture of the location. For example, in demyelinating lesions of the white matter, hypertrophied astroglial processes appear to be oriented in a direction that is parallel to the nerve fibers. Since gliosis in these circumstances are in keeping with the morphological architecture, it has been referred to as *isomorphic gliosis* (Penfield, 1932a,b).

Influencing factors for Astroglial Reactivity

It was obvious to Penfield that all forms of astroglia respond to injury in a stereotypical fashion involving hyperplasia, hypertrophy and fibrous changes. However, the specific influence that initiates the process of astroglial reactivity remained an enigma. From his work with Buckley (1928), it was clear to him that the retention or removal of injured cerebral tissue simulated the ideal circumstances to identifying the influencing factors. He surmised that the same influencing factors might be the contributing principle by which gliosis may be occurring under other varying conditions. He proposed that these influencing factors which produce gliosis may be a "chemical substance elaborated by the focal or widespread death of cerebral tissue". He also suggested that "in inflammatory processes the astroglia may perhaps be stimulated by the destroyed nervous tissue rather than by the inflammatory toxin" (Penfiled, 1932a,b). He disregarded the role of these compound granular corpuscles or amoeboid microglia as been contributory to the process of astrogliosis, since these amoeboid microglia or professional phagocytes were only ascribed with the function of debris clearance.

In this respect, this thesis advances the hypothesis that the presence of these amoeboid brain macrophages (of either intrinsic CNS or extrinsic systemic blood-borne origin) have a prominent role in influencing the process of anisomorphic astrogliosis in traumatic injury.

Present Perspective - *Microglia* [3]

Origin of Microglia

Resident endogenous cells of the adult CNS that express markers specific for mononuclear phagocytes are microglia. They are found throughout the CNS but are more abundant in the adult grey matter and comprise between 5% to 20% of the total neuroglial cell population (Peters et al., 1991; Kreutzberg, 1987). The origin of microglia, whether neuroectodermal or blood-borne, has been a long and protracted controversy over the decades since its discovery (Cammermeyer, 1970; Ling, 1981; Oehmichen, 1982; Jordan and Thomas, 1988; Ling and Wong, 1993).

Presently, much evidence suggest that Rio-Hortega (1919a), who first clearly identified these cells in the CNS parenchyma by silver impregnation, was correct in ascribing microglia to be of blood-borne monocytic origin. Several lines of evidence have been provided by modern investigators to support this notion. Firstly, there is biochemical, immunocytochemical and ultrastructural evidence on the basis of major histocompatibility antigens from chimera studies (Ting et al., 1983, Hickey and Kimura, 1988; Hickey et al., 1992). Secondly, the transition of invading monocytes into amoeboid microglia and their final transformation into ramified microglia in the developing CNS can be followed by autoradiography (Imamoto and Leblond, 1978), histochemical (Boya et al., 1979; Murabe and Sano, 1982a, Kaur et al., 1984; Fujimoto et al., 1987) and immunohistochemical methods (Hume et al., 1983; Perry et al., 1985, Perry and Gordon, 1987; Ling et al., 1990, 1991). Thirdly, by analyzing nuclear morphological changes, carbon-labelled monocytes can be followed ultrastructurally into microglia (Ling, 1979). Finally, from studies utilizing the transplant of embryonic mouse retina into the midbrain of newborn rats (Klassen and Lund, 1987), the transplanted tissues demonstrated the presence of host rat

3. The reader is directed to Perry and Gordon (1991) for a comprehensive review.

monocytes that had invaded the murine tissue and differentiated into microglia when detected by rat immunocytochemical markers (Perry and Lund, 1989).

Microglia in Development

During development (particularly from the late prenatal to early postnatal period), invading monocytes enter the CNS parenchyma (Miyake et al., 1984) but their portal of entry has been difficult to define. Perry and colleagues (1985) have observed their presence in large numbers around the choroid plexus, the lateral ventricular walls and white matter - an observation first provided by Rio-Hortega who interpreted the choroid plexus and the pia to be the portals of entry. Mammalian retinal tissue with its highly organized simple structure and peripheral location has currently surfaced as a more amenable tissue preparation for the study of microglia origin. Here too, most investigators have observed the migration of monocytes in advance of the invading vasculature from which they leave and confirmed their transition to amoeboid microglia and later to the ramified form with maturity (Ashwell, 1989; Ashwell et al., 1989; Boya et al., 1987a, 1991; Chugani et al., 1991; Hume et al., 1983; Sanya and De Ruiter, 1985).

There now appears to be a majority opinion that microglia originate form monocytes, of bone-marrow derivation, and populate the CNS after it has been vascularized (Kershman, 1939; Polak et al., 1982; Oehmichen, 1983; Dolman, 1985). Despite the substantial evidence provided for a haematogenous origin, however, there are others who suggest that microglia are derived from the neuroectoderm (Kitamura et al., 1984; Matsumoto and Fujiwara, 1986; Sminia et al., 1987; Hao et al., 1991)

The majority opinion also favours that upon entry into the CNS parenchyma early in development, monocytes transform into amoeboid phagocytosing cells or amoeboid microglia. These amoeboid microglia have been identified to be colocalized with cells undergoing either morphological changes or degeneration (Hume et al., 1983; Perry et al.,

1985; Ferrer et al., 1990; Ashwell, 1991; Ling and Tan, 1974; Valentino and Jones, 1981). This transient accumulation of amoeboid microglia appears to contribute to CNS cytoarchitecture during development. They serve this function by clearing neuronal debris occurring as a result of natural physiological cell death or by the removal of transitory projections of the white matter without killing the parent cell body (Cowan et al., 1984; Innocenti et al., 1983a, 1983b). The developmental timing for the influx of these invading monocytes appears to occur in parallel with natural physiological cell death or apoptosis, a process important in CNS development (Oppenheim, 1991; Williams and Herrup, 1988), as the birth of the CNS occurs with a high level of redundancy. This redundancy stems from the need for the CNS to establish highly specific cellular interactions and connectivity in comparison to other organ systems. Therefore the presence of these phagocytosing amoeboid macrophages is probably a very necessary developmental event for the achievement of a functional and efficient CNS cytoarchitecture.

Perry and colleagues (1985) have postulated that the occurrence of cell death may act as chemoattractants for the recruitment of monocytes into the CNS. In contrast, more recent quantitative studies of monocytic invasion into the developing retina revealed the presence of macrophages prior to the onset of retinal ganglion cell death (Ashwell et al., 1989; Ashwell, 1989), followed by the entry of larger number of monocytes with the onset of retinal ganglion cell death. These authors propose that cell death may not be the only stimulus for the recruitment of monocytes. However, with the development of the eye there are other earlier phases of cell death prior to that of retinal ganglion cell death (Silver and Sidman, 1980).

At present, the mechanisms involved in the recruitment of monocytes into the parenchyma of the CNS remain elusive. The transformation of these cell into amoeboid microglia and their presence within the parenchyma of the CNS is strictly limited to the developmental phase of life under normal physiological circumstances with the exception of areas lacking a blood-brain barrier (Perry and Gordon, 1988; Ling and Wong, 1993;

Pow et al., 1989). Amoeboid microglia phenotypically appear to be similar to macrophages in possessing a broad flat morphology with pseudopodia, but undergo down-regulation of membrane glycoprotein when transforming into the quiescent ramified microglia of the maturing postnatal rat brain (Wu et al., 1994).

Microglia in Maturity

Ramified microglia in the mature CNS occur developmentally from a sequential conversion of monocytes to amoeboid microglia and finally to the ramified phenotype. These normal mature CNS microglia have a small cell body (5-10μm) emanating radially with several thin branching processes covered with fine bristle-like protrusions, and hence the name ramified microglia. In addition, since these ramified microglia do not look or act like activated macrophages (differences between resting and activated microglia will be described below) in the quiescent CNS, they have also been referred to as resting microglia. Ramified microglia also appear to undergo little or no turnover in comparison to macrophages in other organ systems which continually undergo constant turnover (Van Furth, 1989).

The downregulated nature of ramified microglia in the mature CNS and its subsequent upregulation when macrophage activity is required sharply differs from macrophages in other organ systems. Interestingly, this downregulated state may be consistent with the notion that the CNS is a relatively immune privileged site. This is supported by the fact that the CNS lies behind a blood-brain barrier which is essentially a molecular sieve that prevents molecules above a certain size from freely entering the CNS parenchyma (Davson et al., 1987). [The anatomical basis of the barrier lies in the fact that the endothelium of brain capillaries have tight junctions between them (Reese and Karnovsky, 1967). A consequence of the barrier is not only that the microenvironment within the brain is different from elsewhere but also that the brain may be largely isolated from circulating cells of the immune system in their quiescent state;

however, it has been noted that activated T-lymphocytes can cross the blood-brain barrier (Hickey et al., 1991).] Under normal conditions, then, the blood-brain barrier effectively prevents immunoregulatory peptides such as IFN-gamma from gaining access to the CNS. The importance of this fact lies in the ability of IFN-gamma to activate tissue macrophages (Vogel et al., 1983; Nathan et al., 1983, 1984; Nedwin et al., 1985) and similarly also activate normal resting microglia (Wong et al., 1984; Frei et al., 1987; Colton et al., 1992). Therefore the downregulated status of ramified microglia may be specifically necessary to differentially protect the CNS from consequent immune mediated cellular damage under normal CNS physiological circumstances.

Ramified microglia are generally considered to be inactive downregulated CNS macrophages, acting as sentinels for CNS insults. However, their large number and even distribution suggest otherwise. The notion of an active role for ramified microglia in the CNS is supported by the following reports: 1) Microglia are found in close association with neuron and synapses (Murabe and Sano, 1982b; Palacios, 1990); and 2) Microglia appear to be actively involved in pinocytosis, a function probably essential in extracellular fluid cleansing and transmitter inactivation, particularly for diffusible neurotransmitters in volume transmission (Booth and Thomas, 1991; Glen et al., 1991; Ramson and Thomas, 1991; Ward et al., 1991).

Summary on Microglia

Microglia are most likely of monocytic lineage and appear within the CNS late in embryonic development. They facilitate the final phase of CNS development via the removal of redundant structures and the clearance of apoptotic debris. This is followed by the transformation of these microglia into the functionally resting ramified phenotype and they remain as such under normal circumstances (Theele and Streit, 1993; Ling and Wong, 1993). *In vivo,* the function of resting microglia has remained a mystery to date except for emerging evidence suggesting a role in pinocytosis.

Present Perspective - *Microglia in trauma*

Injury or insult to the CNS results in the rapid transformation of the quiescent resting ramified microglia into an activated state capable of mounting an immune response within the CNS (Streit and Graeber, 1988; Graeber and Streit, 1990; Perry and Gordon, 1988). This conversion is perhaps most strikingly associated with morphological and phenotypical changes that occur in a progressive fashion to include hypertrophy, proliferation, the upregulation of previously present and new surface antigens, and finally phagocytosis. However, the upregulation of resident ramified microglia may not always lead to the full transformation of these cells into phagocytosing brain macrophages also referred to as reactive microglia.

Activated and Reactive Microglia

It appears that sub-lethal neuronal damage, such as the transection of the facial nerve (Graeber et al., 1988; Rieske et al., 1989), leads to activation of microglia but not their conversion into phagocytosing brain macrophages - reactive microglia (Streit et al., 1988). The activated microglia appear swollen or hypertrophied ramified cells and are characterized by a larger cell body with shorter, stouter processes (Graeber and Streit,

1990; Streit and Kruetzberg, 1988). These activated microglia also increase their expression of CR-3 complement receptors (Graeber et al., ,1988) and MHC class I antigen (Streit et al., 1989a; Streit et al., 1989b). Under such circumstances, activated microglia have been reported to perform "synaptic stripping" without phagocytosis (Blinzinger and Krutzberg, 1968; Gehrmann et al., 1990). Therefore this activated microglial activity of synaptic stripping has been postulated to be a beneficial physiological repair process involved in neuronal cell energy conservation by terminating transmission (Nakajima and Kohsaka, 1993).

In contrast, neuronal cell death elicits maximal microglial upregulation as demonstrated in experimental models involving: (1) the injection of ricin into the facial nucleus (Streit and Krutzberg, 1988); (2) the parenchymal injection of kainic acid (Murabe et al., 1981; Kaur et al., 1992; Anderson et al., 1991) or ibotenic acid (Coffey et al., 1990); and (3) transient ischemic episodes (Brierley and Brown, 1982; Gehrmann et al., 1992; Morioka et al., 1992). Neuronal death leads to the transformation of ramified microglia into the phagocytosing brain macrophage phenotype also referred to as reactive microglia. These cells are typically small spheroid cells, but can also exhibit rod-shaped and pleomorphic or amoeboid-like morphologies and lack ramified processes (Akiyama et al., 1988; Brierley and Brown, 1982; Streit and Krutzberg, 1988). Reactive microglia are fully activated macrophages with an increased expression of CR3 complement receptors and MHC I and II antigens (Graeber et al., 1990; Streit et al., 1988, 1989; Streit, 1989). These cells are also capable of respiratory burst and general cytotoxity (Banati et al., 1993; Thomas, 1992).

Microglia/Macrophages in Anisomorphic Injuries

In traumatic situations, where the vasculature is disrupted with damage to the blood-brain barrier, blood-derived cells infiltrate the CNS parenchyma. In these cases, there is upregulation and proliferation of two groups of potentially phagocytic cell

populations - intrinsic microglia and the blood-derived monocytes which transform into macrophages upon entry into the CNS parenchyma. Presently, when these two cell populations are present at a lesion site simultaneously, they cannot be differentiated from each other. However, Giulian (1987) distinguishes between the intrinsic and blood-borne macrophages on the simultaneous basis of morphology and the expression of an acetylated low-density lipoprotein receptor. Until more precise and definite markers to differentiate between these two cell populations at traumatic lesion sites are available, all references to these cell will be as microglia/macrophages. The validity of making such a proposition lies in the ability of reactive microglia to characteristically function like blood-borne macrophages.

The one criticism for the collective use of microglia/macrophages in traumatic injuries may arise from the work of Giulian and Baker (1985, 1986). They demonstrate that amoeboid microglia or reactive microglia are different from blood monocytes or peritoneal macrophages in that microglia can release peptide factors that control the growth of astroglia and under certain conditions release cytotoxins. This criticism awaits future confirmation in other traumatic injury paradigms.

Microglia in Neuropathology

There is now increasing evidence in the literature from both *in vitro* and *in vivo* studies that implicate the presence of reactive microglia as contributory to neurological damage. This is attested to by their activated and/or reactive presence (as determined by an increased expression of MHC antigens) in a wide variety of neuropathology ranging from neural degeneration (Streit and Graeber, 1988) to an involvement in CNS infections (Vazeux et al., 1987; Micheals et al., 1988) and participation in autoimmune neurological diseases (Cuzner et al., 1988; Matsumoto et al., 1989).

What are the roles of activated and reactive microglia in the CNS?

Recent work confirms their role as resident tissue macrophages of the CNS (Dickson et al., 1991; Graeber and Streit, 1990; Jordan and Thomas, 1988; Colton and Gilbert, 1987) as characterized by their macrophage-like phenotype with respect to phagocytosis, tumour cytotoxicity, and superoxide anion production. Furthermore, a number of laboratories working with *in vitro* isolated microglia have identified the ability of stimulated microglia to secrete a number of noxious and cytotoxic agents (Piani et al., 1991; Giulian et al., 1993; Banati et al., 1993). These *in vitro* studies raise the possibility that microglia *in vivo* may facilitate neuronal degeneration via the secretion of all or some of their secretory products.

However, the presence of reactive microglia may also only reflect a further contribution to the already existent insult via a cellular immune mediated mechanism (Fontana et al., 1987; Giulian and Robertson, 1990). This suggestion is in concordance with *in vivo* studies utilizing animal models of autoimmune neurological disease (Matsumoto et al., 1989; Hickey and Kimura, 1988). In addition, besides diseases with a likely autoimmune etiology, microglial MHC expression has also been found in Parkinson's, Pick's and Huntington's diseases, Parkinsonism-dementia of Guam, amyotrophic lateral sclerosis and the Shy-Drager syndrome (McGeer et al., 1988). Microglia therefore appear to be an integral component of the immune response mounted by the CNS and contribute to this response through antigen presentation and lymphocyte activation (Frei et al., 1987; Hickey and Kimura, 1988). In essence every neurological condition has an immune component, which may be significant, in its natural history.

Summary of Microglia in Pathology

The transformation of ramified microglia into activated and reactive forms occur in a sequential stepwise fashion with apparent correlation between morphology and the functional state. Signals that regulate this transition are unclear and appears to be

dependent on the presence of neuronal death as verified by experimental models.

Present Perspective - Astroglia

Astrocytes are now considered to be a principle macroglial cell type in the CNS and participate in a variety of physiological and pathological processes. Astrocytes fall broadly into either the protoplasmic or fibrous categories identified by the earlier pioneers. The distribution of these two categories of astrocytes was identified with ease by Ramón y Cajal's gold chloride-sublimate technique. According to Vaughn and Pease (1967), as well as Mori and Leblond (1969), the gold chloride-sublimate stain deposits gold on the astroglial filaments contained in astrocytes and thereby facilitated the light microscopic evaluation of this neuroglial cell type. The protoplasmic astrocytes have numerous thick, short processes and are generally located in the cortical grey matter, caudate nucleus, putamen and the granular layer of the cerebellum (Tedeschi, 1970). Fibrous astrocytes have long, slender cytoplasmic processes and are chiefly found among bundles of myelinated fibres (white matter) as well as around blood vessels and in the sub-ependymal areas (Duffy 1983).

Astrocyte Function

Astrocytes were initially considered to be important in the mechanical support of other CNS components, but it is now apparent that astrocytes play a number of other important diverse and dynamic functions: a) form growth tracts to guide neuronal migration during early development (Hatten, 1985, 1990; Rakic 1971, 1985); b) supply trophic factor support for neurons prior to the establishment of postsynaptic connections (Schwartz and Mishler, 1990); c) storage of glycogen as an energy stockpile (Magistretti, 1988); d) maintenance of ionic homeostasis for the welfare of neuronal excitability (Lux et al., 1986; Walz, 1989; Sykova et al., 1992); e) neurotransmitter metabolism (Kimelberg and Katz, 1985; Kimelberg and Pelton, 1983; Shain et al., 1986); f) detoxification of

deleterious substances (Hertz et al., 1992); g) secretion of trophic factors (Mallat et al., 1989; Ferrara et al., 1988; Yoshida et al., 1991; Han et al., 1992; Rudge et al., 1992); h) immunomodulatory functions (Giulian, 1988; Giulian et al., 1988; Hertz, 1990); and i) participation in repair and the formation of scar tissue following neuronal loss (Duffy, 1983; Pfeiffer et al., 1977, Nieto-Sampedro, 1988).

Astrocyte Intermediate Filament

Both classes of astrocytes contain numerous 10 nm intermediate filaments which are composed primarily of glial fibrillary astrocytic protein [GFAP] (Bignami and Dahl, 1974); protoplasmic astrocytes contain less GFAP than their fibrous counterparts and this is presumably why the latter are more easily detectable in the normal brain by immunohistochemistry for GFAP. Moreover, the increased expression of and/or immunohistochemical staining detection of GFAP usually points to the occurrence of neurological insult.

GFAP is a highly conserved 50 kDa intermediate filament protein, of the type III subclass, found primarily within astrocytes (Eng et al., 1971, Bignami et al., 1972). Similar to other intermediate filaments, it is composed of three distinct regions which include an amino-terminal HEAD region, a central ROD region, and a carboxy-terminal TAIL region. The HEAD region appears to be critical in their maintenance as intermediate filaments, since phosphorylation of the amino acids in this region leads to disassembly of GFAP filaments into a soluble form (Inagaki et al., ,1990).

What is the function of GFAP?

In general, the principal function of all intermediate filaments is to provide mechanical support to the cell and its nucleus. Intermediate filaments form a "basket" around the nucleus and extend out in gently curving arrays to the periphery of the cell. Furthermore, the relatively slow metabolic turnover rate for GFAP is consistent with this

structural role (DeArmond et al., 1984, 1986; Smith et al., 1984). Beyond this generalization, the functions of intermediate filaments are unknown and their tissue specificity suggest some specialized function. To understand the function of GFAP, two groups of investigators utilised human astrocytoma cell lines ($GFAP^+$ U251 and $GFAP^-$ SF-126) where they either suppressed GFAP expression (U251: Weinstein et al., 1991) with an anti-sense GFAP RNA or induced GFAP expression (SF-126: Rutka et al., 1993) with a GFAP cDNA. These two independent studies revealed that the expression of GFAP in their *in vitro* system was necessary for proliferation arrest, cellular differentiation, and stable process formation in response to co-culture with neurons.

To study the *in vivo* role of GFAP in physiological and pathophysiological conditions, Pekny and associates (1995) generated a mutant strain of mice which were devoid of GFAP. They utilized targeted mutations directed at the embryonic stem cell level to disrupt the GFAP gene. Contrary to past predictions for GFAP, these GFAP deficient mice exhibited a normal pre- and postnatal development to reach fertile adulthood without any overt anatomical, histological, or psychomotor abnormalities. Furthermore, Pekny et al., (1995) report that in response to CNS injury these mutant mice respond in like fashion to that of wild-type animals with scarring 4 days after injury. These findings do not, however, rule out the absence of subtle changes in GFAP deficient mice as a consequence of trauma. Therefore, the function of GFAP still remains obscure.

Glial Fibrillary Acidic Protein

GFAP was initially discovered when it was purified from scar tissue found in a patient with a demyelinating disease, Multiple Sclerosis (Eng et al., 1970). Antibodies raised to this protein isolated from fibrous astrocytes (Eng et al., 1971) proved to be a useful immunohistochemical marker for astrocytes (Bignami et al., 1972) and neoplastic cells of glial lineage (reviewed by McLendon and Bigner, 1994). GFAP-IR has also been demonstrated outside the CNS in non-astrocytic cells that include Schwann cells (Dahl

et al., 1982), glia like cells in the myenteric plexus (Jessen et al., 1984, 1985), Kupffler cells in the liver (Gard et al., 1985), pleomorphic adenomas of the salivary gland (Nakazato et al., 1982; Stead et al., 1988) and a metastatic renal cell carcinoma (Budka et al., 1986).

Developmental studies have shown a caudal to rostral GFAP gradient by Northern analysis that is consonant with normal brain development (Lewis and Cowan, 1985; Landry et al., 1990). In the adult murine CNS, the highest concentration of GFAP mRNA is located in the *glial limitans* followed by decreasing amounts in the white and grey matter as revealed by in situ hybridization (Lewis and Cowan, 1985). GFAP-IR in the early postnatal animal can first be identified after birth predominantly on subependymal astrocytes and later in the corpus callosum followed by the glial limitans at postnatal day 5. GFAP-IR in the mature CNS is particularly strong in the astrocytes which form the *glial limitans,* found at the surface of the brain, followed by fibrillary astrocytes in white matter.

The method of tissue processing appears to be important as aldehyde fixatives have proven to be less optimal for the uncovering of GFAP epitopes in comparison to proteolytic digestion (Eng et al., 1983; McLendon et al., 1986). In this regard, although protoplasmic astrocytes of the grey matter contain low amounts of GFAP, they do not stain for GFAP when fixed in formaldehyde for extended periods of time (DeArmond and Eng, 1984). Under these conditions, independent of the injury stimulus, elevated GFAP-IR is routinely employed as a marker of astroglial reactivity. When astrocytes of either morphology (protoplasmic or fibrous) become reactive following insults to the CNS, they can be distinguished from the normal astrocytes by their large size, longer thicker processes, and increased immunohistochemical detection for GFAP (Bignami and Dahl, 1976).

As rodents age beyond the second year of life, levels of GFAP MRNA and GFAP content increase 2-3 fold above that of young adults particularly in the hippocampus, striatum and cortex (Goss et al., 1991; Nichols et al., 1993). In the hippocampus, the increase in GFAP content with senescence does not appear to be correlated with an increase in the number of total astrocytes (Gordon and Morgan, 1993). This suggests that astroglial reactivity with aging is less likely to be attributed to either proliferation or migration and may rather be mediated via the activity of local hormones (Landfield et al., 1978) or altered neuronal activity (Nichols et al., 1993).

Type 1 and 2 Astrocytes

Two types of GFAP+ astrocytes have been identified under *in vitro* conditions from the developing rat optic nerve (Raff et al., 1983). Type-1 astrocytes with a fibroblast like morphology can be stimulated to proliferate with either bovine pituitary extract or epidermal growth factor (EGF) and do not bind tetanus toxin or the monoclonal antibody A2B5 (both of which bind to specific polysialogangliosides). In contrast, Type-2 astrocytes with a neuron-like morphology bind tetanus toxin and A2B5 but cannot be stimulated to proliferate with either pituitary extract or EGF. Raff and colleagues (1984) proposed that oligodendrocytes and type-2 astrocytes differentiate from a common progenitor cell. This bipotential oligodendrocyte- type-2 astrocyte (O-2A) progenitor cell can differentiate into either oligodendrocytes or type-2 astrocytes if cultured in serum or 10% fetal calf serum respectively (Temple and Raff, 1985).

Developmentally, type-1 astrocytes are the first to appear *in vivo* at embryonic day 16 (E16). In the rat optic nerve O-2A progenitor cells begin yielding ologodendrocytes at birth (E21) and later type-2 (GFAP+, A2B5+) astrocytes bewteen postnatal day 7 - 10 (Miller et al., 1985). The presence of proliferating O-2A progenitor cells have also been confirmed in the adult rat optic nerve (Ffrench-Constant and Raff, 1986).

Is reactive gliosis a property of a distinct subpopulation of astrocytes?

This seminal question was addressed by an article titled with this question by proponents of the O-2A cells (Miller et al., 1986). They report that under normal conditions the majority of the white matter tract astrocytes are of the type-2 (GFAP+, A2B5+) variety. In injury models involving either a stab lesion to the corpus callosum or the transection of the optic nerve, however, the majority of reactive astrocytes were type-1 (GFAP+, A2B5-). Interestingly, quantitative studies following the transection of the optic nerve revealed a 10 fold decrease in the number of type-2 (A2B5+) astrocytes and a 25% increase in the number of type-1 (A2B5-) astrocytes. Therefore it was concluded that reactive astrocytes and their contribution to a glial scar in white matter tract was mainly a function of type-1 astrocytes.

Whether type-2 astrocytes and O-2A progenitors play a significant role *in vivo* remains controversial as their identification and characteriological assessment were done principally on the rat optic nerve. The use of the A2B5 marker to label type-2 astrocytes *in vivo* has also come under disrepute, since the A2B5 ganglioside, released by dying cells, can non-specifically adsorb onto the surface of cells. Therefore this thesis will not address the role of type-2 astrocytes or its O-2A progenitor cells in the study of reactive astrogliosis.

Present Perspective - *Astroglial Reactivity*

The stereotypical and rapid response by astroglia to a variety of neurological insults is referred to as astrogliosis or astroglial reactivity, a feature that is maintained by many different species. These insults include those produced by physical trauma (Amaducci et al., 1981), light-induced photoreceptor degeneration of the retina (Eisenfeld et al., 1984), cellular immunological insult such as experimental allergic encephalomyelitis (Smith et al., 1983), experimental allergic uveitis (Wacker et al., 1977; Chan et al., 1985), hyperthermia (Miller et al., 1987; Sharma et al., 1992), electrically induced seizure

(Steward et al., 1991), administration of toxic substances (Isacson et al., 1987; O'Callaghan et al., 1990; Goodlett et al., 1993), demyelination such as multiple sclerosis (Traugott and Raine, 1985), infection with Cruetzfeld-Jacob disease (Manuelidis et al., 1987), biochemical alteration due to a genetic defect (Eisenfeld et al., 1984) and spinal cord transection (Reier et al., 1986).

Astroglial reactivity, in addition, can also occur from the administration of toxic substances like colchicine, kainate and triethyl tin methyl mercury (O'Callaghan, 1988; Sancesario and Kreutzberg, 1986; Dusart et al., 1991), dichloromethane (Rosengreen et al., 1986a), ethanol (Goodlett et al., 1993), ibotenic acid (Isacson et al., 1987), xylene (Rosengren et al., 1986b), trimethyl tin (Reuhl et al., 1983, 1985; O'Callaghan et al., 1988), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Reinhard et al., 1988; O'Callaghan et al., 1990; O'Callaghan and Seidler, 1992), 1,1,1-trichloroethane (Rosengren et al., 1985), 6-hydoxydopamine (Rataboul et al., 1989) and 3,3'iminodipropionitrile (Llorens et al., 1993).

Characteristics of Astroglial Reactivity

Traditionally, astroglial reactivity refers to the hyperplasia and hypertrophy of astrocytes associated with fibrous changes to the cell (Penfield, 1932b). In addition, other changes include an increase in the number of mitochondria, Golgi membranes, endoplasmic reticulum, glycogen content and various enzyme levels (Latov et al., 1979; Smith et al., 1983; Mathewson and Berry, 1985; Maxwell et al., 1990a, 1990b; Nathaniel and Nathaniel, 1981; Norenberg et al., 1994). Eddleston and Mucke (1993) have extensively reviewed other changes to the reactive astrocyte including levels of various proteases.

In addition, to GFAP, normal astrocytes also express carbonic anhydrase, glutamine synthethase and S-100 (Cammer and Tansey, 1988; Cammer and Downing, 1991; Cocchia, 1981). Therefore, some investigators in the literature have chosen to use one of the above proteins as markers of astroglial reactivity. (Cammer and Tansey, 1989; Cammer and Downing, 1991). Of these, carbonic anhydrase and S-100 are not astrocyte specific and can be located on oligodendrocytes (Ludwin et al., 1976; Cammer et al., 1985; Cammer, 1990; Penky et al., 1995) and in the case of S-100 in certain neuronal membrane structures as well (Cocchia, 1988; Ghandour et al., 1981). In the case of Glutamine synthethase, although it has been reported in both normal and reactive astrocytes (Norenberg, 1983; Cammer et al., 1989, 1990), many recent studies report unchanged levels for this enzyme in astroglial reactivity (Jacque et al., 1986; Condorelli et al., 1990; Li and Bartlett, 1991; Hardin et al., 1994).

Therefore, in recent years GFAP-IR has superseded the use of these other markers for identifying astroglial reactivity since the initiation of GFAP expression can occur rapidly and specifically in astroglia. This rapid response can be initiated within an hour as illustrated by transgenic studies utilizing a GFAP promoter to drive a *LacZ* transgene (Mucke et al., 1991).

GFAP and Astroglial Reactivity

A convenient determination of astrogliosis is presently based on the appearance of reactive astrocytes by GFAP immunostained sections (Norton et al., 1992). GFAP-IR, however, is not quantitative. This is because the intensity of GFAP-IR may not correlate with GFAP content. The increased expression of GFAP-IR following insults usually accompanies an increased GFAP synthesis (Hozumi et al., 1990a; Vijayan et al., 1990). However, GFAP-IR can be observed even when GFAP synthesis is not enhanced. The early stages of experimental allergic encephalomyelitis (EAE) (Smith et al., 1983) reveal the presence of astroglial reactivity by intense GFAP-IR without a concomitant increase

in GFAP content (Smith et al., 1983, Goldmuntz et al., 1986; Aquino et al., 1988, 1990). Similarly, a rapid increase in GFAP staining has been reported 30 minutes after a cryogenic lesion to the rat CNS (Amaducci et al., 1981). Studies by Aquino and coworkers (1988, 1990) have demonstrated that the increased GFAP-IR in EAE is unrelated to the presence of an increased soluble pool of GFAP or the enhanced exposure of GFAP epitopes from proteolysis. Technically, it did not appear to be due to variations in the affinities of different GFAP antibodies. Ultrastructural analysis has however revealed the presence of disrupted glial intermediate filaments in astrocytic processes secondary to edema (Kimelberg et al., 1982). Eng and colleagues (1989) therefore propose that the resulting edema in EAE caters for the enhanced exposure of GFAP epitopes. This exposure occurs via the disassociation of intermediate filaments without an increase in GFAP content. However, while enhanced GFAP-IR is a sensitive and specific marker of an astroglial reaction, it could occur either from an increase in GFAP synthesis or GFAP intermediate filament disassociation.

What is the function of the increased level of GFAP content seen in astroglial reactivity?

The increase can occur early on following mechanical trauma (Hozumi et al., 1990a; Vijayan et al., 1990) or much later in time in EAE (Aquino et al., 1988). In normal rodent brains, astrocytes of the glial limitans and the hippocampal formation show increased levels of GFAP mRNA and IR in comparison to other brain structures (Bignami and Dahl, 1974; Kalman and Hajos, 1989; Landry et al., 1990; Lewis and Cowan, 1985; Mucke et al., 1991). Does this observation on the heterogenous levels of GFAP expression in the normal brain correlate with a general increase in functional demands placed upon these subsets of astrocytes that are strongly GFAP-IR? The answer awaits further experimental evaluation in the normal brain and the results will shed light on the function of astroglial reactivity.

Cellular Response of Astroglial Reactivity

The origin of the increased number of GFAP-IR cells at traumatic lesion sites was commonly assumed in the past to be due to proliferation. In the last decade, the source of these GFAP-IR cells have been intensely debated as to whether they represent proliferation, migration or phenotypic change of local astrocytes. Double labelling studies using GFAP-IR with tritiated thymidine, bromodeoxyuridine [BRDU] or proliferating cell nuclear antigen [PCNA] (Latov et al., 1979; Graeber et al., 1988; Takamiya et al., 1988; Miyake et al., 1988, 1989, 1992) have shown that in response to acute lesions, hyperplasia, when present, is usually minimal and confined to the immediate areas bordering the traumatic lesion (reviewed in Norton et al., 1992); however, some studies have shown proliferation of astrocytes in adult animals to be significant (Topp et al., 1989; Janeczko, 1989). Proliferation of astrocytes, however, can not be excluded from chronic pathological processes where it contributes to astrocytosis or hyperplasia.

Cellular hypertrophy, another commonly recognized astroglial response, is generally taken to reflect a healthy process initiated by an increased demand in relation to metabolism and protein synthesis. However, under certain conditions astroglial swelling appears as hypertrophy under immunohistochemical detection. This may occur in a pathological process that affects the astrocytes such as hepatic encephalopathy (Norenberg, 1986). Therefore, astroglial swelling should be definitively distinguished from astroglial hypertrophy at the ultrastructural level when in doubt.

The process of astroglial reactivity following injuries to the CNS appears to be dynamic and continues to evolve with time. It is conceivable that an increase in cytoskeletal proteins within reactive astrocytes may facilitate wound repair by stabilizing the tissue surrounding the lesion site in trauma. These reactive astrocytes help to wall of areas of tissue necrosis, in an attempt to exclude non-neuronal cells from the CNS parenchyma. A long term reaction of this astrocytic reaction is the formation of a glial

scar. This glial scar, depending on the type of injury may be composed of: (1) many different cell types and myelin debris; (2) collagen dense bundles and extracellular matrix proteins; and (3) multiple layers of abnormal basal lamina (Reir et al., 1983). Traditionally, this glial scar has been looked upon as undesirable as it may generate foci of electrical instability and epilepsy (Pollen and Trachtenberg, 1970).

Inhibition versus Neurotrophism

The CNS does not regenerate effectively after injury (Ramón y cajal, 1928).

Studies by Aguayo and colleagues (1982) demonstrate that this outcome is not due to an intrinsic inability of these central neurons to regenerate but rather to the environment present within the CNS. It has been proposed that the glial scar may form a barrier that could mechanically hinder regenerative processes such as neurite outgrowth (Reier et al., 1983; Reier, 1986). Ultrastructural analysis of glial scars revealed the arrest of regenerating axons in the immediate vicinity of reactive astrocytes (Liuzzi and Lasek, 1987). Immunohistochemically, putative inhibitory factors have been detected on reactive astrocytes (McKeon et al., 1991; Laywell et al., 1992) but the identification of their cellular sources remain to be achieved.

It is difficult to prove that dense gliotic scars mechanically block axonal growth because: (1) growth cones have the ability to degrade extracellular matrix components through the release of proteases (Guenther et al., 1985; Pittman, 1985; McGuire and Seeds, 1990); and (2) in vitro evidence suggest that astrocytes themselves are not necessarily inhibitory to regeneration (Manthrope et al., 1986; Hatten et al., 1991). David and colleagues (1990) have shown that reactive astrocytes do not prevent chick dorsal root ganglion cells from extending neurites over glial scars in optic nerve explants. Moreover, the in vivo experiments of Kawaja and Gage (1991) showed that in the presence of NGF (produced by transplanted fibroblast), reactive astrocytes were able to provide a substrate

for the growth of sympathetic neurites.

More recent evidence suggests that the initial phase of astroglial reactivity may be an attempt by these cells to promote CNS recovery rather than to act as an impediment. This concept is based on *in vivo* observations that include: 1) neurotrophic factors are produced around the locus of a traumatic lesion (Nieto-Sampedro et al., 1982, 1983; Manthrope et al., 1983; Needles et al., 1984; Whittemore et al., 1985) and the source appears to be reactive astrocytes (Nieto-Sampedro et al., 1983); and 2) in previously acallosal adult mice, a nitrocellulose membrane embedded with early postnatal murine astrocytes was able to provide a terrain suitable for axons to traverse the cerebral midline to reform the corpus callosum (Silver and Ogawa, 1983). Furthermore, *in vitro* experiments suggest that astrocytes have many neurotrophic properties. Co-culture studies of astrocytes and neurones have shown that cultured astrocytes: 1) are conducive substrates for the growth of neurons *in vitro* (Banker, 1980; Noble et al., 1984; Fallon et al., 1985); and 2) synthesize a large number of neurotrophic factors (Muller et al., 1984; Rudge et al., 1985; Muller et al., 1987; Varon et al., 1987; Hatten et al., 1988). Some of these neurotrophic factors may affect neurons directly whereas others could benefit neurons indirectly through the support of other non-neuronal cells.

While there is little evidence to implicate a direct detrimental effect by reactive astrocytes on the nervous system, it is conceivable that an impairment of astroglial function could exacerbate neuronal dysfunction. This pathogenic scenario may exist in several pathological conditions. These include: (1) hepatic encephalopathy (Norenberg, 1986); (2) scrapie, in which prions appear to be accumulate first in astrocytes (Diedrich et al., 1991); and (3) AIDS dementia, where viral or macrophage-derived products could interfere with astroglial function such as neurotrophic support and/or the elimination of excitotoxins (Brenneman et al., 1987, 1988; Cheng-Mayer et al., 1987; Lipton, 1992; Pulliam, 1993).

Summary

Injury to the CNS results in astroglial reactivity that is easily identified by GFAP-IR. The prominence and rapid initiation of astroglial reactivity in brain pathology indicates that reactive astrocytes play important roles in the CNS. Clarification of the functions of reactive astrocytes requires the identification of molecular mediators involved in astrogliosis. The manipulation of these modulators can provide clues to comprehend the nature of astrogliosis and have implications for CNS recovery. It is therefore essential to recognize that the nature of molecular mediators may differ depending on the type of injury (e.g. chronic demyelinating degenerative disease versus acute trauma) and the status of the blood-brain barrier. When the blood-brain-barrier is breached (anisomorphic injuries) (Mansour et al., 1990; Fernaud-Espinosa et al., 1993), astrocytes are likely to encounter a milieu substantially different from the environment seen when the blood-brain-barrier is maintained intact as in isomorphic injuries (e.g. facial nerve resection model) (Graeber et al., 1988; Fernaud-Espinosa et al., 1993). This thesis focuses on astrogliosis in anisomorphic injuries, and tests the postulate that inflammatory cytokines are mediators of astrogliosis.

Cytokines and Astroglial Reactivity

Definition of Cytokines

Cytokines are defined as a heterogenous group of large polypeptide immunoregulators. They have traditionally been associated with the immune system and its initiation of an inflammatory response. These immunoregulators are of a variety of types displaying both stimulatory and inflammatory characteristics. In addition, cytokines also possess specific neuromodulatory activities in the normal CNS and thereby establish bidirectional communication between the nervous and immune systems (reviewed by Plata-Salaman, 1991).

These immunoregulatory peptides include the interleukins (IL), interferons (IFN), the various colony stimulating factors (CSF), and the tumour necrosis factors (TNF). The above distinction for cytokines are outlined in order to differentiate them from non-immune related cell secreted products such as epidermal growth factor, fibroblast growth factor and nerve growth factor. Early studies on cytokine biology performed on lymphocytes and macrophages have helped to elucidate the following variety of autocrine and paracrine mechanisms: Cytokines (1) directly alter cell proliferation and/or differentiation; (2) induce the secretion of another cytokine and thereby induce an immune cascade; (3) modulate the effects of other cytokines on the same target to achieve inhibitory, additive or synergistic activity; (4) control the synthesis of extracellular matrix proteins important to cell growth and tissue repair. Ultimately, the functional biological specificity of a cytokine is dependant on the target cell and the microenvironment in which it operates.

Sources of Cytokines

Traditionally cytokines were thought to be derived from lymphocytes and macrophages of the peripheral immune system. However, cytokines can now be shown to be produced by numerous cell types that are not traditionally part of the immune system. This includes neurons and astroglia. Constitutive expression and activity of cytokines in the CNS are generally low, but can be increased in response to "tissue stress" (Fontana et al., 1993; Hopkins and Rothwell, 1995) by either invading pathogens or various forms of tissue damage (Tchelingerian et al., 1993; Wesselingh et al., 1990).

Sites of cytokine synthesis within the CNS are largely dependent on the nature of the stimulus. Since the CNS is not an immune-privileged site, macrophages, T cells and neutrophils can potentially invade the brain and provide a rich local source of inflammatory cytokines. Unlike the peripheral nervous system, entry of inflammatory mononuclear cells into the CNS appears to be delayed until several hours after injury and

neutrophils rarely invade (Perry et al., 1993). Therefore, under circumstances where the CNS vasculature is not damaged, the early expression of cytokines is presumably a response by resident CNS microglial cells. Support for this notion has been provided from in vitro microglial studies (Giulian et al., 1986; Hetier et al., 1988). Although there are probably multiple mechanisms of cytokine induction within the CNS, it is likely that cytokine cascades exist as is apparent in the sequential induction of TNF-α, IL-1, and IL-6 during infection of the CNS (Waage et al., 1989). These cytokines also share the property of further inducing their own synthesis as well as that of others so as to perpetuate a disease process. On the other hand this cytokine cascade may amplify the synthesis of cytokines for a limited time period and then downregulate, returning cytokines to normal low levels, via the presence of inhibitory immunoregulatory factors. The presence of these inhibitory endogenous mechanisms, for example in the form of IL-10 (Mosmann, 1994), IL-1 receptor antagonist (Eisenberg et al., 1990; Hannum et al., 1990) and soluble TNF-α receptors (Seckinger et al., 1988), come as no surprise since the overproduction of cytokines can lead to severe damage. Thus the major challenges faced in studying cytokine regulation within the CNS involves understanding the mechanisms by which it acts.

Cytokines in Astroglial Reativity

Anisomorphic injuries to the adult mammalian CNS generates a cascade of complex cellular events that includes the activation of intrinsic resident microglia, the infiltration of blood-borne monocytes to become brain-macrophages and astroglial reactivity (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). The presence of both microglia and blood-derived macrophages can contribute to increased levels of cytokine presence at the lesion site. Indeed, increased levels of IL-1, IL-6 and TNF-α are detected in the brain following trauma (Nieto-Sampedro et al., 1987a, 1987b; Taupin et al., 1993; Tchelingerian et al., 1993)).

The presence of increased levels of cytokines and astroglial reactivity raises the question of their inter-relationship *in vivo*. In particular, can cytokines have a role in modulating astroglial reactivity that follows traumatic injury? A role for cytokines in mediating the occurence of astrogliosis is supported by the observation that the intraoccular injection of IL-1, γ-IFN and TNF-α resulted in the increased adherence of inflammatory cells to the vascular endothelium and evoked astrogliosis in rabbits (Brosnan et al., 1989). Other studies have demonstrated an increase in the extent of astrogliosis in the adult rodent brain by the administration of IL-1 (Giulian et al, 1988), IL-2 (Watts et al., 1989) and IFN-γ (Yong et al., 1991). Although, the extent to which astrocytic hyperplasia contributes towards astrogliosis remains controversial (Cavanagh, 1970; Latov et al., 1979; Miyake et al., 1988, 1989, 1992; Takamiya et al., 1988; Janeczko, 1988,1991; Topp et al., 1989), the increased *in vitro* proliferation of primary human adult astrocytes to TNF-α (Barna et al., 1990) and IFN-γ (Yong et al., 1991, 1992) remains a valuable indicator of cytokine involvement. In addition there is 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). Moreover, receptors for several cytokines [e.g. IL-1, IL-6, IL-7, TNF-α, IFN-α, IFN-B, IFN-gamma, and colony stimulating factors] have been identified on astrocytes (Rubio and Felipe, 1991; Ban et al., 1993; Sawada et al., 1993; Tada et al., 1994) and the list is likely to increase.

Summary

Wounds to the brain and spinal cord result in damage to the parenchyma of the CNS and the disruption of the blood brain barrier. The acute neurodegeneration and repair of the CNS that follows involves a complex interactive cascade between microglia and astroglia. In addition, inflammatory cells of the blood-borne lineage also participate as the blood-brain barrier is disrupted.

This highly complex scenario is further extended by recent observations that demonstrate cytokine to play important functions in the course of astroglial reactivity. This notion is also supported by reports demonstrating elevated levels of IL-1 and TNF-α in human patients with head injury (McClain et al., 1987; Goodman et al., 1990). Therefore the hypothesis implicating cytokines with modulating astroglial reactivity warrants examination. The results of this study will benefit future neurological management of trauma patients.

Objectives of Thesis

The objectives of my thesis are:

Objective 1. **To define the role of cytokines as mediators of reactive astrogliosis following CNS trauma.**

To achieve this aim, a neonatal mouse brain injury paradigm was utilized as injuries inflicted during embryonic or neonatal life give rise to minimal astrogliosis, if any at all. This minimal astrogliosis is characterized by the lack of scar formation 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 et al., 1984).

Reasons postulated for the apparent lack of astrogliosis include the relative immaturity and plasticity of astrocytes and neurons and the lack of myelin in neonatal animals. Since the immune system in neonatal animals is relatively immature compared to adult (Hobbs, 1969; Abo et al., 1983; Lu and Unanue, 1985; De Paolie et al., 1988; Hannet et al., 1992), there may be an insufficient supply of cytokines at traumatic lesion sites. This consequent lack of cytokine production may thus constitute a probable cause for the lack of astroglial reactivity following neonatal CNS injuries.

To explore this postulate, the aim of the study in chapter 2 was to inflict cortical damage to the neonatal brain to: (1) document the resultant extent of astroglial reactivity and (2) determine whether the extent of astroglial reactivity could be increased by administering cytokines.

Objective 2. **To characterize the chronological description of neonatal astroglial reactivity and inflammatory cell recruitment following trauma.**

The aim of this study in chapter 3 was to: (1) confirm the ability of the neonatal CNS to respond with extensive astroglial reactivity; and (2) determine the differential representation of inflammatory cells to account for astroglial reactivity.

To elicit astroglial reactivity in these studies we utilized an acute (stab) and chronic (nitrocellulose membrane implant) injury paradigms. This extensive quantitative analysis of neonatal astroglial reactivity was performed at the level of GFAP-IR, GFAP content and GFAP mRNA levels. Similar quantitation was performed in adult animals for purposes of comparison. Furthermore, ultrastructural analysis was also performed in neonates for astroglial reactivity and the presence of inflammatory cells.

Objective 3. **To determine whether IL-10, a cytokine synthesis inhibitory factor, could curtail astroglial reactivity in adult mice.**

The objective of this study (in chapter 4) was to attempt attenuation of astroglial reactivity by inhibiting microglia/macrophage function. The premise is that if cytokines modulate astroglial reactivity, then the inhibition of cytokine synthesis and availability would lead to attenuation of astroglial reactivity.

Experiments for this study involved the use of an adult aspiration corticectomy model that by nature of the injury would elicit astroglial reactivity. Attempts at attenuating this astroglial response was achieved by the application of IL-10 via Gelfoam to overly corticectomy sites. The analysis of astroglial reactivity was conducted with the use of GFAP-IR and the gold-chloride sublimate technique for astrocytes. In addition, the activated status of microglia/macrophages was determined by indirect immunoflourescence for Mac-1 (a complement type 3 receptor antibody). Finally, we address whether or not

IL-10 could decrease the level of a microglia/macrophage - derived cytokine, TNF-α, within the injured mouse brain.

Summary

The central hypothesis of this thesis implicates a role for cytokines in modulating astroglial reactivity. Experiments to test this hypothesis were designed on the basis of biochemical, immunocytochemical and ultrastructural criteria.

The experiments outlined in this thesis provide compelling evidence for cytokines in astroglial reactivity.

★★★

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CHAPTER 2

REACTIVE ASTROGLIOSIS IN THE NEONATAL MOUSE BRAIN AND ITS MODULATION BY CYTOKINES

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PREFACE

While the presentation of astrogliosis is common to injuries occuring in the adult CNS, injuries inflicted during embryonic or neonatal life have been observed to produce minimal astrogliosis. Since the immune system in neonatal animals is relatively immature compared to adults, the aim of the present study was to inflict damage to the neonatal brain, to document the resuiltant extent of astrogliosis, and to deterine whether this extent could be increased by exogenously administered cytokines.

ABSTRACT

Reactive astrogliosis is a characteristic response of astrocytes to inflammation and trauma of the adult central nervous system. 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 post-natal day 3 mice resulted in a tremendous astrogliotic response 4 days later, as measured by GFAP immunoreactivity and GFAP content. In contrast, a neonatal stab wound produced limited astroglial response when compared to the adult stab wound. Utilising the neonatal stab wound model, cytokines were micro-injected 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 central nervous system (CNS) (Latov et al., 1979; Smith et al., 1983; Mathewson and Berry, 1985; Maxwell et al., 1990a,b). A long term 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-gamma (γ-IFN) (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-alpha (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., 1990; Yong et al., 1991a; 1992a), 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 (Osterberger and Wattenberberg, 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 mouse resulted in extensive GFAP-IR and increased GFAP content when measured 4 days 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; Maxwell et al., 1990a; Hozumi et al., 1990).

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 days after, cytokine-treated animals had extensive astrogliosis. All cytokines tested [γ-IFN, IL-1, IL-2, IL-3, 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 dependant 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 CD1 mouse pups (of either sex from standard sized litters) obtained from a commercial source (Charles River Canada, Montreal) 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 square piece of nitrocellulose membrane (Schleicher & Schuell,Keene, NH) previously boiled in 3 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 days). 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 h before being returned to their nursing mothers.

Creation of scissors-stab injury in adult mouse brains

Female CD1 retired breeders (Charles River Canada, Montreal) were anesthetized 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 h 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 μL/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-alpha (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 and United Biotechnology Incorporated, Lake Placid, NY).

***Qualitative and quantitative assessment of GFAP-IR* in situ**

All animals were killed by CO_2 euthanasia 4 days following surgery; this time point was chosen to reflect our findings (Moumdjian et al., 1991, Yong et al., 1991a) 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 minutes and fixed in 70% ethanol for 20 minutes. After washing with phosphate buffered saline (PBS), each section was treated for 2 h with 3% ovalbumin (Sigma) as a blocking step prior to incubation with a rabbit anti-GFAP polyclonal antibody (1:100, Dako Corp) for 6 h at room temperature. Following a brief

rinse with PBS, a goat-anti-rabbit immunoglobulin conjugated to FITC (1:75, Jackson) was introduced for 1 h. Negative controls for immunohistochemistry was replacement of the primary antibody with the diluting medium for antibody, HHG (i.e. 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 2.5 X 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 criterion 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 criteria 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 10 X 0.30 NA objective in order to facilitate image acquisition. Images were also obtained (at scan -8) with a 40 X 1.3 NA oil immersion objective for all groups, as a means to verify 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 days 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 volumes 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 (Biorad) which utilized bovine serum albumin as a standard. Six milligrams of protein extract was further diluted in sample buffer (50 mM Tris-HCL (pH 6.8), 2% SDS, 10% Glycerol, approximately 0.1-0.05% Bromophenol Blue) and boiled for 5 min at 100°C. Samples (in 20 µl volume) were electrophoresed on a 10% SDS-PAGE for 45 min at 200 V constant voltage, with different quantities of purified bovine GFAP (Boehringer-Manheim) as standards.

After electrophoresis, the samples were transblotted to a piece of Immobilon P membrane (Millipore) at 100 V constant voltage for 1 h 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 h 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 h. 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 were 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 were 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 (Fig. 2.4 and 2.6).

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 et al, 1992b). In all experiments, cells were treated once with test agents (see Table 2.3) and maintained for 4 days; 1 μCi of ^{3}H-thymidine was administered during the last 16 h 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. 2.1) 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 days (NC-implant) evoked extensive astrogliosis as determined by the area of GFAP-IR (Fig. 2.1 and 2.3). To reconcile this observation with the multitude of reports that have documented minimal

astrogliosis in neonatal animals following a CNS stab injury (Osterberger and Wattenberberg, 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 postnatal day 3 mice. Four days later, GFAP-IR was minimal (Fig. 2.2); in contrast, a similar Scissors-stab injury to the adult mouse brain (Fig. 2.2) resulted in extensive gliosis with a spatial distribution described by Mathewson and Berry (1985). Furthermore, in neonatal animals stabbed with a piece of NC membrane which was then removed (NC-Stab), GFAP-IR was also minimal (Fig. 2.2), 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 quantitated the area of the cortex containing GFAP-IR astrocytes in the different injury models. Figure 2.3 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 postnatal day 3 pups. Similar quantitation 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. 2.3).

Protein extracts from the resected areas circumscribing the lesion sites were electrophoresed on SDS-PAGE (Fig. 2.4). 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 2.1).

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 (Fig. 2.1 and 2.2).

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. 2.1) and GFAP protein content (Fig. 2.4), 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 2.1), 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 2.2, 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 (Hemmi et al., 1989; Gray 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 2.2).

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., 1991a, 1991b, 1992a). 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. 2.5).

Figure 2.3 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 ± 27 $x10^3$ μm^2) compared to the Scissors-stab injury alone (mean area of 30 ± 2 $x10^3$ μm^2). Comparisons between rmγ-IFN and its 0.2% BSA vehicle shows that the deposition of 20 units of rmγ-IFN (mean area of 634 ± 54 $x10^3$ μm^2) further increased the extent of astrogliosis by 3.5 fold ($p< 0.01$, Student's t-test).

Measurements of GFAP content (Fig. 2.6) of 10 mg samples circumscribing the cytokine-injected Scissors-stab site shows that when expressed as a ratio of normal brains, GFAP content 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 be reflective of astrogliosis on the basis of GFAP-IR (3.5 fold increase) following rmγ-IFN treatment (Fig. 2.3)

Proliferative response of neonatal astrocytes to cytokines.

We further addressed the contribution of cytokines towards proliferation of astrocytes, a frequent finding of astrogliosis (Cavanagh, 1970; Latov et al., 1979; Takamiya, et al., 1988; Topp et al., 1989; Janeczko, 1988, 1991), by testing for their *in*

vitro mitogenic capabilities. ^{3}H-thymidine measurements revealed that only rmγ-IFN and rhIL-1 could alter proliferation in an anti-mitotic fashion (Table 2.3). 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 presentation of astrogliosis following injury and inflammation to the adult CNS is a stereotypical occurrence recognised by increased GFAP-IR, a longstanding neuropathological hallmark (Latov et al., 1979; Smith et al., 1983; Mathewson and Berry, 1985; Aquino et al., 1988; Takamiya et al., 1988; Maxwell et al., 1990a; Hozumi et al., 1990; 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 at all (Osterberger and Wattenberberg, 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). Possible reasons attributed to the minimal gliotic response in neonatal injury have included the high degree of plasticity within the neonate's relative immature neural environment, or the lack of myelination. Since 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) and given the relative immaturity of the immune system in neonates compared to adults (Hobbs, J., 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. To test this postulate, we sought to evoke astrogliosis in neonatal animals by administering cytokines to the brain following injury.

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 postnatal day 3 mouse brain for 4 days. What was observed, however, was a tremendous GFAP IR (Fig. 2.1). In contrast, an iris scissors stab wound to the neonatal brain (Fig. 2.2), 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 2.1). 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 a NC membrane, which was then removed immediately), minimal astrogliosis was observed 4 days later (Fig. 2.2). 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. 2.3). Whatever the explanation, the conclusion

is that reactive astrogliosis as measured by GFAP IR and GFAP content (Fig. 2.4) can occur in the neonatal brain, and that it is dependant on the type of injury inflicted (Table 2.1).

In the NC-implant model, astrogliosis was characterized by both an increased synthesis of GFAP intermediate filaments and hypertrophy of the astrocytic cytoplamic processes. The functional role for the increase in this intermediate filament is not known. Smith and associates (1986) have reported that reactive astrocytes could migrate on to a NC-implant within 24 - 48 hours 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-sulphate proteoglycan and cytotactin which were present in adult astrocytes (McKeon et al., 1991). These findings suggest that neonatal reactive astrocytes may have potential regenerative properties.

To further attempt to test the hypothesis implicating cytokines as contributors towards astrogliosis, we chose to utilize the neonatal stab model with its inherent minimal gliotic response. A single micro-injection 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 2.2), similar to that seen in adult stab wound models by GFAP immunoreactivity (Fig. 2.2) (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 2.2), an observation that is in accordance with reports indicating a species-specific interaction between γ-IFN and its receptor (Hemmi et al., 1989; Gray 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 reconstituted by cytokine administration.

The quantification of the extent of astrogliosis evoked by rmγ-IFN (634 ± 54 x10^3 μm^2) revealed a 3.5 fold increase over that of vehicle treated controls (180 ± 27 x10^3 μm^2) as determined by GFAP-IR (Fig. 2.3 and 2.5). 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. 2.6). 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 days postinoculation (dpi), a period where 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 to 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 and colleagues (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, Simon 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 mγ-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., 1991a,b, 1992a).

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 2.2), our *in vitro* studies implicate an anti-mitotic effect by rmγ-IFN (Yong et al., 1992a) and IL-1, without any significant effects by the other cytokines (Table 2.3). 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-IFN was inhibitory for proliferation of neonatal or adult mouse astrocytes, rhγ-IFN was a mitogen for fetal and adult human astrocytes (Yong et al., 1992a).

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 induce extensive astrogliosis. These results implicate immunoregulatroy cytokines as important contributing factors to the production of astrogliosis following an injury to the CNS.

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Table 2.1: Astrogliosis in neonatal NC-implant injury.

Values are mean ± SEM with number of samples shown in parenthesis. 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.

$^{\star}p<0.05$ compared to all other groups, using 1 way ANOVA with Duncan's multiple comparisons.

Table 1. Astrogliosis in neonatal NC-implant injury

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

Table 2.2 Cytokines qualitatively increase the extent of GFAP-IR following neonatal Scissors-stab injury.

Brain sections were analysed blind and the extent of GFAP-IR in the ipsilateral cortex tabulated from a scale of + (minimal) to ++++ (extensive). On average, 4 brain sections per animal were analyzed blind.

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	++
Murine γ-IFN	6	++++
IL-1α,β	6	++++
IL-2	4	++++
IL-6	6	++++
TNF-α	4	++++
M-CSF	4	++++

Table 2.3 Proliferative response of Neonatal Mouse Astrocytes to Cytokines *in vitro*.

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

[a]Confirms published results (Yong et al., 1992a) that mouse but not human γ-IFN produces decrease in proliferation rate at 10, 100, and 1000 U/ml.

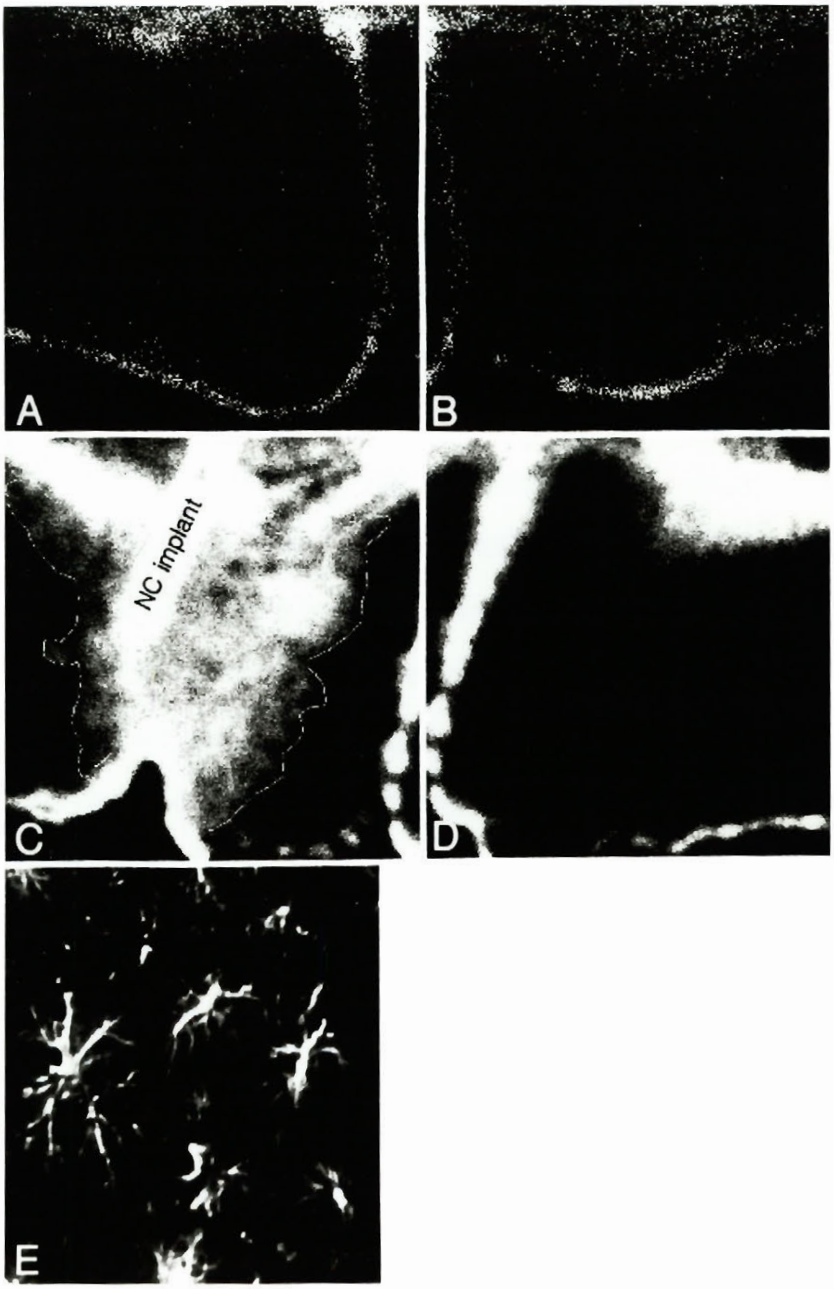
[b]This non-cytokine growth factor was used as a positive mitogenic control.

★$p<0.05$ compared to controls (1 way ANOVA with Duncan's multiple comparisons).

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)*

Figure 2.1: Implant of NC membrane intro postnatal day 3 mouse pups for 4 days 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), or in the contralateral hemisphere of NC-implant pups (D), GFAP-IR was detected only in the the corpus callosum (top orientation of each frame) and glia limitans (bottom of each frame). Images in panels A to D were acquired by confocal laser scanning microscopy (described in detail in text) using a 2.5x objective. Panel E is a higher magnification of the traced area in panel C, acquired using a 40x objective, to denote the morphology and reactive nature of the astrocytes.


A
B
NC implant
C
D
E

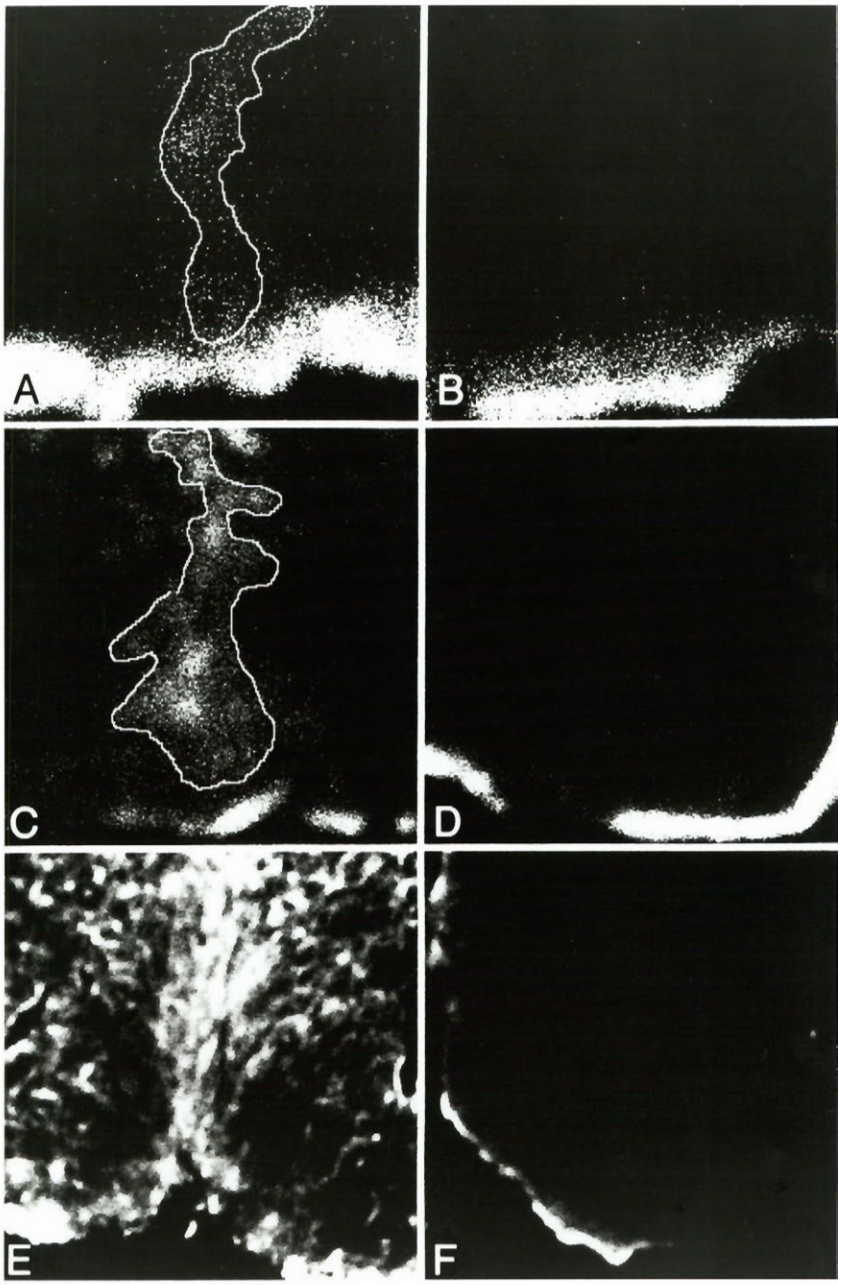

A
B
C
D
E
F

Figure 2.3: Quantitative comparisons of the cortical area covered by GFAP-IR astrocytes in the different injury paradigms in mouse pups. Injury was inflicted on postnatal day 3 and animals were sacrificed 4 days later. Adult Scissors-stab animals have been included for reference. Each dot represents the image acquired from a single brain section. Two brain sections per animal, taken from the lesion site in all cases, from 7 animals per group, were analysed. Scissors-stab (mean GFAP-IR area of $30 \pm 2 \times 10^3$ μm^2) or NC-stab (mean area of $53 \pm 5 \times 10^3$ μm^2) to neonates elicited little astrogliosis when compared to neonate NC-implant (mean area of $1016 \pm 37 \times 10^3$ μm^2) or adult scissors-stab injuries ($496 \pm 22 \times 10^3$ μm^2). The introduction of 2 µl of 0.2% BSA to the neonatal scissors-stab wound site resulted in increased GFAP-IR (mean area of $180 \pm 27 \times 10^3$ μm^2) compared to scissors-stab alone, while 20 U of γ-IFN in 0.2% BSA enhanced astroglial reactivity even further (mean area of $634 \pm 54 \times 10^3$ μm^2).

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

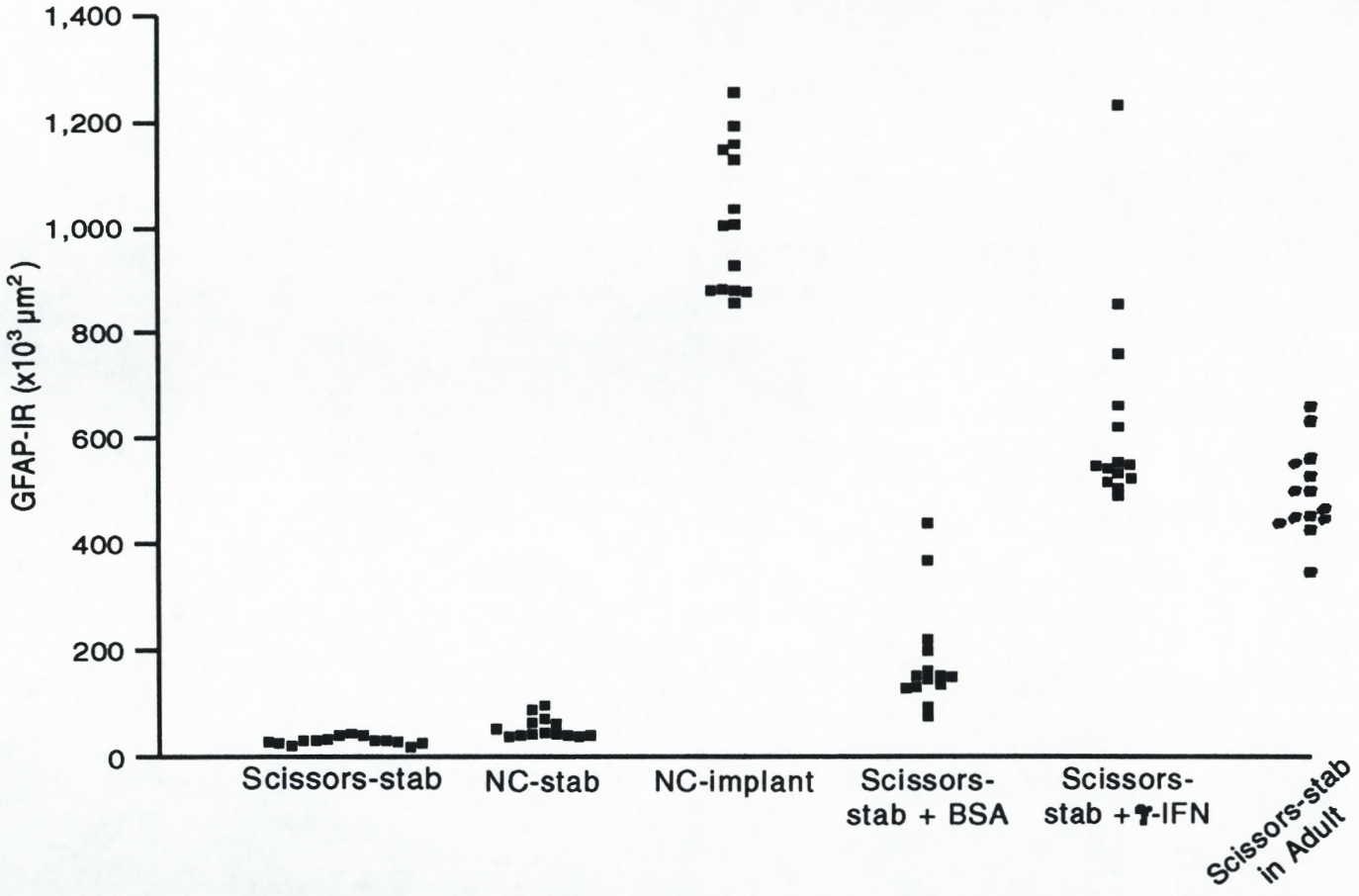


Figure 2.4: GFAP content of tissues (approximately 10 mg wet weight) circumscribing the lesion site in the different injury paradigms in neonatal animals. GFAP content was read off as a standard curve generated by different amounts of purified GFAP (10 - 100 ng). The mouse GFAP bands ran at a slightly 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/g total protein, which corresponds to normal values reported by Goodlett et al., 1993.


Normal
Scissors-stab
NC-stab
NC-implant
GFAP 10 ng
GFAP 25 ng
GFAP 50 ng
GFAP 100 ng

Figure 2.5: Increased GFAP-IR in mouse pups treated with γ-IFN following a scissors-stab wound (C,D) compared to vehicle (0.2% BSA) treatment (A,B). Panels A and C are at the lesion site while panels B and D are hemispheres contralateral to the lesion. Quantitation of the area of GFAP-IR in each group is displayed in Figure 2.3; on average, rmγ-IFN increased GFAP-IR by 3.5 fold over its BSA vehicle control.

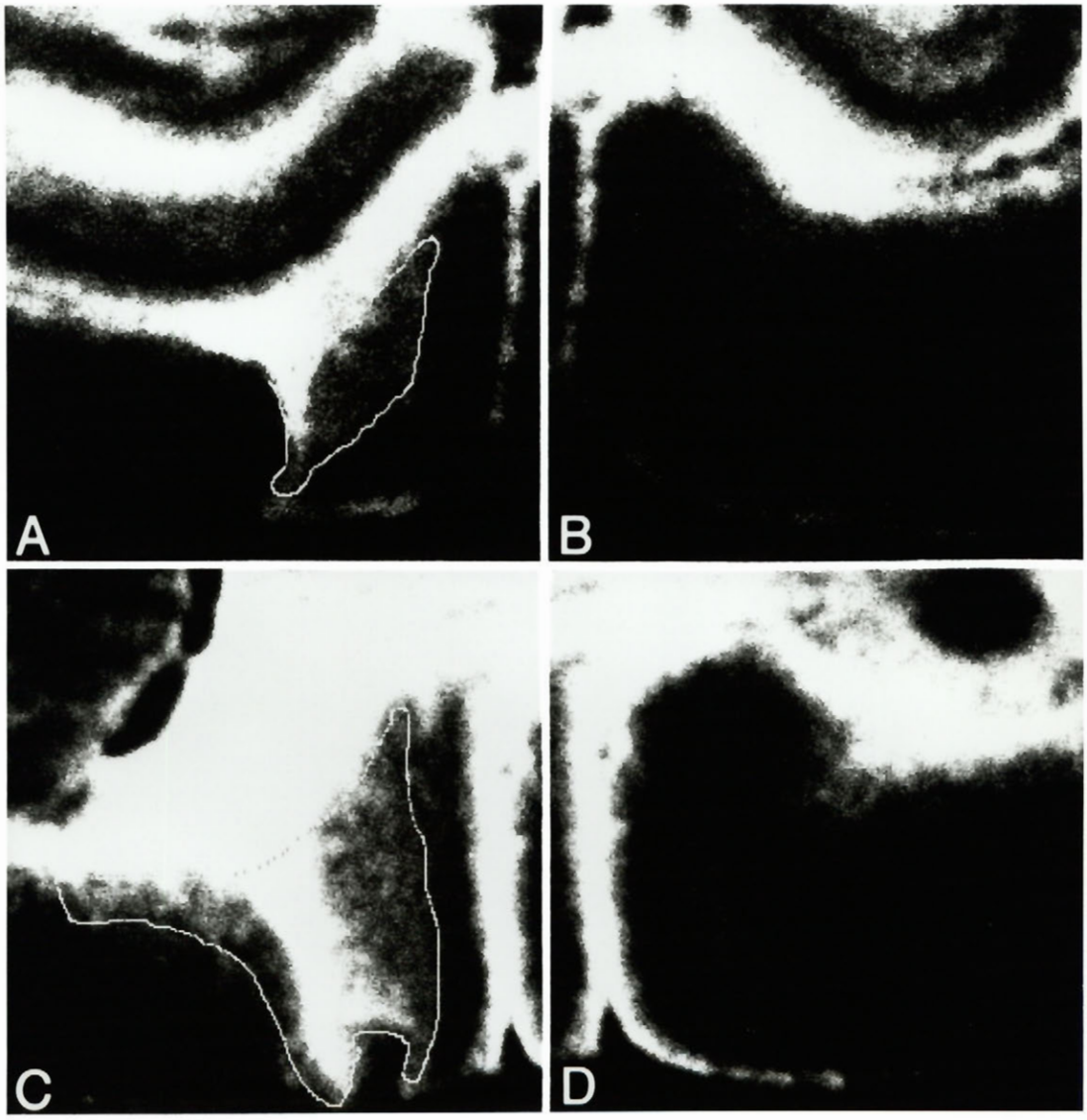
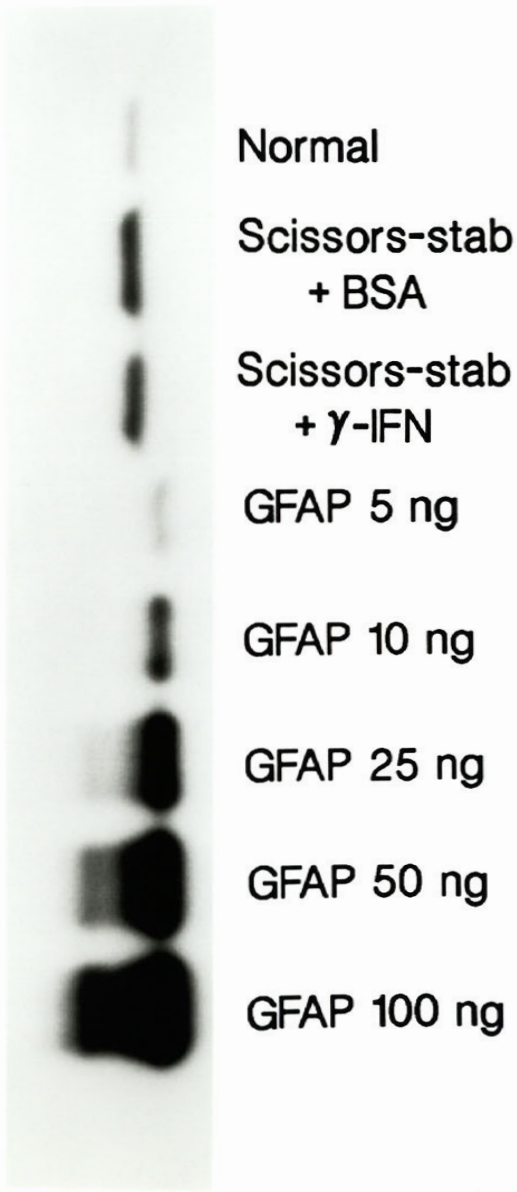

A
B
C
D

Figure 2.6: 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.


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

CHAPTER 3

NEONATAL ASTROGLIAL REACTIVITY: DISCORDANCE OF GFAP mRNA AND PROTEIN CONTENT WITH IMMUNOREACTIVITY, AND ITS DEPENDENCE ON MACROPHAGE PRESENCE

Vijayabalan Balasingam, Kathleen Dickson,
Anthony Brade, and Voon Wee Yong

PREFACE

To elucidate the mechanisms for the evolution of cells into reactive astrocytes, we sought to determine: (1) if GFAP-IR accompanying neonatal CNS injury could be correlated to concurrent changes in GFAP mRNA, GFAP content and ultrastructural changes as seen following adult traumatic injuries; and (2) whether there was a differential representation of inflammatory cells to account for the difference in neonatal GFAP-IR following either the acute NC-stab and chronic NC-implant injury.

ABSTRACT

Unlike insults to the adult, most injuries to the neonatal CNS result in minimal GFAP immunoreactivity (IR), a hallmark of an astroglial reaction. We have previously reported that the minimal GFAP-IR following a stab injury to the brains of postnatal day 3 mice could be converted into extensive GFAP-IR by the single application of inflammatory cytokines, or by the chronic implantation in the brain for 4 days of a piece of nitrocellulose (NC) membrane. In the current study, we sought to characterize further the astrocytic response following acute NC-stab or chronic NC-implant injury, and to elucidate the mechanisms for the evolution of cells into reactive astrocytes in the neonatal CNS. An NC-implant injury in the neonate resulted in elevation of GFAP mRNA and content alongside increased GFAP-IR. Unexpectedly, the acute NC-stab injury to neonates also resulted in elevated GFAP mRNA and content; however, the augmented biochemical GFAP profile did not result in extensive astroglial reactivity as evidenced by GFAP-IR and ultrastructural criteria. As increases in GFAP mRNA and content, but absence of GFAP-IR, is also observed during normal development, the results suggest that an acute insult to the neonatal CNS accelerated astrocytic maturation but that additional factors are required in the cascade leading to astroglial reactivity. Examinations of inflammatory cells show Mac-1 positive macrophages to be present in neonatal NC-implant but not NC-stab animals, suggesting that macrophage-derived factors are necessary for the evolution of cells with increased GFAP mRNA and content into reactive astrocytes.

INTRODUCTION

Astroglial reactivity is a prominent occurrence following injury to the adult central nervous system (CNS). In this phenomenon, astrocytes undergo hypertrophy and increase their number of mitochondria, glycogen content and various enzyme levels (Nathaniel and Nathaniel, 1981; Norenberg, 1994; Landis, 1994). Furthermore, the detection by

immunohistochemistry of glial fibrillary acidic protein (GFAP), a specific marker of differentiated astrocytes, is facilitated. As astrocytes of the normal cortical parenchyma do not display immunoreactivity (IR) for GFAP although containing this protein (Bignami and Dahl, 1974), elevated GFAP-IR is routinely employed as a marker of astroglial reactivity independent of the injury stimulus (Norton et al., 1992). The increased expression of GFAP-IR following insults usually accompanies GFAP synthesis; however, GFAP-IR can be observed even when GFAP synthesis is not enhanced, presumably due to the dissociation of GFAP intermediate filaments which expose previously hidden GFAP antigenic epitopes (Aquino et al., 1988; Eng et al., 1989). Whether the converse can occur, i.e. GFAP-IR remaining unaltered while GFAP synthesis increases following injury, is unknown.

In contrast to the prominent response in adults, minimal astroglial reactivity follows injuries to the *perinatal* CNS (Sumi and Hager, 1968; Bignami and Dahl, 1976; Berry et al., 1983, Maxwell et al., 1990b; Hatten et al., 1991). However, if an adequate stimulus is provided to the neonatal CNS, a reactive astroglial response can ensue. In this regard, we have previously reported that the minimal GFAP-IR following a stab injury to the brains of postnatal day 3 mice could be converted into extensive GFAP-IR by the single microinjection of inflammatory cytokines, or by the implantation in the brain for 4 days of a piece of nitrocellulose (NC) membrane (Balasingam et al., 1994). We have postulated that the chronic nature of the NC-implant injury recruited either intrinsic CNS or extrinsic systemic inflammatory mononuclear cells to the lesion site; the consequent release of cytokines then contributed to the extensive GFAP-IR similar to the microinjection of various cytokines into the neonatal brain.

The aim of the present study was to determine if GFAP-IR accompanying neonatal CNS injury could be correlated to concurrent changes in GFAP mRNA, GFAP content and ultrastructural changes as seen following adult traumatic injuries (Hozumi et al., 1990a, 1990b; Maxwell et al., 1990a; Vijayan et al., 1990). Furthermore we have utilized

the acute (NC-stab) or chronic (NC-implant) neonatal injury paradigms, with minimal and extensive GFAP-IR respectively, to determine whether there was a different representation of inflammatory cells to account for the occurrence of GFAP-IR. The results demonstrate that GFAP mRNA and GFAP content increase rapidly and significantly in both the acute and chronic neonatal injury paradigms. However, elevated GFAP-IR and ultrastructural features of reactive astrocytes were observed only in the chronic (NC-implant) injured animals. Finally we demonstrate the increasing accumulation of macrophages with time in and on the NC membrane of the chronic injury, suggesting that macrophage derived factors may provide the necessary stimuli for the evolution of astrocytes with increased GFAP mRNA and GFAP content into reactive astroglia.

MATERIALS and METHODS

Experimental animals

Newborn mice (of either sex from natural litters) and adult retired female breeders of the CD1 strain (4 to 6 months old) were obtained from a commercial source (Charles River Canada, Montreal, Quebec, Canada). Animals were housed on a 12/12 h light/dark cycle with ad libitum access to food and water. All experimental procedures were approved by the institution's animal care committee and were in accordance with the guidelines instituted by the Canadian Council of Animal Care.

Neonatal NC-stab and NC-implant unilateral injuries were conducted on the left hemisphere as previously described (Balasingam et al., 1994). In brief, postnatal day (P)3 mouse pups were subjected to inhalational methoxyflurane anaesthesia and the skin overlying the skull was then cut with a scalpel. The soft skull was cut with a pair of fine iris-scissors and a dry 1 mm^2 piece of nitrocellulose (NC) membrane (pore size: 8 μm; Schleicher and Schuell, Keene, NH) that was previously boiled to remove surfactant

(Rudge et al., 1989) was inserted into the cortex. For all NC-stab injuries, the NC membrane was removed immediately, while for the NC-implant injury, the NC membrane was left in the cerebral cortex of the animal for the duration of the experiment.

For adult brain injuries, female CD1 adult mice were anaesthetized with an intraperitoneal injection containing a mixture of Ketamine (200 mg/kg) and Xylazine (10 mg/kg). The animals were immobilised in a stereotaxis frame followed by a midline incision and a unilateral circular (2-mm-diameter) craniectomy over the left hemisphere. The NC-stab and NC-implant injuries were inflicted as described for neonates but with a larger 4 mm^2 piece of NC.

***Qualitative and quantitative assessment of GFAP-IR* in situ**

For GFAP immunohistochemical studies, neonatal and adult animals were deeply anaesthetized with either inhalational methoxyflurane or a lethal dose of intraperitoneal chloral hydrate respectively to facilitate intracardial exsanguination with periodate-lysine paraformaldehyde (PLP) solution. The whole brain was removed from the animal and postfixed for 6 h in PLP, then soaked in 25% sucrose overnight for cryoprotection. Twenty micrometer coronal sections were obtained on gelatin coated slides and subjected to immunofluorescence for GFAP as previously described (Balasingam et al., 1994). Examination of astrogliosis was restricted to the cortical regions with the use of a high resolution confocal laser scanning microscope (CLSM) (Leica Lasertechnik, Heidelberg, Germany) equipped with an argon-krypton laser.

Quantitative assessment of GFAP-IR for the different injury paradigms 4 days post injury (dpi) was performed on the dorsal cortex ipsilateral to the lesion site and its corresponding contralateral area with a 4 x 0.08 objective. The scanned images were reconstructed from the average of 16 passes per raster line with a pinhole aperture set to 40% in order to achieve high signal to noise ratio in the confocal plane (Fig. 3.1c-3.1f).

A montage of all scanned images per sample was created to facilitate the calculation of: (1) total number of GFAP-IR astrocytes in the cortex surrounding the lesion site; (2) the area encompassing GFAP-IR astrocytes. Density of GFAP-IR astrocytes were calculated from the values obtained above and expressed in terms of area (mm^2) (Table 3.1).

Quantification of GFAP content

GFAP protein extraction and analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out via a modified protocol as previously described (Balasingam et al., 1994). Cortical samples circumscribing the lesion site were resected (with wet weight approximating 10 mg in neonates and 20 mg in adults) and each sample was homogenized in 50 volumes of 100 mM phosphate buffer containing 6 M urea at 4°C. The homogenates were analyzed for total protein content with a BCA protein assay kit (Pierce) that utilized bovine serum albumin as a standard.

After electrophoresis, the samples were transblotted to an Immobilon-P membrane (Millipore), followed by incubation with 2.5% skim milk in PBS (Blotto) as a blocking step. The membrane was then incubated in an anti-GFAP antibody solution (Dako; diluted 1:1000 in Blotto) for 2 h at 4°C, followed by a PBS rinse, and incubated in ^{125}I-Protein A (1 μCi diluted in 10 ml of Blotto) for 2 h. Following exposure of membranes to a phosphorimager screen, GFAP concentrations were quantified on a phosphorimager (Molecular Dynamics) using ImageQuant software (Fig 3.3b, 3.4b). When comparisons between different membranes were necessary, GFAP content of the test samples were represented as a ratio of GFAP content from the pooled normal cortices on the same SDS-PAGE.

Quantification of GFAP mRNA

Cortical lesion site was resected with wet weights approximating 20 mg in neonates and 40 mg in adults. Resected cortices from 5 neonates or 4 adults were pooled from each time point following injury. Pooled resected cortical tissue from uninjured age-matched animals served as controls. Samples were homogenized in 1 ml of TRIZOL™ extraction reagent (GIBCO BRL, Gaithersburg, MD) and total RNA was obtained following manufacturer's instructions.

For Northern blot analysis total RNA (10 ug per sample) was electrophoresed on agarose formaldehyde gels in MOPS buffer and transferred to nylon Hybond membranes with negative pressure using a Vacu-Gene blotting apparatus. RNA was immobilized via UV-crosslinking and blocked with prehybridization buffer (50% formamide, 2 X SSC, 5 X Denhardt's solution, 0.1% SDS and 250 μg/ml denatured salmon sperm DNA) for 1 h at 43°C. Hybridization was performed for 16 h at 43°C in the same buffer. The mouse GFAP cDNA probe was a gift from Dr. Nicholas Cowan. The cDNA probes were labelled with [α-^{32}P]dCTP using the Klenow fragment of DNA *pol I* and random DNA hexamers (BRL) to a specific activity of approximately 1 X 10^9 cpm/μg of probe. After hybridization, the blots were washed at 43°C with 2 X SSC and 0.1% SDS followed by 0.5 X SSC and 0.1% SDS. To account for differences in RNA loading, membranes were reprobed with an 18S cDNA probe and exposed to a phophorimager screen. Bands were visualized with a PhosphorImager and quantified with ImageQuant software.

Ultrastructural analysis

Three neonatal animals each at 4 dpi were used for both the acute (NC-stab) and chronic (NC-implant) paradigms with three appropriate age-matched controls. Animals were anaesthetized with inhalational methoxyflurane and exsanguinated while perfused intracardially with 2% glutaraldehyde in cacodylate buffer and stored in the same fixative

overnight. The brains were postfixed in Palade's osmium tetroxide, dehydrated in sodium benzoate, and embedded in epoxy resin. Small pieces of tissue, approximately 2 mm^2 in cross-sectional and 2-3 mm in longitudinal extent were flat-embedded in Epon. Prior to trimming, 3 semi-thin (1 μm) sections per animal were stained with a Toluidine Blue solution (0.2-0.5% containing saturated borate and mounted with Permount) for rapid light microscopical examination to locate the cortical lesion track site (Fig. 3.6) and the selection of appropriate cortical areas for ultrastructural analysis. Epon blocks were trimmed to a cross-sectional area approximately 0.5 mm by 0.6 mm to encompass the lesion site and the nitrocellulose membrane where appropriate.

Ultra-thin (60 - 80 μm) sections were collected on a copper grid (with a total 40% open area that has an individual grid field area of 49 μm by 49 μm) and stained with uranyl acetate and lead citrate for ultrastructural analysis on a JEM-100 CX-2 electron microscope (J.E.O.L. Ltd., Tokyo, Japan). Only cross-sections displaying clear nuclear morphology were used for cell counts. A total of 10 - 15 ultra-thin sections per animal were analyzed. To obtain cell densities the total number of cells counted were expressed as a ratio of 50 grid spaces, which is the equivalent number of grid spaces that encompass a 0.5 mm by 0.6 mm section.

Mac-1 immunohistochemistry

The primary antibody used was a monoclonal rat anti-mouse antibody to Mac-1 (complement receptor, type three) antigen derived from hybridoma supernatant. Immunohistochemistry was performed at room temperature on either twenty micrometer brain sections (obtained as described for GFAP-IR) or on the NC-implant membranes that were removed from decapitated animals. In brief, samples were initially fixed in 70% ethanol for 20 min followed by a PBS wash and a blocking step with chicken egg albumin (3%) for 30 min. This was followed by an overnight incubation with the primary antibody (5 μg/ml) and then sequentially incubated with biotinylated anti-rat

immunoglobulin (1:100, Dako Corp.) for 1 h, and in 1% ABC reagent (Vector Laboratories, Burlingame, CA) for 1 h. Immunofluorescence was revealed by an incubation with streptavidin conjugated to rhodamine (1:100, Dako) for 1 h followed by dehydration and cover-slipped with Permount (Fisher Scientific, Pittsburg, PA). Images were obtained with 40 X 1.3 NA oil immersion objective on a CLSM.

RESULTS

GFAP Immunohistochemistry

We confined our inspection of astrocytic reactivity to the cortical regions, since the cortex of normal animals is negative for GFAP-IR from P5 although containing astrocytes and GFAP; in normal brains, GFAP-IR is seen in the corpus callosum, external glial limitans, and regions of the hippocampus (Bignami and Dahl, 1974, Schiffer et al., 1986). At 2 dpi, the neonatal acute (NC-stab) injury generated minimal GFAP-IR; this GFAP-IR was detected in structures resembling processes of reactive astrocytes and not in cell bodies on either side of the lesion (Fig. 3.1A). In contrast, the chronic neonatal (NC-implant) injury produced GFAP-IR astrocytes in the cortical parenchyma surrounding the implant; GFAP-IR was observed in cells with irregularly shaped cell bodies and processes (Fig. 3.1C). There was also evidence of astrocytic migration into the pores of the NC-implant. However, necrosis was not noted in either of the neonatal models and the density of GFAP-IR structures was higher in the deep cortical layers and decreased towards the pial surface. In adult animals, both the acute and chronic injury paradigms elicited, at 2 dpi, numerous reactive astrocytes with a stellate morphology that were GFAP-IR in both cell soma and processes on either side of the lesion surrounding the necrotic area; processes of these astrocytes were preferentially oriented towards the lesion (Fig. 3.1E).

By 4 dpi, the neonatal acute (NC-stab) injury still displayed minimal GFAP-IR and these were observed in structures resembling processes and not cell soma of reactive astrocytes (Fig. 3.1B). These reactive processes were present on either side of the stab lesion with higher densities in the deep cortical layers. In contrast, the neonatal chronic (NC-implant) injury revealed numerous closely packed GFAP-IR astrocytes within the deep and superficial layers of the cortex. At the vicinity of the lesion, GFAP-IR astrocytes and their processes had completely surrounded and penetrated the pores of the NC-implant (Fig. 3.1D). In adult animals by 4 dpi, regardless of the type of injury (either acute or chronic), numerous hypertrophied GFAP-IR astrocytes could be observed (Fig. 3.1F). These adult GFAP-IR astrocytes with enlarged cytoplasm and thickened processes were more densely packed at lesion bordering sites but were more evenly spaced in the distal parenchyma.

We quantitated the area of the cortex that contained GFAP-IR astrocytes in the acute and chronic injury models at 4 dpi for both the neonatal and adult animals; the density of GFAP-IR astrocytes within the affected cortical areas was also tabulated (Table 3.1). The computation re-confirmed the maximal increase in areas occupied by GFAP-IR in the chronic neonatal injury, and minimal GFAP-IR for the acute neonatal injury (Balasingam et al., 1994). In addition, the density of GFAP-IR astrocytes was significantly higher in the neonatal chronic (NC-implant) injury when compared to either of the adult acute or chronic injury paradigms. Thus when the neonatal brain does become reactive following an injury stimulus (NC-implant), it appears to mount a response that is greater than that of adults.

At 21 dpi, the neonatal chronic (NC-implant) injury contained little scattered GFAP-IR in the parenchyma immediately surrounding the lesion site; however, there was significant presence of GFAP-IR within the pores of the NC membrane (Fig. 3.2A). In the neonatal acute (NC-stab) injury, GFAP-IR was absent in the cortex and the parenchyma was similar to that of age-matched controls. For the adult injury paradigms,

GFAP-IR seen in the parenchyma distant to the lesion site at early time points had resolved with time and was now restricted to areas immediately bordering the insult. Thus transient GFAP-IR occurs in parenchymal areas following anisomorphic lesions to both the neonatal and adult CNS.

GFAP mRNA and GFAP content expression during development

The synthesis of GFAP in the developing neonate has been determined by previous studies on the basis of immunohistochemistry (Raju et al., 1981; Bignami and Dahl, 1974; Bovolenta et al., 1984), GFAP mRNA and content (Malloch et al., 1987; Tardy et al., 1990). Our initiative to document GFAP mRNA and protein contents was necessary in order to understand the nature of neonatal cortical astroglial reactivity occurring on a developmentally dynamic background.

GFAP content was detectable at birth from mouse brain cortical homogenates (Fig. 3.3B). During the first postnatal week, GFAP content increased steeply to levels approximately 30 fold higher than that present at birth (Fig. 3.3A). Beyond the first week, increases in GFAP content were gradual and reached adult values by P24. Northern blot analyses for GFAP mRNA levels also revealed a developmentally increasing profile, peaking at 6 fold that of birth values by the end of the first postnatal week (Fig. 3.3A). The developmental increase in GFAP mRNA could be seen preceding that of GFAP content (Fig. 3.3A)

Injury inflicted GFAP mRNA and GFAP content

Unexpectedly, the acute (NC-stab) injury model, with its inherently minimal astroglial response, as suggested by GFAP-IR, showed a significant temporal increase in GFAP mRNA (Fig. 3.4) and GFAP content (Fig. 3.5). As predicted, the neonatal chronic (NC-implant) injury model with its extensive GFAP-IR displayed a correspondent increase

in GFAP mRNA (Fig. 3.4) and GFAP content (Fig. 3.5) similar to that observed in chronic injured adult models. Elevation of neonatal mRNA for GFAP by either the acute or chronic injury paradigm was observed by 6 hours following injury; GFAP mRNA peaked around 3 dpi and was returning to baseline values by 7 dpi. Interestingly, the magnitude of elevation for GFAP mRNA was more marked in adults than in the neonate (Fig. 3.4).

Elevation of neonatal GFAP content from either of the acute or chronic injury paradigms peaked around 4 dpi, and thereafter, decreased to approach age-matched control values by 21 dpi (Fig. 3.5). For purposes of statistical analysis, samples from the maximal response time point at 4 dpi in neonates were electrophoresed on a single wide-mini-SDS-PAGE (Fig. 3.5B); the single membrane had 5 individual samples from the 2 injury paradigms (NC-stab and NC-implant) together with 5 individual age-matched controls. Statistical determinations revealed that both injury paradigms resulted in GFAP content levels that were significantly different from controls (elevation of 5.4 and 9.2 fold from controls for the NC-stab and NC-implant injuries, respectively). This confirms the large temporal increase in GFAP content elicited by the neonatal NC-stab injury that was not matched by an appropriate increase in GFAP-IR.

Ultrastructural analysis

Given the discrepancy between GFAP-IR with GFAP mRNA and GFAP content for neonatal acute injuries above, electron microscopy (EM) analyses were conducted to determine the presence or absence of reactive astrocytes as determined by ultrastructural criteria. Such analyses would also reveal which of the GFAP changes (IR, protein content, or mRNA) were more representative of astroglial reactivity following neonatal CNS injuries.

Initial screening by light microscopic analyses revealed the presence of a lesion track site (Fig. 3.6) created by the insertion of the nitrocellulose membrane in both the acute and the chronic injury paradigms. At the pial surface, the glial limitans was reconstituted with a hypercellular region on either side of the lesion track site in the cortical molecular layer. In deeper cortical regions the track site is bordered by cells that run parallel to the track site and which appears to wall-off the track site. In comparison to the acute injury paradigm, the chronic (NC-implant) paradigm is more hypercellular and elicits a recruitment of cells on to the nitrocellulose membrane and in the surrounding parenchymal regions.

For the purposes of quantitative electron microscopy analysis, the different neuroglial cells were identified primarily by nuclear morphology on the basis of the following criteria (Ling et al., 1973; Parnavelas et al., 1983; Vaughan, 1984; Peters et al., 1991): (1) Protoplasmic astroglia of the cortex have a round to oval shaped palely stained nucleus with a characteristic nuclear envelope adorned by a continuous lining of heterochromatin (Fig. 3.7A); (2) Reactive astroglia have irregularly shaped nuclei and the nuclear envelope may display deep folds. The karyoplasm has a fairly even density with increased heterochromatin clumping immediately adjacent to the nuclear envelope and multiple small nucleoli are present (Norenberg, 1994). Furthermore, upon transformation from the protoplasmic to the reactive state, the cytoplasm contains enlarged cisternae of rough endoplasmic reticulum and an abundance of mitochondria with an increase in the number of glycogen particles (Cheng et al., 1994) (Fig. 3.7B,C); (3) Microglia have an irregular, elongated nucleus containing densely stained clumps of heterochromatin on a palely stained karyoplasmic background (Fig. 3.8A). In addition, reactive microglia/macrophages have lysosomes and phagocytotic vacuoles in the cytoplasm (Fig. 3.8B); (4) Neurons have a large, round nucleus with a typically clear vesicular appearing karyoplasm lacking in chromatin particles. The nucleus generally occupies most of the cell body with a thin husk of cytoplasm surrounding it (Fig. 3.8C).

In unlesioned control brains, the general ultrastructural features of most cortical astrocytes were consistent with that of protoplasmic astroglia. Like protoplasmic astrocytes in other parts of the CNS, the cytoplasm is not extensive and forms a thin rim around the nucleus (Fig. 3.7A). Microglia present in these brains were generally not found to be reactive (Fig. 3.8A).

In the neonatal acute (NC-stab) injury at 4 dpi, the occasional reactive astrocyte and enlarged astrocytic process (Fig. 3.9A), as well as the rare phagocytosing macrophage (Fig. 3.9B), were found along the lesion track site (Fig. 3.6). In addition, on either side of the lesion, the majority of astrocytic nuclei were non-reactive and had the normal characteristic oval euchromatic nuclei (Fig. 3.7A). When the occasional reactive astrocyte was present in the neonatal stab injured brain (Fig. 3.7B), they did not reflect the characteristics typical of that seen in adult brain lesions as per the criteria described earlier.

In contrast, the neonatal chronic (NC-implant) injury at 4 dpi had numerous easily identifiable reactive astrocytes within the implant (Fig. 3.9C) and in the surrounding parenchyma (Fig. 3.9E). Interspersed among these reactive astrocytes were actively phagocytosing macrophages (Fig. 3.9D) and microglia (Fig. 3.9F). The reactive astrocytes in this lesion had enlarged nuclei with an irregular outline and frequently contained multiple nucleoli and increased amount of chromatin (Fig. 3.7C).

The total number of normal and reactive astroglia and microglia/macrophages in the two different neonatal injury paradigms are represented graphically in Fig. 3-10. The chronic (NC-implant) paradigm, with the same trauma burden in comparison to the acute (NC-stab), imposes a high debris burden by its chronic presence and elicits an adequate stimulus for the recruitment of reactive microglia/macrophages with consequent extensive astroglial reactivity. Conversely, the acute (NC-stab) paradigm does not pose a high debris burden sufficient for the recruitment of reactive microglia/macrophages and results in

minimal astroglial reactivity. These results point to the fact that despite the identical trauma (NC injury), the increased presence of reactive microglia/macrophages are key to the development of astroglial reactivity. Moreover, injury to the developing neonatal brain can stimulate the local proliferation of astrocytes without their transformation into reactive astrocytes if a paucity of microglia/macrophages exist.

Fibroblast were also identified at sites bordering the lesion track site of either injury paradigm at more superficial aspects of the cortex rather than the deeper layers. Inflammatory cells (e.g. T-lymphocytes) other than that of the mononuclear phagocyte lineage were also not identified in either of the injury paradigms.

Mac-1 immunochemisty

On gross examination of the cortex, in both the neonatal acute and chronic lesion sites, haemorrhagic clots were found shortly after injury as has been reported for anisomorphic injuries (where the blood brain barrier is disrupted) in adult animals (Matthewson and Berry, 1985). Unlike the adult where haemorrhagic clot clearance required 8 days (Matthewson and Berry, 1985, Maxwell et al., 1990a), haemorrhagic clots in the neonatal lesion were cleared by 2 dpi (Sumi and Hager, 1968; Balasingam and Yong, unpublished observations). This was suggestive of the presence of an active phagocytic system in the neonate that was activated vigorously to clear tissue debris. In an attempt to localize these phagocytes, we used an antibody against Mac-1 that marks the type 3 complement receptor (CR3) (Springer et al., 1974; Beller et al., 1982; Sanchez-Madrid et al., 1983). The major function of CR3 is phagocytosis of opsonized particles by specific binding of C3b of the complement component C3 (Bruck et al., 1990). CR3 is widely expressed on cells belonging to the mononuclear phagocyte system and is detectable even in early fetal life (Flotte et al., 1983). Within the *normal cortical* CNS parenchyma, Matsumoto and colleagues (1985) reported that Mac-1 positive cells were not detectable until P28, while others (Perry et al., 1985; Ohno et al., 1992) stated that

immunoreactivity for Mac-1 in the CNS was very weak before P10. Therefore Mac-1 appeared ideal for the detection of mononuclear cells that might be recruited to the CNS upon injury for the purposes of phagocytosis.

When brain sections from the neonatal NC-stab model were subjected to Mac-1 immunofluorescence, no positive cells were recognized at the lesion site from the onset of injury to 4 dpi. In the neonatal NC-implant injury model, Mac-1 immunostaining in the cortical parenchyma was also not observed. However, Mac-1 immunofluorescence could be detected in the border between NC-implant and brain. By 2, and certainly at 4 dpi, immunofluorescence bordering the NC-implant was very intense for Mac-1.

We removed the NC membrane from the cortex of implanted animals and subjected the membrane to Mac-1 immunohistochemistry. Large round Mac-1 positive macrophages were found adherent to the membrane as early as 1 dpi (first point examined, Fig. 3.8A). The numbers of Mac-1 positive macrophages on the NC-implant membranes increased with time and was abundant on the surface of the membrane by 2 dpi (Fig. 3.8B). Immunohistochemical detection for T cells did not reveal any $CD4^+$ or $CD8^+$ T cells in both the parenchyma and the NC-membranes in neonatal animals (Balasingam and Yong, unpublished observations).

In adult animals, regardless of the injury model, Mac-1 positive macrophages were present at the lesion site as early as 1 dpi (data not shown) and their numbers increased with time as previously reported (Kitamura et al., 1972; Maxwell et al., 1990a). These Mac-1 positive cells were focally located at the lesion sites of either injury model.

DISCUSSION

For many types of CNS injuries, the process of astroglial reactivity is dynamic and continues to evolve with time leading to a densely interwoven "glial scar" which has classically being thought to be undesirable (Reier et al., 1983; Liuzzi and Lazek, 1987). More recent evidence, however, suggests that the process of astroglial reactivity may actually be an attempt by these cells to promote CNS recovery as shown by their tremendous neurotrophic potential. In this regard, astrocytes produce a wide range of neurotrophic factors and are conducive substrates for growth of neurons *in vitro* and *in vivo* (Silver and Ogawa, 1983; Smith et al., 1986; Kliot et al., 1990; Landis, 1994; McMillan et al., 1994; Rudge et al., 1994; reviewed in Yong, in press). To better address the role of reactive astrocytes in facilitating or impeding regeneration, an understanding of the molecular mediators of astroglial reactivity, and the ability to manipulate its occurrence and extent would be beneficial. We have previously reported that the minimal GFAP-IR following a neonatal stab (acute) wound could be converted to an extensive GFAP-IR by the single introduction of several inflammatory cytokines or by the chronic implantation of a NC-membrane (Balasingam et al., 1994). The availability of the anisomorphic neonatal acute and chronic injury paradigms with differential degrees of GFAP-IR can facilitate the evaluation of cytokines in mediating astroglial reactivity as well as the neurotrophic role of reactive astrocytes. The goal of this study was therefore to document other astrocytic changes (GFAP mRNA and GFAP content) not conducted in the earlier analysis (Balasingam et al., 1994) following either acute or chronic injury in neonatal animals; these changes were compared to better described occurrences in adult injury. Furthermore, ultrastructural evidence of astroglial reactivity and the presence of inflammatory mononuclear cells were investigated.

As in the better described adult models, and in correspondence with numerous reports involving a variety of adult CNS insults (Amaducci et al., 1981; Mathewson and Berry, 1985; Schiffer et al., 1986), GFAP-IR was extensive in our adult acute and chronic injury paradigms (Table 3.1). The increased GFAP-IR (Fig. 3.1) was matched by corresponding elevations in GFAP mRNA (Fig. 3.4) and GFAP content (Fig. 3.5), in accordance with previous reports (Hozumi et al., 1990a, 1990b; Vijayan et al., 1990). Neonatal mice inflicted with the chronic injury paradigm elicited extensive GFAP-IR (Fig. 3.1) as previously reported (Balasingam et al., 1994). Corresponding increases in GFAP mRNA (Fig. 3.4) and GFAP content (Fig. 3.5) were also produced, highlighting the concept that a significant astroglial response can occur in neonatal animals if a sufficient stimulus is provided. In this regard, the use of alcohol (Goodlett et al., 1993), ocular enucleation (Trimmer and Wunderlich, 1990), or the application of basic fibroblast growth factor (Eclancher et al., 1990) have also served as adequate injury stimuli for a neonatal astroglial response.

For the neonatal acute injury model, minimal GFAP-IR (Fig. 3.1) was obtained as previously described (Balasingam et al., 1994); Unexpectedly, GFAP mRNA (Fig. 3.4) and GFAP content (Fig. 3.5) were significantly elevated above that of age-matched controls. To our knowledge, this is the first description where the increased GFAP mRNA and GFAP content following an insult were not reflected by GFAP-IR, which has been a hallmark to detect an astroglial reaction. The converse has been demonstrated in Experimental Autoimmune Encephalomyelitis (Aquino et al., 1988), i.e. increased GFAP-IR can occur without elevated GFAP content, presumably due to the disassembly of GFAP filaments by edema to expose antigenic sites (Eng et al., 1989).

Is the elevation of GFAP mRNA and GFAP content or the increase in GFAP-IR the best predictor of astroglial reactivity following neural trauma? To address this we resolved to the electron microscope. Ultrastructurally, the neonatal acute injury revealed an occasional reactive astrocyte (Fig. 3.9). In contrast the neonatal chronic injury revealed

the presence of abundant reactive astrocytes at the lesion site (Fig. 3.10) with nuclei that are enlarged and irregular in outline containing increased amount of heterochromatin and multiple small nucleoli (Fig. 3.7). These nuclear changes (Norenberg, 1994) usually precede cytoplasmic changes (Cheng et al., 1994). Therefore, an increase in GFAP-IR is a better predictor of astroglial reactivity as identified by ultrastructural criteria.

The discordance between the absence of GFAP-IR and an increase in GFAP mRNA and GFAP content is also observed during normal development, where, despite a steep 30 fold rise in GFAP content within the first postnatal week (Fig. 3.3), none of the astrocytes in the cortical murine parenchyma were GFAP-IR. These data thus suggest the intriguing possibility that an acute injury to the neonatal brain has accelerated the normal developmental synthesis of GFAP and/or the proliferation of astrocytes (Janeczko, 1998, 1991). Certainly, our ultrastructural analysis reveals a 2 fold increase in the number of protoplasmic astrocytes at the lesion site of the acute injury paradigm in comparison to controls (Fig. 3.10); these did not evolve into reactive astrocytes. It would be interesting to examine other markers of brain maturation following a stab injury to test this possibility.

What factors are then important in converting the increased GFAP mRNA and GFAP content into detectable GFAP-IR? We suggest that macrophage-derived factors regulate this transition, since ultrastructural analysis revealed only the occasional macrophage in the neonatal acute injury; in contrast, the neonatal chronic injury displayed ultrastructurally an abundance of actively phagocytosing macrophages at the lesion site (Fig. 3.10). Moreover, actively phagocytosing macrophages were also identified by Mac-1 immunohistochemistry in chronic NC-implant injuries as early as 1 dpi (Fig. 3.8) and these increased in numbers by 2 dpi (Fig. 3.11). It is probable that the chronic presence of the NC-membrane provided a sufficient stimulus to evoke an immune cascade with the recruitment of both endogenous and exogenous inflammatory mononuclear cells to the

lesion site. In contrast, T cells were not found by immunohistochemical and ultrastructural methods.

The postulate that macrophage-derived cytokines regulate the transition of cells into reactive astrocytes is also supported by other line of evidence. Trauma to the adult CNS has long being known to involve the recruitment of intrinsic and extrinsic inflammatory mononuclear cells (Giulian, 1987; Perry et al., 1993; Taupin et al., 1993; Woodroofe et al., 1991), and levels of macrophage-derived cytokines (IL-1, and TNF-α) are known to be elevated following trauma (Taupin et al., 1993; Woodroofe et al., 1991; Nieto-Sampedro et al., 1987; Nieto-Sampedro and Berman, 1987). Moreover, receptors for several cytokines [e.g. IL-1, TNF-α, interferons, and colony-stimulating factors] have been identified on astrocytes (Rubio and Felipe, 1991; Ban et al., 1993; Sawada et al., 1993; Tada et al., 1994). Furthermore, the administration of IL-1, TNF-α, or IFN-gamma (a potent stimulator of macrophage activity) into the adult CNS facilitates a reactive astroglial response (Giulian et al., 1988; Brosnan et al., 1989; Watts et al., 1989; Yong et al., 1991). Similarly, the minimal GFAP-IR observed following a stab injury to the neonatal brain can be converted to extensive GFAP-IR by the single microinjection of macrophage-derived-factors like IL-1 or TNF-α (Balasingam et al., 1994). We have recently found that agents that inhibit macrophage function (IL-10 and macrophage inhibitory factor) can attenuate an astroglial response in adult CNS trauma via a decrease in TNF-α mRNA levels (Balasingam and Yong, submitted). Giulian and colleagues (1990, 1993) have previously described the suppression of mononuclear phagocytes after ischemic injury with consequent improved recovery and a decreased number of GFAP-IR cells around the lesion.

Initially, the neonatal chronic and either of the adult injury paradigms exhibited extensive GFAP-IR at 4 dpi. This resolved with time to leave lesion bordering astrocytes (Fig. 3.2) or a "glial scar". Similar transient parenchymal GFAP-IR have also been reported for isomorphic injuries (Streit and Kreutzberg, 1988; Anders and Johnson, 1990;

Canady and Rubel, 1992). Presently the functional significance of transient GFAP-IR is unknown. However, with reports implicating neurotrophic factor production by reactive astroglia, occurring at earlier time points following injury (Nieto-Sampedro et al., 1982, 1983; Ip et al., 1993), and the ability of neonatal reactive astrocytes to serve as substrates for the growth of neurons *in vivo* (Silver and Ogawa, 1983; Kliot et al., 1990), it is possible that the transient GFAP-IR is indicative of a neurotrophic phase of reactive astrocytes and that subsequent "scar"-forming astrocytes have lost their neurotrophic activity. Further investigation is warranted on the neurotrophic role of astroglial reactivity.

In conclusion, the data presented here sheds light on several aspects of astroglial reactivity. Following an injury to the CNS, GFAP-IR, but not elevated mRNA or protein, will best reflect the presence of astroglial reactivity. In acute neonatal brain insults, increased GFAP mRNA and proteins can be elicited without concurrent increases in detectability of GFAP-IR; these GFAP profiles are seen also in normal development, raising the possibility that an acute injury to the neonatal CNS accelerates developmental GFAP synthesis and/or proliferation rather than astrocytic reactivity. Macrophage-derived factors appear necessary for the evolution of increased GFAP mRNA and protein into a reactive GFAP-IR astrocyte. Finally, the acute and chronic neonatal injury paradigms now provide us with two simple contrasting experimental models to study the biochemical consequences of injury in the developing CNS towards neurotrophism.

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Table 3.1: Quantitative measurements of GFAP-IR in mouse cortex following NC brain injuries.

Values are mean ± SEM compiled from 3 brain sections per animal, and n of 3 animals per group. The number of GFAP-IR cells (and therefore, density) following NC-stab in neonates was not determined since the soma of the cells could not be reliably detected by GFAP-IR (Figure 3.1).

$^{\star}p<0.05$ compared to all other groups (ONE WAY ANOVA with Duncan's multiple comparisons).

Table 1. Quantitative measurements of GFAP-IR in mouse cortex following NC brain injuries

Specimen	Number of GFAP-IR cells	Area occupied GFAP-IR cells ($\mu m^2 \times 10^4$)	Density of GFAP-IR (number/mm^2)
Neonatal stab	Not done	7.6 ± 0.7	Not done
Neonatal implant	$1866 \pm 63^{\star}$	$1080 \pm 42^{\star}$	$173 \pm 1.6^{\star}$
Adult stab	862 ± 45	693 ± 14	124 ± 3.9
Adult implant	671 ± 13	593 ± 5.5	113 ± 1.2

Figure 3.1: GFAP-IR in the cortex of neonatal mice at 2 (A,C) and 4 (B,D) days following NC-stab (A,B) or implant (C,D) injury. An NC-stab injury to the adult brain is also shown for comparison at 2 (E) and 4 (F) days post-injury. Note that at 2 dpi, the stab injury in neonates resulted in little GFAP-IR; this GFAP-IR was in processes and not cell bodies. In contrast, at 2 dpi, the NC-implant in neonates had caused cell soma and processes to be discernible by GFAP-IR. Stab or implant injuries to adult animals at 2 dpi revealed many cells that were GFAP-IR in both the cell body and processes. At 4 dpi, many GFAP-IR astrocytes were evident in the NC-implant, but not stab, injury in neonates. The density of GFAP-IR cells following NC-implant in neonates was higher than that in adults and this is reflected in the computations shown on Table 3.1. Magnification for frames A and C, the bar represents 50 μm while for all other frames, the bar represents 80 μm.

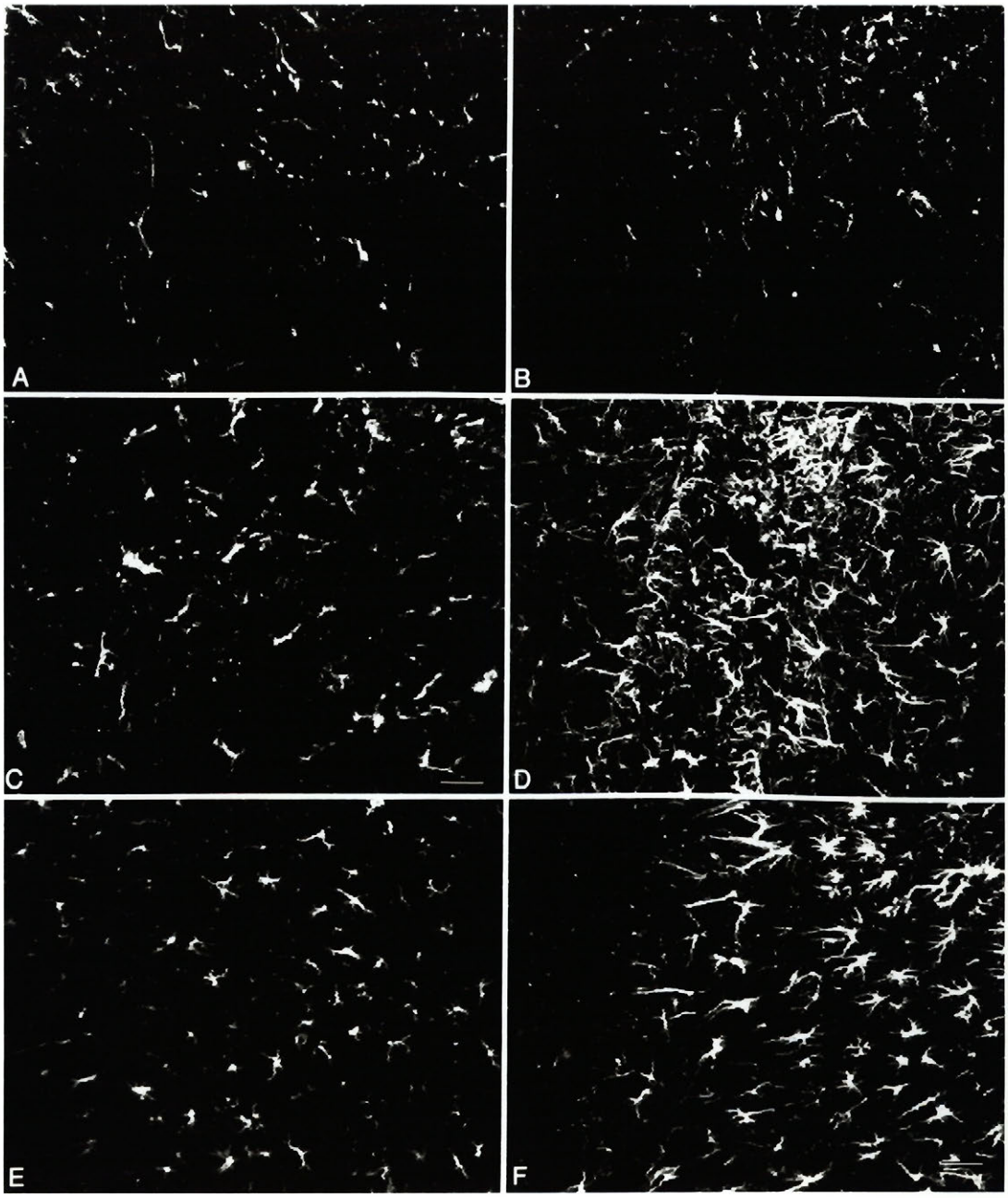

A
B
C
D
E
F

Figure 3.2: GFAP-IR at 21 days following implant injury in neonate (A) and adult (B). In neonatal animals, GFAP-IR astrocytes were on, and in, the NC-implant, while for adults reactive astrocytes could be observed bordering, but not in, the NC-implant. When the NC membrane was removed from the brain of adult animals and then stained for GFAP, stellate cells could be seen on the implant (C). The cells in panel C resembled newly formed membrane glial limitans accessoria (Mathewson and Berry, 1985) indicative of scar-forming reactive astrocytes that have attempted to wall off the lesion site.

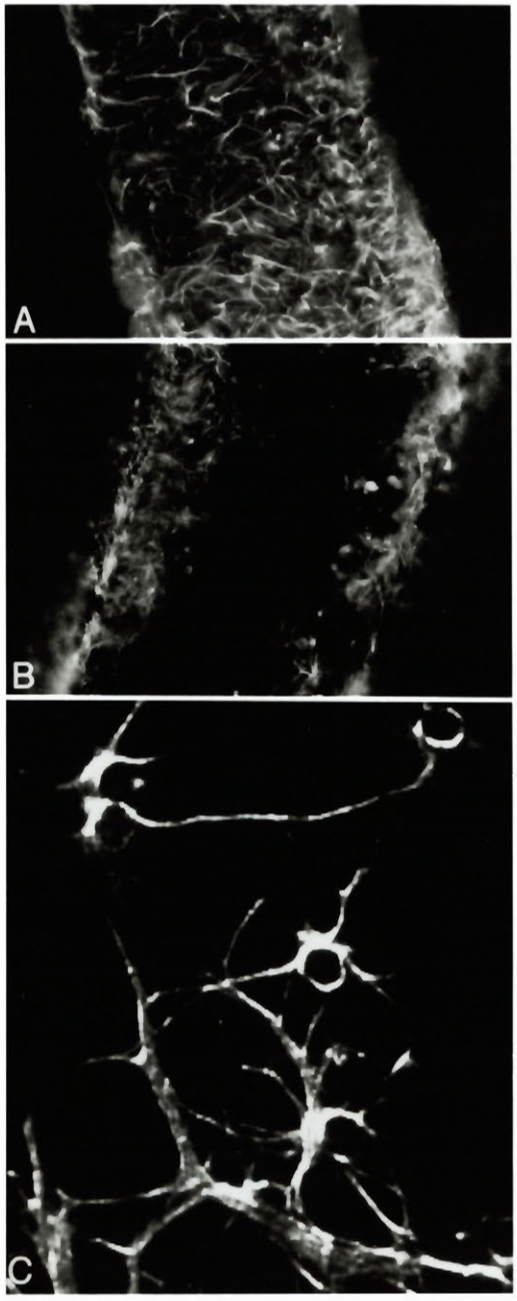
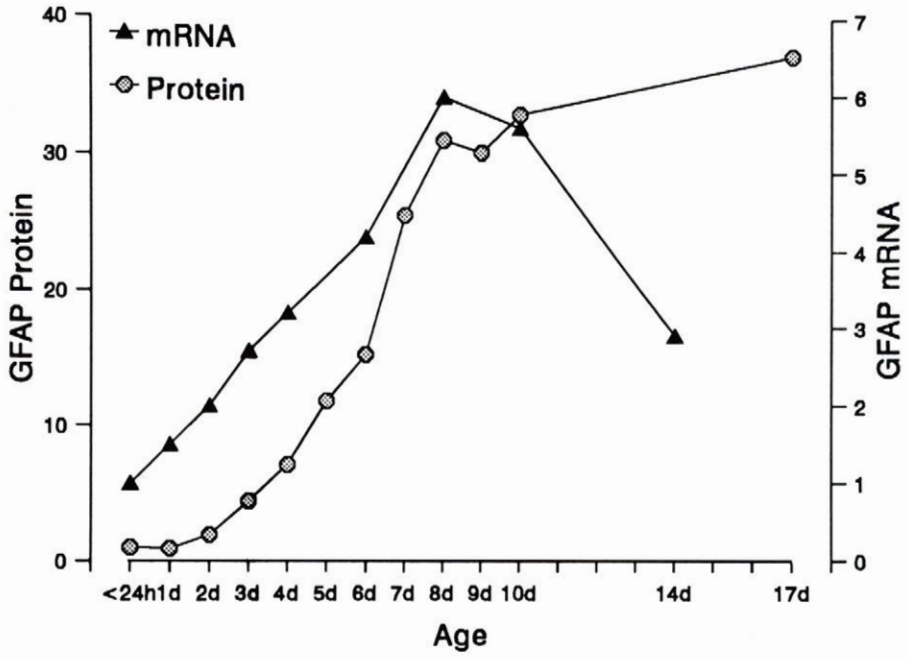

A
B
C

Figure 3.3: GFAP mRNA and protein in the mouse cortex increases rapidly during early neonatal life. In the top panel, results have been expressed as a ratio of that of newborn pups of less than 24 hours old. For mRNA, the GFAP readings were normalized to GADPH mRNA to control for loading, and then expressed as ratios to that of newborn pups. In the bottom panel, the developmental increase in GFAP protein is shown as a representative gel.

GFAP mRNA And Protein During Development

(expressed as a ratio of that of newborns less than 24 hours old)



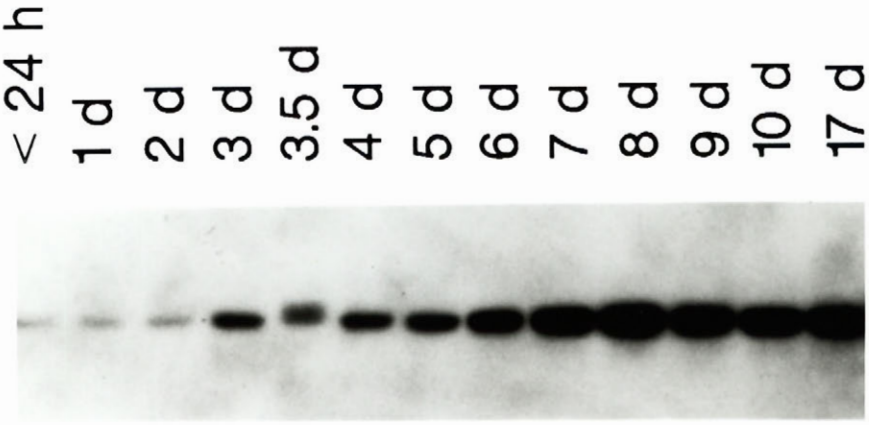
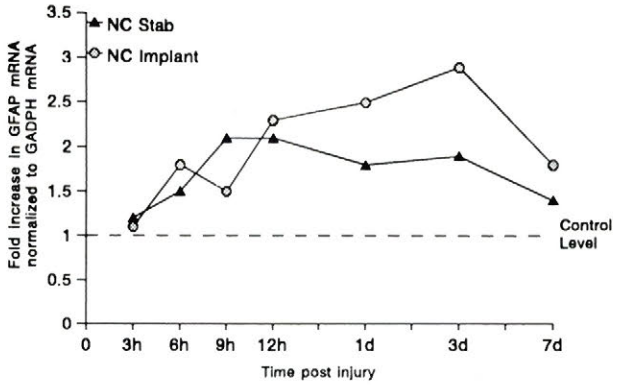


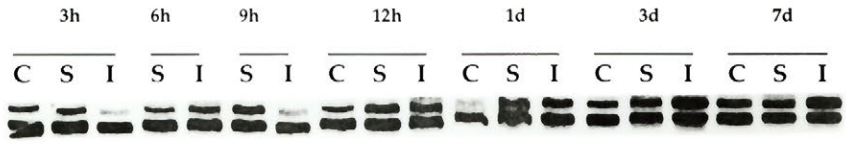
Figure 3.4: GFAP mRNA levels are elevated in all injury paradigms in neonates (a) and adults (C). In panels A and C, the GFAP mRNA values had been normalized to their corresponding GADPH mRNA values, and then expressed as a ratio to that of age-matched controls. In panel B, a representative gel for neonatal samples (GFAP transcript is the upper band while GADPH mRNA is the lower one) is shown (S = stab, I = implant, and C = age-matched controls).

GFAP mRNA Levels In Mouse Cortex Following NC Stab Or Implant Injury

A. Neonatal Mice



B. Northern Blot Analysis Of Neonatal Mouse Samples



C. Adult Mice

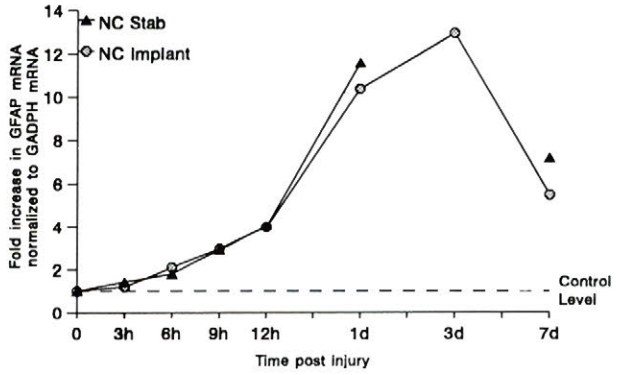
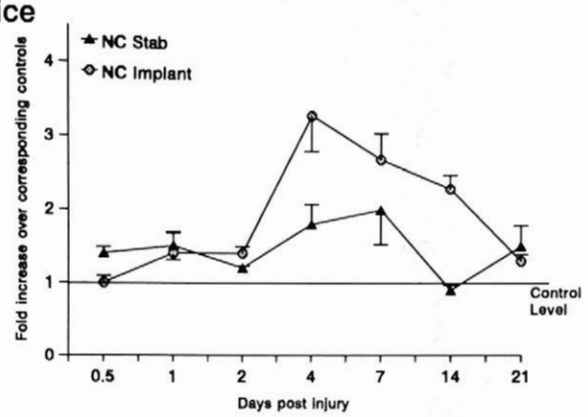


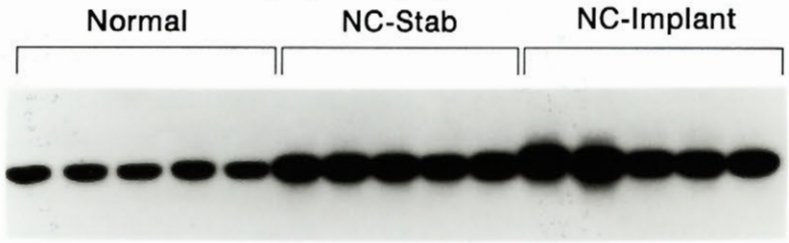
Figure 3.5: GFAP protein content in the mouse cortex following stab or implant injury to neonatal (A) or adult (C) mice. For panels A and C, each time point consisted of 5 injury brain samples and these were run on the same gel with a pooled age-matched control specimen and GFAP standards. The GFAP level of each injury sample was then expressed as a ratio of that of the pooled control. In panel B, individual injury brain specimens were run alongside individual (rather than pooled) control cortices for statistical purposes. By ONE WAY ANOVA with Duncan's multiple comparisons ($p < 0.05$), the elevation of GFAP protein by NC stab (5.4 fold) and implant (9.2 fold) was statistically significant from controls; similarly, results of the NC implant and stab were also statistically significant.

GFAP Protein Content In Mouse Cortex Following NC Stab Or Implant Injury

A. Neonatal Mice



B. Neonatal Mice at 4 days post-injury



C. Adult Mice

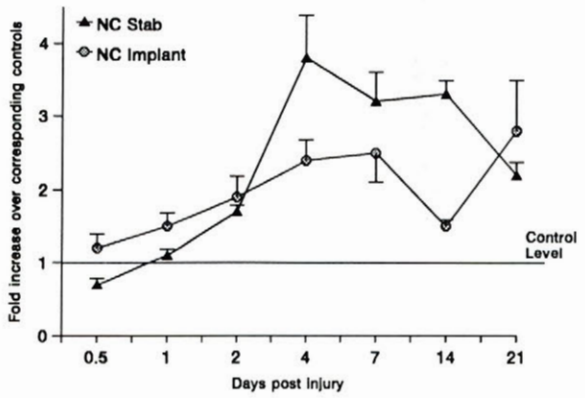
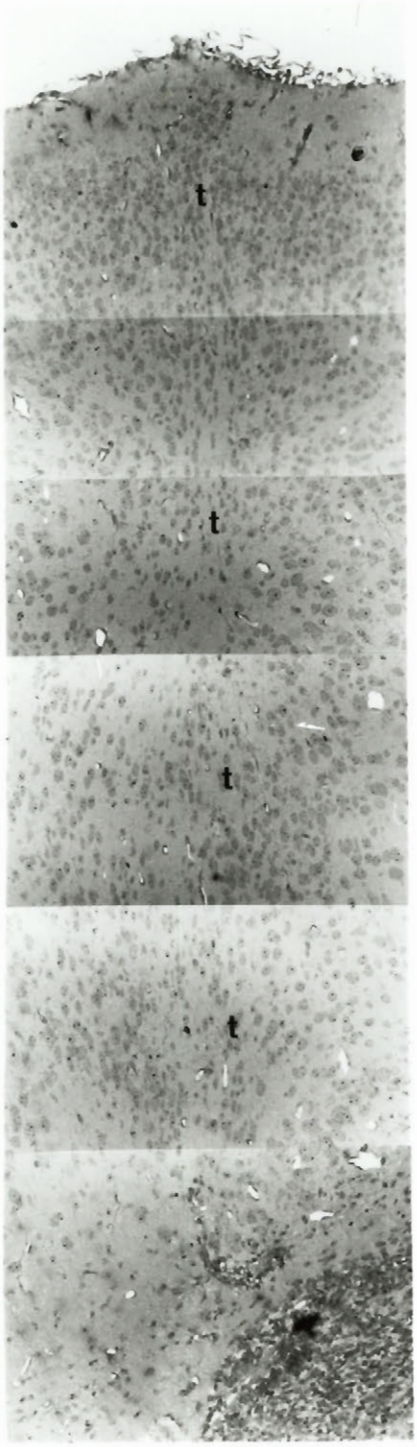


Figure 3.6: Representative photographic montage of the lesion track site in the acute (NC-stab) paradigm on the left and the chronic (NC-implant) paradigm on the right. The track site has been identified by the letter 't'. NC represents the nitrocellulose membrane implant of the chronic injury (left). (Original magnification X 400)


t
t
t
t

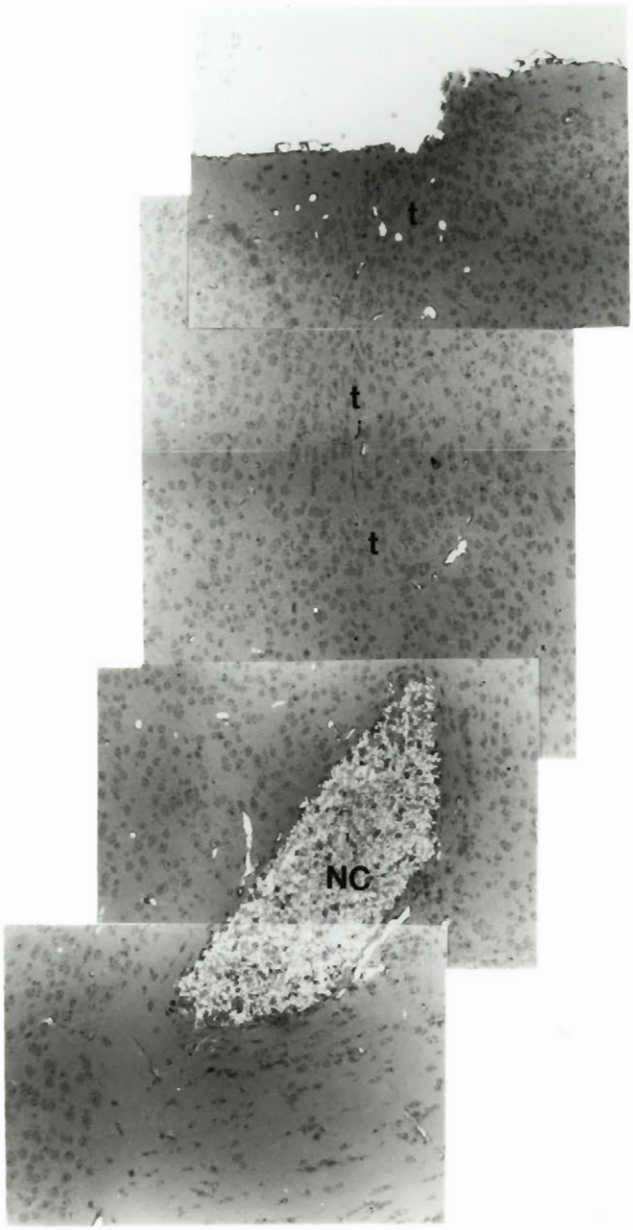

t
t
t
NC

Figure 3.7: Representative electron micrographs of neonatal cortical astrocytes in the normal and reactive state. A depicts a protoplasmic astrocyte, while B and C represent reactive astrocytes in the acute (NC-stab) and chronic (NC-implant) injury paradigms respectively. (Original magnification = 8000 X)

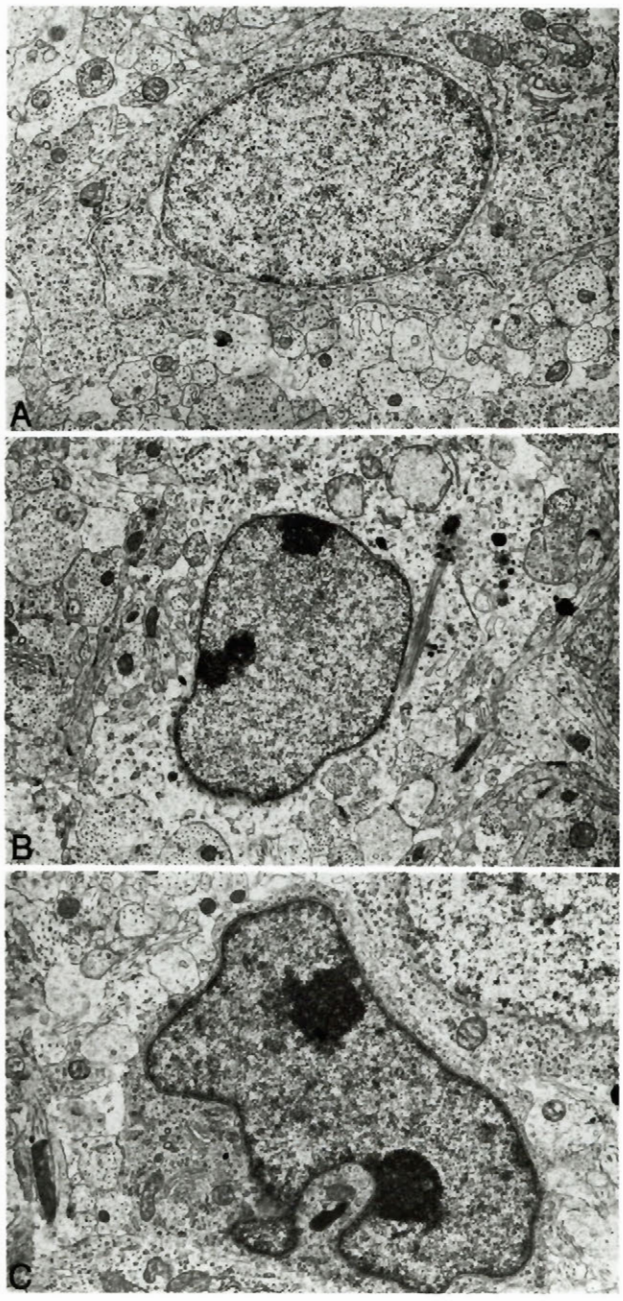

A
B
C

Figure 3.8: Representative electron micrographs of neonatal normal (A) and reactive (B) microglia and a neuronal cell (C). A depicts a quiescent normal looking microglia in the cortical parenchyma, while B represents an activated reactive microglia involved in phagocytosis. The cell depicted in C represents a typical neuronal cell. (Original magnification = 5000 X)

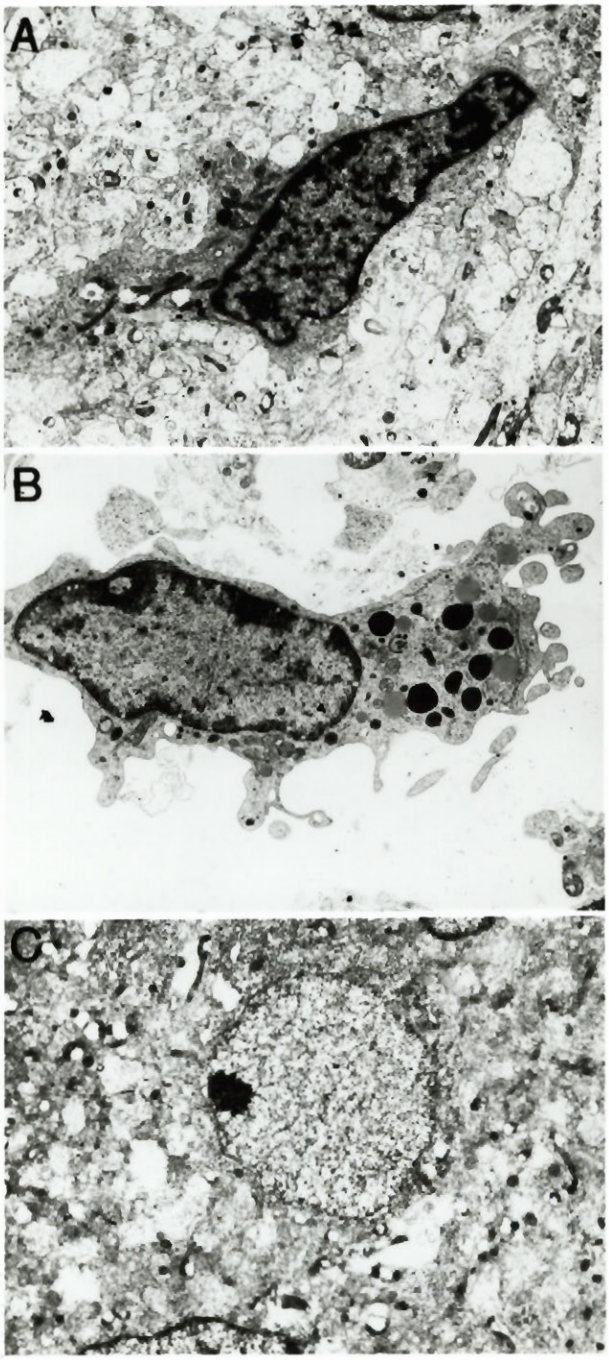

A
B
C

Figure 3.9: An electron micrograph of a 4 dpi neonatal acute and chronic lesion to illustrate the presence of astrocytic processes (AP), macrophage (M), microglia (MI), and reactive astrocytes (RA). The neonatal acute (NC-stab) injury paradigm reveals the presence of occasional reactive astrocyte (A) and macrophage (B). In contrast, the neonatal chronic (NC-implant) injury reveals the parenchymal presence of numerous reactive astrocytes and astrocytic processes (C) and microglia (D). Within the NC-implant, reactive astrocytes (E) and macrophages (F) were seen bordering the pores of the NC-membrane; these pores were visualized as large irregular void areas. (Original magnification = 2700 X)

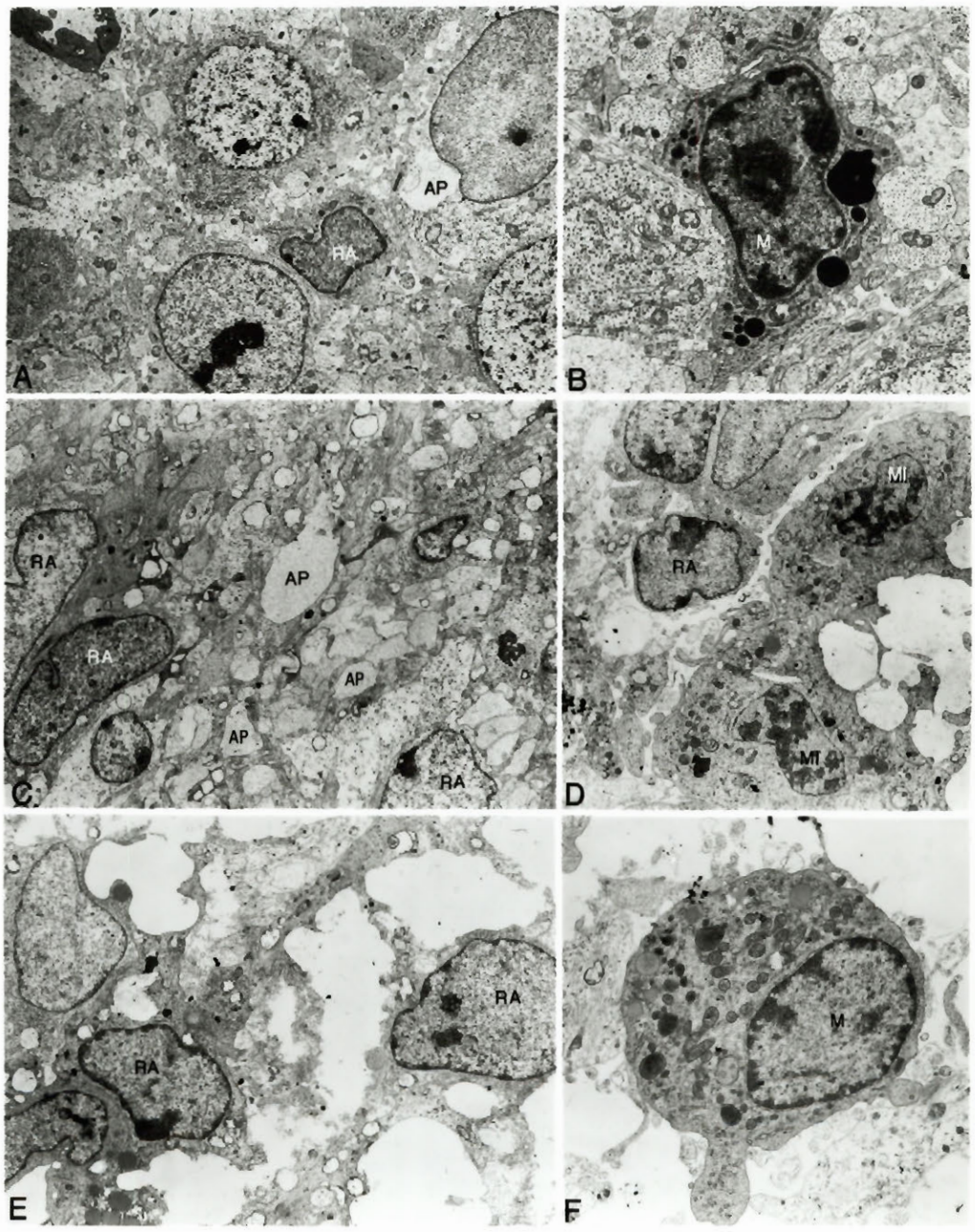

AP
RA
A
M
B
RA
AP
AP
AP
RA
C
MI
RA
MI
D
RA
RA
E
M
F

Figure 3.10: Relationship between astrocytes and microglia/macrophages in the normal and injured neonatal mouse brain.

At the ultrastructural level, the number of reactive astrocytes correlate significantly with the numbers of reactive microglia/macrophages. The different cell types are identified and counted as described in the text. Each histogram is the mean from 3 animals, where for each animal, an average of 10 brain sections were evaluated. The stab injury resulted in a near doubling of normal astrocytes compared to the uninjured brain; the implant injury decreased the number of normal astrocytes, but increased by about 6 fold the total number of astrocytes, the majority of which are reactive.

In the figure insert, regression analysis of the 3 individual implant animals and of the 3 individual stab animals, demonstrate a significant correlation ($P<0.05$) between the number of reactive astrocytes with microglia/macrophages.

$^{\star}p<0.05$ relative to normal uninjured controls (ONE WAY ANOVA with Duncan's multiple comparisons).

Relationship Between Astrocytes And Microglia/Macrophage In The Normal And Injured Neonatal Mouse Brain

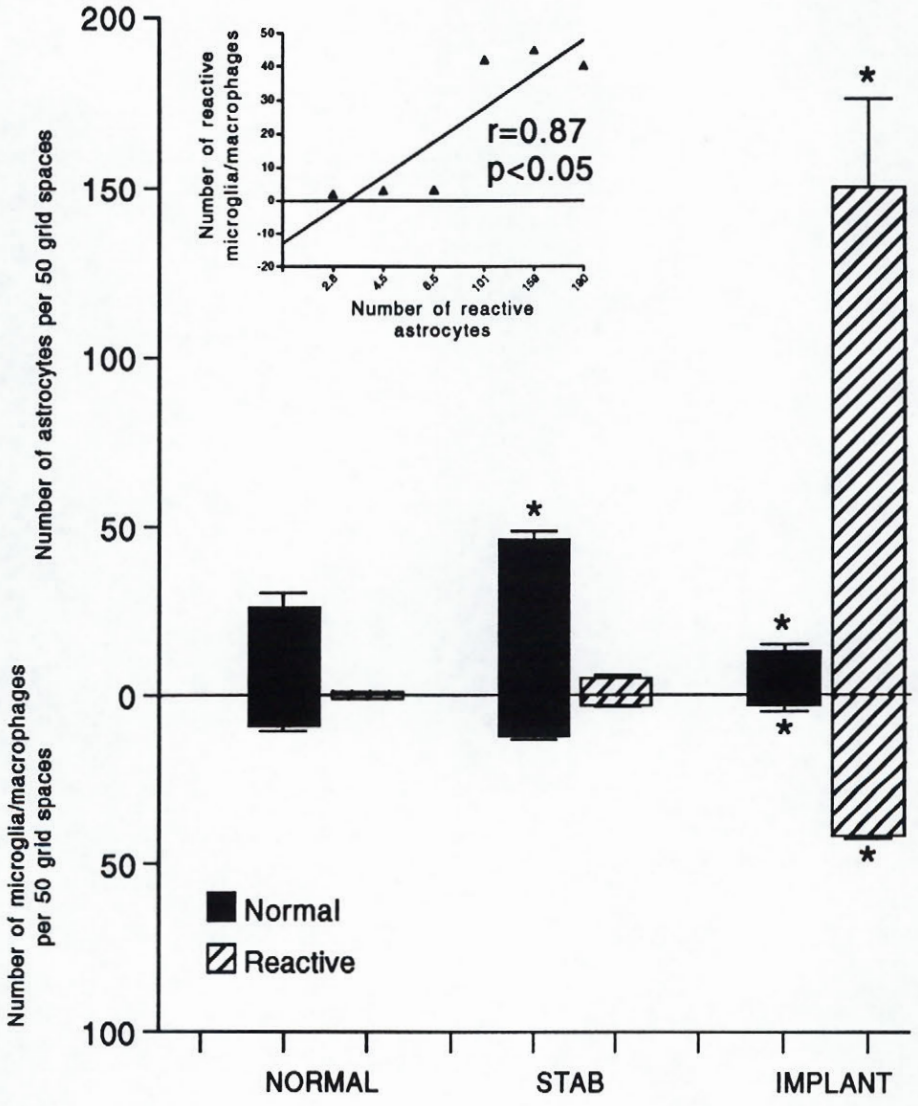
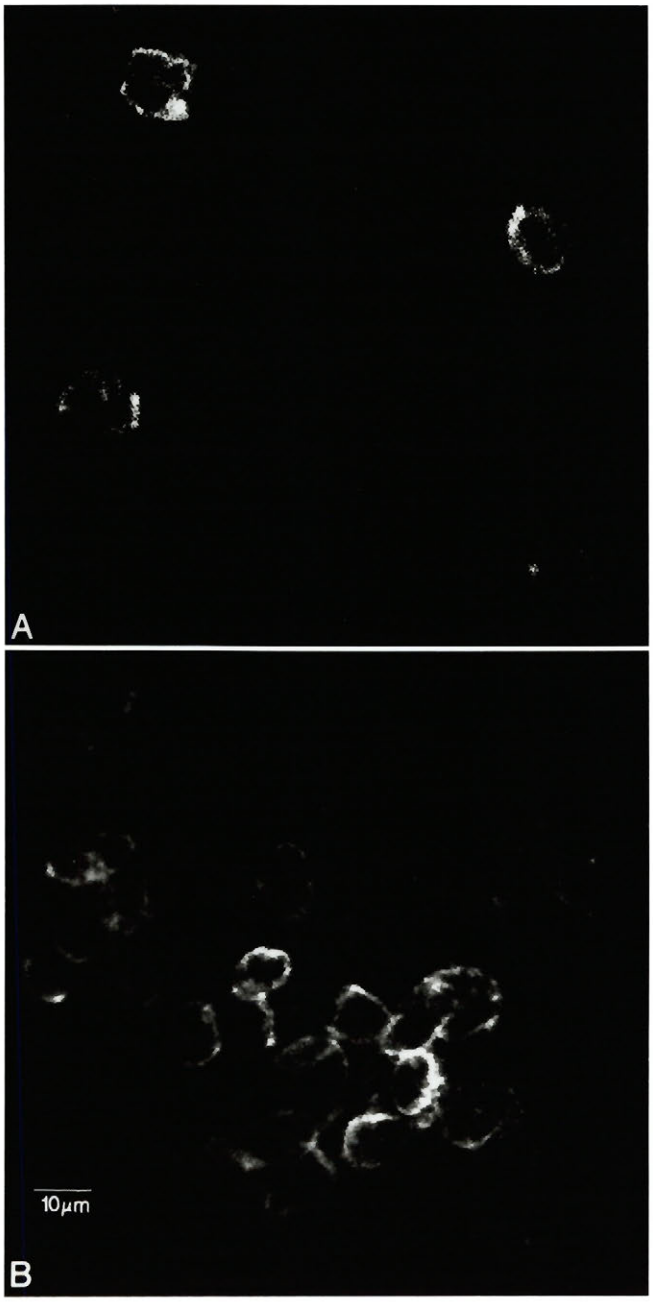


Figure 3.11: Staining of macrophages located on NC-implants which were removed immediately upon decapitation at 1 dpi (A) and 2 dpi (B) respectively. Bar = 10 μm.


A
10μm
B

CHAPTER 4

ATTENUATION OF ASTROGLIAL REACTIVITY BY INTERLEUKIN-10

Vijayabalan Balasingam and Voon Wee Yong

PREFACE

Astroglial reactivity is a characteristic manifestation of brain pathology following anisomorphic insults to the CNS. The aim of the study was to determine if astroglial reactivity could be attenuated by interleukin-10, a potent inhibitor of cytokine synthesis by macrophages/microglia, given the evidence implicating the recruitment of macrophages/microglia, and especially their secreting cytokine products as mediators of astroglial reactivity:

ABSTRACT

Prominent responses that follow brain trauma include the activation of microglia, the recruitment of blood-derived macrophages and astroglial reactivity. Based on evidence that cytokines produced by macrophages/microglia may cause astrocytes to become reactive, the aim of this study was to determine if astroglial reactivity could be attenuated by interleukin (IL)-10, a potent inhibitor of cytokine synthesis by macrophages/microglia. Four days following the local application of IL-10 to the site of corticectomy in adult mice, the number of reactive astrocytes (by 60%) and their state of hypertrophy was reduced when compared to vehicle controls. In the majority of IL-10-treated mice, but not in any vehicle controls, the tissue in the immediate vicinity of IL-10 application contained viable but non-reactive astrocytes. The mechanism by which IL-10 attenuates astroglial reactivity is likely through the reduction of cytokine production by macrophages/microglia since, based on Mac-1 immunohistochemistry, the macrophages/microglia of IL-10 brains had a decreased activation state compared to vehicle-treated controls. As well, another macrophage/microglia deactivating agent, MIF, also reduced astroglial activity in vivo. Furthermore, IL-10 had no direct effect on purified astrocytes in culture, indicating that its in vivo action on astroglial reactivity is likely through indirect mechanisms. Finally, while injury resulted in the substantial rise of TNF-a mRNA levels, this elevation was significantly inhibited by IL-10. The results confirm the importance of immune mediated mechanisms in astroglial reactivity. The ability to manipulate the extent of astrogliosis should provide means of addressing the neurotrophic or inhibitory role of reactive astrocytes in neurological recovery.

INTRODUCTION

Astroglial reactivity is a characteristic manifestation of brain pathology following many types of insults to the adult central nervous system (CNS) (Norton et al., 1992, Eng and Grinikar, 1994). Reactive astrocytes become larger, extend thicker longer processes, increase their level of detection by immunohistochemistry of glial fibrillary acidic protein (GFAP), and up-regulate their number of mitochondria, glycogen content and various enzyme levels (Nathaniel and Nathaniel, 1981, Norenberg, 1994). A long term result of this astrocytic reaction can be the formation of a densely interwoven glial scar at the lesion site which has been thought to be detrimental to axonal regeneration (Reier et al., 1983; Liuzzi and Lazek, 1987). On the other hand, more recent evidence suggests that astroglial reactivity may actually be an attempt by these cells to promote CNS recovery; in particular, neurotrophic factors are produced around the locus of CNS lesions especially in the early phase of astroglial reactivity (Nieto-Sampedro et al., 1982; 1983; Needels et al., 1986; Ip et al., 1993; reviewed by Yong, in press). Thus, the ability to manipulate the extent and the occurence of astroglial reactivity can have implications for CNS regeneration.

Anisormorphic injuries in the adult, involving trauma with disruption of the blood-brain barrier, result in the activation of intrinsic microglia and the recruitment of systemic inflammatory mononuclear cells, including monocytes (which become macrophages upon entry into the tissue), to the lesion site (Kitamura et al., 1972; Boya et al., 1986; Giulian, 1987; Tsuchihushi et al., 1981; Moorshead and van der Kooy, 1990; Leong and Ling 1992). These inflammatory cells are a rich source of pro-inflammatory cytokines as determined by the elevated content of the macrophage-derived cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-a following trauma (Woodroofe et al., 1991; Yan et al., 1992; Taupin et al., 1993; da Cunha et al., 1993; Quan et al., 1994). The rise in the recruitment of macrophages/microglia is followed by the appearance of local

astroglial reactivity (Giulian et al., 1989; Balasingam et al., submitted). Further evidence to implicate macrophages/microglia, and especially their secretory cytokine products, as mediators of astroglial reactivity is provided by the observation that the administration of IL-1, TNF-a, or interferon-g (IFN-g, a potent stimulator of macrophage activity) into the site of CNS trauma increases astroglial reactivity beyond that induced by trauma alone (Giulian et al., 1988; Brosnan et al., 1989; Watts et al., 1989; Yong et al., 1991). In addition, while an acute injury (e.g. stab) to the *neonatal* brain creates minimal astroglial reactivity in contrast to adults (Sumi and Hager, 1968; Berry et al., 1983; Barrett et al., 1984; Maxwell et al., 1990), appropriate insults can elicit extensive astroglial reactivity (Balasingam et al., 1994); the occurence or absence of reactive astrocytes in neonatal animals is correlated, respectively, with the presence or lack of activated macrophages/microglia at the lesion site (Balasingam et al., submitted). Finally, the minimal astroglial reactivity following a stab injury to the *neonatal* brain can be converted to extensive reactivity by the single microinjection of macrophage-derived cytokines (IL-1 or TNF-a) (Balasingam et al., 1994).

Given the above evidence implicating macrophages/microglia in astroglial reactivity, we have addressed whether astroglial reactivity could be attenuated by inhibiting cytokine production by macrophages/microglia. We selected IL-10 for this purpose since IL-10, also named cytokine synthesis inhibitory factor, is a potent inhibitor of cytokine secretion by macrophages/microglia (Bogdan et al., 1991; de Waal Malefyt, 1991a; Fiorentino et al., 1991a; D'Andrea et al., 1993; Deleuran et al., 1994; Mosmann, 1994). We demonstrate that the administration of IL-10 attenuates the astroglial reactivity that follows corticectomy in the adult mouse brain.

MATERIALS and METHODS

Experimental animals

Adult retired female breeders of the CD1 strain (4 to 6 months old) were obtained from a commercial source (Charles River Canada, Montreal, Quebec, Canada). Animals were housed on a 12/12 h light/dark cycle with ad libitum access to food and water. All experimental procedures were approved by the institution's animal care committee and were in accordance with the guidelines instituted by the Canadian Council of Animal Care.

Animals were anaesthetized with an intraperitoneal injection containing a mixture of Ketamine (200 mg/kg) and Xylazine (10 mg/kg). The animals were immobilised in a stereotaxis frame followed by a midline incision and a unilateral circular (approximately 3 mm diameter) craniectomy over the left hemisphere. A 25-30 mm^3 volume of cortical tissue was removed by vacuum aspiration and the wound cavity was packed with Gelfoam (The Upjohn Co., Kalamazoo, MI; 50 mm^3 volume when dry) soaked in 20 µl of test agent. The skin was closed with 4.0 sutures. Animals received IL-10 (PeproTech Inc, Rocky Hill, NJ) of specified doses: 25 U, 50 U, 100 U, and 200 U. Control animals received Gelfoam containing phosphate buffered saline (PBS), which was the vehicle for IL-10. This method of administering test agents by using Gelfoam has been previously described (Yong et al., 1991), and is thought to produce a slow release of agent into the parenchyma. Animals were allowed to survive for 4 days postoperatively.

Cajal's gold chloride sublimate method for astrocytes

This classical method was utilised for its ability to demonstrate three types of astrocytes: the normal protoplasmic astrocyte present in grey matter (e.g. cortex) (Fig. 4.1A), the fibrous astrocyte present in normal white matter (e.g. corpus callosum) (Fig. 4.1B), and the reactive astrocyte (Fig. 4.1C) that can be derived from either protoplasmic or fibrous astrocytes through hypertrophy and extension of processes. In contrast, the more current method of GFAP immunoreactivity (IR) readily reveals normal fibrous astrocytes and reactive astrocytes, but not normal protoplasmic astrocytes in the cortex (Bignami and Dahl, 1976; Yong and Balasingam, 1995). According to Vaughn and Pease (1967), as well as Mori and Leblond (1969), the Cajal gold chloride sublimate stain deposits gold on the astroglial filaments contained in astrocytes, thereby facilitating the light microscopic evaluation of the different types of astrocytes.

Brains from decapitated animals were fixed in formalin-ammonium bromide (2% ammonium bromide and 15% formalin in water) (v/v) solution for 24 hrs. Frozen sections were cut at 20 μm and received in distilled water containing 10 drops of formalin. After 2 washes in distilled water the sections were laid flat in the gold chloride sublimate solution (2% mercuric chloride and 0.25% of aqueous gold chloride solution) (v/v) for 24 hrs. Sections were removed, washed in water followed by 5% sodium thiosulphate, and washed in water again; each wash was of 5 min duration. Sections were mounted on clean albuminized slides, allowed to dry, and was mounted with Eukitt (O Kindler Gmbh & Co, Freiburg, Germany).

Assessment of GFAP-immunoreactivity (IR) in situ

Flourescent GFAP-IR

Animals were anaesthetized and exsanguinated intracardially followed by post-fixation and cryoprotection of the brain as reported earlier (Balasingam et al., submitted). Twenty μm coronal sections were obtained on gelatin coated slides and subjected to immunofluorescence for GFAP as previously described (Balasingam et al., 1994). Three representative sections per animal were photographed with a 10 x 0.30 NA objective on a Reichert Polyvar 2 (Leica) microscope for quantitative purposes. Only GFAP-IR astrocytes with processes and a clearly stained soma were counted.

Non-flourescent GFAP-IR

Brains were removed after decapitation and fixed in 10% formalin overnight and embedded in paraffin. Six μm coronal sections were obtained, deparaffinized in xylene, rehydrated in a decreasing gradient of ethyl alcohol and placed in water. Endogenous peroxidase was quenched with a 3% H_2O_2 solution and the sections were incubated with Protein Block (Immunon, Pittsburg, PA) prior to incubation with a rabbit anti-GFAP polyclonal antibody (1:100, Dako Corp.) for 30 min at room temperature. Sections were then incubated with a biotinylated secondary antibody followed by streptavidin peroxidase and revealed by AEC chromogen (prepared as per manufactures instructions, Immunon, Pittsburg, PA). All sections were rinsed in Tris buffer prior to each of the succeeding steps which were carried out for 30 min at room temperature. Sections were counterstained with Harris haematoxylin and coverslipped with Gelvatol.

Mac-1 immunofluorescence

The primary antibody used was a monoclonal rat anti-mouse immunoglobulin to Mac-1 (complement receptor, type three) antigen. Immunohistochemistry was performed at room temperature on 20 μm brain sections (obtained as described for GFAP-IR). In brief, samples were initially fixed in acetone for 20 min, blocked with chicken egg albumin (3%) for 30 min, and incubated overnight wiht the primary antibody (10 μg/ml) at room temperature. Sections were then sequentially incubated with biotinylated anti-rat immunoglobulin (1:100, Dako Corp.) for 1 h, followed by ABC reagent (Vector Laboratories, Burlingame, CA) for another 1 h. This was followed by a biotin amplification procedure (Adams, 1992) consisting of a 10 min incubation with 0.2% biotinylated tyramide (Dupont) in a 0.1% H_2O_2 solution; the time factor here was critically observed to avoid excessive background. Immunofluorescence was revealed by incubation with streptavidin conjugated to Texas Red (1:100, Dako Corp).

Assessment of in vitro astrocytic response to IL-10

Proliferation and GFAP content

The procedure for the culture of neonatal astrocytes from postnatal day 1 CD1 mouse pups (Charles River Canada, Montreal, Canada), and assessment of proliferation, has been described in detail elsewhere (Yong et al., 1992). In all experiments, cells were treated once with test agents and maintained for 4 days. Proliferation was assessed by the administration of 1 μCi of ^{3}H-thymidine during the last 16 hr of the experiment (Yong et al., 1992). GFAP protein extraction was performed after the 4 d incubation period with either IL-10 (10 U/ml) or PBS and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis as previously described (Balasingam et al., 1994). The concentration of IL-10 used (10 U/ml) was 10 times higher than the in vitro EC50 concentration for co-stimulation (with IL-4) of MC-9 cells.

FACScanning

Astrocytic cultures treated with IL-10 or PBS for 4 d were trypsinized and resuspended in HHG (1mM HEPES buffer, 2% horse serum, 10 % goat serum in Hank's balanced salt solution). The cells were washed in Hank's balanced salt solution (HBSS), fixed in 95% acid alcohol for 20 minutes and blocked with 3% chicken egg albumin prior to incubation with a rabbit anti-GFAP polyclonal antibody (1:100; Dako Corp) for 30 min. This was followed by a wash and a final incubation with secondary goat anit-rabbit immunoglobulin conjugated to FITC (1:100; Jackson). Cells were washed twice with a solution containing HBSS and 2% fetal calf serum (FCS), followed by resuspension in FACS buffer (2% FCS and 0.1% sodium azide in HBSS). Intensity measurements for GFAP-IR in these cell cultures were analyzed with a Fluorescent Activated Cell Sorter flow cytometer (FACScan, Becton Dickinson) utilizing a LYSYS II software (Becton Dickinson) gated for 5000 events.

Reverse transcriptase - polymerase chain reaction (RT-PCR) for TNF-α mRNA

TNF-a mRNA levels were chosen to represent the level of cytokine production by macrophages/microglia. The oligonucleotide primers used for RT-PCR of TNF-a were as follows: forward primer,5'AGCACAGAAAGCATGATCCG and reverse primer, 5'TGAAACCTCAGTAACCAGAG (Sheldon Biotechnology, Montreal) (Renno et al., 1995). The expected PCR product is of 701 bp and represents the entire coding sequence for murine TNF-a. Total RNA was isolated using Trizol^{R} (Gibco/BRL, Grand Island, New York) (Balasingam et al., submitted) from samples resected from around the corticectomy site (about 20 mg wet weight). One μg RNA was reverse-transcribed and amplified in a single step process (Singer-Sam et al., 1990) with the following modifications: 200 μM dNTPs, 1 μM primers, 2 mM $MgCl_2$, 2 U of AMV RT (Gibco/BRL), 1 U of TAQ polymerase (Gibco/BRL), 33 U RNA Guard (Pharmacia Biotech), 1x PCR buffer (Gibco/BRL) and 0.5 μCi/ml of a-^{32}P-dCTP (ICN, Costa Mesa, CA) in a total reaction

Cetus) at 50°C for 15 min followed sequentially by a cyclic phase at 94°C for 45 s, 60°C for 45 s, and then 72°C for 1.5 min for a total of 26 cycles. RNA from ANA-1 cells (a mouse macrophage cell line) was co-amplified and used as standard to determine the linearity of the PCR reaction. Samples were electrophoresced on a 6% non-denaturing polyacrylamide gel, dried under vacuum, visualized by autoradiography, and analysed by ImageQuant software on a phosphorimager.
system.

RESULTS

Cajal's Gold Chloride Sublimate method for astrocytes

A blinded qualitative inspection of astroglial reactivity revealed the ability of IL-10 (200 U) to attenuate astroglial reactivity (Fig. 4.2). Reactive astrocytes detected in the parenchyma of vehicle treated animals by this metal impregnation technique were characterized by the presence of hypertrophied cell soma and abundant processes (Fig. 4.1C and 3A). IL-10 treated animals revealed an attenuation in astrocyte hypertrophy and also had reduced numbers of reactive astrocytes (Fig. 4.3B).

GFAP-immunohistochemistry

We proceeded to investigate whether there was a dose-dependent relationship to the effects of IL-10 in attenuating astroglial reactivity. GFAP-IR was chosen as a read-out in order to utilize a second detection system for astroglial reactivity. Furthermore, GFAP-IR is normally absent in the normal murine cortex (Bignami and Dahl, 1976; Yong and Balasingam, 1995) although this gray matter area contains protoplasmic astrocytes and GFAP.

In a blinded analysis of GFAP-IR , vehicle treated controls had the largest degree of astroglial reactivity (Fig. 4.4), in accordance with results obtained by the Cajal gold chloride sublimate technique. The effect of IL-10 on astroglial reactivity was dose-dependent with significant inhibition at the higher doses employed (Fig. 4.4).

A striking observation by GFAP-IR is the presence of reactive astrocytes that border the lesion in vehicle treated controls (Fig. 4.3C) but not in IL-10 treated animals (Fig. 4.3D). The non-GFAP-IR rim of tissue, present immediately adjacent to the cortical wound in IL-10 treated animals, extended from the glial limitans to the corpus callosum. This rim was seen in 8 out of 10 IL-10-treated (200 U) animals, and 0 out of 10 vehicle-treated controls ($p< 0.001$, Student's T test).

The presence of a non-GFAP-IR rim in IL-10 treated animals, but not in controls, could be indicative of a suppression of astroglial reactivity by IL-10 close to the site of application, since non-reactive astrocytes in the cortex are normally not GFAP-IR. Alternatively, the rim could represent necrotic tissue. To address the latter, paraffin embedded sections were stained for GFAP and revealed by AEC chromagen, and then counterstained with haematoxylin. Vehicle treated controls showed the presence of viable tissue and GFAP-IR reactive astrocytes in areas adjacent to the lesion (Fig. 4.3E). Similarly, the lesion-bordering tissue of IL-10 animals was viable; however, in contrast to vehicle-treated controls, there were much fewer GFAP-IR astrocytes in IL-10-treated animals (Fig. 4.3F)

The extent of astroglial reactivity following either vehicle or IL-10 (200 U) treatment was quantitated by measuring the total number of GFAP-IR astrocytes present on either side of the cortical lesion. This computation confirmed the qualitative results that IL-10 attenuated astroglial reactivity; indeed, IL-10 reduced the number of reactive astrocytes per 20 μm brain section by 60% when compared to vehicle controls (Table 4.1).

Mac-1 Immunohistochemistry

The Mac-1 antibody marks the type 3 complement receptor (CR3) (Springer et al., 1974; Beller et al., 1983; Sanchez-Madrid et al., 1983) which functions in the phagocytosis of opsonized particles. CR3 is widely expressed on cells belonging to the mononuclear phagocyte system. Comparisons between vehicle (Fig. 4.3G) and IL-10 (Fig. 4.3H) brains showed that Mac-1-positive cells were more densely packed, and more intensely stained, around the lesion site in controls than in the IL-10 brains, indicative of a suppression of macrophage/microglial reactivity in IL-10 animals. The results were reproduced in multiple sections taken from 3 animals in each group.

IL-10 did not directly alter astrocyte function in vitro

Astrocytes have receptors for a large number of cytokines [e.g. IL-1, Il-6, IL-7, IFN-a/b, IFN-g, TNF-a] (Rubio and Felipe, 1991; Ban et al., 1993; Sawada et al., 1993; Tada et al., 1993; Aranguez et al., 1995) and can be directly affected by these cytokines. Thus, TNF-a, IL-1 and IFN-g can alter the proliferation rate (Giulian and Lachman, 1985; Barna et al., 1990; Selmaj et al., 1990; Yong et al., 1992; Balasingam et al., 1994) and GFAP mRNA transcription (Oh et al., 1993) of astrocytes in vitro. To determine whether the effects of IL-10 on astrocytic reactivity in vivo could be due to a direct action on astrocytes, we determined whether IL-10 could modulate astroglial activity using purified murine astrocytes in culture.

Measurements of ^{3}H-thymidine incorporation revealed the inablilty of IL-10 to affect astrocyte proliferation when compared to its PBS vehicle (Fig. 4.5B). Similarly, there was no change in GFAP content following IL-10 treatment of astrocytic cultures for up to 4 days (Fig. 4.5C). Finally, FACScan analysis revealed no difference in intensity levels for GFAP-IR between vehicle and IL-10 cultures (Fig. 4.5A).

Macrophage inhibitory factor also decreases astroglial reactivity.

To further address the role of macrophage/microglia in astroglial reactivity, another agent known to inhibit macrophage function was employed. This agent, macrophage inhibitory factor (MIF), is an immunoglobulin derived tripeptide Thr-Lys-Pro (Tuftsin fragment 1-3, Sigma, St. Louis) that has in vitro inhibitory properties on the release of lysosomal enzymes, phagocytosis and the production of superoxide anion by macrophage and monocytes (Auriault, 1983). In vivo, the inhibition of microglial activity by MIF has been shown to influence the elimination of axotomized ganglion cells (Thanos et al., 1993).

Our investigation revealed that the single application of 500 μM of MIF significantly reduced astroglial reactivity when compared to vehicle-treated controls (Fig. 4.2). However, the effect of MIF was not as marked as that of IL-10.

RT-PCR analyses for TNF-α mRNA levels

IL-10 is a cytokine synthesis inhibitor of cells that include macrophages/microglia. To verify that the effects of IL-10 on the attenuation of astroglial reactivity was through the inhibition of cytokine synthesis, areas surrounding the corticectomy site were resected out and subjected to RT-PCR analyses for mRNA levels of a macrophage-derived cytokine, TNF-a.

Figure 4.6 demonstrates that the TNF-a mRNA level of non-injured animals, using a 26 cycle PCR, is near the limit of detection. However, by 12 h following injury (the earliest time point examined), TNF-a transcripts were significantly increased. The administration of IL-10 attenuated by 30-40% the rise in TNF-a levels produced by injury; this was statistically significant at 24 hours following the application of IL-10. Thus, IL-10 in vivo reduced the injury-elicited elevation of the cytokine TNF-a.

DISCUSSION

The activation of intrinsic microglia, and the recruitment of blood-derived macrophages to the lesion site, are among the earliest cellular responses that follow anisomorphic injuries to the adult central nervous system (Kitamura et al., 1972; Tsuchihushi et al., 1981; Boya et al., 1986; Giulian et al., 1987; Moorshead and van der Kooy, 1990; Leong and Ling, 1992; Balasingam et al., submitted). Because of the lack of markers to differentiate between microglia and macrophages, these mononuclear phagocytes are often collectively referred to as macrophages/microglia. Guilian and colleagues (1989) have used chloroquine and colchicine to suppress the function of invading macrophage/microglia, and have reported a reduction of astroglial reactivity following traumatic injury. Similar suppression of macrophage/microglia activity also prevented the deterioration of hindlimb motor function after ischemic injury to the spinal cord (Giulian and Robertson, 1990). Since chloroquine and colchicine are non-specific inhibitors of macrophage/microglia, it was reasoned that more specific inhibitors of macrophage/microglia would provide a more effective means to attenuate the development of reactive astrocytes and reveal the role of macrophage/microglia in astroglial reactivity. In this report, we have used IL-10, a potent cytokine synthesis inhibitor, as an immunosuppressant to inhibit the function of macrophages/microglia following an adult aspiration corticectomy model, in an attempt to attenuate astrocyte reactivity. Such studies would improve the understanding of mechanisms that produce astroglial reactivity, and would also provide for means to examine the neurotrophic consequences of the reactive astrocyte.

The results of this report demonstrate that IL-10 decreased astroglial reactivity in a dose-related manner (Fig. 4.4). In this regard, there was both a decrease in the number (Table 4.1) and the hypertrophic state of reactive astrocytes (Fig. 4.3). Immediately adjacent to the site of IL-10 application, vaible astrocytes were non-reactive in contrast

to those in vehicle-treated animals, suggesting that IL-10 has ablated the evolution of astrogliosis in its immediate vicinity. Further away from the site of IL-10 administration, reactive astrocytes could be found, probably reflecting the decreased diffusability of IL-10 to these regions.

The reduction in astroglial reactivity is unlikely to be due to a direct effect of IL-10 on astrocytes since IL-10 had no effect on astrocyte cultures in vitro in terms of their proliferation, GFAP content or GFAP-IR (Fig. 4.5). What then is the mechanisms of IL-10 in attenuating astroglial reactivity? To address this, it would be instructive to review relevant aspects of IL-10 biology. IL-10 is a product of B cells, TH0 and TH2 cells, and of macrophages late in their activation (O'Garra et al., 1990; Moore et al., 1990; Mosmann et al., 1990; de Waal Malefyt et al., 1991a). The immunosuppressive effect of IL-10 can be mediated via a number of avenues: firstly, IL-10 diminishes the antigen presenting capacity of monocytes/macrophages to T lymphocytes (Fiorentino et al., 1991b), by inhibiting both the constitutive and induced expression of MHC class II antigens (de Waal Malefyt et al., 1991b; Frei et al., 1994; Rott et al., 1994). Secondly, IL-10 impairs the ability of monocytes/macrophages to provide costimulatory signals for activation of resting T cells in vitro. Ding and colleagues (1992, 1993) have demonstrated that this impairment is mediated by the suppression of a macrophage membrane bound antigen, B7/BB1, a costimulator of T cell antigens CD28 and CTLA-4. Thirdly, activated monocytes/macrophages can also produce IL-8 (Yoshimura et al., 1987; Sylvester et al., 1990) and IL-12 (Kobayashi et al., 1989; D'Andrea et al., 1992), the function of which is inhibitable by IL-10 (Tripp et al., 1993; Mosmann, 1994). IL-8 is a chemotactic factor for neutrophils (Baggiolini et al., 1989) and T lymphocytes (Larsen et al., 1989). The suppression of this chemoattractant activity would decrease the recruitment of inflammatory cells to the lesion site. IL-12, or natural killer (NK) cell stimulatory factor, is a strong inducer of IFN-g production by NK and T cells (Chan et al., 1991, 1992); thus, the inhibition of IL-12 synthesis/function can contribute to the attenuation of an immune cascade. Finally, as stated earlier, IL-10 is a potent inhibitor of the synthesis of

several cytokines by leukocytes.

Several lines of evidence would suggest that the effects of IL-10 in attenuating astroglial reactivity is through the inhibition of cytokine production by macrophages/microglia. Firstly, macrophage/microglia-derived cytokines alone (IL-1, TNF-a) can convert the degree of astrogliosis in *neonatal* animals following trauma from minimal to extensive (Balasingam et al., 1994). In adult animals, these cytokines enhance the astrogliosis that is the usual result of CNS injury (Giulian et al., 1988; Brosnan et al., 1989); IFN-g, a potent activator of macrophage/microglia, also increases the extent of astroglial reactivity in adult animals (Yong et al., 1991). Secondly, IL-10 treatment decreased the density and intensity of Mac-1-positive macrophages/microglia (Fig. 4.3), suggesting the reduced function of this cell type. Thirdly, another agent known to decrease macrophage/microglia function, macrophage inhibitory factor (MIF), also reduced astroglial reactivity (Fig. 4.2). Finally, we demonstrate that the level of mRNA for TNF-a, a cytokine which can induce astroglial reactivity, is indeed reduced in the extracts of brain that surround the injury site of IL-10 mice (Fig. 4.6).

Previously, the consequences of astroglial reactivity were thought to be undesirable as glial "scars" were found to inhibit axonal growth or regeneration (Reier et al., 1983; Liuzzi and Lazek, 1987), interfere with remyelination (Raine and Bornstein, 1970), or be the sites of electrical instability and epilepsy (Pollen, 1970). While these detriments are possible consequences of the long-term glial "scars", more recent studies indicate that the process of astroglial reactivity, especially in the early stages, may actually represent an attempt by astrocytes to promote recovery. This concept has evolved from studies (reviewed in Yong, in press) that indicate that cultured astrocytes synthesize a range of neurotrophic factors, that astrocytes are condusive substrates for the survival and growth of neurons in vitro, that neurotrophic factors are produced around the locus of a lesion and that the source of these appears to be reactive astrocytes, and in vivo data that astrocytes can promote regeneration under very specific circumstances. To resolve the

dichotomy of glial "scars" being impediments to recovery and the numerous neurotrophic properties of astrocytes, the control of astroglial reactivity that follows injury would be beneficial. Thus, the finding in this study that IL-10 can attenuate astroglial reactivity should add to the efforts to define the functions of reactive astrocytes.

In conclusion, the results show that astroglial reactivity can be attenuated by the administration of IL-10, which likely acts by inhibiting the production of cytokines by macrophages/microglia. The findings point to the importance of immune mediated mechanisms to brain pathology in general, and to astroglial reactivity in particular. The ability to manipulate the extent of astrogliosis can now provide means of addressing the neurotrophic or inhibitory role of reactive astrocytes in neurological recovery.

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Table 4.1: IL-10 Decreases the number of reactive astrocytes in murine cortex

Confocal microscopy was used to analyse GFAP-IR in the murine cortex as previously described (Balasingam et al., 1994). Images compiled from the entire ipsilateral coronal section was printed and then reconstructed on paper to obtain the entire ipsilateral coronal section. From the reconstructed images (magnified approximately 1000x), the number of reactive astrocytes (GFAP-IR) in the cortex was manually counted. Values displayed above are mean ± SEM of the total number of reactive astrocytes per brain section in the cortex; 3 brain sections per animal, taken from the corticectomy region, were analysed.

*$p<0.001$ compared to PBS vehicle (Unpaired Student's T-test).

Table 1. IL-10 Decreases the Number of Reactive Astrocytes in Murine Cortex

Treatment	# of sections/# of mice	# of GFAP-IR cells per 20 μm brain section
PBS Vehicle	15/5	927 ± 55
IL-10, 200 U	18/6	385 ± 20*

Figure 4.1: Cajal's gold chloride sublimate technique to identify normal and reactive astrocytes. Some astrocytes in all 3 frames are indicated by arrows. **A**: Protoplasmic astrocytes in the grey matter of the uninjured cortex. The darkly stained round structures are neuronal soma. In order to visualize normal protoplasmic astrocytes in the cortex, it is necessary to over-stain brain sections and this results in the neuronal cell bodies being darkly labelled. In contrast, upon injury, astrocytes can be easily revealed without the need for over-staining (e.g. in frame C; thus, neuronal cell soma in C is not prominent). **B**: Fibrous astrocytes in the uninjured corpus callosum. C: Reactive astrocytes in the cortex following traumatic injury. X 400.

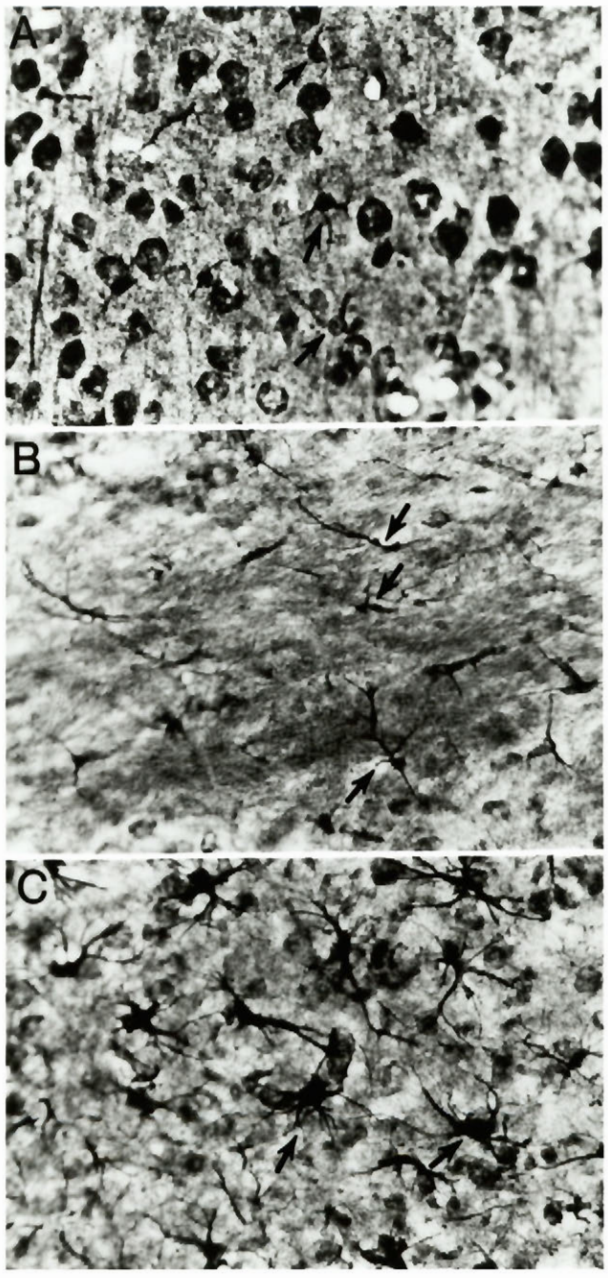

A
B
C

Figure 4.2: Cajal-stained brain sections were analysed blind and the extent of astroglial reactivity in the ipsilateral cortex was tabulated from a scale of 1 (no reactivity) to 5 (extensive reactivity). Values are mean ± SEM and three brain sections per animal was analysed; parentheses indicate the total number of sections examined. $^{H}p<0.05$ compared to injured mice given PBS vehicle (ONE WAY ANOVA with Duncan's multiple comparisons).

Dose Response Of IL-10 On Astroglial Reactivity:
Semi-Quantitative Analyses Using GFAP Immunoreactivity

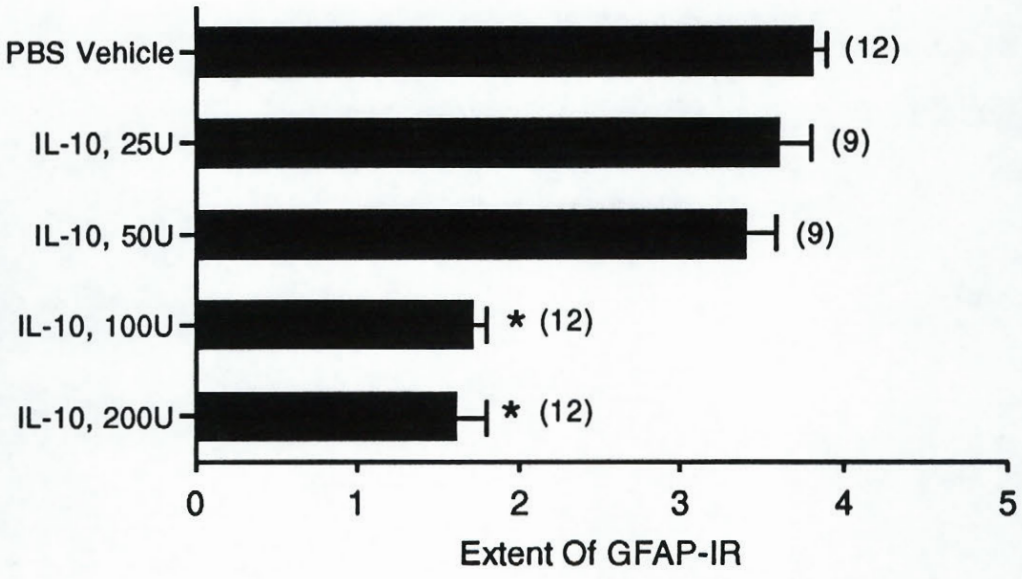
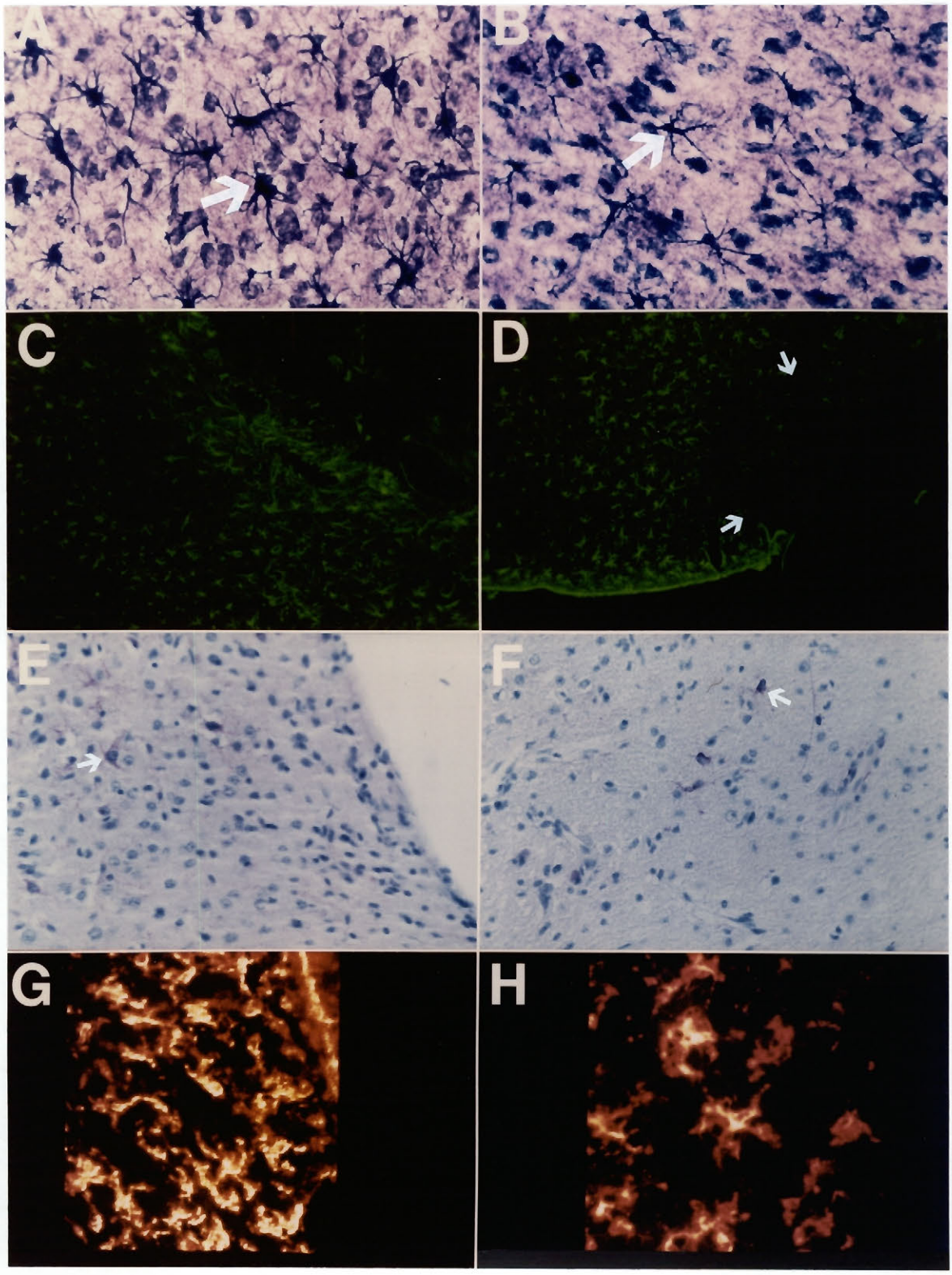


Figure 4.3: Cortical brain sections of corticectomy animals treated with PBS vehicle (A, C, E, G) or 200 U of IL-10 (B, D, F, H). Cajal's gold sublimate technique (x 400): **A** shows increased number and hypertrophism of reactive astrocytes in vehicle treated animals while **B** shows decreased number of, and less hypertrophied, reactive astrocytes in IL-10 mice. Fluorescent GFAP-IR (x 100): **C** demonstrates the presence of reactive astrocytes that abut the lesion border in vehicle treated animals while **D** displays a paucity of reactive astrocytes (indicated with arrows) at sites that border the lesion of IL-10 animals. Non-flourescent GFAP-IR revealed by AEC chromagen and counterstained with Haematoxylin (x 300): **E** indicates the presence of viable tissue and GFAP-IR reactive astrocytes at lesion-bordering sites of vehicle treated animals while **F** shows the presence of viable tissue and a paucity of GFAP-IR astrocytes at similar sites of IL-10 subjects. Mac-1 immunohistochemistry (x 400): **G** displays the presence of intensely stained, and dense, microglia/macrophages at the lesion site of vehicle treated animals when compared to similar area in IL-10 brains (**H**); yellow denotes areas of most intense fluorescence. The intensity of Mac-1 staining at the lesion site of each brain section, as assessed by confocal microscopy, has been normalized to Mac-1 fluorescence in the contralateral, non-injured, cortex, so as to allow for comparisons between sections.


A
B
C
D
E
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G
H

Figure 4.4: Brain sections were analysed blind and the extent of astroglial reactivity in the ipsilateral cortex was tabulated from a scale of 1 (no reactivity) to 4 (extensive reactivity). Values are mean ± SEM and 3 brain sections per animal were analysed; parentheses indicate the total number of sections examined.

*$p<0.05$ compared to PBS-vehicle (ONE WAY ANOVA with Duncan's multipple comparisons).

Cajal's Gold Sublimate Stained Brain Sections: Semi-Quantitative Analysis Of Astroglial Reactivity

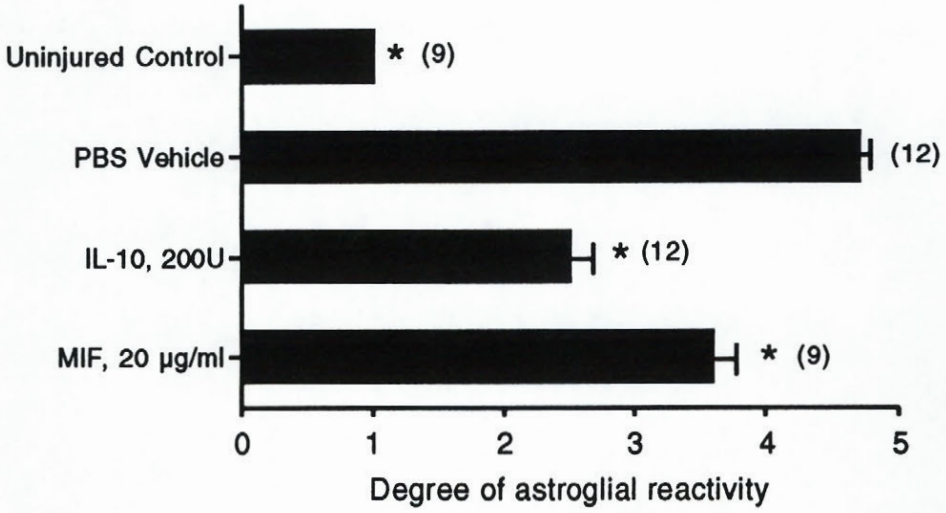
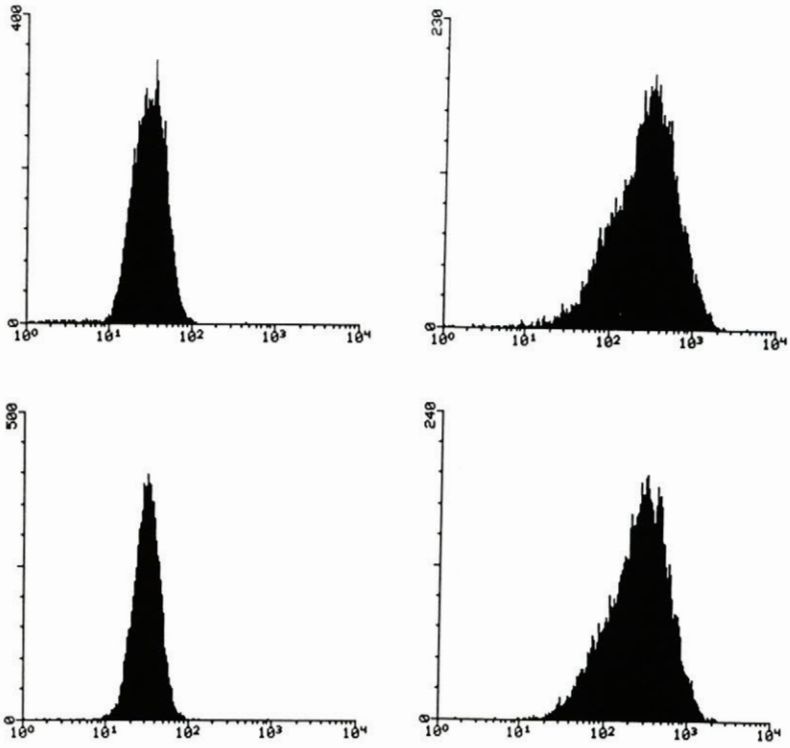


Figure 4.5: Effects of IL-10 on astrocytes in vitro. In group A, flow cytometer analysis revealed no differences in intensity levels for GFAP-IR between either vehicle or IL-10 treatment. The left panel of each treatment is the fluorescent intensity of cells stained with secondary antibody alone while the right panels are of cells labelled for GFAP followed by the secondary antibody. The mean intensity fluorescence for GFAP following IL-10 in the example shown (2 days treatment) is 322.6 while that for vehicle is 342.8. This lack of change was also observed for 4 days of treatment, and was demonstrated for two other sets of cultures. In group B, the proliferation rate of astrocytes (mean ± SEM) did not differ following either vehicle or IL-10 treatment for 2 or 4 days as determined by ^{3}H-thymidine incorporation; cpm denotes counts per minute. The lower rate of proliferation at 4 days compared to 2 days in both the vehicle and IL-10 groups is related to the attainment of confluency at 4 days. In C, a representative GFAP Western (band appears at 49 kDa) of 15 μg of total cell extract is shown for cells treated for 2 days with IL-10 (phosphorimager volume of 406203) or PBS (phosphorimager volume of 419485). This lack of change was reproduced in three other sets of cultures, following 2 or 4 days of treatment.

Effects of IL-10 on astrocytes in vitro

A. GFAP-IR by flow cytometry



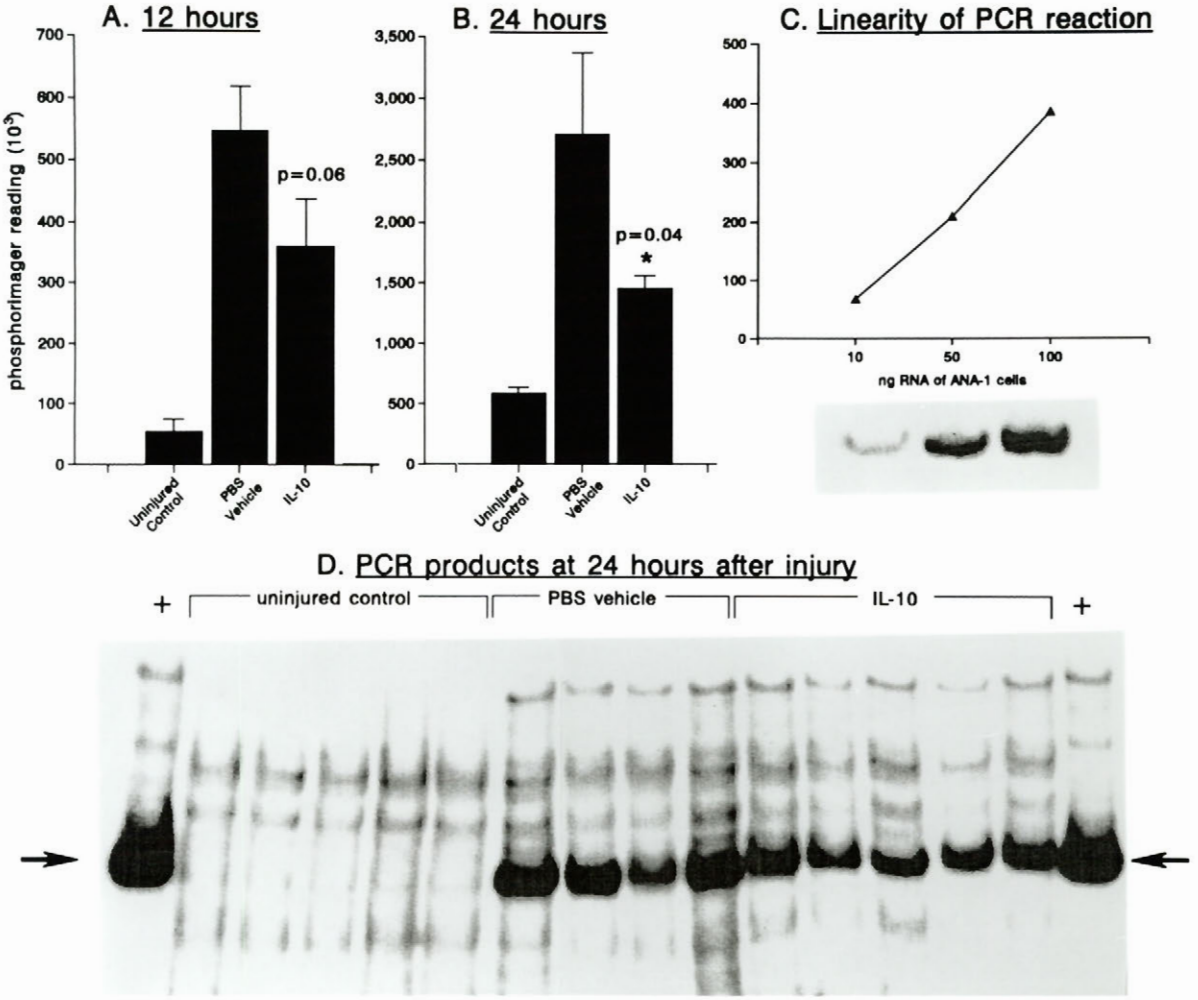
B. Proliferation Rate (cpm)

	Day 2	Day 4
Vehicle	10853 ± 1068	6671 ± 109
IL-10	11329 ± 1018	7727 ± 412

C. GFAP content

Figure 4.6: Semi-quantitative RT-PCR analyses for TNF mRNA in the adult mouse brain. Brain tissue surrounding the corticectomy site was dissected out (about 20 mg wet weight), placed in Trizol^{R} (Gibco), and total RNA extracted as per manufacturer's instructions. RNA samples were then subjected to RT-PCR as described in the text. Panels A and B demonstrate that TNF-α signals increase rapidly following injury; this increase can be attenuated by IL-10. P values refer to Student's t-test comparisons between IL-10 and PBS controls. In Panels A and B, each bar is mean ± SEM of 4 or 5 samples. Values are in phosphorimager units; the difference in the phosphorimager scale for panels A, B and C is due to the different background in each case, each panel representing a separate gel. In panel C, the linearity of the PCR reaction at 26 cycles is confirmed using ANA-1 cells, a mouse macrophage cell line. In Panel D, the PCR reaction products are shown using samples collected at 24 hours following injury. The 701 bp TNF-α cDNA product is indicated by the arrow; + lanes at either end of the gel represents ANA-1 samples, which were used as positive controls for RT-PCR.

TNFα Levels In Adult Mouse Brain At 12 And 24 Hours Following Corticectomy: Effects Of IL-10



CHAPTER 5

SUMMARY OF EXPERIMENTAL WORK

Astroglial reactivity is a prominent and characteristic response of astrocytes to adult brain pathology. However, injury to the embryonic or neonatal CNS has been reported to produce minimal astroglial reactivity, if any at all (Sumi and Hager, 1968; Bignami and Dahl, 1976, Berry et al., 1983; Maxwell et al., 1990). Reasons postulated for the lack of neonatal astroglial reactivity have included the relative immaturity and plasticity of neonatal astrocytes and neurons as well as the lack of myelin.

Since the immune system in the neonatal animal is relatively immature compared to the adult (Hobbs, 1969, Abo et al., 1983; Lu and Unanue, 1985; De Paoli et al., 1988; Hannet et al., 1992), we have postulated that the consequent lack of cytokine production in the injured neonatal CNS might explain the probable lack of astroglial reactivity following neonatal CNS injuries. This notion is further supported by the observation that injury to the CNS involves the activation of intrinsic microglia and the recruitment of blood-borne macrophages when the blood-brain barrier is damaged (Kitamura et al., 1972; Boya et al., 1986; Guilian, 1987; Tsuchihashi et al., 1981; Morshead and van der Kooy, 1990; Leong and Ling, 1992). These inflammatory cells are a rich source of inflammatory cytokines as determined by the elevated content of macrophage-derived cytokines [IL-1, IL-6 and TNF-α] following trauma (Woodroofe et al., 1991; Taupin et al., 1993) and in head injury patients (McClain et al., 1987; Goodman et al., 1990).

This thesis explores the role of cytokines in mediating astroglial reactivity. We provide evidence: *Objective 1*) to establish the ability of the neonatal CNS to mount an astroglial reaction to injury and the ability of cytokines to elicit and enhance astroglial reactivity; *Objective 2*) to document the necessity for the presence of reactive microglia/macrophages with their synthetic ability for cytokines to evoke astroglial reactivity; and *Objective 3*) to attenuate astroglial reactivity in the adult CNS by

suppressing the synthesis of microglia/macrophage cytokines.

With regards to our first objective, we demonstrate in chapter 2 (Balasingam et al., 1994) that the neonatal CNS is capable of manifesting astroglial reactivity. In summary, the neonatal CNS can elicit an extensive astroglial response if a sufficient injury stimulus (chronic NC-implant) is provided. In addition, the neonatal CNS stab wound paradigm, with its inherent minimal astroglial reactivity, can be converted to display extensive astroglial reactivity by the single microinjection of cytokines. Furthermore, the ability of a cytokine to alter proliferation of astrocytes *in vitro* does not appear to predict its capability in enhancing astroglial reactivity *in vivo*.

To confirm the necessity for the presence of reactive microglia/macrophages to evoke astroglial reactivity as stated in objective 2, we document the temporal occurence of astroglial reactivity in relation to microglia/macrophage presence in chapter 3 (Balasingam et al., 1995, submitted). This study was conducted with the use of the chronic (NC-implant) and the acute (NC-stab) injury paradigms that evoked extensive and minimal GFAP-IR respectively. The nature of the neonatal chronic NC-implant injury paradigm as a sufficient injury stimulus to evoke astroglial reactivty was correlated to the increasing presence of reactive microglia/macrophages. Interestingly, the acute (NC-stab) injury displayed minimal GFAP-IR and reactive microglia/macrophage presence but had a rapid and significant increase in GFAP mRNA and GFAP content. This observation involving the increase in GFAP at the biochemical level, but not at the level of immunohistochemical detection, suggest an acceleration of astroglial development. This notion is supported by the observation that during normal neonatal development, GFAP content increase rapidly although its presence is below the level of immunohistochemical detection in brain slices.

Furthermore, the extensive cortical astroglial reactivity observed at 4 days post-injury in either the neonatal chronic or adult injury paradigms resolved with time to leave only lesion bordering astrocytes or a glial scar. The functional significance of this transient astroglial reactivity is presently unknown. We postulate that the transient astroglial reactivity is related to neurotrophism following injury as it does not contribute to the formation of a glial scar.

Finally, we accomplished the third objective of attenuating astroglial reactivity by inhibiting cytokine synthesis by microglia/macrophages. IL-10, a potent macrophage deactivating and cytokine synthesis inhibitory factor, was used in chapter 4 (Balasingam and Yong, submitted) to suppress the activity of recruited microglia/macrophages present at the injury site. This suppression resulted in the attenuation of astroglial reactivity, which was evidenced by a decrease in the number and the hypertrophic state of reactive astrocytes. Furthermore, the majority of IL-10 treated animals, in comparison to saline treated controls, showed the presence of viable but non-reactive astrocytes immediately adjacent to sites of drug administration. TNF-α mRNA levels were alos significantly reduced by IL-10. Finally another macrophage deactivating factor, macrophage inhibitory factor, was also able to reduce astroglial reactivity *in vivo* although it was not as potent as IL-10.

These experimental observations, taken together, strongly implicate the importance of immune mediated mechanisms in astroglial reactivity.

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CHAPTER 6

GENERAL DISCUSSION

Reactive astrogliosis is a prominent response that follows every type of CNS insult. Chronologically,the process of reactive astrogliosis initially involves microglial activation followed by the presence of astroglial reactivity (Rio-Hortega and Penfield, 1927; Giulian et al, 1989; Graeber and Kreutzberg, 1988; Chapter 3). In this respect, astroglial reactivity is taken to mean the phenotypic transformation of local astrocytes by hypertrophy and fibrous change as determined classically by the gold-chloride sublimate technique or currently by GFAP-IR. It is now clear that the presence of these reactive microglia/macrophages in anisomorphic injuries are necessary for the occurrence of astroglial reactivity (chapter 3).

Cytokines and Astroglial Reactivity

Reactive microglia/macrophages present at anisomorphic lesion sites are involved in debris clearance and provide a rich source for inflammatory cytokines (Giulian et al., 1986; Nieto-Sampedro et al., 1987a,b; Hetier et al.,1988). The ability of these inflammatory cells to synthesize cytokines in abundance has been verified by the elevated content of macrophage derived cytokines [IL-1, IL-6 and TNF-α] following trauma in experimental animals (Woodroofe et al., 1991; Taupin et al., 1993; Chapter 4). In addition, it has been reported that head trauma patients also show increased cerebrospinal fluid levels of TNF-α (Goodman et al., 1990) and IL-1 (McClain et al., 1987). This thesis demonstrates that the availability of these cytokines at traumatic lesion sites can contribute to the presence of astroglial reactivity (Chapter 2).

The above evidence suggests that the inhibition of microglia/macrophage function, with the concomitant suppression of cytokine synthesis, should attenuate astroglial reactivity. To achieve effective suppression of reactive microglia/macrophages, IL-10, a

potent macrophage deactivating and cytokine synthesis inhibitory factor (reviewed by Mosmann, 1994) was selected. The use of IL-10 attenuated lesion bordering astroglial reactivity in an adult aspiration injury paradigm (Chapter 4).

Mechanisms for Cytokine Activity

Cytokines initiate astroglial reactivity by first binding to their specific cytokine receptors on astroglia. In this respect, astrocytes are generously endowed with a large number of cytokine receptors [e.g. IL-1, IL-6, IL-7, IFN-α/B, IFN-γ, TNF-α] (Rubio and Felipe, 1991; Ban et al., 1993; Sawada et al., 1993; Tada et al., 1994). The intracellular mechanism by which these cytokines initiate astroglial reactivity currently remains unknown.

At the present time we could speculate that cytokines influence the induction of several immediate early genes (IEG) after traumatic injury (Sheng and Greenberg, 1990; Jenkins et al., 1993). The products of these IEGs, such as *c-fos* and *c-jun*, are nuclear regulatory proteins that can interact through the leuzine-zipper to form transcriptionally active complexes (Curran and Franza, 1988; Sassone-Corsi et al., 1988; Abate et al., 1990a, b, c) that can specifically bind to AP-1 binding sites or TPA (12-O-tetradecanoylphorbol-13-acetate) responsive elements (Morgan and Curran, 1991).

A consequence of the increased AP-1 binding, can be the increased expression of GFAP mRNA levels (Kindy et al., 1991, 1992; Nozaki and Beal, 1992; Gubits et al., 1993). In addition, IEGs are also involved in the late activation of neurotrophin mRNAs (a subject to be discussed later) such as nerve growth factor [NGF] (D'Mello et al., 1990; Hengerer et al., 1990; Lindvall et al., 1992; Hsu et al., 1993) and brain derived neurotrophic factor [BDNF] (Lindvall et al., 1992; Hsu et al., 1993).

Types of Astroglial Reactivity

Traumatic injury to the CNS of either the neonatal or adult can result in two forms of astroglial reactivity: (1) an acute phase transient astroglial reactivity; and (2) a chronic phase scar forming astroglial reactivity.

In adult anisomorphic injuries, astroglial reactivity that occurs at sites removed from the immediate lesion bordering areas are transient and dissipate with time over a two week period (Mathewson and Berry, 1985; Chapter 3). In contrast, astroglial reactivity that occurs at lesion bordering sites remain GFAP-IR long after transient astroglial reactivity has dissipated. These lesion bordering reactive astrocytes are referred to as chronic phase astroglial reactivity. It is these chronic reactive astrocytes that contribute to: (1) the formation of the new membrane glial limitans accessoria [Fig. 3-2] (Mathewson and Berry, 1985); and (2) the traditional scar forming reactive astrogliosis. In this regard, treatment with IL-10 attenuated the presence of these potentially scar forming lesion bordering reactive astrocytes (Chapter 4).

Transient astroglial reactivity has also been reported for isomorphic injuries (Streit and Kreutzberg, 1988; Anders and Johnson, 1990; Canady and Rubel, 1992). Similarly, extensive *neonatal* astroglial reactivity, when elicited with an adequate injury stimulus (e.g. chronic NC-implant: chapter 3; alcohol: Goodlett et al., 1993), is generally transient in the CNS parenchyma.

Therefore it appears that astroglial reactivity as determined by astrocytic hypertrophy can occur at damaged (lesion) and undamaged (remote) sites. In regions where neurons are not permanently damaged, astroglial reactivity is transient or reversible (Petito et al., 1992; Balasingam and Yong, unpublished observation). This transient astroglial phenomenon can also be demonstrated at the GFAP mRNA level in both electrolytic lesions (Steward et al., 1990) and in short term electrically induced seizure

models (Steward et al., 1991). In contrast, astrocyte hypertrophy and persistent GFAP-IR are confined to regions that have experienced neuronal necrosis and subsequent degeneration debris.

The neonatal chronic NC-implant injury paradigm (Chapter 2 and 3) is one that elicits comparable neuronal trauma to the neonatal NC-stab injury paradigm. Yet, the presence of this NC-implant model elicits extensive astroglial reactivity in comparison to the NC-Stab in the neonate. This discrepancy probably arises from the ability of the NC-implant to simulate a scenario involving a large *debris burden.* The need to clear this debris burden stimulated an immune cascade involving the recruitment of a large number of microglia/macrophages with consequent cytokine synthesis. The presence of this large cytokine supply elicited an extensive transient astroglial reactivity with minimal long-term chronic reactive astrocytes within the CNS parenchyma surrounding the NC-implant. In contrast, the NC-implant itself was filled with persistent GFAP-IR chronic reactive astrocytes (Fig. 3-2). Similar injury experiments when conducted in the adult did not reveal a difference in the outcome of astroglial reactivity because the neuronal *debris burden* in the adult NC-stab paradigm was sufficient to elicit an adequate inflammatory response.

Neurotrophism and Transient Astroglial Reactivity

Presently the function of transient astroglial reactivity is unknown. The reported occurrence of transient astroglial reactivity in a number of injury models and its association with cytokine presence *in vivo* suggest important functions other than forming a glial scar. Evidence will be put forth in this discussion from both an *in vitro* and *in vivo* perspective to propose a neurotrophic role for the transient phase of astroglial reactivity.

There is evidence to suggest that following cortical injury there is a short time-dependent increase in certain trophic factors at lesion sites (Nieto-Sampedro et al., 1982; Needles et al., 1986) that support neuronal survival or neurotrophism and neurite promoting activity. This increase in neurotrophic activity by the different lesion models was confirmed by both the *in vitro* survival of peripheral and central neurons (Nieto-Sampedro et al., 1982, 1983; Manthrope et al., 1983, Whittemore et al., 1985; Needels et al., 1985) and the enhanced survival of embryonic transplants into wound cavities (Nieto-Sampedro et al., 1982, 1983, 1984; Manthrope et al., 1983). However, since these neurotrophic factors were found to be maximal at injury sites over a well defined acute period and these locations contained an abundance of reactive astrocytes, it was postulated that reactive astrocytes were the source of these neurotrophic factors (Nieto-Sampedro, 1983).

Analysis of injury extracts, from tissue bordering adult aspiration CNS lesion sites, have identified the presence of ciliary neurotrophic factor [CNTF] (Carnow et al., 1985) and basic fibroblast growth factor [bFGF] (Finkelston et al., 1988; Frautschy et al., 1991). In addition, insulin-like growth factor [IGF] (Komoly et al., 1992), nerve growth factor [NGF] (Lorez et al., 1989; Bakhit et al., 1991) and bFGF (Gomez-Pinilla et al., 1992) have also been identified in a number of other CNS injury paradigms. The neurotrophic properties and the neurite promoting activities of these different growth factors have all been confirmed at the *in vitro* level [e.g. bFGF (Walicke et al., 1986; Morrison et al., 1987; Unsicker et al., 1987); CNTF (Skaper and Varon, 1986; Saadat et al., 1989; Arakawa et al., 1990; Ip et al., 1991; Oppenheim et al., 1991; Larkfors et al., 1994)].

Recently, CNTF, which was capable of rescuing lesioned facial motor neurons from degeneration (Sendtner et al., 1990, 1991) was demonstrated to be present in reactive astrocytes (Ip et al., 1993). These CNTF expressing reactive astrocytes were present at lesion bordering sites in an adult corticectomy injury paradigm. Ip et al., (1993) also reported that the expression of CNTF was elevated for twenty days post-trauma and

appeared to correlate with the temporal framework for astroglial reactivity described by Berry et al., (1983). Although the identification of various neurotrophins at CNS lesion sites and the localization of CNTF to reactive astrocytes is not evidence for the role of transient astroglial reactivity, it is a strong suggestion.

In vitro astrocytic cultures are able to synthesize a number of the neurotrophic factors discovered at CNS lesion sites and their production can be further augmented by the treatment of these cultures with cytokines (reviewed in Yong, in press). A case in point is the augmentation of NGF synthesis in astrocyte cultures by IL-1, IL-4, IL-5, IL-6 and TNF-α. Observations such as these raise the possibility that the presence of cytokines at traumatic lesion sites not only modulate astroglial reactivity but also augment neurotrophic factor synthesis presumably during the phase of transient astroglial reactivity.

Regenerative Failure of the CNS

There is evidence contrary to that which supports a neurotrophic role for reactive astrocytes and which suggests that astroglial reactivity directly impairs recovery of neurological function (Aguayo et al., 1981; Reier et al., 1983). These claims are made on observations that focus on the characteristics of chronic reactive astrocytes that form glial scars. A sampling of such literature state the following: (1) glial scars mechanically hinder neurite extension (Reier et al., 1983; Reier, 1986); (2) ultrastructural analysis of glial scars demonstrate that reactive astrocytes arrest regenerating axons in their immediate vicinity, possibly by eliciting physiological stop mechanisms (Liuzzi and Lasek, 1987); and (3) the immunohistochemical detection of inhibitory molecules on either chronic reactive astrocytes (McKeon et al., 1991; Laywell et al., 1992) or their extracellular matrix (Pindzola et al., 1993), but the identification of their cellular sources remain to be achieved. In summary, these studies suggest that scar forming reactive astrocytes either mechanically or molecularly hinder regenerative processes and runs contrary to the evidence supporting neurotrophism during the early phase of astroglial reactivity.

Perhaps chronic astroglial reactivity impairs regeneration by their inability to maintain a conducive neurotrophic environment for a sufficient duration of time. The loss of these neurotrophic properties that were present during the phase of transient astroglial reactivity may be contributed to by the loss of key extracellular stimulatory signals. Interestingly the presence of the neurotrophic phase of transient astroglial reactivity also corresponds to the presence of inflammatory reactive microglia/macrophages at these lesion sites. It may therefore be possible that the cessation of cytokine synthesis by these active inflammatory cells at lesion sites may contribute to the decrease in the synthesis of neurotrophic factors by reactive astrocytes. This notion is supported by the fact that cytokines are capable of augmenting neurotrophic production by astrocytes and also possess neurotrophic and neuritogenic properties (Schwartz et al., 1994; reviewed in Yong, in press).

Neurotrophic activities of Microglia/Macrophages

The role of microglia/macrophages in contributing to the neurotrophism required at CNS lesion sites appear to be controversial. On the one hand the synthesis of cytokines by these cell types is seen as beneficial for reasons elaborated earlier in facilitating neurotrophism directly and indirectly via neurotrophin production by astroglia. In addition, these cells are also capable of synthesizing bFGF (Shimojo et al., 1991; Araujo et al., 1992) and NGF (Mallat et al., 1989). Interestingly, abortive axonal sprouting nevertheless occurs with the recruitment of these microglia/macrophages at CNS lesion sites (Geddes et al., 1986; Haga et al., 1989).

These microglia/macrophages, when harvested from lesioned CNS sites, can release yet unidentified factors that permit the conversion of non-permissive cryostat sections of adult rat optic nerve to become a permissive substrate for axonal growth (David et al., 1990). Furthermore, when microglia/macrophages during development are actively involved in phagocytosis for the cytoarhitectural organization of the maturing

CNS, their presence is not related with any pathology (Perry et al., 1985; Ashwell, 1991; Ling and Wong, 1993). Instead, they contribute to neurite extension as seen in the plexiform layers of the developing mouse retina (Hume et al., 1983).

Neurotoxicity by Microglia/Macrophages

Microglia/macrophages, with potential neurotrophic properties when present at CNS lesion sites, can also influence a number of neurotoxic mechanisms. These cells have the ability to synthesize deleterious reactive oxygen intermediates [ROI] such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) or nitric oxide [NO] (Giulian and Baker, 1986; Woodroofe et al., 1989; Colton and Gilbert, 1987; Banati et al., 1991; Zielasek et al., 1992; Corradin et al., 1993) which can be augmented by the cytokines IFN-γ and TNF-α (Hu et al., 1995). Oxygen radicals can alter protein structures, lipid membranes and deoxynuclei acids which in turn disrupt the cellular function and integrity of target cells (Halliwell and Gutteridge, 1985). Neuronal death as a result of ROI presence has been confirmed by *in vitro* co-culture studies of neurons and microglia/macrophages (Chao et al., 1992; Thery et al., 1994).

Reactive microglia/macrophages also synthesize a class of small (<500 D) stable nonproteinaceous neurotoxic molecules (Giulian, 1993). The neurotoxic activities of these neuron poisons appear to work via the N-methyl-D-aspartate (NMDA) receptor and can be blocked by NMDA receptor antagonist (Vaca and Wendt, 1992; Giulian et al., 1993a).Since the overproduction of these neurotoxic agents by microglia/macrophages can be deleterious to neuronal survival at lesion sites, it is not surprising that these microglia/macrophages are deactivated shortly after the removal of degenerating debris.

Summary

The principle cellular source of cytokines, following the aftermath of CNS lesions, appear to be reactive microglia/macrophages. These cells, when activated to provide debris clearance, also secrete a number of different neurotoxins in addition to the beneficial cytokines. Therefore, the inactivation of these reactive cells also heralds the decrease in cytokine availability. Importantly, the acute phase of astroglial reactivity, possibly involved in neurotrophic factor production as a response to cytokine presence, may actually be an attempt to facilitate neuronal survival in the realms of a hostile neurotoxic environment generated by reactive microglia/macrophages. In other words, astroglial reactivity competes with neurotoxic microglial/macrophage reactivity for control of neuronal survival (Giulian, 1993; Giulian et al., 1993b).

Therefore the goal of traumatic CNS lesion management should ideally involve the clean removal of all damaged tissue. The removal of such damaged tissue will result in a decreased debris burden and ultimately recruit fewer microglia/macrophages (an observation first noticed by Penfield and Buckely, 1928). Astroglial reactivity will still occur at both remote and lesion bordering sites. On the basis that remote occurring astroglial reactivity is transient and non-scar forming it is postulated that this type of astroglial reactivity is neurotrophic. Experiments to confirm this postulate have been suggested in chapter 7. Ideally then, the lesion bordering scar forming reactive astrocytes should be attenuated in light of their ability to contribute difficulties such as the generation of sites of electrical instability leading to seizure foci (Pollen and Tractenberg, 1970). In this respect, IL-10 application after the clean removal of damaged CNS tissue offers an excellent option to maintain transient astroglial reactivity at remote sites with the attenuation of lesion bordering chronic reactive astrocytes.

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CHAPTER 7

FUTURE PERSPECTIVES

It is striking to realize that how much of today's neurobiology research is directed toward answering questions that were first asked about a century ago. Much of what seemed mysterious then has become clear, not because our generation is smarter than the ones before us, but because we have gadgets they missed. There is, however, one area in which we seem to be almost as confused as they were: it is that of the functions of glial cells.

George G. Somjen, 1988

The remainder of this thesis will discuss leads or areas for future investigation that are likely to be rewarding.

Neurotrophism

While the physiological significance of astroglial reactivity following an insult to the CNS is unclear, there is an abundance of experimental evidence to suggest a neurotrophic role for these reactive astrocytes *in vitro* and *in vivo* (Silver and Ogawa, 1983; Smith et al., 1986; Kliot et al., 1990; David et al., 1990; Landis, 1994; McMillan et al., 1994; Rudge et al., 1994; reviewed in Yong, in press). In addition, there is much *in vitro* evidence to indicate that cytokines can influence astrocyte cultures to produce neurotrophic factors (reviewed in Yong, in press). Therefore it appears that the simultaneous presence of cytokines, reactive astrocytes and neurotrophic factors around the locus of a CNS lesion may be interrelated.

In this regard it was postulated (Chapter 3) that the transient astroglial reactivity in either isomorphic or anisomorphic injuries may be related to neurotrophic factor production. This notion is strengthened by reports that demonstrate neurotrophic factor production by reactive astroglia, occurring at earlier time points following injury (Nieto-Sampedro et al., 1982, 1983; Ip et al., 1993) and the ability of neonatal reactive astrocytes to serve as substrates for the growth of neurons *in vivo* (Silver and Ogawa, 1983; Kliot et al., 1990).

It is proposed that the neurotrophic role of the transient astroglial reactivity may be verified by utilising a modification of the neurite growth technique of David et al (1990). The modified neurite growth paradigm should involve preparing unfixed cryostat sections of cortical tissue containing reactive astrocytes as substrates for cell cultures (Carbonetto et al., 1987; David and Mittal, 1993) followed by seeding with appropriate cell types. Initially, PC12 cells may be used to verify the ability of these substrates to facilitate cell attachment and thus to test the adhesive properties of reactive astrocytes. Secondly, the ability of these substrates to facilitate neurite extension can be evaluated by placing chick dorsal root ganglia (DRG) near the substrates to test for the ingrowth of neurites into the reactive astrocyte containing substrate. These experiments should be conducted in both the neonatal and adult animal chronic (NC-implant) injury paradigms at various time points to determine whether neurotrophins correlate with the occurrence of reactive astrocytes.

Because reactive areas of the brain would be expected to contain both reactive astrocytes and microglia/macrophages (Chapter 3 and 4), it would be necessary to separate out the possible neurotrophic contribution by either astrocytes or microglia/macrophages. While microglia/macrophages are known to elaborate neurotoxins (Giulian and Baker, 1986; Colton and Gilbert, 1987; Woodroofe et al., 1989; Banati et al., 1991; Zielasek et al., 1992; Vaca and Wendt, 1992; Corradin et al., 1993; Giulian, 1993), this cell type can also be neurotrophic (Geddes et al., 1986; Haga et al., 1989; Mallat et al., 1989; Shimojo

et al., 1991; Araujo et al., 1992) under certain situations. While it will be difficult to differentiate whether reactive astrocytes and/or microglia/macrophages in a brain slice is neurotrophic for PC12 cell adhesion or DRG neurite ingrowth, light microscopic analyses of the fine interactions between the PC12/neurite and astrocyte/microglia may help to elucidate this point; cells/neurites may avoid contact with microglia/macrophages if this cell type is not beneficial.

Nitric Oxide

Cytokines capable of initiating astroglial reactivity are also able to facilitate the in vitro astrocyte production of nitric oxide (NO) (Lowenstein and Synder, 1992; reviewed in Murphy et al., 1993). NO is synthesized by NO synthase (NOS) from the amino acid L-arginine (Stuehr et al., 1991). A number of different varieties of NOS have been described (Stuehr and Griffith, 1992). A constitutive type (cNOS) which is Ca^{2+}/calmodulin-dependent has been characterized for the vasculature, platelets and the rat brain (Bredt et al., 1991; Synder and Bredt, 1991). In addition, a cytokine-inducible Ca^{2+}-independent type (iNOS) was described for murine macrophages (Marletta et al., 1988; Stuehr and Marletta, 1987) and characterized in a macrophage cell line (Xie et al., 1992; Lowenstein et al., 1992; Lyons et al., 1992). Both cNOS and iNOS are flavoproteins that are dependant on NADPH as a cofactor for reactivity with enhancement of its enzymatic activity with tetrahydobiopterin (McCall and Vallance, 1992). The physiological and pathological role of nitric oxide (NO) in the CNS has recently been reviewed by Bruhwyler and colleagues (1993).

Presently, the distribution of nicotinamide adenine dinucloetide phosphate diaphorase (NADPH-d) enzymatic activity, after appropriate aldehyde fixation of tissue, is taken as an immunohistochemical indicator of NOS activity (Vincent and Kimura, 1992). In this regard, Sestan and Kostovic (1994) point out the rediscovery of NADPH-diaphorase activity in the brain as early histochemists had previously described the weak

staining of NADPH-diaphorase activity in astrocytes of the human, rat and rabbit CNS (Osterberg and Wattenberg, 1962) with more intense staining in reactive astrocytes (Osterberg and Wattenberg, 1962; Rubinstein et al., 1962; Smith, 1963; O'Connor and Laws, 1963).

In light of the emerging evidence implicating that reactive astrocytes can produce neurotrophic factors, it is worth investigating if astrocytic iNOS is beneficial in facilitating neurotrophism. This notion is based on the present scepticism in the literature with regards to the ascribed neurotoxic role for NO (Boje and Arora, 1992; Chao et al., 1992; Dawson et al., 1991). Yamamoto et al., (1992) have demonstrated that the inhibition of NOS increased the size of cerebral ischemia. This area of increased damage was localized to the area surrounding the infarction zone (Nedergaard et al., 1986) and could be reversed by the co-administration of L-arginine (Rees et al., 1989). It was concluded by Yamamoto et al., (1992) that the inhibition of NOS might therefore interfere with *in vivo* NO-mediated neuroprotective cellular events that include local vasodilation, platelet aggregation, and synaptic transmission. Moreover, Wu (1993) has demonstrated that NOS can be induced in the adult CNS after traumatic injury to the spinal cord and the initial 2 week phase of NOS expression was coincident with an increase in the number and extension of neurites by lesioned motor neurons. Furthermore, there is evidence for NO involvement in neuronal function such as long-term potentiation (Bohme et al., 1991), long-term depression (Ito, 1989; Shibuki and Okada, 1991) and during the later phase of neuronal development involving the formation, segregation, and shaping of the neuropil (Gally et al., 1990).

To assess whether astroglial NO is beneficial to neurotrophism, experiments using the coculture of astrocytes and neurons should be designed. These co-culture studies can provide efficient avenues to assess neuronal survival following manipulation of NO content *in vitro*. Furthermore, it is possible to address the role of *in vivo* astrocyte produced NO in trauma studies by administering NOS inhibitors intraperitoneally prior

to and following CNS trauma. Two possible proven NOS inhibitors in the cerebral ischemia literature are L-N^G-nitroarginine (Nowicki et al., 1991) and N^G-nitro-L-arginine methyl ester (Buisson et al., 1992). The potential neurotrophic potential of NOS inhibitor treatment can then be analysed via immunohistochemical markers for astroglial reactivity and in situ hybridization analysis for the potentiation of neurotrophin mRNA levels.

Finally, the inhibition of NOS *in vivo* prior to the creation of brain injury can provide us with answers to whether the NO produced by astrocytes (as stated earlier, cytokines can facilitate NO production by astrocytes *in vitro*) may indeed be a final mediator of astrocytic reactivity following injury.

Osteopetrotic (op/op) Mutant Mice and Astroglial Reactivity

Astroglial reactivity in this thesis is dependant on the presence of microglia/macrophages with their ability to provide large sources of inflammatory cytokines. To verify the necessity for the presence of microglia/macrophages, it would be ideal to utilize an animal model that is deficient in these two populations. Osteopetrotic (*op/op*) mice, in addition to osteopetrosis, are characterized by a severe generalised deficiency of osteoclast (Wiktor-Jedrzejczak et al., 1981; Marks et al., 1976) and macrophages (Wiktor-Jedrzejczak, et al., 1982). This deficiency is due to a total absence of a macrophage growth factor, colony-stimulating factor (CSF)-1 (Wiktor-Jedrzejczak, et al., 1990; Felix et al., 1990), resulting from a point mutation in the CSF-1 gene (Yoshida et al., 1990). This congenital defect can be corrected by the exogenous administration of recombinant CSF-1 (Felix et al., 1990; Wiktor-Jedrzejczak, et al., 1991; Kodama et al., 1991).

This *op/op* mutant animal possesses between 3% and 30% of the total number of macrophages observed in normal littermates, depending on the tissue being examined (Naito et al., 1991; Wiktor-Jedrzejczak et al., 1992a). These small observable resident

macrophage populations are presumably produced under the influence of factors other than CSF-1, such as granulocyte-macrophage (GM)-CSF and interleukin 3 (Wiktor-Jedrzejczak et al., 1992b). This dramatic decrease in the macrophage population, by 70% to 97% in the *op/op* mice, appears to have no appreciable *in vivo* effects on either phagocytosis or humoral and cellular responses following exposure to a T-cell dependent antigen (e.g. sheep red blood cells) and therefore in this respect are similar to controls.

In response to endotoxin stimulation normal animals usually respond with detectable levels of TNF-α (Old, 1985; Meager et al., 1989) and G-CSF (Whetton and Dexter, 1989). *Op/op* mice, on the other hand, at 2 to 4 months of age when challenged with endotoxin display a profound 10 fold decrease in releasing TNF-α with delayed kinetics when compared to normal animals (Wiktor-Jedrzejczak et al., 1992a). However, *op/op* mice do not remain in their macrophage deficiency state and can recover with age. Begg and colleagues (1993) demonstrated that by 5 mo of age the marrow cellularity was comparable to that of normals followed by an increase in the percentage of macrophages to normal levels by 9 mo. Their macrophage observations were made on the basis of F4/80, a specific murine macrophage marker, for which function is presently unknown (Austyn and Gordon, 1981). It is therefore evident that during early postnatal development, but not at later life, the *op/op* mice has a deficient number of TNF-α secreting peripheral macrophages.

Microglia, the brain macrophages, have been postulated to be derived from mesodermal monocyte/macrophage progenitors that are CSF-1-dependent. Thus, it would be fair to assume that microglia of *op/op* mice may exhibit some microglial defects. Examination of microglia populations in *op/op* mice brains from 4 to 5 weeks of age, however, revealed no discernible difference between that of littermate controls (Chang et al., 1994). One possible explanation for this apparent discrepancy would involve the scenario where non-hematopoietic cells resident to the CNS are capable of furnishing necessary levels of M-CSF for microglia maturation.

In this regard, Thery et al., (1990) using Northern blot analysis of mouse cerebral RNA were able to show the presence of CSF-1 transcripts from as early as embryonic day (ED) 14 to postnatal day (PD) 14. Chang et al., (1994), via S1 nuclease analysis, also confirmed the presence of both CSF-1 and C-fms, the cellular receptor for CSF-1, from ED to PN15. Troutt and Lee (1989) confirmed the presence of CSF-1 transcripts in adult brain. The presence of CSF-1 mRNA of astroglial origin was detected by using polymerase chain reaction (PCR) analysis on astrocyte cultures (Wesselingh et al., 1990). Liu and colleagues (1994) also demonstrated that the production of CSF-1 by human fetal astrocytes can support the maturation of microglia. In this regard, the op/op mice can initiate astroglial reactivity to anisomorphic chronic (NC-implant) injuries as early as P21 (Balasingam and Yong, unpublished observations) probably on the basis of a mature microglia population despite the deficient systemic TNF-α producing macrophage status.

The brains of these *op/op* mice are similar in hemispheric shape and contour to normals with the exception of decreased brain weight. Light microscopic analysis of the CNS cytoarchitecture revealed normal well-demarcated grey-white matter interfaces, basal ganglia structures, and cortical layers. Furthermore, uninjured *op/op* mice did not reveal an increase for GFAP-IR that would be indicative of an astroglial response to pathology (Chang et al., 1994; Balasingam and Yong, unpublished observations).

The experiment that warrants conduct involves one where cerebral injury (NC-implant) is inflicted upon young *op/op* neonates at PD3. This experimental situation will provide: 1) the lack of recruitable, mature systemic cytokine producing macrophages and 2) the presence of a maturing microglia cell population. Controls should be normal animals given the same NC-implant injury where the recruitment of macrophages is expected. Under such circumstances the role of cytokines in modulating astroglial reactivity can be verified in a physiological system. This experiment will warrant the needs of analyzing tail derived DNA immediately after the birth of mice in order to identify *op/op* mutants from their normal littermate controls. Otherwise, mutants can only

be recognized at three weeks of age, from their normal littermates, by their toothless phenotype and dome-shaped skull.

Following injury, these injured op/op mice should be verified for the presence of GFAP IR, protein content and mRNA levels in comparison to controls. In the event that there is a lack of GFAP-IR, the neuropathological hallmark of astroglial reactivity, further experiments should be performed to: 1) analyze the activation status of the microglia/macrophage population at the lesion site, 2) measure the content of inflammatory cytokines such as IL-1 and TNF via a cytotoxicity bio-assay, and 3) determine the increase in transcription of various cytokine mRNA levels. The contrary result of an increase in GFAP-IR will also prove to be useful in verifying the choice of the *op/op* mice as an inadequate model for GFAP-IR with respect to its microglia deficient state.

Proliferation of Astrocytes

Injury to the adult system appears not to involve a proliferative phase in the evolution of astroglial reactivity as shown by a number of quantitative studies using autoradiography, bromodeoxyuridine and proliferating cell nuclear antigen (Miyake et al., 1988, 1989, 1992); however, some studies have shown that proliferation of astrocytes in adult animals to be significant (Topp et al., 1989; Janeczko, 1989). Furthermore, injury to the neonatal system acts as a stimulus for proliferation of astrocytes as revealed by ultrastructural studies in Chapter 3. While astrocytes *in vivo* in this thesis were characterized using GFAP and ultrastructural criteria, it may be informative to assess the extent of proliferation in each of the models described (including injuries where cytokines such as IFN-γ or IL-10 were administered). From the ultrastructural results of Chapter 3, it would appear that significant proliferation of astrocytes would be detected in the neonatal but not the adult animals. Proliferation of astrocytes *in vivo* may be achieved by combining GFAP-IR with ^{3}H-thymidine autoradiography, or with immunohistochemistry

for cell proliferation markers (BRDU, PCNA).

Cytokine Content Measurement

The content of various cytokines at traumatic lesion sites is an important area for investigation. The ability to identify the different cytokines present at traumatic lesion site will aid us in determining the role of these cytokines in contributing towards the various cellular aspects that follow trauma to the CNS. Any effort to identify the various cytokine players at the lesion site will have to be done with the understanding that these immunoregulatory peptide are inducible proteins that are probably transiently stored. The difficulty in identifying these cytokines stems generally from their interactive nature with other cytokines, their characteristic overlapping biological activities and the fact that they are probably not released in isolation but in conjunction with other factors such as a heterogenous mixture of enzymes, proteins, hormones and other cytokines.

Cytokine detection at trauma sites can be initiated with the use of cytotoxic bio-assays (Taupin et al., 1993). Although this is a useful method of analyzing cytokine production it may not be sensitive to low levels of cytokine secretion. Furthermore, these methods assume that the bulk-release of cytokines in any one given condition is initiated by a single cellular phenotype with similar or identical cytokine secreting performance. However, despite these criticisms, this method can be useful as pilot studies to identify those cytokines that appear to be major players in a given scenario *in vivo*. Alternatively, a wide variety of immunostaining techniques have been developed at both the light and electron microscopic level to detect the presence of cytokine production in cells (reviewed by Lewis, 1991). Although these techniques appear to provide means of identifying intracellular presence of cytokines, extrapolating that the self-same cell produced these cytokines can be dubious. This is based on the fact that, despite the transient storage of cytokines after translation, immunohistochemically detectable cytokine may represent an internalized product that originated from another cell. Such criticism warrants the

necessity to not only detect the final cytokine product but also the ability to identify the presence of increased transcription for their corresponding mRNA. This approach should initially be attempted with the use of sensitive detection methods like RT-PCR on total RNA obtained from brain tissue (e.g. in Chapter 4).

On confirmation of the presence of cytokine mRNA, it would be ideal to locate the cytokine mRNA in question via *in situ* hybridization technique in an effort to localize the cell populations involved. *In situ* hybridization is useful as it theoretically allows: 1) for the simultaneous identification of more than one cytokine per cell; and 2) the use of conventional immunohistochemical methods to identify the phenotype of the producer cell (Ogilvie et al., 1990).

Whatever the technique used to detect cytokine levels, it would be interesting to determine whether the increased levels of a particular cytokine can be temporarily and spatially correlated with the occurrence of astroglial reactivity in both the neonate and adult animals.

Identification of Inflammatory Cell Types

The presence of activated microglia/macrophages and their ability to produce various macrophage-derived cytokines (reviewed in Chapter 4) may conceivably recruit other inflammatory cell types to the lesion site. The recruitment of these other inflammatory cells may also contribute to the existent cytokine immune cascade and probably occurs a few days after injury. One avenue of detection used in immunological circles for the identification of inflammatory cells is the use of a fluorescent activated cell sorter (FACS). In the case of CNS trauma inflicted by the different injury paradigms in this thesis, the area of tissue involvement is very focal. Therefore to benefit from the use of the FACS, pooled resected tissue from the focal injury site should be used. This technique could also be utilized to determine the status of the microglia/macrophage

population at these injury sites by the use of flourecsent tagged activation markers for molecules such as MHC class I and II antigens and others.

Finally, the presence of inflammatory cell types, and whether their presence correlates spatially and temporally with the evolution of astrogliosis, can also be determined using RT-PCR for cell type specific markers.

Transgenic and Knock-out Mice[1]

The pathophysiological roles of cytokines in astroglial reactivity can be further addressed if there were means to increase or decrease the content of cytokine levels in the brain. Unfortunately, the exogenous administration of recombinant cytokines or neutralizing antibodies to respectively increase or decrease cytokine levels in the brain cannot be done without injury. The solution then lies in the technology that allows for the endogenous manipulation of cytokine levels. This involves the generation of genetically modified 'designer' animals via specific germ line mutations which involve: (1) Transgenic animals with ectopic overexpression of cytokine genes in neural specific structures; and (2) Gene knock-out animals which possess targeted gene deletions for specific cytokines.

These models can potentially provide a valuable means to advancing our understanding of the CNS pathobiology of cytokines for several reasons: (1) The transgenic approach is a reliable method for overproducing a single cytokine *in vivo*; (2) Transgenic mice provide an optimal method for analyzing the consequences resulting from the overexpression of a single cytokine; and (3) It is possible to restrict cytokine

1. For an account of techniques used in generating specific germ line mutations *in vivo*, the reader is referred to Aguzzi et al. (1994), and Galli-Taliadoros et al. (1995). To appreciate this technology the reader is directed to the book titled 'Overexpression and knock-out of cytokines in transgenic mice', edited by Chaim O Jacob.

production to select CNS cell types by utilizing tissue-specific regulatory elements to drive transgene expression.

Transgenic methodology has its own limitations. Normally, cytokines are produced transiently and rapidly in response to an external stimuli. However, this is not the case with transgenic models, where the cytokine transgene is constitutively expressesd. In addition, observed alterations in the physiology of the transgenic animal in the experimental design may not always be directly attributable to the transgene-derived cytokine, since, cytokines by nature are pleiotrophic in action and can also initiate cascades of other cytokines.

In the future we will be able to solve some of these problems by crossing transgenic mice with gene knock-out mice to help further elucidate the role of cytokines in astroglial reactivity

Differential Display

Astroglial reactivity is a pathophysiological process that involves a chronological stage specific transformation of a quiescent cell into a reactive form. These reactive astrocytes could either undergo transient astroglial reactivity if located distant to the lesion site or participate in the formation of a glial scar scaffold if present immediately around the lesion site. Presently the function of these two end points of astroglial reactivity is unknown. However, the choice of outcome is probably driven by a complex interaction of a multitude of factors. Ultimately, these different reactive states are driven by changes in gene expression of the individual cell. It is therefore worthwhile to commence a comparative study of gene expression between both the normal and the two types of transient and permanent reactive astrocytes. Efforts directed in this direction will greatly enhance the understanding of the various biological processes, and particularly that of cytokines, which govern the reactive state. In this regard, subtractive hybridization,

differential display and single cell amplification may be viable techniques in highlighting the differential gene expression in the normal and reactive states.

Subtractive Hybridization

Presently, comparative studies in gene expression utilize mRNA subtraction hybridization techniques (Lee et al., 1991) and the protocols are well described by Travis et al. (1990). This approach can be very time consuming and involves developing large cDNA libraries for both the normal and reactive astroglia. Moreover, in practice this technique requires a second subtraction hybridization step at lower densities to differentiate colonies with low signal levels form background. Furthermore, subtractive hybridization is a kinetic reaction in which not all the cDNA that is common to both the normal and reactive states will be subtracted out. This scenario is reflected in the isolation of mRNA common to both the normal and reactive states, such as GFAP mRNA, which is not subtracted because of the enriched levels in the reactive state. Another example of a similar situation would be the detection of multiple isolates of abundant mitochondrial mRNAs (Watson et al., 1992; Adams et al., 1991) for example and not necessarily novel expression of genes during reactivity.

mRNA Differential Display

Another alternative would be to use the recently developed reverse transcription (RT)-PCR method of mRNA differential display that involves the detection, separation and cloning of individual mRNA that are differentially expressed (Liang and Pardee, 1992; Liang et al., 1992). This is an emerging technique that has the potential to supersede subtractive hybridization as the method of choice for differential screening. This method involves the use of minimal mRNA from which cDNAs are generated with the use of short arbitrary random primers via RT-PCR. This is followed by the rapid detection of differential mRNA content as cDNA bands on DNA denaturing gels.

The major advantages of the differential display technique are: 1) the small amount of mRNA required for the method; 2) the ability to simultaneously display the enriched or under-expressed mRNAs in normal versus reactive astrocytes, unlike subtraction hybridization where only uniquely expressed mRNA in one type of state is visualized; 3) the capability to detect mRNA coded by novel gene structures that may otherwise be lost as double-stranded molecules during subtractive hybridization; 4) the possibility of cloning individual bands revealed by this technique for use as probes in Northern (RNA) and Southern (DNA) blottings; and 5) the ability to perform rapid analysis in comparison to subtractive hybridization. The disadvantages for this technique lies in the assumption that all cDNAs present will be amplified by PCR, however, this is not true. The ability of the PCR to amplify cDNA is highly dependent on the availability of binding sites for the random primers chosen, otherwise amplification of specific cDNA and its detection as a discernible band may not occur. In this respect, it is beneficial to conduct pilot differential display experiments with single arbitrary 10-mer primers before the addition of others as needed to increase the amplification of mRNA. Time reductions in conducting these experiments will be achieved if cDNA bands can be rapidly sequenced and identified with comparison to known sequences contained in GenBank and EMBL DNA databases. The one major concern with these PCR-based technique is the issue of linearity with reference to amplification, and awaits clarification with future experience.

Single Cell Differential Screening

Differential screening can now also be performed at the single cell level. This achievement represents the combined efforts of both the molecular biologist and the electrophysiologist in generating PCR-based (Lambolez et al., 1992) and cRNA-based (Van Gelder et al., 1992; Eberwine et al., 1992; Mackler et al., 1992) probes. This experimental paradigm essentially involves performing electrical recordings to identify the cell type followed by the aspiration of cytoplasmic contents with patch-clamp microelectrodes for ***in situ*** cDNA synthesis and amplification. Lambolez et al., (1992)

effectively amplified cDNA for GFAP mRNA from single glial cells in organotypic cerebellar cultures via PCR. Amplification of mRNA can also be performed by the generation of cRNA by *in vitro* transcription with bacteriophage RNA polymerases. These radiolabelled cRNA probes can then be used to screen either cDNA libraries randomly (Eberwine et al., 1992) or Southern blots containing cDNA fragments of interest for the purposes of expression profiling experiments (Eberwine et al., 1992; Mackler et al., 1992).

The major advantage of using the cRNA-based system lies in the minimization of the issue concerning the linearity of amplification encountered in PCR-based experiments. In general, single cell amplification analysis can also be combined with the differential display technique. For PCR-based experiments, labelled double stranded cDNA can be generated from the same primers utilized in differential display. In the case of cRNA-based experiments, antisense cRNA can be generated by *in vitro* transcription followed by cloning to obtain their cDNA equivalents. Finally the cDNAs from either the PCR-based or cRNA-based experiments are electrophoressed on a DNA sequencing gel for comparisons. However, the major obstacle for single cell amplification lies in the availability of cell RNA, as a cell contains 1 pg or less of mRNA.

Experimental Design

Experiments involving differential display warrant the ability of obtaining purified mRNA from specific cell types: normal and reactive astrocytes. The use of neonatal cultured astrocytes may not provide a representative picture of gene expression in the study of astroglial reactivity, although they have been used in the past. Firstly, the process of retrieving CNS tissue for culture studies could be viewed as a traumatic process that might conceivably activate the gene machinery. Secondly, despite the availability of the *in vitro* scratch model of astrogliosis designed by Yu and colleagues (1993), it is difficult to conceive of this *in vitro* model as being adequate for the reason stated above together with the absence of other cellular players that are important *in vivo*. Thirdly, the nature

of astrocytes responding to CNS insults in the adult may not be similar to that of the neonate. Despite these criticisms, it may prove valuable to initially utilize these *in vitro* models to obtain preliminary differences besides gaining valuable experience in conducting these experiments.

The culmination of these technologically demanding experiments lies in the ability to extract cell RNA from both normal and reactive astrocytes *in vivo* for physiological relevance. This may be possible by utilizing tools of electrophysiology to detect astroglia in slices immediately *ex vivo* followed by the extraction of cytoplasmic contents as described earlier. In this regard, reactive astrocytes may be easily identified by electrophysiological tools on the NC-implant membrane immediately *ex vivo* particularly in the neonatal animals. Experience from such experiments may eventually lead to the ability to identify reactive astrocytes in slices immediately *ex vivo*. The identification of differentially regulated mRNA can then be identified by sequencing the generated cDNA; probes may also be generated for use in Northern, Southern and *in situ* hybridization experiments. Evidence from such experiments can help facilitate the identification of temporal sequences involved in astroglial reactivity beginning from the activation of early immediate genes. Advances in this direction will later also facilitate the identification of differences between transient astroglial reactivity and astroglial reactivity leading to scar formation in the adult and the role of cytokines in this process.

Final Comments

The results of this thesis strongly implicate inflammatory cytokines elaborated by microglia/macrophages as being causative for the evolution of astroglial reactivity. Future experiments have been suggested to test the possible neurotrophic role of reactive astrocytes.

This ability to determine the beneficial role of astroglial reactivity and the mechanisms that lead to scar forming astroglia reactivity will ultimately determine the nature of future clinical therapy. If the presence of a prolonged activated immune cascade is identified to be responsible for the creation of a glial scar at sites immediately bordering lesion sites, then designing anti-inflammatory treatment may be conceived to reduce the inflammatory process in the CNS. The prospect of devising such a treatment to inhibit the immune cascade involving cytokine synthesis is particularly attractive because it is likely to require a short period of treatment which should not lead to long-term side-effects.

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