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IN VITRO CHARACTERIZATION OF A CELL SURFACE MOLECULE EXPRESSED BY CERTAIN CELLS OF NEUROECTODERMAL AND MESENCHYMAL ORIGIN

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February, 1994

A Thesis submitted to
The Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

ISBN 0-315-94686-5

To my parents

ABSTRACT

Adhesive interactions between neurons and astroglia are likely to play an important role during central nervous system development. Using a monoclonal antibody (designated MAb 1A1) raised against purified neonatal rat astrocytes, I have characterized some of the in vitro functional and biochemical properties of a potentially novel cell surface molecule. By indirect immunofluorescence, the antibody labels subpopulations of astrocytes (flat type-1 astrocytes and Bergmann glia) and cells derived from the mesenchyme (leptomeninges and fibroblasts). The latter showed $1A1^+$ immunoreactivity only when grown to confluency. Cell-cell contact and extracellular matrix molecules were found to play a role in the regulation of 1A1 antigen expression on leptomeninges. 1A1 Fab fragments inhibited the binding of neuron to astrocyte and astrocyte to astrocyte, but not of neuron to neuron in an in vitro adhesion assay, thus indicating that this surface antigen may function as a cell adhesion molecule on astrocytes. Addition of Fab fragments of MAb 1A1 also reduced leptomeningeal cell adhesion. Further functional antibody perturbation experiments indicated that this surface molecule mediates neurite outgrowth on astrocytes and neuronal migration on Bergmann glia via a heterophilic-binding mechanism. Both immunoprecipitation and immunocytochemical analysis showed the timing of 1A1 antigen expression in postnatal rat cerebellum to coincide with the developmental period of granule cell migration along Bergmann glia. On SDS-PAGE, the immunopurified 1A1 surface molecule migrated as a single molecular weight band of $\approx$ 135 kd and appeared to be poorly glycosylated. Based on its unique cell-type distribution, functional properties and biochemical analysis, this 135 kd glycoprotein is likely to be distinct from other known cell adhesion molecules expressed on astrocytes.

RÉSUMÉ

Les interactions adhésives entre les neurones et les astroglies jouent probablement un rôle important pendant le développement du système nerveux central. Avec un anticorps monoclonal (désigné MAb 1A1) dirigé contre les astrocytes purifiés de rat néonatal, j'ai caractérisé in vitro quelques unes des propriétés fonctionnelles et biochimiques d'une nouvelle molécule de surface. Par immunofluorescence indirecte, l'anticorps marque des sous-populations d'astrocytes (plats type-1 astrocyte et glie de Bergmann) et les cellules dérivées du mésenchyme (leptoméninges et fibroblastes). Ces dernières ont montré une réaction positive seulement quand elles atteignaient confluence. Il a été démontré que les molécules impliquées dans les contacts entre cellules et celles de la matrice extracellulaire modulent l'expression de l'antigène 1A1 sur les leptoméninges. Les fragments Fab 1A1 ont inhibé l'attachement du neurone à l'astrocyte et de l'astrocyte à l'astrocyte, mais pas les attachements de neurone à neurone dans un test d'adhésion in vitro, ce qui indique donc que cet antigène de surface peut fonctionner comme une molécule d'adhésion cellulaire sur les astrocytes. L'addition de fragments Fab de MAb 1A1 a aussi réduit l'adhésion des cellules des leptoméninges. D'autres expériences fonctionnelles d'inhibition utilisant l'anticorps 1A1 indiquent que cette molécule de surface permet la croissance de neurites sur les astrocytes et la migration neuronale sur la glie de Bergmann par un mécanisme de liaison hétérophile. Les analyses d'immunoprécipitation et d'immunocytochimie ont démontré que l'expression de l'antigène 1A1 dans le cervelet du rat postnatal coïncide avec la période de développement des cellules granulaires migrant le long de la glie de Bergmann. Sur SDS-PAGE, la molécule de surface 1A1 immunopurifiée a migré en une bande simple d'un poids moléculaire de $\approx$ 135 kd et est apparue faiblement glycosylée. Compte tenu de sa distribution cellulaire unique, ses propriétés fonctionnelles et l'analyse biochimique, cette glycoprotéine de 135 kd est probablement distincte des autres molécules d'adhésion cellulaires connues qui sont exprimées sur les astrocytes.

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ACKNOWLEDGEMENTS

I wish to express my profound appreciation to all those who have helped me throughout this work in innumerable ways.

I thank my research supervisor, Dr. Samuel David, for his continuous support and interest. I truly appreciate his help and advice offered over the years. I express my gratitude to Dr. Paul C. Holland for his kind assistance during this project. He has been a valuable advisor and a member of my thesis committee along with Drs. Robert J. Dunn and Jean-Pierre Julien, both of whom I wish to thank.

This study would not have been possible without the technical assistance of Mrs. Ourania Tsatas who helped with some of the culture work. Many antibody reagents and cell lines used in this study were provided by other investigators mentioned within the text, whom I also thank.

I acknowledge the statistical advice of Drs. Rhonda Amsel and Lawrence Joseph in Chapter 3. I also acknowledge the funding received from the Rick Hansen Man in Motion Legacy Fund and the departmental FCAR training award.

I am indebted to my friends and especially my family for their unrelenting love, support and encouragement.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
Ca^{2+}	Calcium ion
CAM(s)	Cell adhesion molecule(s)
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid(s)
CNP	2',3'-cyclic nucleotide-3'-phosphodiesterase
CNS	Central nervous system
°C	Degrees Celcius
dBcAMP	Dibutyryl cyclic adenosine monophosphate
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate
DIV	Days in vitro
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Embryonic day
EC1	Extracellular domain 1
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGL	External granular layer
EGTA	[Ethylenebis (oxyethylenenitrilo)] tetraacetic acid
EHS	Engelbreth-Holm-Swarm
Fab	Antigen binding fragment
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FN	Fibronectin
g	Gram
GFAP	Glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
^{125}I	Iodine-125
Ig	Immunoglobulin
IGL	Internal granular layer
IL-1	Interleukin-1
kd	Kilodalton
MAb	Monoclonal antibody
MAbs	Monoclonal antibodies
MEM	Minimal Eagle's medium
MEM-H	Minimal Eagle's medium with HEPES
mg	Milligram
Mg^{2+}	Magnesium ion
MHC	Major histocompatibility complex
ML	Molecular layer

mm	Millimeter
mM	Millimolar
mRNA	Messenger ribonucleic acid
MSD1	Muscle specific domain
MW	Molecular weight
NF	Neurofilament
NGF	Nerve growth factor
NGS	Normal goat serum
6-OHDA	6-hydroxydopamine
p	Probability
pH	Hydrogen ion potential
P	Postnatal day
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PLL	Poly-L-lysine
PNS	Peripheral nervous system
PSA	Polysialic acid
^{35}S	Sulfur-35
SAM(s)	Substrate adhesion molecule(s)
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TBS	Tris-buffered saline
TGF-β	Transforming growth factor-β
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
TSP	Thrombospondin
μCi	Microcurie
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar
VASE	Variable alternatively spliced exon

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Chapters 2 and 3 have been published in Molecular and Cellular Neurosciences. Chapter 4 has been submitted to The Journal of Neuroscience Research (see page xiii). All aspects of the work presented in this thesis were done by me.

OVERVIEW

Astrocytes are thought to play important roles in influencing axon growth during development and after injury to the adult CNS. The objective of my research was to identify and characterize a novel astrocyte cell surface molecule involved in neuron-astrocyte interactions. For this purpose, monoclonal antibodies were generated using cultured rat astrocytes as the immunogen. Several positive hybridomas were obtained. One of these (monoclonal antibody 1A1) appears to recognize a novel astrocyte cell surface molecule. In this thesis, I report the functional and biochemical characterization of a distinct astrocyte surface molecule (1A1 antigen). It has been shown previously that N-CAM, N-cadherin, and integrins of the β_1 subclass mediate neurite outgrowth on monolayers of astrocytes (Reichardt et al., 1989). The methods used to characterize and study the 1A1 antigen were similar to those used for other adhesion molecules, such as N-CAM. I address, in this thesis, the following questions: i) what is the cellular distribution of the 1A1 antigen in both neural and non-neural tissues? ii) the species specificity of the 1A1 antigen, iii) the role of the 1A1 antigen in neurite outgrowth and neuronal migration in the developing CNS, iv) the regulation of expression and adhesive functions of the 1A1 antigen in non-neural (leptomeninges) cells in vitro, and v) whether the 1A1 antigen is a novel molecule based on the in vitro and biochemical characterization?

As background for this work, I will first review studies dealing with astrocytes, and developmental events that are associated with these cells. Emphasis will be placed on adhesion molecules involved in mediating neurite outgrowth and neuronal migration on astrocytes. In addition, since the 1A1 antigen is also expressed by leptomeningeal cells, studies on the origin and role of leptomeninges will also be reviewed.

CLAIMS FOR ORIGINALITY

The research described in this thesis has focussed on the in vitro characterization of a potentially novel cell surface molecule, designated as 1A1. The following points represent original data presented in this thesis using the MAb 1A1.

1. The 1A1 antigen is a cell surface molecule expressed only by astrocytes (flat type-1 astrocytes and Bergmann glia) in the CNS and by certain mesenchymally-derived cells (leptomeninges and fibroblasts).

2. Its expression differs in neuroectodermal and mesenchymally-derived cells.

3. The epitope recognized by the MAb 1A1 is species-specific and is expressed in rat but not mouse, chick or human tissues.

4. The 1A1 antigen is a cell surface glycoprotein with a molecular weight of about 135 kd on SDS-PAGE.

5. The 1A1 antigen has no interchain disulfide bonds and lacks the tripeptide HAV cell recognition sequence found in cadherins.

6. The 1A1 antigen has adhesive functions mediating neuron-astrocyte, astrocyte-astrocyte, and leptomeningeal cell adhesion.

7. The 1A1 antigen is involved in neurite outgrowth on astrocytes, and glial-guided neuronal migration along elongated (Bergmann) glia in vitro.

8. Cell-cell and cell-matrix interactions play a role in the regulation of 1A1 antigen expression in leptomeninges.

PUBLICATIONS RESULTING FROM MY WORK WHILE IN THE Ph.D. PROGRAM AT McGILL UNIVERSITY

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Other publications not directly related to my thesis work

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Chapter 1: *Literature review*

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In this chapter, I will cover some of the general features of astrocytes, such as the identification and origin, because my thesis work deals with the characterization of an astrocyte surface molecule. An overview of the structure and functions of the major families of cell adhesion molecules (CAMs) as well as the extracellular matrix (ECM) glycoproteins and their receptors, that are associated with astrocytes will also be presented. The main focus of the latter section will be to discuss the adhesion molecules found on astrocytes that might play a role in neurite outgrowth and neuronal migration. This will be followed by a brief review of leptomeningeal cells, because the fourth chapter of my thesis deals with the characterization of the 1A1 antigen on leptomeningeal cells.

1.1 Historical basis of astrocytes

In 1846 Rudolph Virchow, while studying sections of human brain tissue, observed an interstitial component morphologically distinct from neurons, which he named "neuroglia" or nerve glue (Virchow, 1846, 1856). The term neuroglia was used by Virchow to identify this component because he believed that it served as a support element for neurons. Named for their "star-shaped" morphology, astrocytes were subsequently identified in tissue sections by metallic-impregnation staining techniques that were introduced by Camillo Golgi and further developed by Santiago Ramón y Cajal in the early twentieth century (reviewed by Tower, 1988). The gold chloride sublimate procedure (Ramón y Cajal, 1913) clearly showed Virchow's support elements in the central nervous system (CNS) as a distinct class of non-neuronal cells which are now classified as macroglia (Peters et al., 1991). Besides astrocytes, oligodendrocytes constitute the other macroglial cell-type in the CNS. Oligodendrocytes and a third type of glia, the microglia, were first defined by Pío del Río-Hortega (1919). These glial

constituents form about half the volume of the brain, and out-number neurons by 10-fold in the mammalian CNS (Pope, 1978). Of these cells, astrocytes constitute the principal macroglial cell in the CNS (Pope, 1978). Astrocytes surround neurons and their processes, and maintain close contact with blood vessels via cytoplasmic processes called endfeet (Peters et al., 1991). Astrocytes also respond to injury by forming dense glial scars (Reier, 1986; Malhotra et al., 1990). Thus, astrocytes play a dynamic function in the brain, and are now known to have several important roles in the mammalian CNS, much more than a supportive role as originally thought (reviewed by de Vellis, 1993). The development of methods for identifying and culturing astrocytes from defined brain regions has provided the possibility of studying the functions of astrocytes in the normal and injured CNS. Furthermore, in view of the growing evidence that the astrocyte population is morphologically, biochemically, and physiologically heterogeneous (Raff et al., 1983a; Chneiweiss et al., 1985; Papasozomenos and Binder, 1986; Patel, 1986; Rosenberg and Dichter, 1987; Barbin et al., 1988; Barres et al., 1988; Hansson, 1988; Batter and Kessler, 1991; McCarthy and Salm, 1991; Batter et al., 1992; Qian et al., 1992), it is likely that region-specific astrocytes subserve functional specialization within the CNS (Wilkin et al., 1990).

1.2 Identification and function of astrocytes

1.2.1 Glial fibrillary acidic protein in astrocytes. Antibodies against specific markers have been utilized to identify neural cells in both mature and developing systems in vivo and in vitro (Raff et al., 1979; Mirsky, 1980). Astrocytes can be identified by their cytoplasmic expression of a specific type of intermediate filament protein, glial fibrillary acidic protein (GFAP) (Bignami et al., 1972). In the CNS, this

protein is characteristic of mature and differentiated astrocytes (Bignami et al., 1980). The mesenchymal-type of intermediate filament protein, vimentin, is normally found in immature astrocytes and in some reactive astrocytes following injury (Pixley and de Vellis, 1984). The transition from vimentin to GFAP usually occurs during astroglial differentiation (Bignami and Dahl, 1985; Schnitzer, 1988; Voigt, 1989) at the time when axon ingrowth has ceased (Bovolenta et al., 1984). In the rat cerebellum, vimentin to GFAP transition was shown to correspond to the onset of myelination (Dahl, 1981). On the other hand, both vimentin and GFAP may also be localized in the same filaments as shown in human glioma cell lines (Sharp et al., 1982; Wang et al., 1984), and may coexist in mature astrocytes in vivo (Schnitzer et al., 1981). Vimentin and GFAP have been reported to coexist in varying proportions in cultured astrocytes (Fedoroff et al., 1983; Chiu and Goldman, 1984; Eng et al., 1986), and in Bergmann glial fibers of the adult mouse cerebellum (Bovolenta et al., 1984). Vimentin may also be present simultaneously with GFAP in other specialized cells of the adult CNS, such as ependymal cells lining the ventricles and Müller cells of the retina (Shaw et al., 1981; Björklund and Dahl, 1985).

Although GFAP immunoreactivity is the most reliable way of identifying cells of astroglial lineage in CNS tissue, GFAP-like immunoreactivity has been found in certain non-CNS cells, such as non-myelinating Schwann cells, lens epithelium, and liver Kupffer cells (Eng et al., 1985; Yen and Fields, 1985). However, biochemical differences were reported in GFAP reactivity in the CNS versus non-CNS tissues. In non-CNS tissues, the GFAP reactive epitope was labile to aldehyde fixatives and could not be detected in paraffin-embedded sections (Yen and Fields, 1985). Thus, GFAP-like immunoreactivity in non-CNS tissues has been reported when employing mild

tissue-processing and more sensitive detection procedures (Yen and Fields, 1985). An exception to this are the glial cells of the enteric nervous system that show intense staining for GFAP, very similar to that seen in astrocytes (Jessen and Mirsky, 1980). However, it has been argued that the enteric glia resemble astrocytes of the CNS with respect to gross and fine structure, and to some extent, in function (Gershon and Rothman, 1991). Certain tumors of non-neural origin (i.e. capillary hemangioblastoma, papillary meningioma and metastasizing renal carcinomas) have also shown the presence of GFAP-like immunoreactivity (Deck and Rubinstein, 1981; Budka, 1986). In the case of the hemangioblastoma, the presence of GFAP is suggested to be due to the absorption or phagocytosis of extracellular GFAP protein by these malignant cells (Deck and Rubinstein, 1981). Recently, Feinstein et al. (1992) have isolated cDNA clones encoding rat GFAP from both CNS (astrocyte) and non-CNS (Schwann cell) cDNA libraries. Their studies revealed differences in the structures of GFAP in the two cell-types, at both mRNA and protein level (Feinstein et al., 1992), and may explain the immunochemical differences observed in CNS and non-CNS GFAP as stated earlier.

The functional role(s) of the intermediate filament protein, GFAP, during CNS development or in the normal adult brain is not known. GFAP is similar in structure to other types of intermediate filament proteins, like desmin, vimentin, and peripherin (Conway and Parry, 1988; Thompson and Ziff, 1989). Recent studies have indicated that GFAP filaments are dynamic structures that may be regulated through posttranslational phosphorylation (Nakamura et al., 1991). Protein phosphorylation generally involves a cascade of protein kinases, including cAMP-dependent protein kinase (Nestler and Greengard, 1983). It has been previously shown that membrane

receptors present on astrocytes can function to regulate phosphorylation of both GFAP and vimentin in cultured astrocytes by affecting intracellular cAMP levels (McCarthy et al., 1985). Alternatively, changes in the GFAP cytoskeleton have been proposed to arise by enzymatic cleavage by Ca^{2+}-activated protease(s) in astroglial cells, as observed with other major classes of intermediate filaments in brain tissues (Schlaepfer and Zimmerman, 1981; Nelson and Traub, 1983). The susceptibility of GFAP to Ca^{2+}-mediated degradation was shown to increase with age (Smith et al., 1984), perhaps reflecting differences in the levels and/or activity of intrinsic Ca^{2+}-activated protease in astrocytes present during development (Smith et al., 1984). Given its dynamic structure, GFAP may thus play a role in glial cell morphogenesis (Nakamura et al., 1991). Metabolic studies with rodent astrocyte cultures have shown that the rate of GFAP synthesis and its cytoplasmic accumulation can be experimentally modulated (Chiu and Goldman, 1985). For example, the transformation of the flat, polygonal astrocyte to the stellate form, by varying the initial plating densities from low to high, is accompanied by an increase in GFAP content (Goldman and Chiu, 1984a). These authors and others also reported an increase in the amount of intermediate filaments in astrocytes after dBcAMP treatment (Fedoroff et al., 1984a; Goldman and Chiu, 1984b). The increase in intracellular content of GFAP was also correlated with morphological changes from flat, polygonal to stellate, process-bearing (Goldman and Chiu, 1984b). Similar increases in expression of GFAP has been shown with monolayer cultures of rat C6 gliomas (Raju et al., 1980). Astrocytes treated with hormones, growth factors or forskolin, or cultured in the presence of neurons have also been found to undergo morphological transformations with remodeling of glial filaments (Hatten, 1985; Morrison et al., 1985; Pollenz and McCarthy, 1986; Culican

et al., 1990; Gasser and Hatten, 1990a). In cultures of purified rat astrocytes, GFAP levels were increased by 2- to 4-fold in the presence of hydrocortisone, putrescine, prostaglandin F-2α and pituitary fibroblast growth factor (Morrison et al., 1985). Treatment of similar cultures with forskolin increased GFAP and vimentin phosphorylation by 4-fold and 2-fold, respectively, and induced a morphological change in cells from polygonal to process-bearing (Pollenz and McCarthy, 1986). A different type of study which involved the suppression of GFAP in astrocytoma cell lines by antisense mRNA demonstrated inhibition in the ability of these cells to form stable processes in the presence of neurons (Weinstein et al., 1991). Neuron-dependent morphological differentiation of astroglia in vitro has been described previously (Hatten, 1985; Nagata et al., 1986; Ard and Bunge, 1988; Culican et al., 1990; Gasser and Hatten, 1990a). Taken together, these in vitro studies suggest that GFAP may be important for the morphological stability of astrocytes. The relatively slow metabolic turnover rate for GFAP is consistent with such a structural role (Smith et al., 1984; DeArmond et al., 1986).

Trauma to the CNS, either by lesioning or in a diseased state, is also associated with a substantial increase in the expression of GFAP in astrocytes (Bignami and Dahl, 1976; Amaducci et al., 1981; Lindsay, 1986; Smith and Eng, 1987; Aquino et al., 1988; Takamiya et al., 1988; Malhotra et al., 1990). Other changes include an increase in the number of mitochondria, Golgi membranes, endoplasmic reticulum, glycogen content and enzyme levels (reviewed by Lindsay, 1986), and in some astrocytes intense staining for vimentin has also been observed following trauma (Dahl et al., 1981a; Pixley and de Vellis, 1984). Thus, astrocytes undergo proliferation and hypertrophy, and extend numerous processes in response to diverse forms of CNS

injury or disease (Reier et al., 1989; Malhotra et al., 1990; David and Ness, 1993). Astrocytes undergoing such changes are termed "reactive" astrocytes. Similar changes also occur in vitro in response to treatment with cytokines, such as IL-1 (Giulian and Lachman, 1985). In young animals, reactive astrocytes may have phagocytic activity immediately following trauma (Fulcrand and Privat, 1977; Nathaniel and Nathaniel, 1981). Reactive astrocytes can also serve as antigen-presenting cells by expressing surface markers typical of cells in the immune system, such as MHC class I and II antigens (Wong et al., 1984; Salamat et al., 1988; Hertz et al., 1990) and ICAM-1 (Frohman et al., 1989). It should be emphasized that regional differences are known to occur with respect to the astrocyte response to injury or disease; for instance, as seen in the case of increased expression of vimentin and GFAP (reviewed by David and Ness, 1993). The differential accumulation of intermediate filament proteins in reactive astrocytes might therefore reflect changes in their functional properties.

The existence of heterogeneity in GFAP mRNA size has been reported in the neonatal mouse brain (Lewis and Cowan, 1985). The cDNA probes encoding mouse GFAP (Lewis et al., 1984) were also used to study GFAP mRNA levels in different regions of the developing mouse brain (Lewis and Cowan, 1985). These authors reported more copies of GFAP mRNA in astrocytes in the white matter of adult animals as compared to gray matter astrocytes, while the greatest content of GFAP mRNA was found in astrocytes of the glia limitans. These results not only suggest heterogeneity among astrocytes from different regions of the brain, but might also reflect functional differences associated with different rates of GFAP synthesis. However, the functional significance for GFAP in astrocytes still remains unclear. Further examination of factors that induce GFAP gene expression in astrocytes and the

application of genomic probes for GFAP in developing and injured CNS tissue should give more insight into the regulatory mechanisms that control GFAP expression. In addition, the possibility of transfecting non-neural cells with cDNAs encoding for GFAP may help to study the precise functional role of GFAP.

1.2.2 Functions of astrocytes. In the normal state, astrocytes have an important homeostatic role in regulating extracellular potassium levels released from electrically active neurons (Gardner-Medwin et al., 1981; Orkand et al., 1981; Walz and Hertz, 1983), with a 10-fold higher potassium conductance reported in the endfoot processes of astrocytes isolated from the optic nerve of the salamander (Newman, 1986). Similar non-uniform potassium conductance was observed in retinal Müller cells (Brew et al., 1986). Furthermore, these authors showed that potassium buffering is mediated by a single type of channel, with more channels being expressed at the endfoot of Müller cells than elsewhere on the cell (Brew et al., 1986), and not by different types of channels at the cell body and the endfoot as suggested previously (Newman, 1985). In this respect, the demonstration of gap junctions in astrocytes is interesting (Brightman and Reese, 1969; Landis and Reese, 1974), since these contact sites may serve for inter-astrocytic communication (Mugnaini, 1986). Astrocytes are coupled ionically and metabolically via gap junctions and have been suggested to form a functional syncytium in vivo (Mugnaini, 1986). Furthermore, there is evidence to suggest that changes in potassium concentration in culture act as signals for controlling astrocyte glycogen stores (Salem et al., 1975; Pentreath, 1982; Cambray-Deakin et al., 1988). The mechanisms by which potassium could influence glycogen synthesis and degradation in astrocytes are not clear, although a role for Ca^{2+} in cerebral glycogen metabolism has been suggested (Ververken et al., 1982). A second specialization of

membrane structure called "assemblies" has been recognized in astrocytes with the freeze-fracture technique (Landis and Reese, 1974; Anders and Brightman, 1979). In particular, the glia limitans and astrocytic processes adjacent to blood vessels manifest a large number of assemblies (Landis and Reese, 1981). It was therefore suggested that assemblies may have a role in the transport of material into or out of the blood and cerebrospinal fluid compartments (Landis and Reese, 1981). A possible role in inhibiting axonal growth and regeneration has also been suggested for these assemblies (reviewed by Reier, 1986). It was shown that developing astrocytes which provide a substrate for neuronal migration and axonal growth lack assemblies, as do astrocytes from the adult amphibian which allow regeneration (Anders and Brightman, 1979; Wujek and Reier, 1984). In contrast, assemblies are numerous in mammalian astrocytic scars, which have been reported to be inhibitory to axon growth and penetration (Wujek and Reier, 1984; Reier, 1986).

Astrocytes also participate actively in the metabolism of various neurotransmitters. The presence of receptors for different neurotransmitters and neuromodulators has been demonstrated on astrocytes, with regional differences (Hansson, 1988). Such regional heterogeneity can be seen, for instance, in the case of glutamate uptake processes (Drejer et al., 1982). Glutamate was shown to induce an increase in free calcium propagating as waves within the cytoplasm of cultured hippocampal astrocytes, and may play a role in rapid signal transmission within the brain (Cornell-Bell et al., 1990). Glutamate released from neurons is converted into glutamine by glutamine synthetase. In the brain, glutamine synthetase is found exclusively in astrocytes and therefore serves as another specific marker for astrocytes besides GFAP (Norenberg and Martinez-Hernandez, 1979). It has been shown that

astrocytes from the neostriatum with predominantly glutamatergic input have a higher uptake capacity for glutamate as compared to astrocytes originating from other brain regions (Fonnum et al., 1981; Drejer et al., 1982). In another study, Patel et al. (1985) reported variations in glutamine synthetase activity in astrocytes from different brain regions. Similarly, McCarthy and his colleagues have very clearly demonstrated, using a combination of autoradiographic and immunocytochemical techniques, the presence of β-adrenergic receptors on the flat, polygonal astrocytes, but not the process-bearing astrocytes (Burgess et al., 1985). These observations are in contrast to the α_1-adrenergic receptor expression which, unlike the β-adrenergic receptor, is associated with the majority of the process-bearing astrocytes (Lerea and McCarthy, 1989). More examples of regional heterogeneity have been found on astrocytes with respect to receptor sites for various other neurotransmitters, such as histamine and dopamine (Murphy and Pearce, 1987).

Other roles of astrocytes include the induction of blood-brain barrier properties in non-neural endothelial cells (Arthur et al., 1987; Janzer and Raff, 1987), and the differentiation of oligodendrocytes by release of PDGF (Raff et al., 1988). Astrocytes also produce other trophic factors necessary for the survival and differentiation of neurons (reviewed by David, 1993). In addition, astrocytes participate actively in repair and regeneration of the CNS tissue (reviewed by Lindsay, 1986; Reier, 1986). During development, astrocytes and their embryonic precursors may have a role in guidance of neuronal migration and axonal growth (Rakic, 1971; Hatten et al., 1984; Smith et al., 1986; Reichardt et al., 1989). This aspect is discussed in more detail in section 1.4.

There is growing evidence pointing to region-specific interactions between

astrocytes and neurons, e.g., astrocytes derived from specific brain regions differentially support neuronal differentiation (Denis-Donini et al., 1984; Chamak et al., 1987; Autillo-Touati et al., 1988; Barbin et al., 1988; Chamak and Prochiantz, 1989; Qian et al., 1992). For instance, the morphology of embryonic mouse mesencephalic dopaminergic neurons was strongly influenced by the regional origin of the astrocytes after 2 days in culture (Denis-Donini et al., 1984). On mesencephalic astrocytes (homotopic cocultures), dopaminergic neurons had complex multipolar, highly branched neurites. In comparison, on striatal astrocytes (heterotopic cocultures), most neurons were mono- or bipolar, and almost devoid of secondary branches (Denis-Donini et al., 1984). The functional variations in astrocytes observed within cell populations derived from the different brain regions may be due to the biochemical and physiological heterogeneity of astrocytes, as already discussed with regard to GFAP expression (Lewis and Cowan, 1985), membrane structural properties (Landis and Reese, 1981; Batter et al., 1992), and neurotransmitter receptor systems (Hansson, 1988). Additionally, astrocyte heterogeneity has been observed with regard to the expression of ion channels (Barres et al., 1988, 1990), surface glycoproteins (Barbin et al., 1988) and adhesion molecules (Chamak and Prochiantz, 1989), as well as in the production of diffusible factors (Qian et al., 1992). Such heterogeneity must underlie the region-specific interactions between astrocytes and neurons, as well as other cells present in the nervous system, including cells of non-neural origin such as endothelial cells.

1.3 Origin and subtypes of astrocytes

The classical concepts about the formation and differentiation of neuroglial cells have changed considerably since the turn of the century. In 1889, Wilhelm His proposed

the existence of two types of primitive stem cells in the neural tube. He suggested that neuroglial cells including astrocytes are derived from columnar epithelial cells or spongioblasts, while neurons arise from a histologically distinct subpopulation of epithelial cells or germinal cells (His, 1889). An opposing view was put forward by Schaper (1897), and later by Sauer (1935), who considered the two cell-types described by His to simply represent different mitotic phases of the same type of cell. Hence, unlike His's earlier perspective, the ventricular zone was considered to be homogeneous in its cellular composition. Evidence from tritiated thymidine autoradiographic studies also supported the concept of the ventricular zone consisting of a homogeneous stem cell population (Fujita, 1963). Thus, it is now widely accepted that glial cells, like neurons, must originate from the same primitive neuroepithelium in the ventricular zone and possibly in the subventricular zone (Privat and Leblond, 1972; Jacobson, 1978). Since few progenitors have been characterized in the ventricular cell layer, mainly due to morphological similarities among the cells within the ventricular zone (Jacobson, 1978), it is not known precisely when the neuroepithelial cells become destined to be glial precursor cells. More recent studies, however, have shown that some astrocytes, and in some cases oligodendrocytes (Choi and Kim, 1985), arise from radial glia which themselves become established during the first half of embryonic life depending upon the species (Choi and Lapham, 1978; Schmechel and Rakic, 1979a; Levitt and Rakic, 1980; Dahl et al., 1981b; Schnitzer et al., 1981; Bovolenta et al., 1984). In adult mammals, radially oriented glia persist only in the retina, cerebellum and hypothalamus, and are called Müller glia, Bergmann glia and tanycytes, respectively (Peters et al., 1991).

Our knowledge about the cellular and molecular mechanisms that induce the

progenitor cell population in acquiring glial characteristics in vivo has been somewhat limited due to the lack of an adequate number of specific markers. Two monoclonal antibodies recognizing distinct surface gangliosides: polysialoganglioside recognized by the monoclonal antibody (MAb) A2B5 (Eisenbarth et al., 1979) and GD3 ganglioside (Goldman et al., 1984) have allowed the identification of glial precursors in cultures of the newborn rat optic nerve and the fetal subventricular zone, as well as in cultures of the developing white matter of the cerebrum and cerebellum (Raff et al., 1983a,b; Miller et al., 1985; Goldman et al., 1986; Curtis et al., 1988; LeVine and Goldman, 1988; Reynolds and Wilkin, 1988). Antibodies to Ran-2 (Bartlett et al., 1981), Rat-401 (Hockfield and McKay, 1985), now defined as a novel intermediate filament protein, nestin (Lendahl et al., 1990), and RC1/RC2 (Misson et al., 1988; Edwards et al., 1990) also bind to antigens expressed by the glial lineage-restricted progenitor cell (Abney et al., 1981; Frederiksen and McKay, 1988; Misson et al., 1991b). Thus, the acquisition of these glial cell-specific antigens might be indicative of an initial step in the divergence of neuronal and glial cell lineages (reviewed by Cameron and Rakic, 1991).

Astrocyte lineages have been studied both in vitro and in vivo in several animals including monkeys, humans, rats, and mice (Fedoroff and Doering, 1980; Levitt and Rakic, 1980; Choi, 1981; Raff et al., 1983b; Goldman et al., 1986; Skoff, 1990). As already mentioned, all astrocytes have been suggested to arise from the proliferating immature neuroectodermal cells which may or may not pass through the transient stage of radial glial cells. That some astrocytes may develop from embryonic radial glial cells has been based upon several lines of evidence (Levitt and Rakic, 1980; Choi et al., 1983; Rickmann et al., 1987; Misson et al., 1991b). This was confirmed using

antibody labeling in vitro (Culican et al., 1990), as well as using vital dye markers in vivo (Voigt, 1989) to directly demonstrate the morphological transformation of radial glial cells into mature GFAP-expressing astrocytes (Voigt, 1989; Culican et al., 1990). During development neurons are guided in their migrations by the radial glial cells (Rakic, 1971). During this migratory phase, radial glia remain as non-dividing cells (Schmechel and Rakic, 1979b; Rakic, 1981). However, once migration is completed, radial glia in most regions start proliferation and differentiate into astrocytes with shorter processes (Schmechel and Rakic, 1979a). The transformation of the bipolar radial cell into the multipolar form of astrocyte may occur directly or with the monopolar radial form as an intermediate state of transformation (Misson et al., 1991b). It has been suggested that the signals that induce the transformation of radial glia to mature forms of astrocytes may come from differentiating neurons, since the morphological change occurs at a time when neuronal migration along radial glial fibers is completed (Misson et al., 1991b). In vitro studies have provided strong support for neuronal influence (Hatten, 1985; Culican et al., 1990), and have further suggested that the neuron-dependent transformation may be membrane-mediated (Hatten, 1987). In addition, during the transformation radial glial cells change their biochemical properties as evidenced by the loss of vimentin, Rat-401 and RC1 immunoreactivity (Bovolenta et al., 1984; Pixley and de Vellis, 1984; Hockfield and McKay, 1985; Culican et al., 1990; Edwards et al., 1990). Changes in the expression of other antigens might also occur during subsequent astroglial cell maturation (Smith et al., 1993). The newly formed astrocytes are also likely to assume different functions.

The possibility of direct transformation of ventricular cells to astroglia was first

suggested by Skoff et al. (1976a) in the developing rat optic nerve. Astrocytes are the first macroglial cells to differentiate in the nerve, first appearing around embryonic day 16 (E16) (Skoff et al., 1976b). Fedoroff and his colleagues (1980, 1984b) have followed the astroglial cell lineage from glioblasts isolated from ventricular and subventricular zones to differentiated astrocytes, and have correlated the stage of cell differentiation with changes in the cytoskeleton and morphology (Fedoroff, 1985). Other studies have also shown that astrocytes can differentiate from neuroepithelial progenitor cells in primary culture (Goldman et al., 1986; Temple, 1989). Raff and his colleagues (1983a,b) used immunocytochemical methods to define two separate astroglial lineages in cultures of the developing rat optic nerve (discussed later). However, several questions remain controversial. It is not known whether the different patterns of gliogenesis observed in vitro correlate to development in vivo, or whether it reflects the plasticity of neural cells in culture. As mentioned earlier, there are differences in astrocytes in various regions of the CNS. How these different astrocytes are developmentally related to each other is not known. For this, the use of a lineage marker to trace clonal development in vivo has been a major step in determining single cell lineages (Sanes et al., 1986; Price et al., 1987; Cepko, 1988; Luskin et al., 1988). The method uses a recombinant retrovirus to insert a foreign gene into the genome of a dividing cell. The product of the gene is then detected histochemically in the progeny of the infected cell. The main criticism with this technique would reside in the timing of the infection. However, if coupled with in situ morphological and the use of existing immunological markers as well as the development of additional novel markers for immunocytochemical studies in vivo, clonal analysis would facilitate our understanding of the unanswered questions related to gliogenesis.

Traditionally, two distinct populations of astrocytes have been identified in the normal vertebrate CNS as a result of studies carried out by Carl Weigert (1895) and Ramón y Cajal (1909, 1913). Weigert studied the distribution of glial fibers and demonstrated numerous fibrils present in astrocytes of the white matter (Weigert, 1895). These astrocytes are referred to as fibrous astrocytes, and have a stellate, process-bearing morphology. The astrocytes found in the gray matter, on the other hand, generally contain fewer fibrils and consequently have been called protoplasmic astrocytes. However, in pathological conditions, protoplasmic astrocytes may acquire dense fibrils (Peters et al., 1991). Unlike fibrous astrocytes, protoplasmic astrocytes have sheet-like processes associated with neurons and blood vessels. Thus, in addition to their in vivo location, the two types of astrocytes differ from each other in their morphology which became apparent with the use of Cajal's gold sublimate method (Ramón y Cajal, 1913). By electron microscopy, fibrous and protoplasmic astrocytes differ in their ultrastructure (Peters et al., 1991). Besides these two types of astrocytes, specialized forms of astrocytes are defined in the normal CNS and include the radially oriented glia mentioned earlier.

Raff and his colleagues (1983a) have further demonstrated two types of astrocytes in cultures of perinatal rat optic nerve using cell-type specific antibodies, and have termed them as type-1 and type-2 astrocytes. The presence of similar type-1 and type-2 astrocytes have also been shown in cultures of neonatal rat cerebrum and cerebellum (Williams et al., 1985; Levi et al., 1986). The most obvious difference between the two subpopulations of astrocytes in culture is that type-1 astrocytes have a fibroblast-like morphology, while type-2 astrocytes are process-bearing (Raff et al., 1983a). Other distinguishing properties of the two astrocytes include: (i) the presence

of specific cell surface antigens, (ii) the timing of differentiation, (iii) the difference in cell lineage, (iv) the distribution in vivo, and (v) the differences in response to growth factors and (vi) to injury (reviewed by Miller et al., 1989; Raff, 1989). For instance, type-1 astrocytes react with MAb Ran-2 (Bartlett et al., 1981), but not with MAb A2B5 (Eisenbarth et al., 1979). In contrast, type-2 astrocytes are labeled with MAb A2B5 (Raff et al., 1983a) and show little or no reactivity with MAb Ran-2 (Raff et al., 1984). Furthermore, cell dissociation and tissue culture studies indicate that type-1 astrocytes appear early in rat development, i.e. at around E16, whereas type-2 astrocytes appear later in the second postnatal week (Miller et al., 1985). Also, studies involving complement-dependent lysis indicate that type-1 and type-2 astrocytes are derived from two distinct astrocyte lineages (Raff et al., 1983a,b; 1984). The Ran-2$^+$ cell population contains precursor cells for type-1 astrocytes, while the type-2 astrocytes are derived from Ran-2$^-$, A2B5$^+$ bipotential progenitor cells (O-2A) that are also capable of differentiating into oligodendrocytes in serum-free medium (Raff et al., 1983b; Saneto and de Vellis, 1985). Subsequent studies on cloned cells strongly supported the view that individual O-2A progenitor cells are bipotential (Temple and Raff, 1985). Extensive in vitro work has further led to the identification of factors released by type-1 astrocytes influencing the proliferation and differentiation of the O-2A progenitor cells (reviewed by Lillien and Raff, 1990). However, there is some controversy in the literature about the existence of type-2 astrocytes in vivo. The morphological and immunocytochemical studies of mitotic cells in the rat optic nerve in vivo have shown that oligodendrocytes and astrocytes originate from separate lineages, and only one wave of astrocyte proliferation was observed around the time of birth (Skoff and Knapp, 1991). Furthermore, since the A2B5 MAb is a poor

marker in vivo, the identification of type-2 astrocyte in vivo has not been established with certainty (Noble, 1991). Despite the uncertainty of the existence of type-2 astrocytes in vivo, in vitro studies clearly indicate that flat, type-1 astrocytes differ from process-bearing type-2 astrocytes in their cell lineage, surface phenotype and other characteristics pointed out earlier.

1.4 Developmental events associated with astrocytes

As mentioned earlier, astrocytes are the first glial cell-type to differentiate during CNS development. Interactions of astrocytes with different types of brain cells during development are therefore important for normal function of the nervous system. Most detailed studies on such intercellular interactions have been carried out between astrocytes and neurons. Astrocytes are thought to influence neuronal development in a variety of ways, such as by secreting trophic factors necessary for neuronal survival, and by guiding migrating neurons and growing axons to their appropriate locations mediated through adhesive mechanisms. In this section, I will review the main findings for astroglial support of neuronal migration along Bergmann glia, as well as discuss some of the evidence for axonal growth on astrocytes in vivo.

1.4.1 Neuronal migration. In the developing cerebellum, the close association of Bergmann glial fibers and granule cells was first noted by Gustaf Retzius (1894). In 1971 Rakic published a Golgi and electron microscopic study on the relationship of the migrating granule cell to the Bergmann glial fibers in the rhesus monkey cerebellum. The close proximity of migrating neurons to radial glial processes has been reported in other regions of the developing CNS as analyzed by three-dimensional reconstructions of serial electron micrographs (Rakic, 1972, 1978, 1981; Rakic et al.,

1974). These studies led to the suggestion that radial glia, that span the thickness of the neural tube, might help guide developing neurons from the ventricular zone to reach their final destinations.

More recently, Hatten and her colleagues have developed an in vitro model system in which glial-guided neuronal migration can be observed in real time using time-lapse video microscopy (Hatten et al., 1984; Edmondson and Hatten, 1987). Their findings (Edmondson and Hatten, 1987; Hatten, 1990) showed that (i) neuronal migration is bi-directional in vitro when the astroglial process is occupied by a single neuron. When two or more cells are migrating along the process, migration becomes oriented, (ii) neuronal migration is saltatory, moving at an average speed of 20-60 μm/hr in vitro, and (iii) neuronal migration involves an active movement of neuronal cell soma along the astroglial process, rather than simply translocating the nucleus through the cytoplasm. Moreover, a specialized junction is found between the migrating neuron and the astroglial process. The cytological features of the migrating neuron in vitro (Hatten et al., 1984; Edmondson and Hatten, 1987; Gregory et al., 1988) closely matched the in vivo findings made by Rakic (1971, 1972). The close apposition of migrating neurons to radial glia has further suggested that membrane adhesion systems provide the molecular basis for neuronal migration in the developing CNS, several of which have been identified by functional antibody perturbation studies (will be reviewed in section 1.5.2). Neuronal migration along Bergmann glia was shown to be mediated by the ECM molecules tenascin/cytotactin and astrochondrin, and the neuronal surface molecule astrotactin (Chuong et al., 1987; Fishell and Hatten, 1991; Husmann et al., 1992; Streit et al., 1993). Other studies have suggested that the mechanism for glial-guided neuronal migration is conserved among brain regions

(Gasser and Hatten, 1990b; Hatten, 1990). In heterotypic recombinations of neurons and glia from mouse cerebellum and rat hippocampus, neurons were shown to migrate on heterotypic glial processes with similar dynamics to that observed in homotypic neuronal migration (Gasser and Hatten, 1990b). More recently, the dynamics of neuronal migration was visualized in situ by implanting labeled neuronal precursor cells in the developing cerebellum (Gao and Hatten, 1993). In addition, studies involving retroviral labeling of neurons demonstrate substantial alignment of labeled migrating cells with $RC2^+$ glial fibers (Misson et al., 1991a). These experiments provide good evidence that astroglia are involved in neuronal migration.

1.4.2 Axonal growth. Silver and his colleagues (Silver and Sidman, 1980; Silver et al., 1982; Silver, 1984) have examined the involvement of astroglial cells in the development of axons. These investigators reported that axons of retinal ganglion cells grow and become segregated into discrete regions of the optic stalk because of the presence of tunnel-like spaces that are lined with neuroepithelial cells thought to be glial precursors. It is along the inner surfaces of these tunnels that axons normally grow (Silver and Robb, 1979; Silver and Sidman, 1980). Failure in the formation of these tunnels as seen in mutant mice results in retinal degeneration (Silver and Robb, 1979). It was concluded from these experiments that the embryonic glia lining the tunnels must offer permissive surfaces that promote axonal growth (Silver and Sidman, 1980). Silver and Rutishauser (1984) also showed that N-CAM is expressed by the cells lining the tunnels. Similarly, the presence of glial cells which form a "sling" or bridge-like structure connecting the two hemispheres was shown to be necessary for the formation of the corpus callosum (Silver et al., 1982; Silver and Ogawa, 1983). The growth of callosal fibers is aborted in animals lacking the glial sling, owing to

mutation or because of surgical manipulation (Silver and Ogawa, 1983). If, however, a sling is provided in the form of a small piece of filter paper implanted into the midline region of embryonic or early postnatal acallosal mice, astroglial cells migrate onto the filter, followed by axons which cross the midline (Silver and Ogawa, 1983).

Others have also reported that glia may serve as an effective substrate for axon growth in the mouse visual system in vivo (Bovolenta and Mason, 1987). These axon-glial interactions are likely to be mediated by adhesion molecules. For example, N-CAM (Silver and Rutishauser, 1984), laminin (Cohen et al., 1987) and L1 (Bartsch et al., 1989) are present in the developing visual system in rodents. Direct evidence that adhesion molecules can mediate neurite growth on astrocytes have been obtained from in vitro studies (Noble et al., 1984; Fallon, 1985a,b; David, 1988; Neugebauer et al., 1988; Tomaselli et al., 1988). Antibody perturbation experiments in vitro have demonstrated that N-CAM, N-cadherin, and integrins of the β_1 subclass contribute to axon growth along glia (Reichardt et al., 1989). It is interesting to note that unlike a common mechanism for glial-guided neuronal migration which was proposed by Hatten (1990), the growth of axons in different regions of the nervous system appears to be regulated by different mechanisms (Denis-Donini et al., 1984). A likely explanation for this may be the spatiotemporal expression of specific adhesion molecules during CNS development.

1.5 Adhesion molecules on astrocytes

Considerable progress has been made in the identification and characterization of cell surface molecules that mediate adhesion of cell-cell and cell-matrix interactions during development. Adhesion molecules on astrocytes include both cell surface molecules and molecules of the ECM that constitute mainly the non-collagenous type

glycoproteins (e.g. laminin and fibronectin). In particular, in recent years a large number of the cell surface adhesion molecules have been identified in the nervous system and are generally grouped into three distinct gene families. These are: (i) the immunoglobulin superfamily, (ii) the cadherin family, and (iii) the integrin family. Many of the molecules that belong to the first two families of cell adhesion molecules are known to mediate cell-cell interactions, while the majority of integrins bind to various ECM molecules and mediate cell-matrix interactions in the nervous system. Accordingly, adhesion molecules have also been commonly classified as cell adhesion molecules (CAMs) that mediate the adhesion of cells to one another, or substratum adhesion molecules (SAMs) that mediate the adhesion of cells to the ECM. However, many CAMs that are associated with the cell surface via a phosphatidylinositol linkage may participate in cell-matrix adhesion and thus the distinction between the two categories of adhesion molecules is not always sharp. For example, although N-CAM is generally considered to be a cell adhesion molecule, some forms of N-CAM are secreted and deposited in the ECM in vivo, and can function as a matrix adhesion molecule through binding to cell surface N-CAM and heparan sulfate proteoglycan (Cole et al., 1985; Cole and Glaser, 1986). This would suggest that the cellular events mediated by CAMs and SAMs may not be fundamentally different. In fact, several adhesion molecules that belong to the cell surface and to the ECM are known to play similar key roles in neural development. Below, I first describe the three families of cell adhesion molecules which are based on their structural similarities, followed by a brief description on the composition and structure of the ECM.

The immunoglobulin superfamily. Members of this family of CAMs all have one or more structural motifs which are similar to those first identified in immunoglobulin

(Ig) molecules, and are called Ig domains. Many members of this family also contain two or more domains similar to those first found in the ECM molecule, fibronectin. These domains are called fibronectin type III (FN type III) repeats. Each of the Ig domains and the FN type III repeats are approximately 100 amino-acids long, the former forming a disulfide-bonded loop. Furthermore, the FN type III repeats are less conserved than the Ig domains which have been found in a variety of molecules involved in adhesive functions (Salzer and Colman, 1989; Grumet, 1991). The neural CAM, N-CAM, was the first adhesion molecule found to contain Ig domains (Cunningham et al., 1987). Other adhesion molecules possessing Ig domains include L1/NILE, Ng-CAM/8D9, Nr-CAM, Contactin/F11/F3, TAG-1/axonin-1, MAG, P_o, DM-GRASP/SC-1, neuroglian, fasciclin II and fasciclin III (reviewed by Rathjen, 1991). Adhesion molecules that belong to this superfamily generally mediate Ca^{2+}-independent cell-cell adhesion of either a homophilic or heterophilic nature (Williams and Barclay, 1988). The signal transduction mechanisms in neurite outgrowth are only now beginning to be understood (Doherty and Walsh, 1992). Much of the information has been generated for N-CAM- and L1-mediated neurite outgrowth. These studies have suggested a common but specific second messenger pathway involving a number of signaling events, that include increased protein tyrosine phosphorylation, pertussis toxin sensitive G protein activation, and stimulation of Ca^{2+} fluxes into the growth cone via L- and N-type Ca^{2+} channels (Doherty and Walsh, 1992; Williams et al., 1992).

The cadherin family. Cadherins are Ca^{2+}-dependent CAMs consisting of four structurally repeated extracellular domains, a Ca^{2+} binding site, and a highly conserved cytoplasmic region that shows 63-89% amino-acid homology between different members of the cadherin family (Magee and Buxton, 1991; Takeichi, 1991; Grunwald,

1993). Also located in the extracellular domain close to the transmembrane region are four conserved cysteine residues (Magee and Buxton, 1991; Takeichi, 1991). Furthermore, the homophilic-binding site through which cadherins interact with each other has been localized to the amino-terminal extracellular domain which contains the HAV (His-Ala-Val) cell adhesion recognition sequence (Blaschuk et al., 1990; Nose et al., 1990). An exception to this structural scheme is the truncated form of cadherin or T-cadherin that lacks the transmembrane and cytoplasmic domains. Instead, T-cadherin is anchored to the plasma membrane by means of a phosphatidylinositol linkage (Ranscht and Dours-Zimmermann, 1991). Cadherins generally mediate cell-cell adhesion by homophilic interaction, although heterophilic binding between different cadherin molecules has been shown (Inuzuka et al., 1991). Three types of cadherins were originally characterized in non-neural (E- and P-cadherin) and neural tissues (N-cadherin). Recently, besides T-cadherin, two other cadherins (B- and R-cadherin) have been identified in the nervous system (Inuzuka et al., 1991; Napolitano et al., 1991). In addition, eight novel cadherin-encoding cDNA fragments were isolated from brain which have been designated as cadherins 4-11 (Suzuki et al., 1991). A related overall structure to cadherins is found in desmoglein and desmocollins which are also members of the cadherin family of CAMs (Goodwin et al., 1990; Holton et al., 1990; Koch et al., 1990; Wheeler et al., 1991). Except for these desmosomal glycoproteins which are associated with intermediate filaments, cadherins associate with actin filaments and cytoplasmic proteins termed catenins (Ozawa et al., 1990; Geiger and Ayalon, 1992). There are at least three major classes of catenin proteins (α, β and τ) and these were shown to interact with both E- and N-cadherin (Knudsen and Wheelock, 1992; Ozawa and Kemler, 1992).

The integrin family. Integrins are all non-covalently linked heterodimers with distinct α and β subunits that function not only as ECM receptors but can also mediate cell-cell adhesion by binding to cell surface molecules of the Ig superfamily, namely ICAM-1, ICAM-2 and VCAM-1 (Hynes and Lander, 1992). So far, 14 α and 8 β subunits have been reported, most of which have been cloned and sequenced (Hynes, 1992; Sastry and Horwitz, 1993). The extracellular termini with globular domains of α and β subunits associate to form functional $\alpha\beta$ heterodimers. This domain binds the RGD (Arg-Gly-Asp) sequence present in a variety of ECM molecules, such as fibronectin and vitronectin, and requires divalent cations for function (D'Souza et al., 1988; Gailit and Ruoslahti, 1988; Ignatius and Reichardt, 1988; Hautanen et al., 1989; Smith and Cheresh, 1990). Although the RGD sequence is the first and perhaps the most common integrin recognition site in the ECM ligands (Ruoslahti and Pierschbacher, 1987), other binding sites have also been reported (reviewed by Hynes, 1992). The ligand specificity of integrins is determined by both subunits or even the cell-type in which it is expressed (Elices and Hemler, 1989). For example, $\alpha_v\beta_1$ integrins bind vitronectin in kidney cells (Bodary and McLean, 1990) but appears to function exclusively as fibronectin receptors in neuronal cells (Vogel et al., 1990). Thus, some integrins have multi-ligand binding capabilities depending on the cell-type. The determinants responsible for this cell specific regulation of integrins are unclear, but may involve posttranslational modifications (Reichardt and Tomaselli, 1991; Hynes and Lander, 1992). Both α and β subunits can have alternatively spliced cytoplasmic domains (Brown et al., 1989; Van Kuppevelt et al., 1989). These cytoplasmic domains are believed to interact with cytoskeletal proteins, such as talin, vinculin, α-actinin and actin, to form cell-substratum or cell-cell contacts (Singer, 1982; Horwitz et al., 1986;

Burridge et al., 1988; Hemler, 1990; Otey et al., 1990). An exception is the β_4 cytoplasmic domain which colocalizes with hemidesmosomes, suggesting a direct linkage to an intermediate filament associated molecule rather than the actin cytoskeleton (Stepp et al., 1990; Suzuki and Naitoh, 1990; Sonnenberg et al., 1991).

The extracellular matrix molecules. Molecules of the ECM found in the basal lamina are commonly classified in three major families which include collagens, non-collagenous glycoproteins, and proteoglycans (reviewed by Carbonetto, 1984; Rutka et al., 1988; Reichardt and Tomaselli, 1991). In the CNS, basal laminae are found beneath ependymal and meningeal linings, in the inner limiting membrane of the retina, as well as surrounding blood vessels (Carbonetto, 1984). The ECM is now defined more broadly to include secreted molecules found outside cells, even though they lack a morphologically organized basal lamina. Several ECM constituents originally identified in non-neural tissues have been found in the CNS. In particular, increased attention has been given to glial-derived ECM molecules and their role in histogenesis (Lander, 1989; Sanes, 1989; Reichardt and Tomaselli, 1991). For instance, embryonic CNS contains laminin (Liesi, 1985), fibronectin (Stewart and Pearlman, 1987), thrombospondin (O'Shea and Dixit, 1988; O'Shea et al., 1990) and tenascin/cytotactin (Crossin et al., 1986; Steindler et al., 1989), most of which are associated with astrocytes (Reichardt et al., 1989). On the other hand, the ECM of the adult CNS has a relatively large amount of proteoglycan (Aquino et al., 1984; Herndon and Lander, 1990) while the expression of glycoproteins, such as laminin and fibronectin, is decreased (reviewed by Rutka et al., 1988). Recent work has further revealed the existence of multiple isoforms of ECM glycoproteins with functional domains that commonly include binding sites for cells, other ECM glycoproteins, and proteoglycans

(reviewed by Reichardt and Tomaselli, 1991). Furthermore, it has been possible to localize specific functions to specific structural domains. The most prominent of these domains is the FN type III repeat found in fibronectin, tenascin, thrombospondin as well as several CAMs (reviewed by Carbonetto and David, 1993). Some ECM glycoproteins and proteoglycans have also been shown to bind diffusible growth factors such as FGF (Yayon et al., 1991) and TGF-β (McCaffrey et al., 1989) as well as proteases (Silverstein et al., 1986). Growth factors in turn can modulate the biological activities of the ECM. For instance, TGF-β has been reported to stimulate mesenchymal and epithelial cells to synthesize fibronectin and collagen, which are subsequently incorporated into the ECM (Choy et al., 1990; König and Bruckner-Tuderman, 1992).

1.5.1 Molecules involved in neurite outgrowth. Several lines of evidence suggest that neurons extend neurites by interacting with other neurites, with glia or ECM molecules. Adhesion molecules involved in axon-axon mediated neurite outgrowth, such as DM-GRASP (Burns et al., 1991), TAG-1/axonin-1 (Furley et al., 1990; Stoeckli et al., 1991), F3/F11 (Gennarini et al., 1991), etc. will not be discussed in this section. Instead, I will focus solely on the adhesion molecules that contribute to neurite growth along glia, namely astrocytes. As already mentioned, neurite outgrowth on astrocytes is largely promoted by N-CAM, N-cadherin, and the integrins which are the cell surface receptors for several ECM glycoproteins. Among the ECM glycoproteins expressed on astrocytes, both laminin and thrombospondin are known to stimulate axonal growth via integrins. Therefore, in this section I have limited the review to the following four adhesion molecules found on astrocytes: N-CAM, N-cadherin, laminin and thrombospondin. Since the literature on some of these

molecules is extensive, emphasis will be placed on their overall structure and distribution. Also, in order to avoid repetition some of the general characteristics of these adhesion molecules will not be mentioned if already discussed within the families of CAMs in the previous section.

Neural cell adhesion molecule. N-CAM is composed of five Ig domains and two FN type III repeats (Cunningham et al., 1987). The single N-CAM gene, which consists of at least 19 exons (Owens et al., 1987), is alternatively spliced to give three major polypeptide isoforms ($\approx$ 115, 135 and 190 kd) that differ in the lengths of their carboxy-terminal (Owens et al., 1987). The smallest isoform lacks the transmembrane domain, and is anchored to the membrane by phosphatidylinositol (He et al., 1986; Sadoul et al., 1986). Additional exons have later been discovered at two locations to generate multiple forms of similar molecular weight (MW) N-CAM polypeptides. These exhibit both tissue and developmental specificity, and include: (i) the variable domain alternatively spliced 30 base pair exon named VASE which is localized in the fourth Ig domain encoded by exons 7 and 8 (Owens et al., 1987; Small and Akeson, 1990), and (ii) four alternatively spliced exons of 3-48 base pair collectively called MSD1 which are localized near the transmembrane domain in the region of the FN type III repeats between exons 12 and 13 (reviewed by Walsh and Dickson, 1989). MSD1 containing N-CAM forms are predominantly expressed by myotubes (Dickson et al., 1987), and do not seem to affect function (Doherty et al., 1990b). In addition, alternative splicing between exons 12-13 also gives rise to a secreted form of N-CAM found in both muscle and brain (Gower et al., 1988). Although little is known about its function, secreted N-CAM could act as either a positive or negative effector of cell adhesion events by mediating cell-matrix interactions. For instance, N-CAM has been

shown to bind heparin (Cole and Akeson, 1989) and collagens (Probstmeier et al., 1992). The expression of VASE, on the other hand, increases during brain development (Small and Akeson, 1990) and has been shown to significantly alter N-CAM function by down-regulating the neurite outgrowth-promoting activity of N-CAM (Doherty et al., 1992b; Liu et al., 1993). Thus, immature and developing cells of the CNS preferentially express N-CAM lacking VASE. Interestingly, VASE is also not expressed in the adult olfactory epithelium, which undergoes continual cellular and axonal growth throughout life (Small and Akenson, 1990).

During rodent brain development, the amount and the polypeptide pattern of N-CAM at the cell surface changes. N-CAM appears early in embryogenesis (Crossin et al., 1985). Its levels relative to total protein increase from late embryonic to early postnatal ages, followed by a decrease in the adult (Chuong and Edelman, 1984; Linnemann and Bock, 1986). Similar changes in N-CAM concentration are observed in monolayer cultures of rat cerebral and hippocampal cells during in vitro development (Bock et al., 1980; Krivko et al., 1993; Smith et al., 1993). N-CAM is expressed differentially by all major cell-types in the CNS. For instance, neurons are known to express mainly the 135 and 190 kd isoforms (Nybroe et al., 1989), astrocytes express mainly the 115 and 135 N-CAM isoforms (Noble et al., 1985), while oligodendrocytes express the 115 isoform (Bhat and Silberberg, 1986). Thus, the largest isoform of N-CAM is restricted to neurons in the CNS, and there is indication that its long cytoplasmic domain is associated with the cytoskeletal protein, spectrin (Pollerberg et al., 1987). Furthermore, this isoform is relatively poor at stimulating neurite outgrowth, and can be converted to the 135 kd isoform of N-CAM with a smaller cytoplasmic domain by intracellular protease activity (Doherty et al., 1992c). The

three brain N-CAM isoforms can be further modified posttranslationally by phosphorylation, sulphation and glycosylation (reviewed by Nybroe et al., 1988). In particular, changes in the polysialic acid (PSA) content of N-CAM appears to influence the adhesive function of N-CAM as well as other adhesion molecules, such as laminin and L1 (Doherty et al., 1990a; Acheson et al., 1991; Zhang et al., 1992). Specific enzymatic removal of PSA was shown to increase adhesion (Hoffman and Edelman, 1983; Rutishauser et al., 1988; Acheson et al., 1991). In contrast, its removal from N-CAM expressed on embryonic chick retinal cells inhibited N-CAM-dependent neurite outgrowth (Doherty et al., 1990a). This effect was partially reversed by anti-L1 antibodies (Zhang et al., 1992). It was suggested that PSA on neuronal N-CAM may favour neurite outgrowth by inhibiting stable clustering of CAMs (Doherty and Walsh, 1992). Another feature of N-CAM glycosylation is the presence of the carbohydrate epitope L2/HNK-1, which is found on 15-20% of the two larger isoforms of N-CAM (Kruse et al., 1984). Adhesion assays using antibodies have demonstrated that N-CAM in brain is involved in neuron-neuron, neuron-astrocyte, astrocyte-astrocyte and oligodendrocyte-oligodendrocyte adhesion (Keilhauer et al., 1985; Bhat and Silberberg, 1988).

N-cadherin. N-cadherin is an integral membrane glycoprotein with an apparent MW of 130 kd. N-cadherin, like other classical cadherins, is encoded by a single gene. It consists of eight potential glycosylation sites (Hatta et al., 1988). However, it is not known whether all or some of these glycosylation sites are glycosylated. N-cadherin was originally identified in embryonic chick neural retina (Grunwald et al., 1982). During early formation of the nervous system, the observed distribution of N-cadherin is very similar to that of N-CAM (Rutishauser, 1986; Takeichi, 1987).

N-cadherin later becomes restricted in its distribution. N-cadherin shows 74% amino-acid sequence homology with R-cadherin which was identified in chicken retina (Inuzuka et al., 1991). Weak heterophilic interactions between N- and R-cadherin have been suggested (Inuzuka et al., 1991; Redies and Takeichi, 1993). Studies of N-cadherin expression revealed that during development of the retina and brain, expression of N-cadherin is significantly down-regulated (Matsunaga et al., 1988; Lagunowich and Grunwald, 1989; Lagunowich et al., 1992) and may be mediated by multiple mechanisms (Roark et al., 1992). The down-regulation of N-cadherin in the retina, for instance, was shown to be due in part to an endogenous metalloproteolytic activity at the cell surface to release NCAD90, a soluble 90 kd amino-terminal fragment of N-cadherin corresponding to the extracellular domain (Roark et al., 1992). Furthermore, the apparent conversion of the 130 kd to the 90 kd form of N-cadherin was inhibited by the metalloprotease inhibitor 1,10-phenanthroline (Roark et al., 1992). These studies led to the conclusion that the 90 kd soluble form of N-cadherin was not generated de novo (Roark et al., 1992). This is in contrast to the soluble form of N-CAM that is generated de novo by differential gene splicing (Gower et al., 1988). Further studies have indicated that the purified NCAD90 retains biological activity and promotes retinal neuron adhesion and neurite outgrowth in a fashion similar to intact N-cadherin as shown previously (Bixby and Zhang, 1990; Paradies and Grunwald, 1993). These results suggest that the cytoplasmic domain may not be necessary for all cadherin functions. The adhesive interactions mediated by T-cadherin that also lacks a cytoplasmic domain (Ranscht and Dours-Zimmermann, 1991), further implicates a function for cadherins in the absence of direct cytoskeletal associations. In addition, recent evidence suggests that cadherins may regulate cell function through second

messenger systems that may be activated by other factors (Doherty et al., 1992a,d). For instance, exogenously added ganglioside GM1 was found to enhance N-cadherin stimulated neurite outgrowth from PC12 cells (Doherty et al., 1992a).

Laminin. Laminin was initially isolated and characterized from the mouse EHS tumor (Timpl et al., 1979). It is a cross-shaped heterotrimer made up of three disulfide-linked polypeptide chains designated as A (400 kd), B1 (225 kd) and B2 (205 kd). The A chain has three globular domains at the amino-terminal each separated by cysteine-rich EGF-like repeats, while the B chains contain two of each structure. In addition, unlike the B chains, the A chain has a large globular domain at the carboxyl end (Sasaki and Yamada, 1987; Sasaki et al., 1988). Laminin is the major ECM glycoprotein of the basement membranes. Other common components of basement membrane include collagen type IV, heparan sulfate proteoglycan and entactin/nidogen, all of which interact with laminin (Mercurio, 1990). Several biologically active sites have been localized to the laminin molecule using proteolytic fragments, antibodies and synthetic peptides. For example, active sites for cell adhesion and neurite outgrowth have been localized to a fragment at the carboxyl end of the molecule (Edgar et al., 1984; Engvall et al., 1986). Other active sites on laminin for cell adhesion have also been identified (Yamada and Kleinman, 1992). Cell surface receptors that mediate cell adhesion or neurite outgrowth by binding laminin include, a high-affinity 67 kd receptor, gangliosides, sulfatides, a family of galactoside-binding lectins, and several integrins (Mecham, 1991). At least eight different integrins have been reported to bind EHS laminin, in particular the β_1 subclass of integrins (Tryggvason, 1993). Laminin can be found distributed diffusely within the brain at early stages of development, and then decreases at later stages (Liesi, 1985;

Letourneau et al., 1988). In vitro studies support the involvement of laminin in cell survival, proliferation and differentiation. It is a potent inducer of neurite outgrowth from both CNS and PNS neurons, and this activity depends on their developmental age (Cohen et al., 1986; Hall et al., 1987; Tomaselli and Reichardt, 1989). Furthermore, laminin can mimic the effects of EGF and NGF (Edgar et al., 1984; Panayotou et al., 1989). Thus, laminin is a multifunctional molecule with sites that bind to other ECM molecules as well as to cells.

During the past few years, however, it has become clear that the protein structure of the EHS tumor laminin is not reflective of laminin found in all tissues. Specifically, the A chain of laminin is altered or missing during embryonic development (Klein et al., 1988) and in many cultured cells, including astrocytes (Kleinman et al., 1987; Liesi and Risteli, 1989). The identification of several novel chains that share structural similarities, has so far demonstrated the existence of at least five additional laminin isoforms, including S-laminin and merosin which are homologues of the B1 and A chains, respectively (Tryggvason, 1993). The functional role and the factors that affect laminin isoforms are only now beginning to emerge.

Thrombospondin. Thrombospondin (TSP) is a 450 kd trimeric glycoprotein of identical subunits. The subunits contain four different structural domains and are connected near the amino-terminus by disulfide linkages (Frazier, 1987). TSP was first found to be secreted by activated platelets (Lawler et al., 1978) and later by various cells in culture, including astrocytes (Asch et al., 1986). Like laminin, TSP is a multifunctional protein with the ability to interact with cell surfaces, Ca^{2+}, many matrix molecules including heparan sulfate proteoglycans, fibronectin, laminin, and collagen types I and V, and plasma proteins such as fibrinogen and plasminogen (reviewed by

Lawler, 1986). The nature of the interaction of TSP with cells are complex and controversial. TSP has been reported to serve both adhesive and anti-adhesive functions (Chiquet-Ehrismann, 1991). Of particular interest is the finding that TSP can promote adhesion and neurite outgrowth from several types of neurons mediated likely by integrins (Tuszynski et al., 1987; Neugebauer et al., 1991; O'Shea et al., 1991). This finding complements recent data indicating the presence of TSP in the developing mouse nervous system (O'Shea and Dixit, 1988; O'Shea et al., 1990). It is now known, however, that at least four structurally and genetically distinct TSP isoforms exist in mammals, and may subserve different functions (reviewed by Bornstein, 1992; Lawler et al., 1993).

Another molecule expressed on astrocytes, which has both adhesive and anti-adhesive functions, is the ECM glycoprotein tenascin (will be reviewed in section 1.5.2). Although tenascin has been reported to be a non-permissive substrate for CNS neurons (Faissner and Kruse, 1990; Crossin et al., 1990), studies have shown that it will promote neurite outgrowth if neurons are cultured on substrates that allow them to adhere (Wehrle and Chiquet, 1990). Furthermore, two distinct sites within the tenascin molecule were found to be responsible for the cell-binding and neurite outgrowth-promoting activity from neurons (Lochter et al., 1991). Whether tenascin mediates neurite outgrowth on monolayers of astrocytes, which are excellent substrates for cell adhesion, has not been established.

1.5.2 Molecules involved in neuronal migration. Functional assays in which antibodies were used to block neuron-glial interactions in vitro have been frequently used to define some of the ligands which bind migrating neurons to astroglial cells. Four molecules, namely astrotactin, AMOG, astrochondrin and tenascin/cytotactin that

were identified with this approach, are reviewed below. Antibodies against these molecules were shown to inhibit granule cell migration along cerebellar Bergmann glial fibers.

Astrotactin. Astrotactin is a glycoprotein expressed on cerebellar granule cells, and has an apparent MW of 100 kd (Edmondson et al., 1988). Fab fragments of astrotactin antibodies blocked granule cell membrane binding to glia, but had no effect on neuron-neuron adhesion (Stitt and Hatten, 1990). Furthermore, astrotactin was shown to function in heterotypic neuron-glial binding via a Ca^{2+}-independent mechanism (Stitt and Hatten, 1990). In vivo studies indicated that the expression of astrotactin is developmentally regulated in the mouse cerebellum. By Western blot analysis, low levels of astrotactin were seen in late embryonic period just prior to granule cell migration, as well as in the adult (Stitt et al., 1991). Astrotactin expression was increased to high levels in early postnatal cerebellum during which active neuronal migration occurs. Immunocytochemical studies also indicated astrotactin immunolabeling to be most intense in migrating neurons. Thus, the timing of astrotactin expression overlaps with the period of granule cell migration along the Bergmann glial fibers (Stitt et al., 1991). By video microscopy, in dissociated cerebellar cultures astrotactin Fab fragments blocked migration along Bergmann glia, while in a similar assay antibodies against N-CAM, L1, integrin β_1 and TAG-1 had no effect on migration (Fishell and Hatten, 1991). The receptor for astrotactin on Bergmann glia is not known. Furthermore, its relationship to other known cell adhesion molecules awaits microsequence data and molecular characterization.

Adhesion molecule on glia. AMOG is an astrocyte cell surface glycoprotein of MW 45-50 kd that is involved in Ca^{2+}-independent neuron to astrocyte, but not

astrocyte to astrocyte adhesion (Antonicek et al., 1987). Immunoaffinity-purified AMOG incorporated into lipid vesicles was shown to bind to subpopulations of neurons (Antonicek and Schachner, 1988), thus demonstrating a direct role of AMOG in adhesion. AMOG expresses the L3 carbohydrate epitope and is developmentally regulated (Antonicek et al., 1987). AMOG is expressed by glial cells during periods of granule cell migration, which was significantly inhibited by monoclonal AMOG antibodies (Antonicek et al., 1987). The cDNA sequence predicts AMOG to be a membrane glycoprotein anchored by a transmembrane segment and a small cytoplasmic tail (Gloor et al., 1990). However, it bears no similarity to either of the known families of CAMs. Mouse AMOG showed 40% amino-acid identity with the β subunit of rat brain Na,K-ATPase (Gloor et al., 1990). It was speculated that binding of AMOG on glial cells to its receptor on neurons may modulate Na,K-ATPase activity directly or indirectly. Thus, AMOG-mediated neuron-glial adhesion could be a new form of cell interaction in which cell recognition is followed by signal transduction, possibly mediated by ion channels (Gloor et al., 1990).

Astrochondrin. Astrochondrin is a recently characterized chondroitin sulfate proteoglycan of $\approx$ 500 kd containing three core protein moieties (260, 360 and 380 kd) that show considerable similarities in their proteolytic peptides (Streit et al., 1993), and therefore are structurally related to each other. It is not known, however, whether the three components are generated by posttranslational modifications or whether they are transcribed from different mRNAs. Additionally, this proteoglycan is expressed by astrocytes in vitro, but not by any other neural cell-type in the murine CNS (Streit et al., 1990), hence the name astrochondrin (Streit et al., 1993). Astrochondrin expresses the L2/HNK-1 and L5 carbohydrate epitopes (Streit et al., 1990) and is

developmentally regulated (Streit et al., 1993). By Western blot analysis, expression of astrochondrin was highest at the time of cerebellar granule cell migration and decreased to low levels in the adult. Antibody perturbation experiments indicated that astrochondrin might be involved in granule cell migration. Process formation of mature astrocytes on laminin and collagen type IV, but not fibronectin was also influenced by antibodies to astrochondrin in vitro (Streit et al., 1993), suggesting that astrochondrin is a ligand for laminin and collagen type IV, but not for fibronectin. This is consistent with the localization of astrochondrin in the basal lamina of meningeal and endothelial cells (Streit et al., 1993). Furthermore, the interactions of basal lamina ECM with astrochondrin were enhanced in the presence of Ca^{2+} and may be dependent on the L5 carbohydrate epitope (Streit et al., 1993).

Tenascin/cytotactin. Tenascin is an ECM glycoprotein first detected in the myotendinous junction (Chiquet and Fambrough, 1984). It was later found to be expressed by a variety of tissues including smooth muscle, lung, kidney, and brain (Grumet et al., 1985). Tenascin in the brain carries the L2/HNK-1 carbohydrate epitope. However, this epitope is absent from the non-neural form (Grumet et al., 1985; Hoffman et al., 1988). A number of structurally related isoforms of tenascin generated by differential splicing have been found with MW between 190 and 240 kd (Spring et al., 1989; Jones et al., 1989; Weller et al., 1991). Tenascin glycoproteins contain a cysteine-rich amino-terminal domain followed by three domains homologous to EGF, FN type III repeat (which contains the RGD peptide sequence), and β and τ chains of fibrinogen (Jones et al., 1988; Pearson et al., 1988; Gulcher et al., 1989). By electron microscopy, tenascin appears as a six-armed structure that is assembled by disulfide bridges at the amino-terminal end, and hence is also referred to as

hexabrachion (Erickson and Inglesias, 1984). In the nervous system, immunoreactivity for tenascin first appears between the neuroectoderm and the notochord (Crossin et al., 1986; Mackie et al., 1988). Furthermore, in vivo and in vitro experiments with amphibian embryos showed that tenascin is produced by the neuroectoderm itself in response to neural induction (Riou et al., 1988). In the developing CNS, tenascin exhibits site-restricted expression (Crossin et al., 1986). It is transiently expressed by immature astrocytes in the somatosensory barrels in the mouse cortex (Crossin et al., 1989; Steindler et al., 1989). Tenascin is also associated with radial glial cells as they guide the migration of neuronal precursor cells (Chuong et al., 1987). The functional roles of tenascin have been assayed in culture using neural (e.g. neural crest cells, neurons and astrocytes) and non-neural (e.g. fibroblasts) cell-types, with evidence for both inhibition and promotion of migration and neurite outgrowth. For instance, perturbation experiments with polyclonal antibodies have suggested that tenascin is involved in neuron-glial binding (Grumet et al.. 1985) and mediates granule cell migration along Bergmann glial fibers (Chuong et al., 1987). Tenascin has also been implicated in neural crest cell migration (Tan et al., 1987; Bronner-Fraser, 1988; Mackie et al., 1988). On the other hand, tenascin was found to be a repulsive substrate for CNS neurons (Faissner and Kruse, 1990) or neurites (Crossin et al., 1990), and inhibited the spreading of fibroblasts (Chiquet-Ehrismann et al., 1988)). Studies with fusion proteins have demonstrated that cell binding and inhibitory properties of tenascin are located on two distinct domains (Spring et al., 1989). Thus, tenascin appears to have multiple functions that may be a consequence of its interaction with different ligands. Although tenascin has been shown to bind to fibronectin with low affinity (Chiquet-Ehrismann et al., 1988), the other prominent ECM component

with which it binds is chondroitin sulfate proteoglycan (Hoffman and Edelman, 1987; Hoffman et al., 1988). Candidates for tenascin receptors on the cell surface have also been identified and include syndecan, a cell surface proteoglycan (Vainio et al., 1989) and integrins (Bourdon and Ruoslahti, 1989). The binding of the latter appears to be mediated by an RGD-dependent mechanism (Bourdon and Ruoslahti, 1989).

Besides the four adhesion molecules described above, there is some indirect evidence to support a role for laminin in neuronal migration. This evidence is based on the fact that laminin is expressed along the radial Bergmann glial fibers during the migratory phase of granule cells (Liesi, 1985; Liesi and Risteli, 1989). Moreover, cerebellar granule cells were shown to migrate rapidly on glass fibers coated with laminin, but less so on glass fibers coated with fibronectin and not at all on collagen (Fishman and Hatten, 1993). Antibody perturbation experiments suggested the migration on this purified laminin substratum to be mediated by integrin β_1 expressed on neurons (Fishman and Hatten, 1993). However, Hatten and her colleagues had previously shown using video microscopy that anti-integrin β_1 antibodies had no effect on neuronal migration along cerebellar astroglial processes (Fishell and Hatten, 1991). Furthermore, these authors reported laminin labeling to be restricted to meninges and blood vessels in the early postnatal cerebellar tissue, and argued that laminin may not be the primary guidance system in vivo because of the low level of EHS laminin expression on cerebellar glial fibers (Fishman and Hatten, 1993). Their findings, however, do not rule out the possibility that other forms of laminin specifically expressed in brain may function in neuronal migration along glial fibers. The availability of additional antibodies directed against glial forms of laminin will allow to test more directly the role of laminin in glial-guided neuronal migration.

1.6 Origin and role of leptomeninges

The meninges covering the CNS of adult mammals consist of three layers: the dura mater, the arachnoid mater, and the pia mater. The two inner coverings, i.e. the arachnoid and the pia, are together termed the leptomeninges. The latter adheres closely to the outer surface of the CNS, but is separated from it by a basal lamina. By electron microscopy, the cells of the pia are often indistinguishable from those of the arachnoid. In fact, both membranes are connected by numerous fine trabeculae traversing the subarachnoid space (Peters et al., 1991).

The embryological origin of the meninges is thought to be mesenchymal (Angelov and Vasilev, 1989); however, the concept of a mesodermal origin of the leptomeninges was debated for some time. In 1924, Harvey and Burr reported that both the pia and the arachnoid are in large part derived from certain ectodermal elements of the neural crest. Their observations were based on transplantation experiments on amphibian embryos in which portions of the CNS, with or without neural crest cells, were transplanted to other areas and observed for the development of the meninges over the neural transplant. Leptomeninges formed in only those embryos in which the neural crest cells had been transplanted. The development of the dura mater, on the other hand, was uneffected by the presence or absence of the neural crest (Harvey and Burr, 1924, 1926). Harvey and Burr concluded from these experiments that the dura probably developed from the mesenchyme, whereas the leptomeninges developed from the cells of the neural crest. Their view, however, received little support and was severely criticized by Louis Flexner (1929). Using similar tranplantation experiments, Flexner came to a totally different conclusion which contradicted the findings of Harvey and Burr. Flexner regarded the meninges as

entirely of mesodermal origin.

The exact origin of the leptomeninges has not yet been fully established. The current hypothesis is that these cells develop from the mesenchyme that may be derived from both mesoderm and neuroectoderm, with variations observed between species and with the level along the neuraxis (O'Rahilly and Müller, 1986). For instance, in birds, contribution from neural crest has been postulated in the development of the forebrain and midbrain leptomeninges, whereas those of the hindbrain and spinal cord have a purely mesodermal origin (LeLièvre, 1976). This is in contrast to leptomeningeal cell development in humans where cells of the neural crest take part in the formation of the pia mater of the occipital part of the hindbrain, and perhaps of the spinal cord (Sensenig, 1951; Gil and Ratto, 1973; O'Rahilly and Müller, 1986). Recently, a morphogenetic study of rat cranial meninges by light and electron microscopy indicated that all three meningeal layers develop from an embryonic network of connective tissue-forming cells (Angelov and Vasilev, 1989). These authors argued that some of the very early studies have suggested a neuroectodermal origin of the leptomeninges because the embryonic neuroectodermal and mesodermal cells are morphologically indistinguishable, and that their ultrastructural data obtained from before E15 demonstrates the connective tissue character of the prenatal rat leptomeningeal cells (Angelov and Vasilev, 1989).

Leptomeningeal cells have a flat, polygonal fibroblast-like appearance and synthesize collagen (Peters et al., 1991). Bundles of collagen fibers are found in the leptomeninges by electron microscopic studies, the amount of which increases after birth (Peters et al., 1991). Collagen fibers are not found in the early stages of leptomeningeal cell development (O'Rahilly and Müller, 1986; Angelov and Vasilev,

1989). Additionally, leptomeningeal cells and meningiomas express various ECM molecules in culture, including fibronectin, laminin, collagen types I, III and IV, and tenascin (Rutka et al., 1986; NG and Wong, 1993; Ajemian et al., 1994). These cells are also known to express the intermediate filament protein vimentin (Kartenbeck et al., 1984). On the other hand, various human meningiomas in culture have been found to display epithelial features, such as well-formed desmosomes (Kartenbeck et al., 1984) and epithelial membrane antigen (NG and Wong, 1993). This may explain the apparent dual (i.e. epithelial and mesenchymal) characteristics of meningiomas which are believed to be derived from the arachnoid (Scheithauer, 1990).

Leptomeningeal cells are known to have diverse functions. Their presence is necessary for the normal development of the CNS (Sievers et al., 1986; Abnet et al., 1991; Hartmann et al., 1992). In vivo experiments which involved the destruction of the meningeal cells by 6-hydroxydopamine (6-OHDA) over the cerebellum and medial cerebral hemisphere of newborn hamsters showed several morphogenetic defects of the CNS tissue resulting in abberent foliation of the cerebellum and abnormalities in the formation of the dentate gyrus, respectively (Doeberitz et al., 1986; Hartmann et al., 1992). Furthermore, this influence of meningeal cells occurred over a short critical ontogenetic period, after which the injection of 6-OHDA had no effect (Sievers et al., 1986; Hartmann et al., 1992). Sievers and his colleagues (1986) have suggested that the meningeal cells participate in CNS development both by stabilizing the ECM of the pial surface, and by organizing the glial scaffold and the lamination of the underlying cortex. There is also evidence to indicate that leptomeningeal cells or their extract can regulate the proliferation of both neuronal stem cells and glial cells (Baraket et al., 1981, 1982). In response to infection, trauma, or neoplastic infiltration,

leptomeningeal cells are also responsible for the extensive proliferation and secretion of basement membrane glycoproteins (Rutka et al., 1986; Russell and Rubinstein, 1989). Furthermore, the ECM glycoproteins produced by leptomeningeal cells in culture were shown to inhibit the growth of glioma cells, and induce their differentiation (Rutka et al., 1987). Whether leptomeningeal cells also inhibit proliferation and enhance differentiation of astrocytes in vivo after insults to the CNS or during scar formation is not known. However, leptomeningeal cells are known to participate in the formation of the new glia limitans (Berry et al., 1983; Reier et al., 1989), and were shown to influence astrocytic gap junctional coupling in vitro (Anders and Salopek, 1989). In addition, leptomeningeal cells are potential macrophages. The flattened polygonal cells of the arachnoid are capable of transformation into rounded cells, and engage in a phagocytic response to foreign antigen (Krisch et al., 1984). Leptomeninges may also function in the normal state as an effective physiological barrier, separating the cerebrospinal fluid from the surrounding milieu (Krisch et al., 1984).

Chapter 2: *Identification of a potentially novel cell adhesion molecule involved in neurite outgrowth on astrocytes*

2.1 Introduction

Cellular interactions in the course of neural development govern morphogenetic events, including cell migration and axonal growth. Such events are in part regulated by the spatiotemporal expression of specific adhesion molecules. Several adhesion molecules have been identified in neural tissue, and include both cell surface molecules and molecules secreted into the ECM (reviewed by Reichardt et al., 1989; Grumet, 1991; Rathjen, 1991). These adhesion molecules are mainly glycoproteins that mediate binding among neural cells via homophilic and/or heterophilic interactions. Cell surface adhesion molecules are broadly classified into at least three distinct gene families based on their structural similarities: the Ig superfamily (Williams and Barclay, 1988; Hunkapiller and Hood, 1989), the cadherin family (Takeichi, 1990; Suzuki et al., 1991), and the integrin family (Hynes, 1987, 1992).

In vivo and in vitro evidence suggest that neurons extend neurites by interacting with other neurites, with glia or ECM molecules. Different adhesion mechanisms operate during neurite growth on these various substrates (Tomaselli et al., 1986; Bixby and Jhabvala, 1990). On a cellular substrate, neurite outgrowth is likely to be regulated by a combination of various CAMs and their neuronal receptors. In vitro studies have shown that astrocytes (Noble et al., 1984; Fallon, 1985a,b; David, 1988; Neugebauer et al., 1988; Tomaselli et al., 1988), unlike oligodendrocytes (Schwab and Caroni, 1988), promote neurite outgrowth from various types of neurons. Several adhesion mechanisms have thus far been identified that promote neurite growth on astrocytes. These include N-cadherin, N-CAM, and integrin-mediated interactions

(Tomaselli and Reichardt, 1989). The functional importance of these interactions depends upon the type of neurons and their developmental stage (Cohen et al., 1986; Neugebauer et al., 1988, 1991; Tomaselli et al., 1988).

In order to identify other adhesion molecules on astrocytes that mediate neuron-astrocyte interactions, the hybridoma technology was utilized to generate function blocking monoclonal antibodies. Using this approach, a MAb 1A1 was generated which recognizes a potentially novel cell surface molecule. In this chapter, I describe (i) the cell-type specificity of the 1A1 antigen, (ii) its biochemical characterization, and (iii) its involvement in adhesion and neurite outgrowth. In subsequent chapters, I will describe studies on the role of this antigen in neuronal migration (Chapter 3) and its expression and role in non-neural cells (Chapter 4).

2.2 Materials and Methods

Generation of the Monoclonal Antibody 1A1

Rat cerebral cortical astrocytes purified in culture were removed from the culture flasks using 5 mM EDTA and injected intraperitoneally into Balb/c mice (3-4 x 10^6 cells/mouse). After two further immunizations, animals were bled and the serum tested for neurite outgrowth inhibitory activity. Spleen cells from mice whose sera showed such inhibitory activity were then fused with mouse Sp2/O myeloma cells as described previously (Fazekas de ST. Groth and Scheidegger, 1980). Hybridoma supernatants were screened by an indirect immunofluorescence assay for cell surface reactivity to live astrocytes. Those showing positive immunoreactivity were retested in an in vitro assay (described later) for their ability to block neurite outgrowth on astrocyte monolayers. One such positive well was subcloned twice by limiting dilution

to generate the MAb 1A1. Immunoglobulins were isolated from ascites fluid and Fab fragments of purified IgG were prepared by papain digestion according to manufacturer's instructions (Pierce). The purity of whole IgG and Fab preparations was tested on 10% SDS-PAGE under reducing conditions. Purified IgG and their Fab fragments were dialyzed extensively, lyophilized, diluted in defined culture medium, and sterile filtered through 0.22-μm filters (Millipore) prior to their use in functional in vitro assays. The MAb 1A1 belongs to the IgG_1 subclass as determined using a mouse monoclonal typing kit (ICN).

Other Antibodies

Whole IgG and monovalent Fab fragments of MAbs JG22, 6C6, Ran-2, and 1A1 were used to study their effects on adhesion and neurite outgrowth on astrocyte monolayers. The first three antibodies purified from ascites fluid served as control antibodies. JG22, which belongs to the same IgG_1 subclass as 1A1, recognizes an epitope on the chick integrin β_1 subunit (Horwitz et al., 1984). It does not bind to rat tissues, and was used to control for non-specific binding. The MAb 6C6 binds to the surface of rat astrocytes (David and Crossfield-Kunze, 1985) but has no blocking effect on neurite outgrowth and neuron to astrocyte adhesion (Chuah et al., 1991). The MAb Ran-2 binds to a cell surface antigen on astrocytes (Bartlett et al., 1981).

The following five rabbit polyclonal antibodies and two mouse MAbs were used for immunocytochemical identification of various neural cell-types: (i) polyclonal anti-GFAP antiserum (diluted 1:1000, obtained from Dr. M.C. Raff, UCL, UK) was used to identify astrocytes; (ii) polyclonal antibody against fibronectin (FN, diluted 1:100, obtained from Dr. S. Carbonetto, McGill University) was used to identify leptomeningeal cells and fibroblasts; (iii) a polyclonal antibody to 2', 3'-cyclic

nucleotide-3'-phosphodiesterase (CNP, diluted 1:150, obtained from Dr. P. Braun, McGill University) was used to identify both immature and mature oligodendrocytes; (iv) a polyclonal antibody to S-100 (diluted 1:100) was used to identify Schwann cells; (v) anti-neurofilament (NF) polyclonal antibody (diluted 1:75, obtained from Dr. W. Mushynski, McGill University) or (vi) a MAb (RT97) directed against a phosphorylated epitope of the 200 kd subunit of neurofilaments (ascites diluted 1:1000, obtained from Dr. J.N. Wood, Sandoz, UK) were used to label neurons; and (vii) the MAb A2B5 (ascites diluted 1:1000) was used to identify process-bearing type-2 astrocytes (Raff et al., 1983a).

Western blot analysis of membrane extracts and immunoprecipitated 1A1 antigen was performed using (i) a rabbit polyclonal antibody generated against a cadherin synthetic peptide (FHLRAHAVDINGNQV) containing the cell adhesion recognition sequence HAV found in cadherins (diluted 1:200, obtained from Dr. O.W. Blaschuk, McGill University), and (ii) a mouse MAb directed against the HNK-1 carbohydrate epitope (diluted 1:200, obtained from Dr. R.J. Dunn, McGill University).

Cell Cultures

(i) *Primary cultures*: Dissociated cultures obtained from different regions (cerebral and cerebellar cortex, hippocampus, dorsal root ganglia, sciatic nerve, and meninges) of the neonatal Sprague-Dawley rat nervous system were used for this study. Dissociation was done as described previously (David, 1988). The tissue was cut into pieces and incubated with 0.125% trypsin (Sigma) for 20-30 minutes at 37°C. Collagenase (0.1%, Sigma) was added along with trypsin (0.125%) for dissociations of dorsal root ganglia and sciatic nerve. Cells were dissociated in the presence of trypsin inhibitor (0.05 mg/ml, Sigma) and DNAase (0.04 mg/ml, Sigma), collected by centrifugation,

and plated onto poly-L-lysine (PLL, Sigma) -coated round glass coverslips (13 mm diameter, Fisher Scientific) at 1-2 x 10^4 cells/cm^2. An additional step of centrifugation through a Percoll density gradient was performed to obtain enriched high density (10 x 10^4 cells/coverslip) cultures of cerebellar granule cells (Hatten, 1985), and plated onto laminin-coated glass coverslips. In addition, explants of newborn rat liver, kidney and spleen, and dissociated cultures of lung, skeletal and cardiac muscle, and aorta were plated onto laminin-coated glass coverslips. Dissociations of the latter tissues were done in 0.125% trypsin and 0.125% collagenase for 90 minutes at 37°C. Cultures were grown in Dulbecco's modified Eagle's medium (DMEM) containing 1% MEM-vitamins (Gibco), 1% penicillin/streptomycin (Gibco) and 10% fetal bovine serum (FBS, Gibco). Cultures were maintained in a moist atmosphere of 5% CO_2 at 37°C and used for immunocytochemistry at various days in vitro. Skin from 10 day chick embryos was dissected essentially as described above. The cells were cultured in flasks and used for immunoaffinity chromatography. For functional assays, 0.5-1 mM $CaCl_2$ was added during trypsinization. Cell viability was measured by Trypan blue dye exclusion.

(ii) *Secondary cultures*: Astrocytes were purified using a modification of the McCarthy and de Vellis (1980) method (Noble et al., 1984). Briefly, dissociated neonatal cerebral cortical cells were plated onto PLL-coated 75 cm^2 tissue culture flasks (Gibco). After 9-10 days, flasks were shaken overnight to remove the top cells. Attached cells were trypsinized and replated at one-third the original density and treated with 0.01 mM cytosine arabinoside (Sigma) for 7 days. These purified astrocytes (95-98%) were used as the immunogen to generate the MAbs as well as for biochemical analysis. Monolayer cultures of astrocytes plated onto PLL-coated glass

coverslips were used for functional assays.

Type-2 astrocytes were purified by shaking primary cultures of the cerebral cortex as described above. The detached cells were centrifuged and replated at a density of 10 x 10^4 cells per coverslip. These cultures were enriched in $A2B5^+/GFAP^+$ process-bearing type-2 astrocytes.

To purify oligodendrocytes (McCarthy and de Vellis, 1980), primary dissociated cerebral cortical cells were grown in 5% FBS instead of 10% FBS. The culture medium was changed to 1% FBS on the fifth day and allowed to grow to confluency. After ten days, flasks were shaken at 37°C for 16 hours in the presence of 5 mM L-leucine methyl ester HCl (ICN). Floating cells were removed, centrifuged, and resuspended in 10% FBS containing media. About 10 x 10^4 cells were plated onto PLL-coated coverslips.

Immunofluorescence Staining

Double Indirect immunofluorescence was carried out using cultures grown on coverslips. All incubations with antibodies were carried out for 30 minutes at room temperature. Cell surface labeling was examined by incubation of live cultures with 1A1 hybridoma supernatant or MAb A2B5. This was followed by incubating cultures with a rhodamine-conjugated goat anti-mouse IgG (G α MIgG-Rh; Cappel, 1:250). Cultures were then fixed and permeabilized with 5% acetic acid in ethanol for 20 minutes at -20°C. Double-labeling was performed to distinguish the various cell-types. Cultures were incubated with cell-type specific rabbit polyclonal antibodies, the binding of which was visualized by a fluorescein isothiocyanate-coupled secondary goat anti-rabbit IgG (Cappel, 1:400). Coverslips were mounted in bicarbonate buffered glycerol (pH 9.0) containing phenylenediamine (Sigma) and viewed with a Leitz

ortholux fluorescence microscope equipped with N_2 (rhodamine), L_2 (fluorescein) and D (Nuclear yellow) filters. No staining was observed in control cultures incubated with 1:100 rabbit non-immune serum or Sp2/O ascites fluid (diluted 1:1000). All antibody dilutions were made in minimal Eagle's medium with 0.02 M HEPES buffer (MEM-H) containing 1% normal goat serum (NGS).

Biosynthetic Labeling of Proteins

Labeling with [^{35}S]methionine was carried out using modifications of previously described protocols (Faissner et al., 1985). Monolayer cultures of astrocytes or rat C6 glioma cells were washed twice with 10 ml of HMCF buffer (150 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 5 mM glucose, 10 mM HEPES, pH 7.4) and incubated in methionine-free RPMI containing 2% dialyzed FBS for 1 hour (pre-incubation period). When tunicamycin (Sigma) was used in the experiments, it was added to the medium during the pre-incubation period at a final concentration of 1.5 μg/ml. After the pre-incubation period, cultures were labeled for 7 hours with 50 μCi/ml [^{35}S]methionine (ICN). Subsequently, cultures were washed twice with HMCF buffer containing 2mM cold methionine and then immediately detergent-solubilized. Detergent solubilization was carried out in 2 ml ice-cold solubilization buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.02% NaN_3, pH 7.6), containing 1% (vol/vol) aprotinin, soybean trypsin inhibitor (50 μg/ml), phenylmethylsulfonylfluoride and N-ethylmaleimide (both at 5 mM), and leupeptin and pepstatin (both at 1 μg/ml). Labeled proteins in the lysate were then incubated with pre-swollen Sepharose-protein A beads (Pharmacia) for 3 hours at 4°C with gentle shaking. For analysis of culture supernatants, cells were left to incubate for another 7 hours in 5 ml of fresh RPMI containing 2% FBS and 2 mM cold methionine following the labeling period. Labeled

proteins in the conditioned media were incubated with Sepharose-protein A beads. In both instances, pre-cleared supernatants were obtained and used for immunoprecipitation.

Cell Surface Labeling with ^{125}I

Lactoperoxidase-catalyzed radioiodination of cultured rat astrocyte or chick skin fibroblast surface proteins was carried out as described (Harlow and Lane, 1988). Briefly, cultures were washed and EDTA extracted (5mM) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution. Washed cells were resuspended in 1 ml of Dulbecco's phosphate-buffered saline (PBS) containing 50 mM KI and 10 mM glucose. To this cell suspension, 500 μCi of $Na^{125}I$ (Amersham) was added and allowed to stand at room temperature for 2-5 minutes. The reaction was initiated by adding 200 μl lactoperoxidase (0.2 mg/ml) and 50 μl glucose oxidase (0.2 mg/ml) in PBS. After 10 minutes, the reaction was stopped by adding 1 ml PBS containing 50 μM thiosulfate, followed by two washes in PBS containing 10 mM KI and three washes in PBS alone. Labeled cells were subsequently solubilized in ice-cold solubilization buffer containing protease inhibitors as described above. Cleared supernatant was subsequently used for immunoprecipitation or immunoaffinity chromatography.

Immunoprecipitation

NP-40 solubilized extracts from purified cultures of neonatal rat astrocytes and rat C6 gliomas were used for immunoprecipitation. One ml aliquots of [^{35}S]methionine or $Na^{125}I$-labeled proteins in solubilization buffer were mixed with 15 μg of MAb 1A1 or MAb JG22 for 2 hours. This was followed by an overnight incubation with 100 μl of Sepharose-protein A beads. Both steps were carried out at 4°C with shaking. Beads

were washed 7 times with NET buffer (150 mM NaCl, 50 mM Tris, 0.1% NP-40, 0.25% gelatin, 0.02% NaN_3, pH 7.6) and then resuspended in 30 µl SDS-sample buffer. Beads were boiled for 5 minutes, centrifuged, and supernatants stored at -70°C for SDS-PAGE analysis.

Immunoaffinity Chromatography

Purified MAb 1A1 or normal rat IgG were coupled to Affi-gel Hydrazide beads according to manufacturer's instructions (Bio-Rad) at a concentration of about 4 mg protein per ml of gel matrix. IgG from non-immune rat sera was used to control for non-specific binding. The antibody columns were stored in solubilization buffer without NP-40 at 4°C until use.

Immunoaffinity purification of the 1A1 antigen was carried out as described previously (Antonicek et al., 1987). Detergent-solubilized protein extracts prepared from cultures of ^{125}I-labeled rat astrocytes or chick skin fibroblasts, were first passed through the normal rat IgG Affi-gel Hydrazide column. The flow-through of this column was then added to the MAb 1A1 affinity column. Columns were washed twice with 100 ml of solubilization buffer containing low (150 mM NaCl) and high (400 mM NaCl) salt concentrations, followed by another wash with 50 ml of low salt buffer. Bound antigen was eluted with 0.2 mM glycine (pH 2.8), neutralized with 1 M Tris buffer (pH 11.5) and lyophilized after subjecting eluant to dialysis. The eluted antigen was analyzed by SDS-PAGE under both reducing and non-reducing conditions.

SDS-PAGE Analysis

Detergent-solubilized proteins and immunopurified antigen were analyzed by SDS-PAGE according to Laemmli (1970) with reagents from Bio-Rad Laboratories.

Samples were run in the presence or absence of reducing agent (0.1 M dithiothreitol, Sigma) on 7.5% acrylamide gel. Labeled proteins were visualized by autoradiography. Kodak X-ray films were exposed at -70°C and developed 3-4 days later. Molecular weights were estimated on the basis of the following markers: myosin, 200 kd; β-galactosidase, 116 kd; phosphorylase β, 97 kd; bovine serum albumin, 66 kd; and ovalbumin, 45 kd (Bio-Rad). To visualize standard proteins, gels were stained with Coomassie blue.

Immunoblot Analysis

After SDS-PAGE, portions of the gel were stained with a silver-staining procedure as described by Morrissey (1981). The remaining proteins were transferred electrophoretically onto nitrocellulose filters. Immunoreactive bands were visualized by the following procedure, all carried out in sequence at room temperature, unless indicated. The filters were washed with Tris-buffered saline (TBS, 25 mM Tris, 50 mM NaCl, pH 8.0) and blocked with 20% swine serum (Cedarlane) in TBS for 30 minutes. Incubation with rabbit polyclonal anti-cadherin antibody (diluted 1:200) or mouse monoclonal anti-HNK-1 antibody (diluted 1:200) for 1 hour was followed by an overnight incubation at 4°C. Filters were then washed with TBS containing 0.05% Tween-20 and incubated for 45 minutes with alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, 1:5000) or biotin-labeled goat anti-mouse IgG and IgM (Bio/Can Scientific, 1:30 000). In the latter case, filters were washed and incubated with avidin conjugated to alkaline phosphatase (Sigma, 1:3000) for 30 minutes. After subsequent washings, filters were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma) for 5-10 minutes. The filters were then dried and photographed. For controls, the nitrocellulose filters were probed with either rabbit

pre-immune sera (diluted 1:200) or Sp2/O ascites fluid (diluted 1:200) followed by incubation with secondary antibodies as described above. All antibody dilutions were made in TBS containing 20% swine serum.

Cell Adhesion Assay

(i) *Cell-cell adhesion*: A short-term adhesion assay was carried out using monolayer cultures of astrocytes or neurons as target cells. Probe cells which were either neurons or astrocytes were labeled with 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethyl-indocarbocyanine perchlorate (DiI, 3 x 10^{-3} mM) for 2 hours at 37°C. Pure populations of cerebellar granule cells were obtained by centrifugation through a Percoll density gradient (Hatten, 1985). Flat type-1 astrocytes were purified from cerebral cortex as described above. Astrocytes were plated at a density of 2 x 10^4 onto PLL-coated 13 mm glass coverslips. These coverslips were used within a week when the astrocytes formed a fully confluent monolayer. Neurons were plated on similar coverslips at a density of 1.5 x 10^5 cells per coverslip so as to yield very high density cultures which were used the following day for the adhesion assay. These monolayers were washed 3 times with DMEM and treated with IgG or Fab fragments of MAb 1A1 or control MAbs for 15 minutes at 37°C prior to adding DiI-labeled probe cells at a density of 2 x 10^3 cells/cm^2 for astrocytes, and 5 x 10^3 cells/cm^2 for neurons. The coverslips were placed on scored Petri dishes in a total volume of 100 μl of tissue culture medium per coverslip. Probe and target cells were incubated for 90 minutes at 37°C. Coverslips were then washed 3 times by gently dipping in MEM-H and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 15 minutes. The coverslips were mounted on slides and the number of DiI-labeled single adherent cells in a given number of fields were counted under a

fluorescence microscope. Phase optics was also used to confirm that the DiI-labeled cells were attached to the cells of the monolayer and not directly to the coverslip.

(ii) *Cell-substratum adhesion*: To assess the effects of MAb 1A1 on the attachment of astrocytes to culture substrata coated with various ECM molecules, 24 well culture plates were used. PLL-coated 24 well plates were incubated overnight at 4°C with the various ECM molecules used at concentrations that were previously found to give maximal attachment of rat astrocytes (Tawil et al., 1990). Fibronectin was used at 50 μg/ml while laminin, collagen (type I), and PLL at 10 μg/ml each. After overnight incubation the dishes were rinsed 3 times with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, and 200 μl of medium containing no MAb or 1A1 MAb (100 μg/ml) in DMEM was added to wells. Cultures of purified astrocytes were washed, trypsinized, and gently triturated to obtain a suspension of single cells. An equal number of cells was added to wells and slowly shaken to distribute cells uniformly. Astrocytes were allowed to attach to the coated wells for 2 hours, washed extensively by aspirating medium, fixed, and visualized with Coomassie blue stain. The number of attached cells were counted in the same number of fields for each experiment using an inverted light microscope.

Neurite Outgrowth Assay

Dissociated hippocampal (E18) or cerebellar cortical (P1) cells were plated onto astrocyte monolayers at a density of 1×10^4 cells per coverslip in serum-free chemically defined medium (Bottenstein and Sato, 1979). These cultures were incubated for 18-20 hours at 37°C in a 5% CO_2 atmosphere in the absence or presence of antibodies. They were then fixed with 4% paraformaldehyde followed by 5% acetic acid in ethanol. Incubations were carried out as described before. Cultures were

labeled with anti-NF antibodies using the MAb RT97. This was followed by a second incubation with G α MIgG-Rh. These cultures were also stained with the fluorescent dye Nuclear yellow (0.001%, Hoechst S769121). The percentage of single neurons extending neurites greater than two cell body diameter in length was estimated using both fluorescence and phase optics. For the cerebellum, small neurons ($\approx$ 10 μm) were counted. These have been described previously to be granule cells (Altman, 1972).

2.3 Results

Monoclonal Antibody 1A1 was Generated using Cultured Rat Astrocytes

In order to identify neurite-outgrowth promoting molecules expressed on astrocytes, cultured astrocytes purified from the neonatal rat brain were used to immunize mice and the hybridoma technology used to generate monoclonal antibodies. Hybridoma supernatants were screened for cell surface labeling of molecules on astrocytes. Those antibodies that labeled astrocytes were then tested for their ability to interfere with neurite outgrowth from developing CNS neurons. In this chapter, the characterization of an adhesion molecule recognized by the MAb 1A1 is reported.

Monoclonal Antibody 1A1 Labels a Subpopulation of Non-neuronal Cells in Rat CNS

The MAb 1A1 binds poorly to tissue sections for immunohistochemical studies. Therefore the cell-type labeling with the antibody was done using dissociated cell cultures. The MAb 1A1 labeled the surface of flat epithelial-like cells in primary dissociated cultures of neonatal rat cerebral cortex (Figure 2.1, A). The majority of these $1A1^+$ cells were identified as astrocytes by their characteristic fibrillar staining

with anti-GFAP antibodies (Figure 2.1, B). The flat GFAP$^+$ astrocytes found in these cultures are likely to be type-1 astrocytes (Raff et al., 1983a). In short-term cerebral cortical cultures, not all flat type-1 astrocytes were 1A1$^+$. This was in contrast to long-term cultures (> 10 days) in which more than 97% of the flat GFAP$^+$ cells were 1A1$^+$. Likewise the majority of the cells ($\approx$ 95%) expressed the 1A1 antigen in secondary cultures of purified astrocytes (Figure 2.1, C and D). In CNS cultures, a distinct population of process-bearing astrocytes (type-2 astrocytes) is found that is derived from a different lineage than the flat type-1 astrocytes (Raff et al., 1983a; Levi et al., 1986) and which express polysialogangliosides recognized by the MAb A2B5 (Raff et al., 1983a). In contrast to the flat astrocytes, the process-bearing type-2 astrocytes purified from neonatal rat cerebral cortex did not express the 1A1 antigen (Figure 2.1, E and F) but were A2B5$^+$ (Figure 2.2). 1A1 immunoreactivity was also found to be restricted to the type-1 astrocyte lineage in explant cultures of the neonatal rat optic nerve and primary cultures of neonatal rat cerebellar cortex (data not shown). In addition to the flat astrocytes, elongated GFAP$^+$ astrocytes which resemble Bergmann glial cells in the cerebellum also showed intense surface labeling with MAb 1A1 (Figure 2.1, G and H). Oligodendrocytes, the other macroglial cell-type in the CNS, were 1A1$^-$ in short-term primary cultures and also in secondary cultures of oligodendrocytes purified from neonatal rat cerebral cortex (Figure 2.1, I and J).

Monoclonal antibody 1A1 did not bind to neurons in primary cerebellar cortical cultures. This was further confirmed by obtaining purified cerebellar granule cells by Percoll density gradient centrifugation. These neurons which were plated at high density were 1A1$^-$ (Figure 2.3, A, C and D). In these cultures, 1A1 immunoreactivity was associated only with astrocytes that make up less than 2% of the cells (Figure 2.3,

A-C). The type-1 astrocytes had a process-bearing morphology in these cultures because of the high density of neurons as reported previously (Hatten, 1985; Culican et al., 1990). Other CNS neurons from the cerebral cortex, retina, and hippocampus also did not express the 1A1 antigen. The MAb 1A1 also did not bind to neurons (Figure 2.4, A and B) or Schwann cells (Figure 2.4, C and D) in primary cultures of neonatal rat dorsal root ganglia or peripheral nerve.

The expression of 1A1 antigen was further examined in cultures enriched for leptomeningeal cells. These FN^+ cells in short-term cultures did not express the 1A1 antigen (Figure 2.5, A and B) and remained $1A1^-$ in long-term cultures as long as the cultures were non-confluent. The only cells that were $1A1^+$ in the non-confluent leptomeningeal cultures were the small number of astrocytes that were present (Figure 2.5, E-G). However, upon confluency leptomeningeal cells became $1A1^+$ (Figure 2.5, C and D). Skin fibroblasts also showed a similar type of regulation in the expression of the 1A1 antigen (data not shown).

The cellular distribution of 1A1 antigen in other non-neural tissues was also examined in explant or dissociated cell cultures by immunocytochemistry. Flat, FN^+ fibroblast-like cells in cultures of spleen and lung were found to be $1A1^+$ (Figure 2.6, A-D). These cells likely belong to the connective tissue capsule and septa found in these organs. A few $FN^+/1A1^+$ cells were also occasionally found in the liver and kidney. Figures 2.6E and 2.6F show an example of $1A1^-$ cells in the kidney. $1A1^+$ cells were not found in cultures of skeletal and cardiac muscle, or aorta (data not shown).

Monoclonal Antibody 1A1 Binds to a Cell Surface Molecule

Two lines of evidence suggest that the 1A1 antigen is found on the cell surface.

(i) Immunocytochemical labeling of unfixed cultures with MAb 1A1 is indicative of a cell surface molecule. The MAb 1A1 showed a punctate staining pattern on the surface of rat astrocytes (Figure 2.1, A, C and G) and leptomeninges (Figure 2.5, C). This type of punctate labeling is characteristic of many surface molecules, such as N-CAM, N-cadherin, and integrins (Noble et al., 1985; Tawil et al., 1990). (ii) The surface localization of 1A1 antigen was further confirmed by radioiodinating live astrocytes with the lactoperoxidase method (discussed later). These experiments clearly indicated that the 1A1 antigen is a cell surface molecule.

Monoclonal Antibody 1A1 Interferes with Adhesion on Astrocytes

To establish whether the 1A1 antigen is an adhesion molecule, its involvement in neuron-astrocyte, and astrocyte-astrocyte adhesion was investigated using a short-term adhesion assay. Highly enriched populations of postnatal day 1 (P1) rat cerebellar granule cells and astrocytes from neonatal rat cerebral cortex were labeled with the fluorescent marker DiI and plated onto monolayer cultures of astrocytes. DiI-labeled neurons or astrocytes were allowed to adhere to astrocyte monolayers for 90 minutes with or without antibodies. The number of single cerebellar neurons attached to astrocyte monolayers in the presence of 1A1 antibodies (Fab fragments at 500 μg/ml) was inhibited by 27% as compared to cultures grown in the presence of control antibody 6C6 (Table 2.1). The MAb 6C6 binds to an astrocyte surface antigen (David and Crossfield-Kunze, 1985) and has been shown previously to have no effect on neurite growth on astrocytes (Chuah et al., 1991), but blocks astrocyte-astrocyte adhesion (unpublished observations). This antibody was therefore used as a control for all functional assays involving neuron-astrocyte interactions. Another antibody, the MAb JG22, was used to control for non-specific binding. JG22 is a chick-specific

MAb against the integrin β_1 subunit (Horwitz et al., 1984) and does not bind to rat tissue. The control antibody JG22 gave results similar to MAb 6C6 in the neuron-astrocyte adhesion experiments. Astrocyte-astrocyte adhesion was also inhibited by 1A1 antibodies (500 μg/ml) by 33% as compared to control cultures in the presence of MAb Ran-2 (Table 2.2). The MAb Ran-2 binds to a surface antigen on rat astrocytes (Bartlett et al., 1981). Neuron-neuron adhesion assayed using Percoll density purified P7 rat cerebellar granule neurons was unaffected by MAb 1A1 (Table 2.3), suggesting that the inhibition by MAb 1A1 in the previous experiments is specific. The blocking effect of MAb 1A1 on neuron-astrocyte and astrocyte-astrocyte adhesion was concentration dependent and required calcium during trypsinization of DiI-labeled probe cells. Although the receptor for 1A1 antigen appears to be sensitive to calcium, the 1A1 antigen itself was not, because labeling of astrocytes in suspension with MAb 1A1 was still observed immediately after trypsinization in the absence of calcium (Figure 2.7, A and B).

Monoclonal Antibody 1A1 Does Not Inhibit Attachment of Astrocytes to ECM Molecules

In order to exclude the possibility that the adhesion to astrocytes mediated by 1A1 antigen is not via ECM molecules or their receptors, the effects of the MAb 1A1 were examined on the attachment of astrocytes to laminin, fibronectin, and collagen (type I). The MAb 1A1 had no effect on the attachment of astrocytes to these substrata (Table 2.4). The MAb 3A3 which recognizes the $\alpha_1\beta_1$ integrin inhibited attachment to laminin and collagen (data not shown) as reported previously (Tawil et al., 1990).

Monoclonal Antibody 1A1 Inhibits Neurite Growth on Astrocytes

Affinity-purified IgG or Fab fragments of 1A1 antibodies were further tested for their

effects on neurite outgrowth from two different populations of rat CNS neurons on monolayers of astrocytes. E18 hippocampal and P1 cerebellar cortical cells were cultured for 18-20 hours on astrocyte monolayers in the absence or presence of MAbs, which included 1A1, 6C6, and JG22 antibodies. The presence of 1A1 antibodies (1 mg/ml) in the culture medium significantly decreased the number of neurons extending neurites greater than two cell body diameter in length (Figures 2.8 and 2.9). Neurite outgrowth from E18 hippocampal neurons was reduced by 29% with 1A1 Fab fragments as compared with control cultures (Figure 2.8). In experiments with P1 cerebellar cortex, the percentage of neurons bearing neurites was reduced by 30% with monovalent Fab fragments of 1A1 antibodies (Figure 2.9). There was no significant difference in the percentage of process-bearing neurons when cultures grown with control antibodies (6C6 and JG22) are compared to those cultured in the absence of antibodies (Figures 2.8 and 2.9).

Biochemical Analysis of 1A1 Antigen by Immunoprecipitation and Immunoaffinity Chromatography

Monoclonal antibody 1A1 recognizes a 135 kd band on SDS-PAGE. Immunoprecipitation and immunoaffinity chromatography were carried out to characterize this antigen because the MAb 1A1 does not recognize the antigen on Western blots. After metabolic labeling of purified rat astrocytes with [^{35}S]methionine, a prominent band at 135 kd was seen in immunoprecipitates obtained with MAb 1A1 under both non-reducing (Figure 2.10, A, lane 1) and reducing conditions. A 135 kd band was also immunoprecipitated with MAb 1A1 following ^{125}I-labeling of live cultured astrocytes (Figure 2.10, A, lane 3). A similar MW band was also immunoprecipitated from confluent leptomeningeal cultures labeled with [^{35}S]methionine (data shown in

Chapter 4). When [^{35}S]methionine-labeling was performed in the presence of tunicamycin, there was a reduction in apparent MW from 135 to 128 kd (Figure 2.10, B, lanes 1 and 2), indicating that the 1A1 antigen contains N-glycosidically linked sugar moieties accounting for about 5% of its weight. To examine whether the 1A1 antigen is found in culture supernatants of purified astrocytes, cultures were pulse-labeled with [^{35}S]methionine and the labeled proteins in the conditioned media were analyzed 7 hours later by immunoprecipitation. A doublet migrating at apparent MW of 100 and 110 kd was seen in immunoprecipitates obtained with MAb 1A1 (Figure 2.10, C, lane 1). Whether these bands represent the secreted form of 1A1 antigen or a degradation product by extracellular proteases is not known. In addition to astrocytes, several glial cell lines were screened for the expression of 1A1 antigen (Appendix 1). Of these cell lines, two showed 1A1$^+$ immunoreactivity (DI TNC$_1$ and C6 gliomas). The majority of rat C6 glioma cells were found to express the 1A1 antigen both by immunocytochemistry (Figure 2.11) and by immunoprecipitation following metabolic labeling with [^{35}S]methionine (Figure 2.10, D, lane 1).

The 1A1 antigen on cultured rat astrocytes was further analyzed by immunoaffinity chromatography. The eluted ^{125}I-labeled fraction from the 1A1 affinity column showed a prominent band migrating at approximately 135 kd under both reducing (Figure 2.12, lane 1) and non-reducing conditions. This band was not seen on SDS-PAGE when ^{125}I-labeled confluent cultures of 1A1$^-$ chick skin fibroblasts were used as control cells (Figure 2.12, lane 3) or when eluants from normal rat IgG affinity column were analyzed (Figure 2.12, lanes 2 and 4).

Relationship of 1A1 antigen to other cell adhesion molecules. To determine whether the 1A1 antigen belongs to the cadherin family or possesses the HNK-1

carbohydrate epitope, the immunopurified protein obtained from solubilized rat C6 glioma cells (Figure 2.13, lane 1) was analyzed by Western blotting. A rabbit polyclonal antibody raised against a synthetic peptide containing the amino-acid sequence HAV, a highly conserved region in most cadherins, recognized a single band at approximately 130 kd in extracts of C6 gliomas (Figure 2.13, lane 2), but did not bind to the purified 1A1 antigen (Figure 2.13, lane 4). A mouse MAb against the HNK-1 epitope failed to react with Western blots of C6 glioma extracts, as well as with the purified 1A1 antigen (Figure 2.13, lanes 5 and 7). The anti-HNK-1 antibody however recognized antigens in extracts of neonatal rat brain (Figure 2.13, lane 8).

2.4 Discussion

This chapter describes the in vitro and biochemical characterization of a rat neural cell surface glycoprotein that is recognized by MAb 1A1. The 1A1 antigen appears to be distinct from other adhesion molecules expressed in the CNS (discussed below). Mouse and chick cells do not react with 1A1 antibodies, except for some labeling observed on 1-2% of MAC-1^+ cells in mouse CNS cultures (data not shown).

The 1A1 Antigen is a Cell Adhesion Molecule

In this study it was shown that the antigen recognized by the MAb 1A1 is involved in mediating neuron to astrocyte, and astrocyte to astrocyte adhesion. The MAb 1A1 induces a reduction of about 30% in both neuron-astrocyte (heterotypic) and astrocyte-astrocyte (homotypic) interactions. Astrocytes also appeared less flattened in the presence of 1A1 antibodies. Antibodies to N-CAM, J1, and AMOG have been shown previously to interfere with neuron to astrocyte adhesion by about 30% (Keilhauer et al., 1985), 50% (Kruse et al., 1985), and 25% (Antonicek et al., 1987), respectively.

In addition, N-CAM antibodies interfere with astrocyte to astrocyte adhesion by about 25% (Keilhauer et al., 1985). These adhesion assays were carried out with antibodies used at 1 mg/ml. The degree of inhibition by antibodies to these adhesion molecules are comparable to that obtained with the MAb 1A1. For instance, similar inhibition as anti-N-CAM antibodies is seen with 1A1 Fab fragments in neuron-astrocyte (34% with 750 μg/ml) and in astrocyte-astrocyte (25% with 250 μg/ml) adhesion. Cell-cell adhesion is important in histogenesis and may regulate many developmental events in the nervous system, such as neuronal migration and axon elongation. In addition to the flat astrocytes, the 1A1 antigen is expressed on radial Bergmann glia which support the migration of granule cells in the developing cerebellar cortex (Rakic, 1971; Stitt and Hatten, 1990). There is evidence that this molecule is developmentally regulated in vivo in the developing cerebellum and is involved in the adhesion and migration of neurons to Bergmann glia in vitro (data shown in Chapter 3). However, its cellular distribution in vivo is not known since the monoclonal antibody binds very poorly to tissue sections.

The 1A1 antigen is not expressed by neurons; therefore, it must be mediating neuron-astrocyte adhesion in vitro by a heterophilic-binding mechanism. The binding mechanisms involved in astrocyte-astrocyte adhesion by this molecule may be homophilic and/or heterophilic. Several CAMs have been identified that mediate both homophilic and heterophilic interactions (Grumet and Edelman, 1988; Lemmon et al., 1989; Kuhn et al., 1991; Mauro et al., 1992; Murray and Jensen, 1992). A significant inhibition in neuron-astrocyte adhesion with MAb 1A1 occurs only when neurons, which are $1A1^-$, are trypsinized in the presence of calcium, suggesting that the receptor for the 1A1 molecule on neurons is sensitive to trypsin in the absence of calcium.

However, 1A1 itself does not show such trypsin sensitivity. Tryptic dissociation of cells in the presence of calcium leaves the calcium-dependent adhesive mechanisms intact, while the calcium-independent ones become inactivated (Ueda and Takeichi, 1976). Therefore, the interaction between the 1A1 molecule and its receptor may involve a heterophilic calcium-dependent mechanism. There are several examples of calcium-dependent heterophilic interactions, such as those mediated by ICAM-1 (Staunton et al., 1990), VCAM-1 (Elices et al., 1990), PECAM-1 (Albelda et al., 1991), and Nr-CAM (Mauro et al., 1992).

The functional inhibition of the 1A1 molecule with antibodies was further shown to affect neurite outgrowth on astrocytes. In the presence of monovalent Fab fragments of MAb 1A1 (1 mg/ml) neurite outgrowth from developing CNS neurons on monolayers of astrocytes is inhibited by 30%. Neurite outgrowth on glial cells (astrocytes or Schwann cells) is mediated by a combination of different adhesion molecules (Bixby et al., 1988; Neugebauer et al., 1988; Tomaselli et al., 1988; Chuah et al., 1991). To date, three distinct adhesive mechanisms have been described that mediate neurite outgrowth from CNS neurons on cultured astrocytes. It was shown that N-cadherin, N-CAM, and integrins are important in process formation from retinal neurons on astrocytes (Neugebauer et al., 1988). Extracellular matrix glycoproteins that are expressed on astrocytes, such as laminin (Liesi et al., 1983) and thrombospondin (Asch et al., 1986), are known to regulate axonal growth via integrins (Neugebauer et al., 1988; Tomaselli et al., 1988; Neugebauer et al., 1991). The neuronal surface molecule L1 has also been shown to mediate neurite outgrowth on Müller glia (Drazba and Lemmon, 1990) and astrocytes (Smith et al., 1990; Chuah et al., 1991). The extent to which these adhesion molecules are involved in mediating

neurite outgrowth varies with the developmental age and type of neurons (Neugebauer et al., 1988; Tomaselli et al., 1988). The study reported in this chapter show that the astrocyte surface molecule recognized by the MAb 1A1, might be another candidate that can mediate neurite outgrowth from developing CNS neurons.

Relationship of 1A1 Antigen to Other Known Cell Adhesion Molecules

The comparison of the 1A1 molecule with other adhesion molecules indicate differences mainly in their cell-type distribution. Most of the known neural adhesion molecules are expressed on neurons, but not glia; these include L1/NILE (Salton et al., 1983; Rathjen and Schachner, 1984), Ng-CAM/8D9 (Grumet et al., 1984b; Lemmon and McLoon, 1986), astrotactin (Edmondson et al., 1988), contactin/F11/F3 (Ranscht, 1988; Gennarini et al., 1989a), TAG-1/axonin-1 (Dodd et al., 1988; Stoeckli et al., 1989), DM-GRASP/SC-1 (Tanaka and Obata, 1984; Burns et al., 1991), and Nr-CAM (Grumet et al., 1991). This is in contrast to the cellular distribution of 1A1 in the CNS, which is restricted to flat type-1 astrocytes and cells that resemble Bergmann glia. In addition to these cells which are derived from the neuroectoderm, the 1A1 antigen is also expressed by leptomeningeal cells and skin fibroblasts which are derived from the mesenchyme when these cells are grown to confluency. Fibronectin$^+$ fibroblast-like cells in confluent cultures of spleen and lung are also found to express the 1A1 antigen. These cells likely belong to the connective tissue capsule and septa found in these organs. A few FN$^+$/1A1$^+$ cells are also occasionally found in cultures of the liver and kidney. 1A1$^+$ cells are not found in cultures of skeletal and cardiac muscle, or aorta (data not shown).

Unlike 1A1, the MAb Ran-2 described previously by Bartlett et al. (1981) labels not only astrocytes and leptomeningeal cells but also Schwann cells (Bartlett et al.,

1981; Mirsky and Jessen, 1984), is trypsin sensitive (Bartlett et al., 1981), and does not block astrocyte to astrocyte adhesion. In addition, unlike MAb 1A1, Ran-2 binds to leptomeningeal cells at low plating density (unpublished observations). Therefore the MAbs Ran-2 and 1A1 are unlikely to recognize the same molecule. Recently, a glial cell adhesion molecule (G-CAM) was identified on rat astrocytes and oligodendrocytes (Geisert et al., 1991). 1A1 differs in its apparent MW (135 kd) from G-CAM which migrates at 106 kd on SDS-PAGE. In addition, unlike G-CAM, 1A1 is not expressed by oligodendrocytes. The astrocyte surface molecule, AMOG, described previously by Antonicek et al. (1987) also differs from 1A1 in its MW (45-50 kd). Other adhesion molecules, like ECM molecules and their integrin receptors, as well as N-CAM and cadherins, have a wider cellular distribution in the nervous system. The 1A1 molecule differs from these adhesion molecules in its selective distribution on astrocytes and leptomeningeal cells. There are several other lines of evidence to suggest that 1A1 is distinct from N-CAM, cadherins, and members of the integrin family of ECM receptors: (i) The 1A1 molecule is isolated from cells as a single MW band of 135 kd, in contrast to the 115, 135, and 190 kd molecular isoforms of N-CAM. Unlike 1A1, the 135 kd form of N-CAM is expressed by both neurons and astrocytes (Noble et al., 1985; Nybroe et al., 1986) and is heavily glycosylated (Cunningham et al., 1987). In addition, N-CAM as well as other members of the Ig superfamily express HNK-1 (Gennarini et al., 1989b; Jessell et al., 1990; Burns et al., 1991; Grumet et al., 1991; Kayyem et al., 1992), whereas 1A1 purified from C6 gliomas lacks this carbohydrate epitope. However, heterogeneity in the expression of the HNK-1 carbohydrate epitope has been previously observed for N-CAM, L1, MAG, and P_o such that only a proportion of each of these adhesion

molecules express HNK-1 (reviewed by Salzer and Colman, 1989). Although the HNK-1 epitope is absent in the 1A1 molecule isolated from C6 glioma cells, it is not known whether this carbohydrate epitope is present on a proportion of 1A1 molecules at different developmental stages in vivo. Therefore these results do not exclude the possibility that 1A1 may belong to the Ig superfamily. (ii) The 1A1 molecule differs from cadherins in three major respects: the absence of the HAV sequence, its binding mechanism, and its insensitivity to trypsin in the absence of calcium. The purified 1A1 antigen does not immunoblot with a polyclonal anti-cadherin antibody raised against a peptide derived from the EC1 domain of mouse N-cadherin, that contains the HAV cell adhesion recognition sequence (Blaschuk et al., 1990; Nose et al., 1990). This three amino-acid sequence is conserved in most cadherins (reviewed by Geiger and Ayalon, 1992) and shown to be involved in neurite outgrowth on astrocytes (Blaschuk et al., 1990; Chuah et al., 1991) as well as in other cadherin-dependent cell adhesion (Blaschuk et al., 1990). It is not known whether there are full length cadherins in the nervous system that lack the HAV sequence. However, a truncated form of cadherin (T-cadherin) that lacks the HAV sequence was recently isolated from the developing chick brain (Ranscht and Dours-Zimmermann, 1991). The 1A1 molecule (135 kd) differs from T-cadherin (95 kd) in its apparent MW on SDS-PAGE. Furthermore, cadherin-mediated adhesion is known to involve a homophilic-binding mechanism, although weak interactions among the members of the cadherin family of CAMs have been reported recently (Takeichi, 1990). The expression of 1A1 on astrocytes but not neurons suggests that this molecule mediates the binding between astrocytes and neurons by heterophilic mechanisms. Neuron-astrocyte adhesion mediated by 1A1 is unlikely to involve a heterophilic interaction between cadherins, since the 1A1 labeling

of astrocytes is not sensitive to trypsin in the absence of calcium. Taken together these observations suggest that the 1A1 molecule is different from the cadherin family of glycoproteins. (iii) 1A1 is not likely to be a member of the integrin family. Integrins are heterodimers made up of two distinct non-covalently associated α and β subunits. In several instances integrin α and β subunits have disulfide bonds which when reduced alter their mobilities on SDS-PAGE (Hemler, 1990; Hynes, 1987). Many of the α subunits yield two fragments under reducing conditions. Under such conditions, the β subunits migrate slower on SDS-PAGE. The single band immunopurified using MAb 1A1 from cultured astrocytes or C6 gliomas does not shift under reducing and non-reducing conditions, suggesting that 1A1 has no interchain disulfide bonds. In addition, the adhesion of astrocytes to laminin, fibronectin, and collagen (type I) substrata was not affected by MAb 1A1. Since proteins of the ECM mediate cell attachment and neurite growth by binding mainly to their integrin receptors, the above experiments show that MAb 1A1 may not be recognizing these ECM molecules or their integrin receptors.

In general, molecules of the ECM are large MW glycoproteins that are secreted and deposited on cells. However, the distinction between whether a molecule belongs to the cell surface or to the ECM is not always clear cut, since many of the CAMs may be expressed both on the cell surface and in the ECM. Secreted forms of N-CAM (Gower et al., 1988), L1 (Faissner et al., 1985), and axonin-1 (Ruegg et al., 1989) have been reported. Monoclonal antibody 1A1 immunoprecipitated a doublet from culture supernatants of purified astrocytes with a lower MW of 100-110 kd than that seen on the cell surface (135 kd). Whether the 1A1 molecule is also a secreted protein requires further analysis.

An attempt was made to purify the 1A1 molecule by immunoaffinity chromatography for further molecular characterization. Several cell lines were screened, and of these the rat C6 gliomas expressed the 1A1 antigen at high levels. C6 glioma tumors were raised in nude rats by injecting cells subcutaneously. Tumors were allowed to grow and membrane preparations obtained for immunoaffinity purification of the 1A1 antigen. Even though a high degree of purity of the 1A1 antigen was obtained on SDS-PAGE, the amount purified with the Affi-gel Hydrazide column was not sufficient for microsequencing.

Concluding remarks

The results presented in this chapter provide evidence for a cell surface glycoprotein that is found exclusively on flat astrocytes (type-1) and Bergmann glia (radial glia) in the rat CNS. It is also expressed by certain cells of mesenchymal origin such as leptomeningeal cells and fibroblasts. Furthermore, this surface molecule contributes to neuron-astrocyte and astrocyte-astrocyte adhesion and neurite outgrowth on astrocytes. These results suggest that the MAb 1A1 recognizes a molecule that appears to be different from other known cell adhesion molecules. Microsequence data and molecular characterization will help establish its relationship to other known adhesion molecules.

Figure 2.1 Glial cells in dissociated cell culture of neonatal rat cerebral and cerebellar cortex. Cultures double-labeled with MAb 1A1 (A, C, E, G and I), and polyclonal antibodies to GFAP (B, D, F and H) and CNP (J). A & B and G & H are primary cultures at 5 days in vitro from cerebral and cerebellar cortex, respectively. C-F, I and J are secondary cultures of purified glia from cerebral cortex. Punctate cell surface labeling with MAb 1A1 is observed only on flat type-1 astrocytes (A, C and E) and Bergmann glia (G). The intensity of 1A1 labeling varies between cells (C). The process-bearing type-2 astrocytes (E and F) and oligodendrocytes (I and J) are $1A1^-$. Although the process-bearing astrocytes in E are negative, the flat astrocyte in this field is $1A1^+$. Bar, 25 μm.

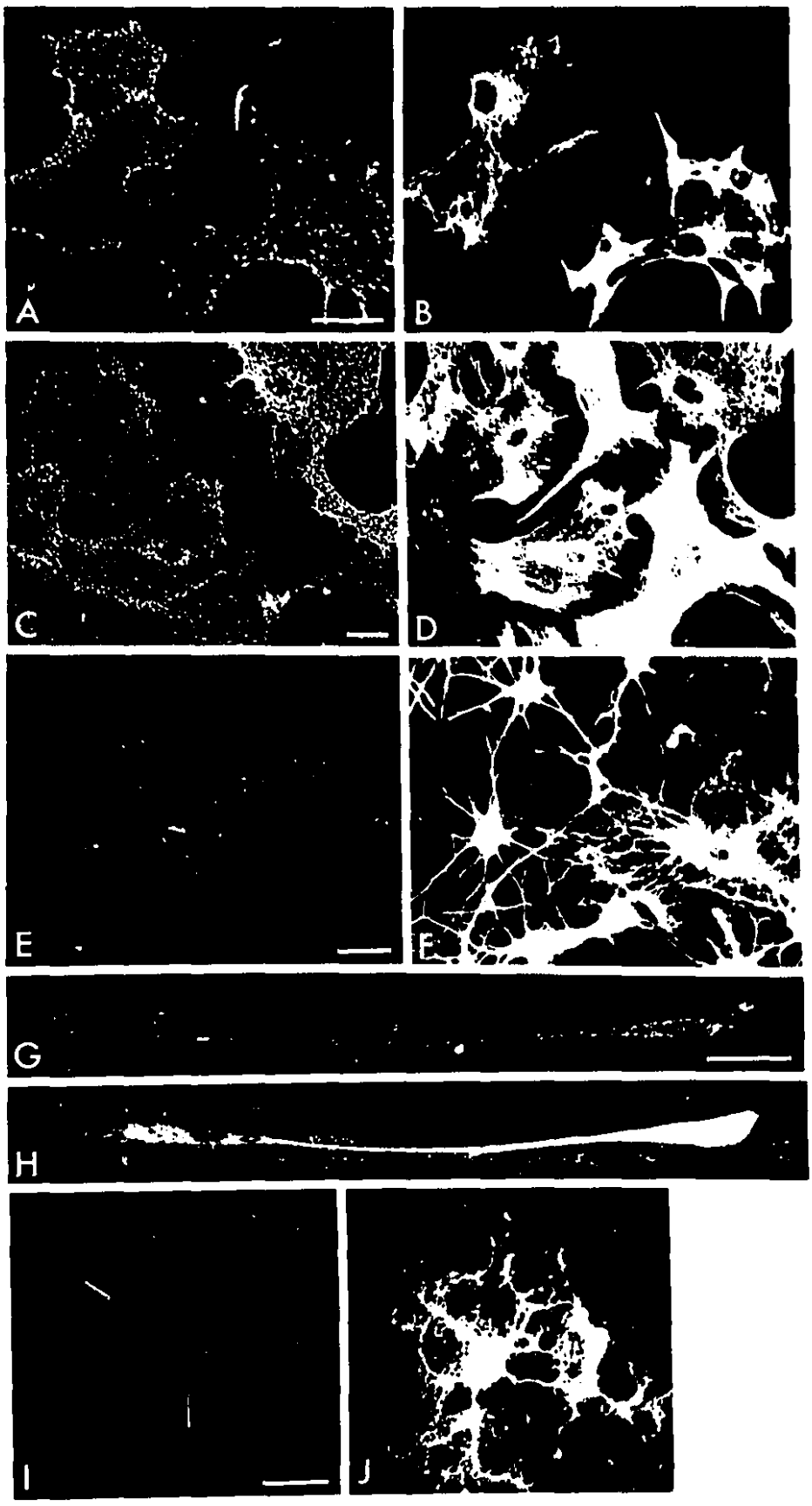

A
B
C
D
E
F
G
H
I
J

Figure 2.2 Secondary culture of purified process-bearing astrocytes from neonatal rat cerebral cortex stained with the MAb A2B5 to demonstrate that these GFAP$^+$ cells (Figure 2.1, F) are type-2 astrocytes. Bar, 25 μm.

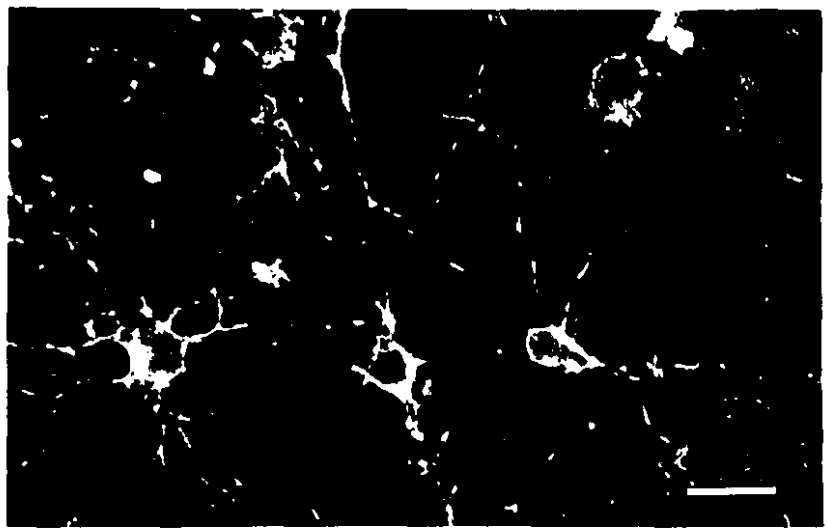


Figure 2.3 Double-immunofluorescence labeling of granule cells at 3 days in vitro purified from neonatal rat cerebellar cortex by Percoll density gradient centrifugation. Cultures labeled with MAb 1A1 (A), polyclonal anti-GFAP (B), Nuclear yellow (C), and anti-neurofilament RT97 (D). Neurons which comprise the majority of the cells are $1A1^-$ (A). The only cells that are $1A1^+$ in these cultures are the few $GFAP^+$ astrocytes (B) that are present. Nuclear yellow staining of the same field is shown (C) for easy visualization of the $GFAP^-$, $1A1^-$ neurons. Sister cultures were stained with anti-NF antibodies (D) to demonstrate that the small, $GFAP^-$ cells are neurons. Bar, 25 μm.

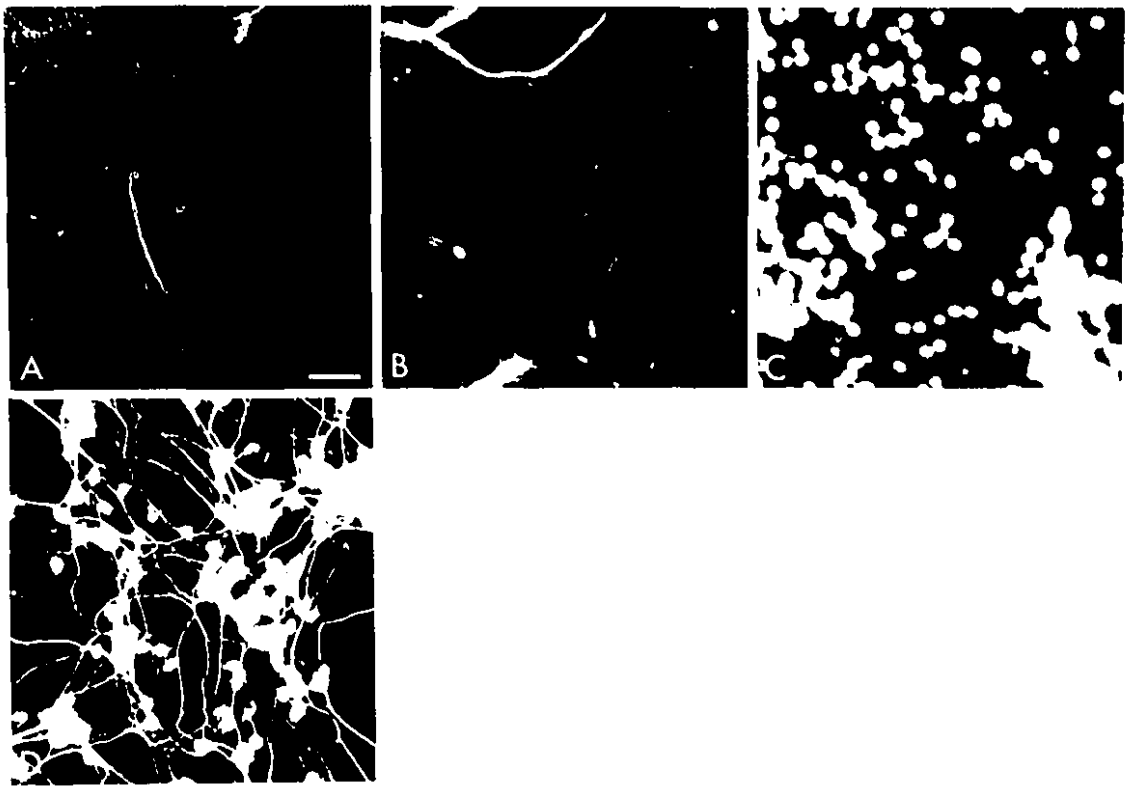

A
B
C

Figure 2.4 Dissociated cell culture of neonatal rat dorsal root ganglia (A and B) and sciatic nerve (C and D) at 7 days in vitro. Cultures double-labeled with MAb 1A1 (A and C), and polyclonal antibodies against neurofilament (B) and S-100 (D). The MAb 1A1 does not bind to peripheral neurons or Schwann cells in these cultures. Bar, 20 μm.

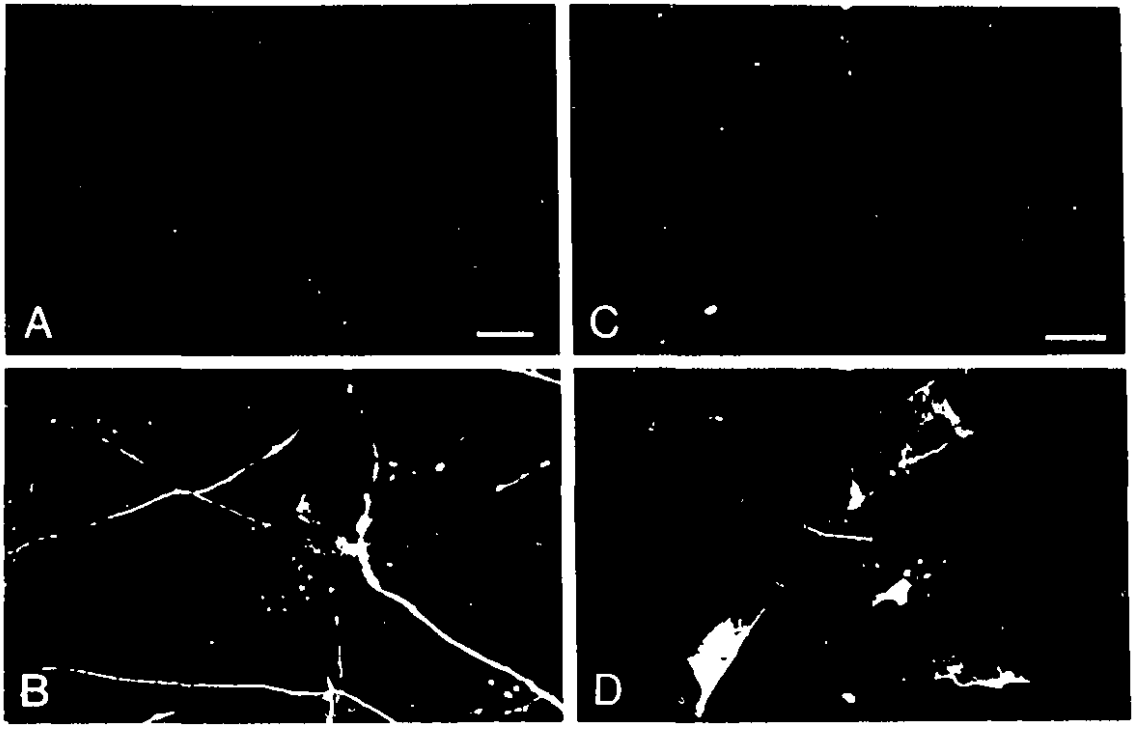

A
C
B
D

Figure 2.5 Immunofluorescence labeling with MAb 1A1 in primary non-confluent (A, B, E, F and G) and confluent (C and D) cultures of the leptomeninges from neonatal rat. In non-confluent leptomeningeal cultures at 4 days in vitro, 1A1 immunoreactivity is not seen (A and E) on FN^+ (B), $GFAP^-$ (F) leptomeningeal cells, but is seen only on $GFAP^+$ astrocytes (E and F). Figure G illustrates Nuclear yellow staining of cells shown in E and F. In confluent leptomeningeal cultures at 6 days in vitro, the FN^+ (D) leptomeningeal cells are $1A1^+$ (C). Bar, 25 μm.

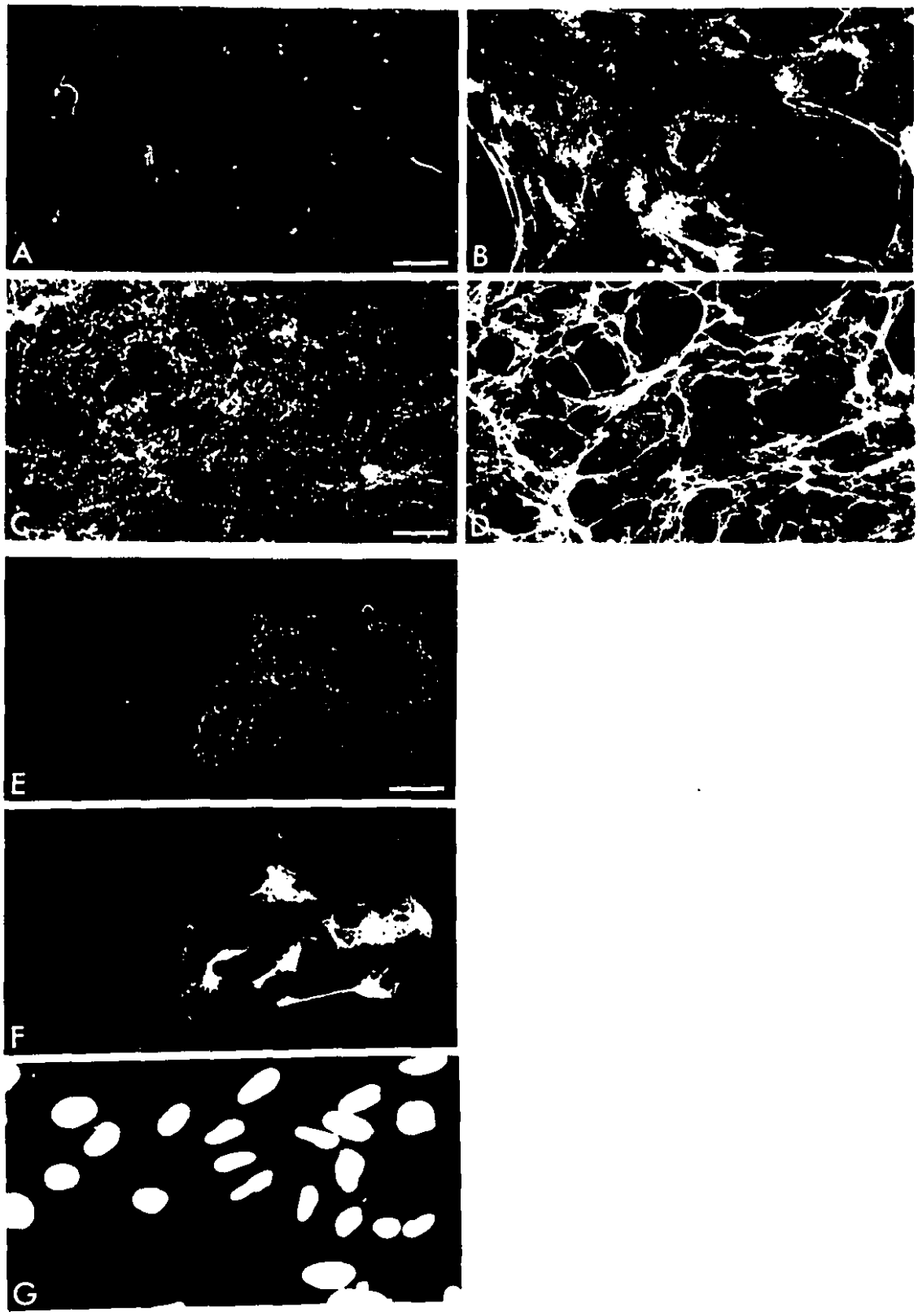

A
B
C
D
E
F
G

Figure 2.6 Immunofluorescence labeling with MAb 1A1 (A, C and E) and Nuclear yellow labeling of the same fields (B, D and F), of explant cultures of newborn rat spleen (A and B), and kidney (E and F), and a dissociated culture of the lung (C and D). $1A1^+$ cells are found in cultures of the spleen and lung. These cells are flat and FN^+ (data not shown), and are likely to be fibroblasts found in the capsule and connective tissue septa in these organs. The explants of the kidney are $1A1^-$ (E and F). The kidney capsule was removed prior to explantation. Bar, 25 μm.

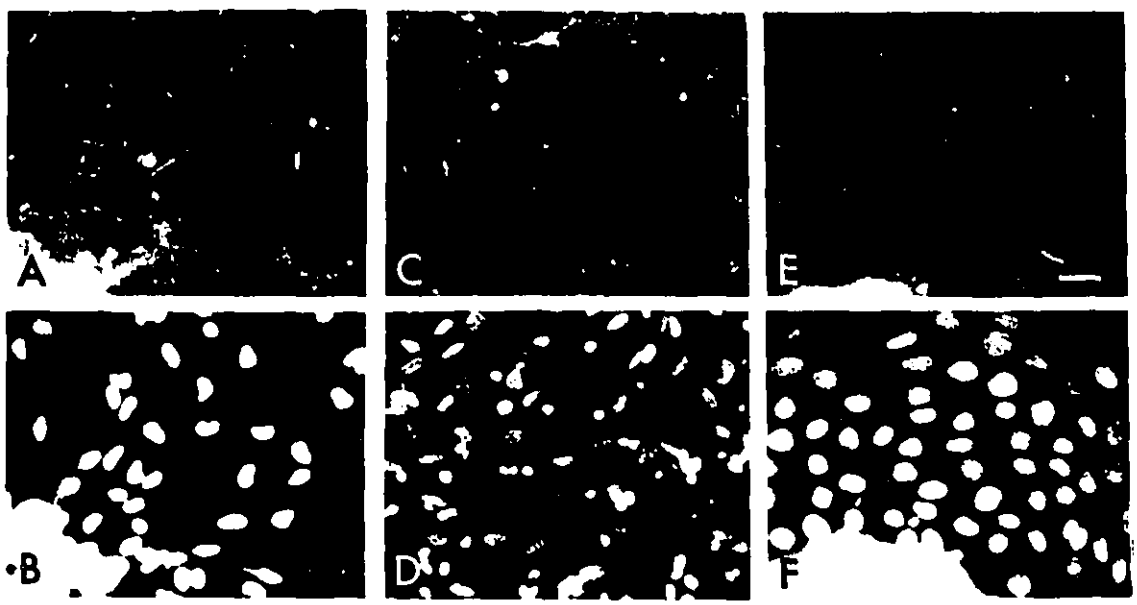

A
C
E
B
D
F

Figure 2.7 Purified flat type-1 astrocytes from neonatal rat cerebral cortex labeled in suspension with MAb 1A1 (A) and Nuclear yellow (B) following trypsinization. Bar, 20 μm.

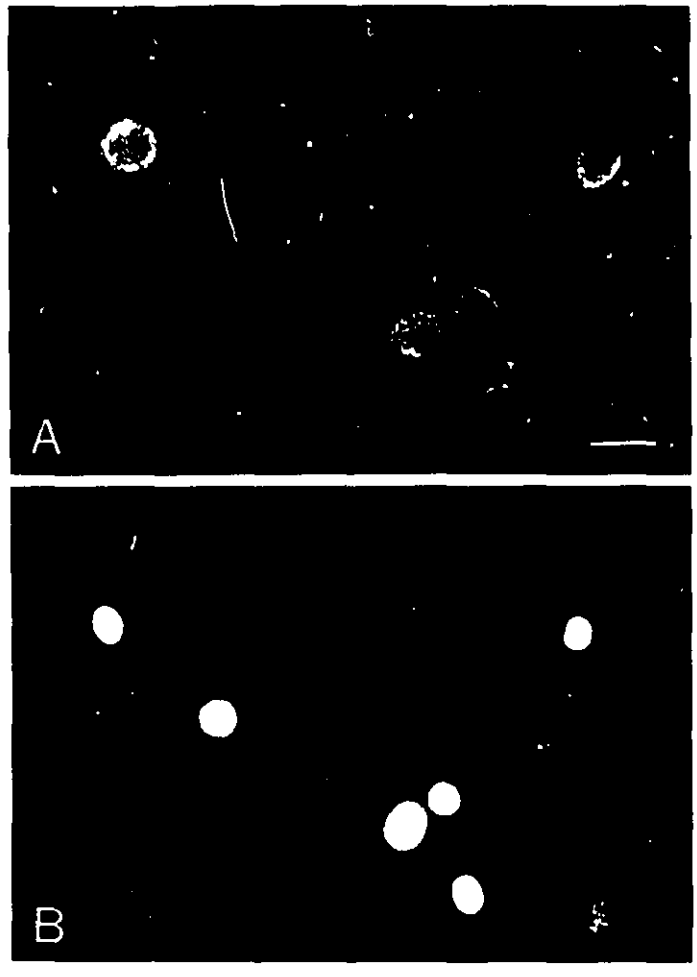

A
B

Figure 2.8 Percentage of hippocampal neurons extending neurites on monolayers of astrocytes. Hippocampal neurons from E18 rats were cultured for 18-20 hours on astrocytes in serum-free chemically defined medium with or without antibodies. Each value represents the mean ± SEM of 2-3 experiments. For each experiment 150-200 cells were counted on duplicate coverslips. A significant inhibition in neurite outgrowth is seen in cultures grown with 1A1 IgG or 1A1 Fab fragments as compared to control cultures (* 42% inhibition as compared with 6C6 IgG; ** 29% inhibition as compared with JG22 Fab fragments). Neurite outgrowth in cultures without antibodies are not significantly different from those cultured in the presence of control antibodies. Significance levels were determined using a Student's t-test; $^{*}p < 0.005$, $^{**}p < 0.01$

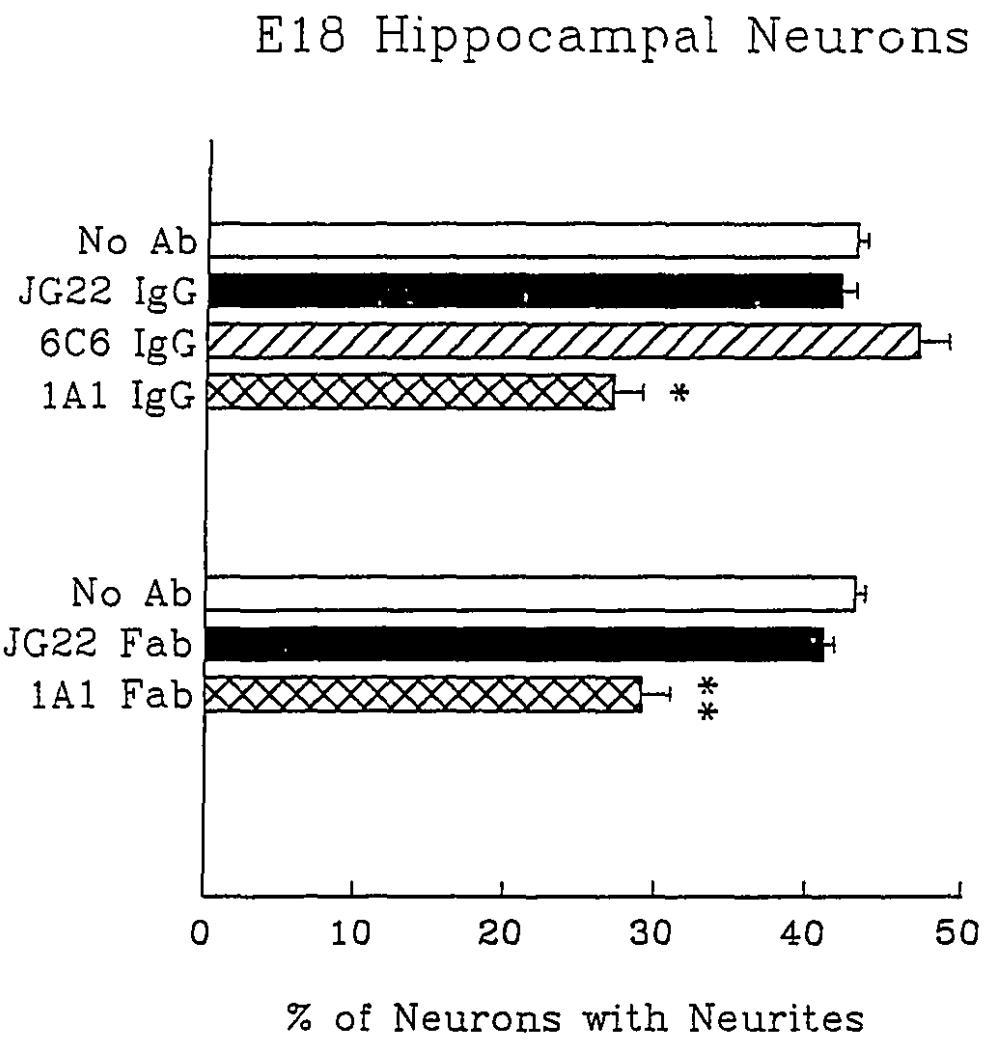

E18 Hippocampal Neurons
No Ab
JG22 IgG
6C6 IgG
1A1 IgG
*
No Ab
JG22 Fab
1A1 Fab
**
0
10
20
30
40
50
% of Neurons with Neurites

Figure 2.9 Percentage of cerebellar neurons extending neurites on monolayers of astrocytes. Cerebellar cortical cells from P1 rats were cultured on astrocytes for 18-20 hours in serum-free media in the presence or absence of antibodies. Each value represents the mean ± SEM of 2-4 experiments. 100-125 cells were counted on duplicate coverslips per experiment. A 30% reduction in neurite outgrowth is seen in cultures grown in the presence of 1A1 antibodies as compared to control 6C6 antibodies. Control cultures (6C6 or JG22) are not significantly different from those cultured without antibodies. Significance levels were determined using a Student's t-test; $^{*}p < 0.001$, $^{**}p < 0.01$

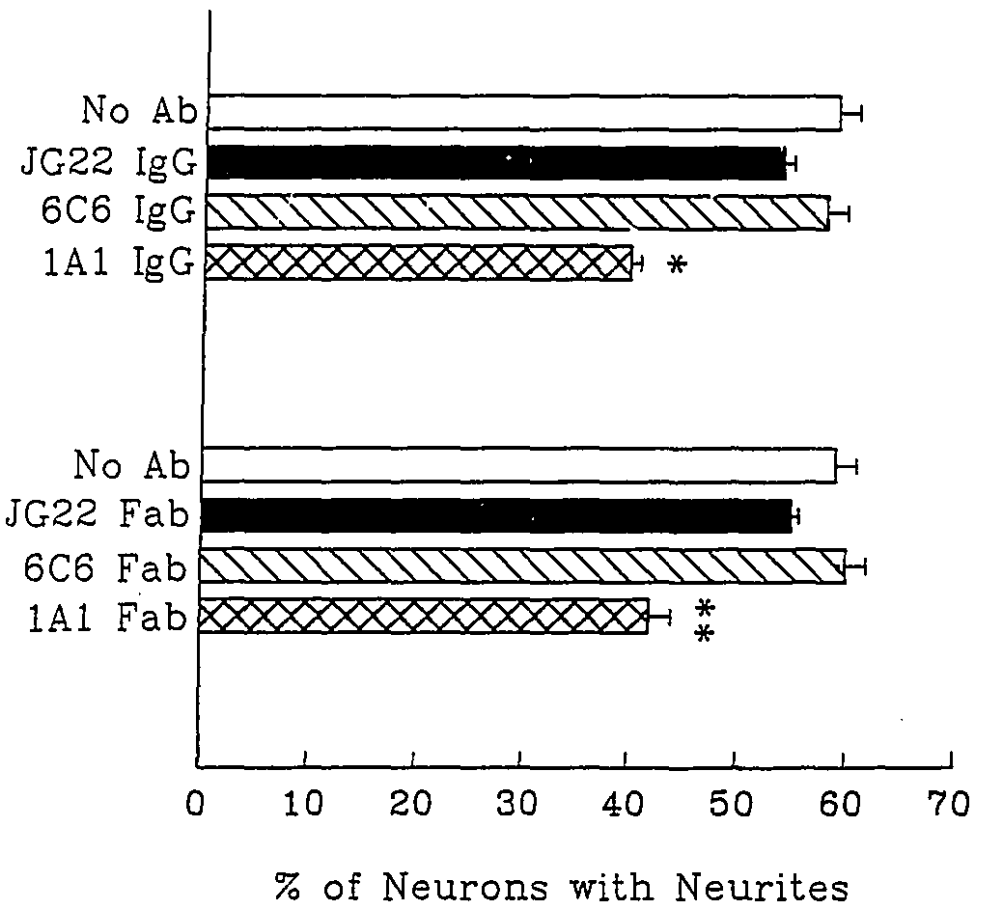

P1 Cerebellar Neurons
No Ab
JG22 IgG
6C6 IgG
1A1 IgG
*
No Ab
JG22 Fab
6C6 Fab
1A1 Fab
**
0
10
20
30
40
50
60
70
% of Neurons with Neurites

Figure 2.10 Autoradiographs of immunoprecipitates obtained with MAb 1A1 (A, lanes 1 and 3; B, lanes 1 and 2; C, lane 1; D, lane 1) and control MAb JG22 (A, lanes 2 and 4; B, lane 3; C, lane 2; D, lane 2) after non-reducing (A, B and C) and reducing (D) SDS-PAGE (7.5%). Molecular weight markers from top are: 200, 116, 97 and 66 kd.

(A) Immunoprecipitates after biosynthetic labeling with [^{35}S]methionine (lanes 1 and 2) and cell surface labeling with ^{125}I (lanes 3 and 4) of purified rat cultured astrocytes. The arrow shows the 135 kd specific band immunoprecipitated by MAb 1A1 (lanes 1 and 3).

(B) Immunoprecipitates from solubilized rat astrocytes after labeling with [^{35}S]methionine in the absence (lane 1) or presence of tunicamycin (lanes 2 and 3). The arrow shows the 135 kd band in lane 1. This band is reduced to 128 kd with tunicamycin-treatment of cultured astrocytes (lane 2). Lane 3 shows immunoprecipitates with control antibody.

(C) Immunoprecipitates from [^{35}S]methionine-labeled proteins in the conditioned media of purified rat astrocytes. Lane 1 shows a doublet at 100-110 kd immunoprecipitated by MAb 1A1.

(D) Immunoprecipitates from solubilized rat C6 glioma cells after metabolic labeling with [^{35}S]methionine. The arrow shows the 135 kd specific band immunoprecipitated by MAb 1A1. The faint bands seen in lane 1 are the non-specific bands.


A
1 2 3 4
C
1 2
B
1 2 3
D
1 2

Figure 2.11 Immunofluorescence labeling with MAb 1A1 (A) and Nuclear yellow (B) in a dissociated cell culture of rat C6 gliomas. The MAb 1A1 shows a punctate staining pattern similar to that seen on flat type-1 astrocytes. Bar, 25 μm.

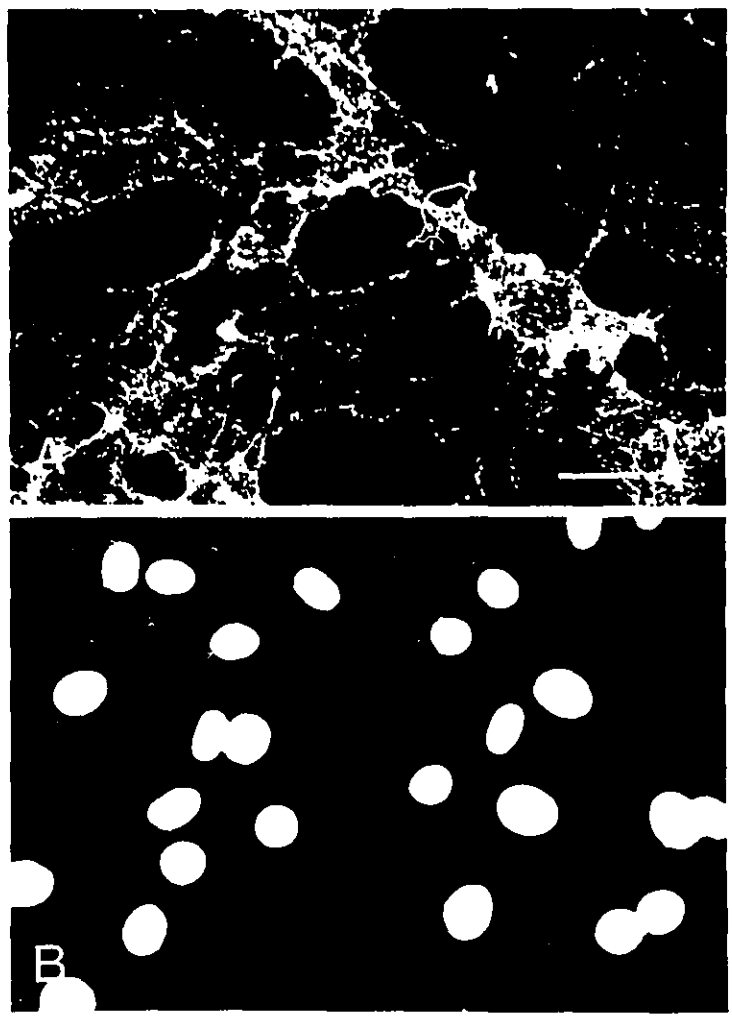

A
B

Figure 2.12 Autoradiographs of ^{125}I-labeled eluants from MAb 1A1 (lanes 1 and 3) and normal rat IgG (lanes 2 and 4) affinity columns using purified rat astrocytes (lanes 1 and 2) or chick skin fibroblasts (lanes 3 and 4) after reducing SDS-PAGE (7.5%). The immunoaffinity purified 1A1 antigen from extracts of rat astrocytes migrates at an apparent MW of 135 kd (lane 1). This band is absent from chick skin fibroblasts (lane 3) that were found to be 1A1$^-$ by immunocytochemistry (data not shown). The MAb 1A1 is rat specific. Molecular weight markers from top are: 200, 116, 97 and 66 kd.

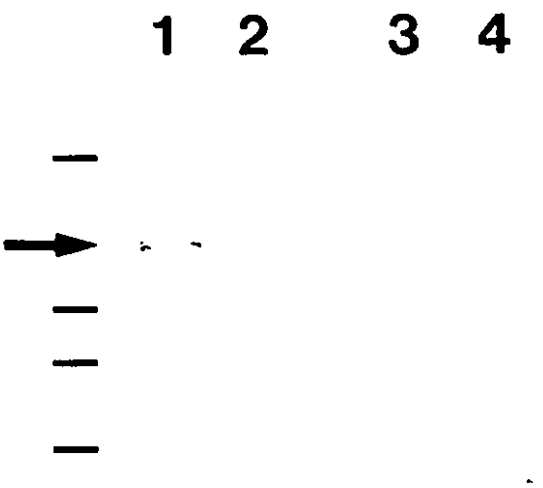

1 2 3 4

Figure 2.13 The 1A1 antigen immunopurified from C6 glioma cells visualized by silver-staining (lane 1), and Western blot analysis of C6 glioma extracts (lanes 2, 3, 5 and 6), of immunopurified 1A1 antigen (lanes 4 and 7), and of neonatal rat brain extracts (lane 8) after reducing SDS-PAGE (7.5%). Molecular weight markers from top are: 200, 116, 97 and 66 kd.

Lanes 2 to 4: Blots probed with either rabbit anti-cadherin antibodies (lanes 2 and 4) or rabbit pre-immune serum (lane 3). The anti-cadherin antibodies recognize a single band at approximately 130 kd in C6 glioma cell extract (lane 2) which is absent in control (lane 3). The immunopurified 1A1 antigen however is not recognized by anti-cadherin antibodies (lane 4).

Lanes 5 to 8: Blots probed with either mouse anti-HNK-1 antibodies (lanes 5, 7 and 8) or Sp2/O ascites fluid (lane 6). The anti-HNK-1 antibodies do not bind to extracts of C6 gliomas and immunopurified 1A1 antigen (lanes 5 and 7, respectively), but react with neonatal rat brain extracts (lane 8). Lane 6 shows the antibody control.

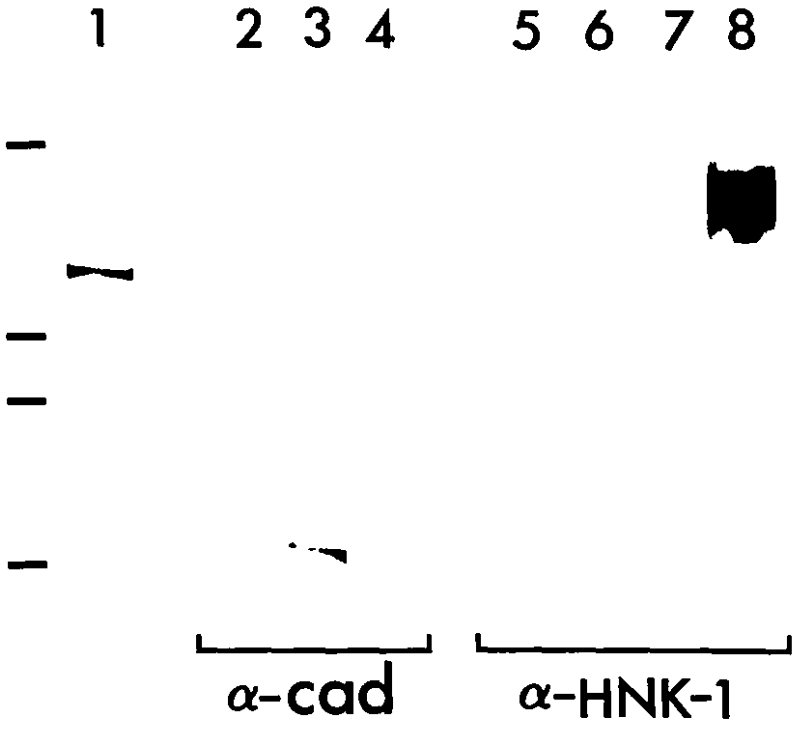

1
2 3 4
5 6 7 8
α-cad
α-HNK-1

Table 2.1 The number of single DiI-labeled neurons attached to astrocytes in a short-term adhesion assay with or without antibodies. In the presence of MAb 1A1, a significant reduction in the attachment of neurons to astrocyte monolayers is seen as compared to control cultures in the presence of MAb 6C6. Bottom results show no blocking effect of MAb 1A1 on adhesion when neurons are trypsinized in the absence of calcium. Cultures grown in the absence of antibodies are not significantly different from those cultured in the presence of control antibodies (6C6 or JG22). Numbers indicate mean values $\pm$ SD. The number of experiments is indicated in parentheses with counts made from duplicate coverslips. Student's t-test was used to assign significance levels; a = not significantly different from control cultures, b = $p < 0.02$, c = $p < 0.01$, d = $p < 0.001$. Percentage inhibition is the difference in cell attachment compared to 6C6 controls.

NEURON TO ASTROCYTE ADHESION

Antibody (μg/ml)	Number of single cells attached in 8 mm^2	Percentage of inhibition
None	179 ± 5 (3)[a]	
JG22 IgG (500 μg/ml)	173 ± 2.5 (3)	
6C6 IgG (500 μg/ml)	174 ± 6 (2)	
1A1 IgG (500 μg/ml)	122 ± 1.5 (2)[c]	30%
(250 μg/ml)	129 ± 8.5 (2)[b]	26%
JG22 Fab (500 μg/ml)	179 ± 2 (2)	
6C6 Fab (500 μg/ml)	175 ± 4 (3)	
1A1 Fab (750 μg/ml)	116 ± 6 (3)[d]	34%
(500 μg/ml)	128 ± 8.5 (2)[b]	27%
(250 μg/ml)	137 ± 2 (3)[d]	22%
(100 μg/ml)	166 ± 6 (2)[a]	5%
None	154 ± 3.5 (2)[a]	
6C6 Fab (750 μg/ml)	160 ± 3 (2)	
1A1 Fab (750 μg/ml)	153 ± 5 (2)[a]	4%

Table 2.2 DiI-labeled astrocytes plated onto monolayers of astrocytes for 90 minutes in the presence or absence of antibodies. A significant inhibition in astrocyte to astrocyte adhesion is seen in cultures grown with MAb 1A1 as well as 6C6 MAb compared to control cultures (Ran-2 or JG22). Numbers indicate mean values ± SD. The number of experiments is indicated in parentheses with counts made from two coverslips per experiment. Student's t-test was used to assign significance levels; a = not significantly different from control cultures, b = $p < 0.02$, c = $p < 0.01$, d = $p < 0.001$. Percentage inhibition is the difference in cell attachment compared to Ran-2 controls.

ASTROCYTE TO ASTROCYTE ADHESION

Antibody (μg/ml)	Number of single cells attached in 5 mm^2	Percentage of inhibition
None	161 ± 4 (4)[a]	
JG22 IgG (750 μg/ml)	151 ± 6.5 (3)	
Ran-2 IgG (500 μg/ml)	159 ± 7.5 (2)	
1A1 IgG (500 μg/ml)	103 ± 1.5 (2)[c]	35%
(250 μg/ml)	112 ± 8.5 (2)[b]	30%
(100 μg/ml)	118 ± 1 (2)[b]	26%
6C6 IgG (500 μg/ml)	113 ± 4 (2)[b]	29%
JG22 Fab (500 μg/ml)	150 ± 8 (2)	
Ran-2 Fab (500 μg/ml)	155 ± 6 (3)	
1A1 Fab (500 μg/ml)	104 ± 3 (2)[c]	33%
(250 μg/ml)	116 ± 4 (2)[c]	25%
(100 μg/ml)	120 ± 2.5 (3)[d]	23%
(10 μg/ml)	138 ± 5 (2)[a]	11%
6C6 Fab (500 μg/ml)	115 ± 5.5 (2)[c]	26%

Table 2.3 The attachment of single DiI-labeled neurons to monolayer cultures of cerebellar neurons in a 90 minute adhesion assay in the presence or absence of antibodies. Numbers indicate mean values ± SD. The number of experiments is indicated in parentheses with counts made from 2-3 coverslips per experiment. Student's t-test was used to assign significance levels; a = not significantly different from control cultures.

NEURON TO NEURON ADHESION

Antibody (μg/ml)	Number of single cells attached in 4 mm^2
None	78 $\pm$ 5.5 (2)
JG22 Fab (500 μg/ml)	69 $\pm$ 5 (2)
Ran-2 Fab (500 μg/ml)	80 $\pm$ 4 (2)
1A1 Fab (500 μg/ml)	73 $\pm$ 9 (2)[a]

Table 2.4 The attachment of purified astrocytes to poly-L-lysine (PLL; 10 μg/ml), laminin (10 μg/ml), fibronectin (50 μg/ml), and collagen (type I; 10 μg/ml) assessed in a 90 minute adhesion assay with and without MAb 1A1. Results from 3 separate experiments were normalized so that attachment to PLL in the absence of antibody is considered as 100%. The MAb 1A1 does not affect attachment of astrocytes to these substrata. Student's t-test was used to assign significance levels.

ATTACHMENT OF ASTROCYTES TO VARIOUS SUBSTRATA

Substratum	Percentage of attached cells as compared to poly-L-lysine control	
	Control (No MAb)	1A1 MAb (100 μg/ml)
Poly-L-lysine	100	101.5 $\pm$ 0.7
Laminin	116.6 $\pm$ 5.0	111.0 $\pm$ 6.3
Fibronectin	121.5 $\pm$ 6.9	119.6 $\pm$ 12.3
Collagen (type I)	105.3 $\pm$ 9.3	102.9 $\pm$ 6.5

Chapter 3: *The role of the IAI molecule in neuronal migration in the developing rat cerebellum*

3.1 Introduction

Neuronal migration occurs in many regions of the developing mammalian CNS, a feature that has been well documented (Ramón y Cajal, 1909; Rakic, 1971, 1972, 1978; Sidman and Rakic, 1973; Austin and Cepko, 1990; Hatten, 1990). Normal histogenesis of the CNS is dependent upon the precise migration of neurons during development, as evidenced in the abnormalities seen in certain neurological mutants (Rakic and Sidman, 1973a,b; Sotelo and Changeux, 1974; Caviness and Rakic, 1978; Hatten et al., 1986). In the cerebellum, granule cell precursors migrate along tangential paths from the most caudal portion of the cerebellar ventricular zone onto the surface of the cerebellum where they form the external granular layer (EGL) during the late embryonic period in rodents (Miale and Sidman, 1961; Altman and Bayer, 1978; Ryder and Cepko, 1992). This cellular migration required to form the EGL might be mediated at least in part via the ECM molecule fibronectin (Hatten et al., 1982; Price and Hynes, 1985). During the early postnatal period in rodents, granule cells in the proliferative EGL migrate down into the cerebellar cortex to form the internal granular layer (IGL) (reviewed by Rakic, 1982). Studies by Rakic (1971) describing contacts between neurons and glia from electron microscopic analysis provided some of the early evidence that granule cells may use the processes of radial Bergmann glia to migrate from the EGL to the IGL. Later, Hatten and her colleagues (Edmondson and Hatten, 1987; Fishell and Hatten, 1991; Rivas et al., 1992) reported the migration of cerebellar granule cells along Bergmann glia using video microscopy in dissociated cell cultures. Although the molecular mechanisms influencing granule

cell migration are not yet fully understood, it has become clear from various in vitro studies that a number of molecular mechanisms, i.e., adhesion molecules, ECM glycoproteins (reviewed by Chuong, 1990), plasminogen activator, and proteases (Moonen et al., 1982; Lindner et al., 1986a; Verrall and Seeds, 1988), may be involved in glial-guided neuronal migration.

In this study, evidence is provided for another cell surface molecule on Bergmann glia recognized by the MAb 1A1 that plays an important role in neuronal migration in the developing rat cerebellum. The MAb 1A1 binds exclusively to flat type-1 astrocytes and Bergmann glia, but not to neurons or other glia in rat CNS cultures (data shown in Chapter 2). This antibody however binds very poorly to tissue sections for immunohistochemical studies. Immunoprecipitation studies indicate that this MAb recognizes a glycoprotein with an apparent MW of 135 kd. Antibody perturbation experiments in which dissociated cerebellar or hippocampal neurons or astrocytes were plated onto monolayers of astrocytes demonstrate that this cell surface molecule is involved in neuron-astrocyte and astrocyte-astrocyte adhesion, as well as in neurite outgrowth on monolayers of astrocytes (Chapter 2).

In this study, immunocytochemical and immunoprecipitation data is provided on the developmental changes in 1A1 antigen expression in the postnatal rat cerebellum. In addition, two functional in vitro assays were employed to investigate the role of this molecule in neuron-Bergmann glial adhesion and neuronal migration.

3.2 Materials and Methods

Antibodies

Monoclonal antibodies 1A1 and 6C6 were used in all functional in vitro studies. Fab

fragments of these MAbs were prepared by proteolytic digest with papain as described in chapter 2. The specificity of these MAbs has been described previously (David and Crossfield-Kunze, 1985; Chuah et al., 1991; Chapter 2). Briefly, both MAbs are known to bind to different epitopes on the surface of astrocytes. The MAb 6C6 served as a control antibody in all functional assays, since it does not have an effect on neuron-astrocyte adhesion (Chapter 2). The MAb JG22, shown previously to be a chick-specific anti-integrin antibody against the β_1 subunit (Horwitz et al., 1984), was used as a control antibody in immunoprecipitation experiments.

Cell Cultures

(i) *Dissociated cultures*: Cerebellar cortex from Sprague-Dawley rats at P7 were dissociated into a single-cell suspension. The dissociation was carried out as described previously (David, 1988). Briefly, the cerebella, freed of all meninges and major blood vessels, were cut into pieces and incubated with 0.125% trypsin (Sigma) for 20 minutes at 37°C. The tissue was subsequently treated with trypsin inhibitor (0.05 mg/ml) and DNAase (0.04 mg/ml, both from Sigma). Dissociated cells were washed and plated on coverslips that were pre-treated with PLL (5 μg/ml, Sigma) at a density of 1.2-1.4 x 10^5 cells/coverslip (13 mm diameter, Fisher Scientific) in DMEM supplemented with 1% MEM-vitamin (Gibco), 1% penicillin/streptomycin (Gibco), 10% FBS (Gibco), and 10% horse serum (Gibco). After a 24 hour incubation period in a humid atmosphere of 5% CO_2 at 37°C, astroglial process extension and the association of neurons with radial glia were observed by immunocytochemistry.

For developmental changes in the expression of the 1A1 antigen in the developing rat cerebellum, 8-10 x 10^4 cerebellar cortical cells were plated onto PLL-coated glass coverslips as described above and used for MAb 1A1 immuno-

cytochemistry after 1 day in vitro. This culture period was required for the full morphological differentiation of the elongated astroglia.

(ii) *Explant cultures*: Cerebella taken from P7 rats were dissected, cleaned as described above, and cut into pieces. The tissue was forced through a metal mesh (pore size ≈ 700 μm) with the plunger of a 1 ml syringe. The tissue pieces were then washed 3 times in culture medium (DMEM containing 5% FBS) by sedimentation at room temperature in a 15 ml Falcon tube. The tissue pieces were transferred to a petri dish (35 mm diameter) containing media and plated onto PLL-coated glass coverslips (15 mm diameter) using a fire-polished pipette. Six to eight similar-sized explants of about 600-700 μm in diameter were placed on each coverslip. About 12 hours after plating, the media was changed to 5% FBS containing Fab fragments of MAbs 1A1 and 6C6, or no antibodies. The cultures were maintained in a 5% CO_2 incubator at 37°C for 3 additional days, after which they were fixed and processed for immunocytochemistry.

Immunocytochemistry

Cultures were labeled either with the MAb 1A1 or with antibodies against neurofilament proteins or GFAP (dissociated and explant cultures). Dissociated and explant cultures of the neonatal rat cerebellar cortex were fixed after 24 and 82 hours in vitro respectively, first with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 15 minutes at room temperature, followed by 5% acetic acid in ethanol for 30 minutes at -20°C. For 1A1 cell surface labeling, cultures were incubated with antibodies (MAb 1A1) prior to fixation. A monoclonal anti-NF antibody (RT97, diluted 1:1000, obtained from Dr. J.N. Wood, Sandoz, UK) was used to identify neurons and a polyclonal anti-GFAP antiserum (diluted 1:1000, obtained from Dr.

M.C. Raff, UCL, UK) was used to identify radial glia. All antibody incubations were carried out for 30 minutes at room temperature containing 1% NGS. The binding of MAbs was visualized with a rhodamine-conjugated goat anti-mouse IgG (Cappel, 1:200), while a fluorescein isothiocyanate-coupled goat anti-rabbit IgG (Cappel, 1:400) was used to visualize polyclonal antibodies. In the last antibody incubation step, the fluorescent dye Nuclear yellow (0.001%, Hoechst S769121) was added in order to visualize the nuclei. Stained cultures were mounted with glycerol in 0.1 M sodium bicarbonate buffer (pH 9.0) and viewed with a Leitz Ortholux fluorescence microscope equipped with N_2 (rhodamine), L_2 (fluorescein) and D (Nuclear yellow) filters.

Neuron-Bergmann Glial Binding Assay

P7 dissociated rat cerebellar cortical cells were plated in the presence or absence of antibodies at a density of 1.2-1.4 x 10^5 cells/coverslip. This plating density yielded an average of seven to eight neurons interacting with each Bergmann glia in control cultures, when examined after 24 hours in vitro. Following the 24 hour incubation, cultures were fixed and immunostained with anti-GFAP antiserum and Nuclear yellow, and the number of neurons attached to each Bergmann glia was estimated. Neurons were identified by immunostaining with anti-NF antibodies. In the developing rat cerebellum at this age, the majority (> 90%) of these neurons are likely to be small granule cells (< 10 μm diameter) as shown previously (Hatten, 1985, 1987). Other types of cerebellar cortical neurons include stellate cells, which are similar in size to granule cells; however, they are born later during development (Altman and Bayer, 1978). Similarly, it was ruled out that the counts of neurons on Bergmann glia comprise the large cerebellar cortical neurons, i.e., Purkinje cells and Golgi cells. The rest of the small pale and basket cells most likely constitute less than 5% of all

cerebellar cortical neurons at P7. Initially, quantification of cell cultures was done using both phase-contrast and fluorescence optics. Since no differences were found using these two techniques, subsequent counts were made using Nuclear yellow and anti-GFAP labeling. A Leitz camera lucida drawing tube was used to trace the length of $GFAP^+$ elongated Bergmann glial cells. The number of small, rounded cells contacting these Bergmann glia was estimated using the Nuclear yellow labeling. Between 60 and 150 radial glial cells were counted per experiment on two to three coverslips. The camera lucida drawings were then analyzed using a Zeiss IBAS-I image analysis system.

Granule Cell Migration Assay

Cerebellar microexplants from P7 rats were prepared as described above and grown in DMEM containing 5% FBS for 84 hours at 37°C in a humid 5% CO_2 atmosphere with or without antibodies. The cultures were topped every 24 hours with 25 μl of fresh medium containing appropriate antibodies. Later they were fixed and incubated with antibodies against GFAP, NF, and Nuclear yellow as described above. The extent of cell migration was estimated by counting the small neurons attached onto $GFAP^+$ cells in four areas located at right angles to each other at the periphery of each explant. Cell counts were done using a 40x objective lens, with a grid in the eye piece that measured an area of $\approx$ 0.025 mm^2. The area containing $GFAP^+$ cells and the number of neurons were estimated. The mean number of neurons was calculated for each explant from four readings. Twenty six to thirty five explants were evaluated in three separate experiments. Only explants of 600-700 μm were quantified. The percentage inhibition of neuronal migration was calculated as the mean number of neurons in explants of control cultures minus the mean number of neurons in cultures

treated with MAb 1A1 divided by the mean number of neurons in control cultures, multiplied by 100.

Immunoprecipitation and SDS-PAGE

NP-40 extracts of rat cerebellar tissue were obtained from various age groups (P1, P10, P20 and P35) and used for immunoprecipitation essentially as described in chapter 2. Briefly, 1 ml aliquots of crude membranes were diluted to a final concentration of 1 mg/ml in ice-cold solubilization buffer (150 mM NaCl, 20 mM Tris, 0.5% NP-40, 0.02% NaN_3, pH 7.6), containing 1% (vol/vol) aprotinin, soybean trypsin inhibitor (50 μg/ml), phenylmethylsulfonylfluoride and N-ethylmaleimide (both at 5 mM), and leupeptin and pepstatin (both at 1 μg/ml). Proteins were then incubated with pre-swollen Sepharose-protein A beads (Pharmacia) for 3 hours at 4°C with gentle shaking. To remove Sepharose-protein A beads, the samples were centrifuged for 2 minutes in an eppendorf microcentrifuge and pellets were discarded. The pre-cleared supernatants were mixed with 10 μg of MAb 1A1 or MAb JG22 for 2 hours, followed by an overnight incubation with 100 μl of Sepharose-protein A beads. Both steps were carried out at 4°C with shaking. Beads were washed 7 times with NET buffer (150 mM NaCl, 50 mM Tris, 0.1% NP-40, 0.25% gelatin, 0.02% NaN_3, pH 7.6) containing protease inhibitors, and then resuspended in 30 μl SDS-sample buffer containing 0.1 M dithiothreitol (Sigma). The samples were placed in a boiling bath for 5 minutes after which $1/10^{th}$ of each sample was loaded onto a 7.5% SDS-PAGE prepared by the method of Laemmli (1970). The rest of the samples were stored at -70°C. Gels were stained with a silver-staining procedure as described by Morrissey (1981). Molecular weights were estimated on the basis of the following markers: myosin, 200 kd; β-galactosidase, 116 kd; phosphorylase β, 97 kd; bovine serum

albumin, 66 kd; and ovalbumin, 45 kd (Biorad).

3.3 Results

Expression of 1A1 Antigen on Bergmann Glia

In primary dissociated cultures of neonatal rat cerebellar cortex, GFAP$^+$ slender, elongated cells showed intense surface labeling with MAb 1A1 (Figure 3.1, A and B). Based on their morphology, these astrocytes are likely to be Bergmann glia that mediate neuronal migration during development (Rakic, 1971; Hatten et al., 1984). Neurons in these cultures did not express 1A1 as reported in chapter 2.

Developmental Regulation of 1A1 Antigen in the Cerebellum

Immunoprecipitations with MAb 1A1 were carried out to analyze the changes in 1A1 antigen during postnatal development of the rat cerebellum, using membrane preparations of the cerebellum from P1, P10, P20 and P35 rats. Similar amounts of soluble protein concentrations of the membrane preparations were used for immunoprecipitations. A 135 kd band was seen in imimunoprecipitates obtained with MAb 1A1 in all four developmental age groups. Densitometric analysis of the gels indicated about a 2-fold increase between P1 and P20 followed by a decrease to the P1 levels at P35 (Figure 3.2). Whether this difference represents an actual change in the expression of 1A1 molecule on Bergmann glia or on the other flat type-1 astrocytes of the cerebellum cannot be known from these experiments. In order to address this question more directly, the number of 1A1$^+$ Bergmann glia was estimated in primary dissociated cultures of the rat cerebellar cortex at two developmental ages. Dissociated cerebellar cortical cultures were obtained from P1 and P7 rats, and labeled with MAb 1A1 24 hours later. This length of culture period was required to obtain glial cells

with a well-defined elongated morphology. About 33% of these Bergmann glia were $1A1^+$ at P1, as compared to 78% at P7 (Figure 3.3). Labeling of Bergmann glia in dissociated cultures of cerebella from older rats could not be studied because of the difficulty in obtaining good cell dissociation at the older postnatal ages.

The MAb 1A1 Inhibits Neuron-Bergmann Glial Binding in Dissociated Cultures

Since cell-cell adhesion is thought to play an important role during migration, the effect of the MAb 1A1 on the binding of neurons to Bergmann glia was assessed. Dissociated cerebellar cells from P7 rats were plated at various densities ranging from 0.5-2.0 x 10^5 cells per coverslip. A plating density of 1.2-1.4 x 10^5 cells resulted in an average of seven to eight neurons being associated with each Bergmann glial-like $GFAP^+$ cell in control cultures (see Figure 3.4). Fab fragments of MAbs 1A1 and 6C6 were added to such cultures at the time of plating, and their effect on neuron-glial binding was analyzed by immunocytochemistry after 24 hours. Neuron-glial interactions were quantitated by measuring the length of the $GFAP^+$ Bergmann glial cells by means of a camera lucida and by counting the number of neurons that were directly associated with these glia. The mean number of small cerebellar neurons interacting with the Bergmann glia decreased by $\approx$ 50% in the presence of 1A1 antibodies (Fab fragments at 500 μg/ml) as compared to control antibodies (Table 3.1 and Figure 3.5, A). Sister cultures were double-labeled with anti-NF antibodies to confirm the neuronal phenotype of the cells attached to the elongated astrocytes (data not shown). Based on their size and nuclear morphology, these $GFAP^-/NF^+$ cells are most likely to be granule cells which constitute the majority of the cerebellar neurons at this age (Altman, 1972; Hatten, 1985). Since the Bergmann glia vary in length considerably in these cultures, the number of neurons attached to a 100 μm length of

Bergmann glia was calculated. Using such an estimation, there was a 48% inhibition in the number of neurons attached to a 100 μm length of Bergmann glia in cultures grown in the presence of the MAb 1A1 (Table 3.1). In control cultures, the length of the Bergmann glia varied from about 50 to 200 μm. The inhibition of neuronal adhesion to Bergmann glia by MAb 1A1 occurred regardless of the size of the Bergmann glia (Figure 3.5, A). When the lengths of the Bergmann glia were plotted in a frequency histogram, it became evident that the MAb 1A1 induced an increase in the number of Bergmann glia shorter than 100 μm in length (Figure 3.5, B). These results suggest that the length of Bergmann glia is influenced by the number of granule cells bound to it.

The MAb 1A1 Inhibits the Migration of Cerebellar Neurons in Microexplant Cultures

The influence of MAb 1A1 on neuronal migration in microexplant cultures prepared from P7 rat cerebellum was tested as previously described (Fischer et al., 1986). In this assay, migration of granule cells occurs over a period of 3 days in vitro (Fischer et al., 1986). The effects on neuronal migration was therefore assessed after 3 days following the addition of 1A1 and control MAb 6C6, or no antibodies (see Figure 3.6). Similar sized explants (600-700 μm in diameter) were analyzed for each experimental group. Neuronal cell counts were made in four areas (each $\approx$ 0.025 mm^2) located at right angles to each other at the periphery of each explant. Areas not occupied by astrocytes were excluded from these measurements. Using these criteria, there was about a 60% decrease in the number of neurons that had migrated out to the periphery of the explants cultured in the presence of MAb 1A1 (Fab fragments at 500 μg/ml) as compared to those of control cultures (Figure 3.7).

3.4 Discussion

The 1A1 Antigen Mediates Neuron-Bergmann Glial Adhesion

Elongated, slender astrocytes, the Bergmann glia, have been shown to support the migration of cerebellar granule cells during development, both in vivo (Rakic, 1971) and in vitro (Hatten et al., 1984; Hatten, 1990; Fishell and Hatten, 1991). It was shown previously that the MAb 1A1 binds to the surface of flat type-1 astrocytes and Bergmann glia, but not to neurons or to oligodendrocytes (Chapter 2). In this study, Fab fragments of MAb 1A1 induced about a 50% inhibition in neuronal adhesion to elongated Bergmann glia in dissociated cultures of P7 rat cerebellar cortex. The molecule recognized by the MAb 1A1, therefore, contributes to the binding of cerebellar neurons to Bergmann glia. These findings are also consistent with the previous findings that the MAb 1A1 blocks the adhesion of neurons to monolayers of flat type-1 astrocytes purified from the neonatal rat cerebral cortex, but has no effect in neuron-neuron adhesion (Chapter 2). These observations suggest that the 1A1 molecule mediates neuron-glial adhesion via a heterophilic-binding mechanism.

In dissociated control cell cultures, the Bergmann glia were found to range in length between 50 and 200 μm. The inhibition of neuronal attachment to Bergmann glia by the MAb 1A1 was seen with Bergmann glia of all lengths. Glial differentiation in vitro is known to be regulated by contact with other glia, or with neurons or their membranes, suggesting that the cellular signal(s) involved is membrane-mediated (Hatten, 1985, 1987; Hatten and Shelanski, 1988; Culican et al., 1990). In the case of heterotypic neuron-glial contact, in cultures of P3-4 mouse cerebellum a neuron to astrocyte ratio of 3:1 is needed to induce changes in astroglial morphology from flat to stellate, process-bearing (Hatten, 1985). In addition, interactions with neurons

influence the development of the mature cytoskeletal composition in radial glia in cultures of E13 mouse cerebral cortex (Culican et al., 1990). Consistent with these reports, it was found that Bergmann glial differentiation is influenced by contact with neurons. The findings indicate that the MAb 1A1-induced decrease in the adhesion of neurons to Bergmann glia also results in an increase in the number of Bergmann glia that are shorter than 100 μm, as compared to control cultures. The data also indicate that four to five neurons per Bergmann glial cell are required to achieve a Bergmann glial length of 100 μm or greater.

The 1A1 Antigen Mediates Neuronal Migration Along Bergmann Glia

To examine whether the timing of 1A1 antigen expression in vivo coincides with the developmental period of granule cell migration along radial glia, immunoprecipitation was carried out using membrane extracts from P1, P10, P20, and P35 rat cerebellum. There was about a 2-fold increase in the amount of 1A1 antigen between P1 to P20, followed by a reduction to the P1 level at P35. However, it is not possible to know from the immunoprecipitation data whether the developmental increase in 1A1 in the cerebellum between P1 and P20 reflects changes in the expression of this molecule by flat type-1 astrocytes or Bergmann glia. The latter would be expected to occur if the 1A1 antigen plays a role in neuronal migration along Bergmann glia. In fact, the immunofluorescence labeling studies confirmed that there was about a 2.5-fold increase, from 33% to 78%, in the number of Bergmann glia that are $1A1^+$ between P1 and P7. Therefore, the 1A1 antigen is present at the appropriate time to be involved in the migration of granule cells along Bergmann glia in vivo.

The adhesion assays discussed earlier indicate that the 1A1 antigen mediates the binding of neurons to Bergmann glia. Adhesive mechanisms have been shown

previously to be involved in the migration of granule cells along Bergmann glia (reviewed by Chuong, 1990; Fishell and Hatten, 1991; Husmann et al., 1992). In the present experiments, the effects of the MAb 1A1 were examined on the behavior of migrating neurons along radial glial processes in microexplant cultures of the P7 rat cerebellum. In this explant model, neurons migrate from the center of the explants to the periphery. Therefore, the maximal effect on the inhibition of neuronal migration was expected to be seen in the peripheral regions of the explants. In this functional assay, the MAb 1A1 induced about a 60% decrease in the number of neurons that migrate out to the periphery of the explant, as compared to control cultures (no MAb- or 6C6-treated cultures).

The migration of neurons is likely to depend on complex interactions involving various CAMs (Lindner et al., 1983, 1986b; Antonicek et al., 1987; Chuong et al., 1987; Lehmann et al., 1990; Fishell and Hatten, 1991; Streit et al., 1993), ECM molecules (Hatten et al., 1982; Liesi, 1985; Chuong et al., 1987; O'Shea et al., 1990; Husmann et al.,1992; Liesi et al., 1992), adhesion inhibitors, and proteolytic enzymes (Moonen et al., 1982; Lindner et al., 1986a), all displaying differential spatiotemporal expression patterns in the developing brain. In order to understand this multi-molecular mechanism of neuronal migration, cellular interactions have been perturbed using antibodies against various molecules at different developmental stages. The evidence available at present suggests that these molecules are likely to influence different stages in the process of neuronal migration (reviewed by Chuong, 1990).

There are at least two major stages in cerebellar granule cell migration that occur postnatally in rodents. These involve the migration of post-mitotic neurons in the EGL just before their entry into the molecular layer (ML), followed by rapid

migration of granule cells from the EGL through the ML along the processes of Bergmann glia into the IGL. Different in vitro assays have been used to study granule cell migration; these include dissociated and microexplant cultures, as well as tissue slice preparations. For instance, by applying functional blocking antibodies to inhibit cell adhesion in tissue slices, it was shown that the cell adhesion molecules Ng-CAM and L1 function in the early stages of granule cell migration, i.e., within the EGL (Lindner et al., 1983; Chuong et al., 1987). Both Ng-CAM identified in chicken (Grumet et al., 1984a) and L1 identified in the mouse (Faissner et al., 1984) are known to be present on post-mitotic neurons. Antibodies to these adhesion molecules might, therefore, influence granule cell migration by inhibiting mainly neuron-neuron adhesion within the EGL where Bergmann glia are absent. Using similar tissue slice preparations, N-CAM, which promotes both neuron-neuron and neuron-glial adhesion (Keilhauer et al., 1985), had a small effect mainly in the early phases of granule cell migration showing only a 13% inhibition as compared to polyclonal anti-L1 antibodies which showed a 33% inhibitory effect (Lindner et al., 1983, 1986b; Chuong et al., 1987). A glial lectin along with one of its neuronal ligands (Lehmann et al., 1990) and the ECM molecule thrombospondin (O'Shea et al., 1990) also influence granule cell migration within the EGL. Inhibition of about 50% and 40% respectively, was observed with polyclonal antibodies against the lectin and thrombospondin using tissue slice preparations (Lehmann et al., 1990; O'Shea et al., 1990).

In contrast to the molecules known to influence granule cell migration through the EGL which lacks Bergmann glia, the ECM molecules, tenascin/cytotactin (Chuong et al., 1987; Husmann et al., 1992) and astrochondrin (Streit et al., 1993), and the neuronal cell surface molecule, astrotactin (Fishell and Hatten, 1991), have been

reported to play a major role in neuronal migration along Bergmann glia in tissue slice or dissociated cell cultures. When tissue slices were treated with anti-cytotactin antibodies, only 30% of granule cells that had entered the ML actually migrated down to the IGL as compared with 81% in control cultures (Chuong et al., 1987). Using a different in vitro assay system, the addition of polyclonal anti-astrotactin antibodies to dissociated cerebellar cortical cultures was shown to reduce the mean rate of neuronal migration along Bergmann glia by 61% (Fishell and Hatten, 1991). Earlier studies have also shown that antibodies against cytotactin as well as astrotactin block neuron-glial binding in vitro (Grumet et al., 1985; Edmondson et al., 1988). However, a recent report indicated that cerebellar histogenesis appeared to be normal in mutant mice with a knock-out of the tenascin gene (Saga et al., 1992). The reasons for the lack of obvious phenotype in these mice is not clear, but may be due to redundancies built into the adhesive systems mediating migration. Hatten and her colleagues further showed that antibodies against N-CAM, L1, and N-cadherin did not have any effect on the binding of cerebellar granule cells or their membranes to primary cultures of P2-4 mouse cerebellar glia (Stitt and Hatten, 1990) and on granule cell migration along Bergmann glia in dissociated cell cultures (Fishell and Hatten, 1991). These findings, therefore, provide additional evidence that N-CAM and L1 are not likely to be involved in the later stages of granule cell migration from the EGL to the IGL along Bergmann glia. These investigators also showed that β_1 integrins do not play a role in granule cell migration along Bergmann glia (Stitt and Hatten, 1990; Fishell and Hatten, 1991). However, there is conflicting evidence as to the role of the ECM molecule laminin in influencing such migration (Antonicek et al., 1987; Liesi et al., 1992). A recently characterized chondroitin sulfate proteoglycan (astrochondrin),

isolated from the murine CNS (Streit et al., 1990), was shown to be involved in cerebellar granule cell migration (Streit et al., 1993). Polyclonal antibodies to astrochondrin inhibited granule cell migration by 28% (Streit et al., 1993). In addition to these adhesion molecules, several other molecules, such as a protease inhibitor, glia-derived nexin (Lindner et al., 1986a), the plasminogen activator-plasmin system (Moonen et al., 1982), have been reported to regulate granule cell migration. Monoclonal antibodies to AMOG, a homologue of the β subunit of the Na,K-ATPase, have also been reported to block glial-guided neuronal migration by 36% (Antonicek et al., 1987; Gloor et al., 1990).

Taken together, the previously published antibody perturbation experiments indicate that the ECM molecules, tenascin/cytotactin and astrochondrin, and the neuronal surface molecule, astrotactin, are the main adhesion molecule candidates involved in neuronal migration along Bergmann glia. In this chapter, evidence is provided for a Bergmann glial cell surface molecule with adhesive properties, recognized by the MAb 1A1, that also mediates neuronal adhesion and migration on Bergmann glia in vitro. In addition, the changes in the expression of this molecule in the developing cerebellum is consistent with it having such a role in vivo.

Figure 3.1 Double-immunofluorescence labeling with MAb 1A1 (A) and polyclonal anti-GFAP (B) in a dissociated cell culture of neonatal rat cerebellar cortex after 24 hours in vitro. The GFAP$^+$ Bergmann glia (B) shows punctate cell surface labeling with MAb 1A1 (A). Bar, 25 μm.

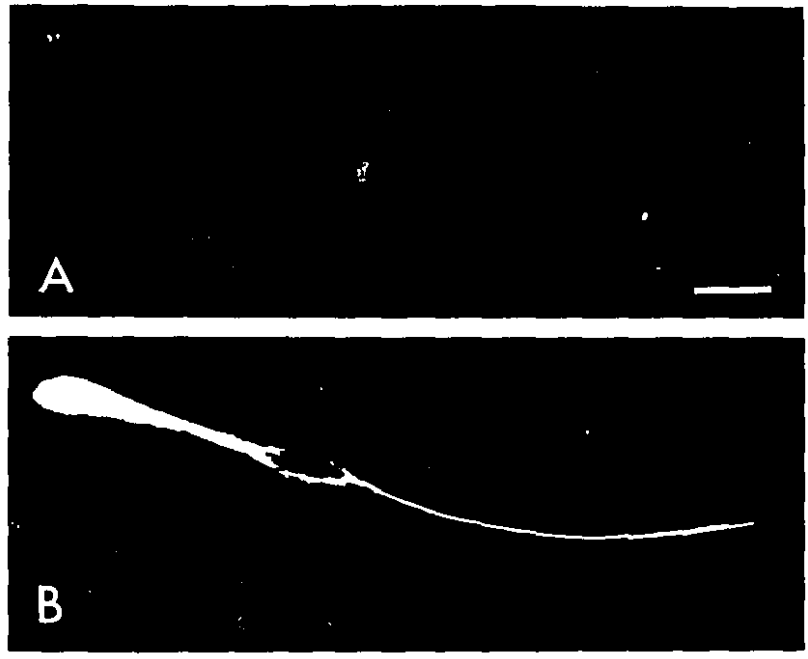

A
B

Figure 3.2 Silver-staining after reducing SDS-PAGE (7.5%) of immunoprecipitates obtained with MAb 1A1 (lanes 1 to 4) using membrane preparations of postnatal day-1, -10, -20 and -35 rat cerebella, respectively. The relative percentage value of each band obtained from densitometric readings is shown in the lower panel. These results indicate that the amount of the 1A1 antigen increases between P1 and P20 followed by a reduction at P35.

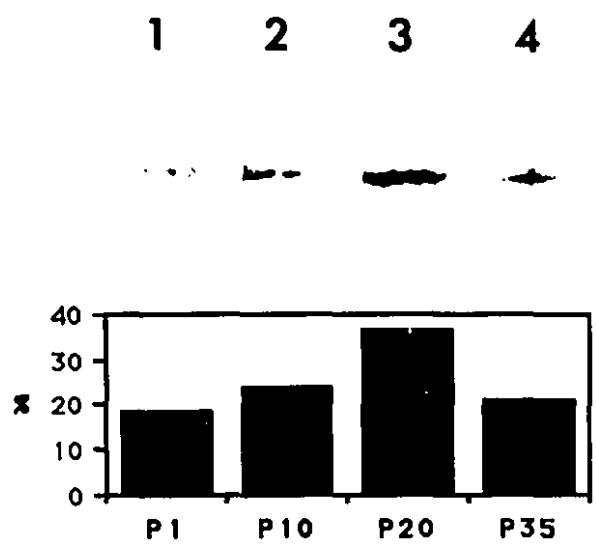

1
2
3
4
40
30
%
20
10
0
P1
P10
P20
P35

Figure 3.3 Percentage of $1A1^+/GFAP^+$ Bergmann glia in primary cultures of the P1 and P7 rat cerebellar cortex. Dissociated cerebellar cortical cells were used after 24 hours in vitro. Each value represents the mean $\pm$ SD of 3 experiments. For each experiment between 100-350 cells were counted on 2-3 coverslips. There is about a 2.5-fold increase in the number of $1A1^+$ Bergmann glia between P1 and P7.

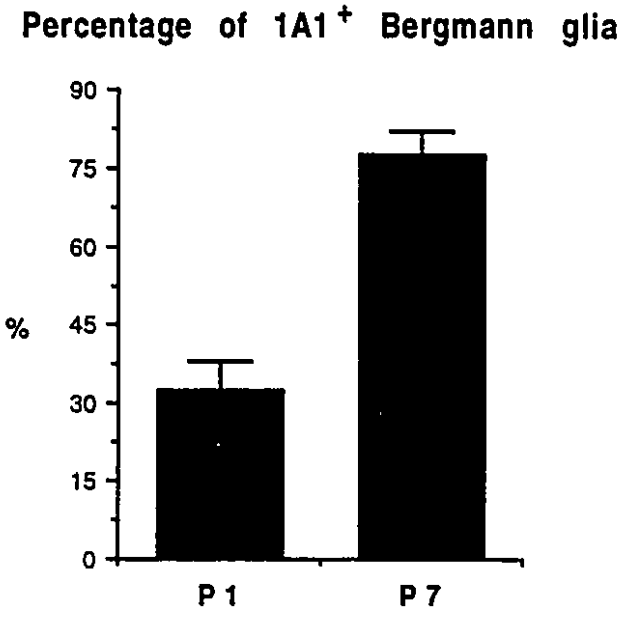

Percentage of 1A1+ Bergmann glia
90
75
60
%
45
30
15
0
P 1
P 7

Figure 3.4 Double-immunofluorescence labeling of mixed cultures of the P7 cerebellar cortex, labeled with MAb 1A1 (A) and polyclonal anti-GFAP (B) after 24 hours in vitro. The elongated $1A1^+$ cell (A) which is also $GFAP^+$ (B) is identified as a Bergmann glia. The $1A1^-/GFAP^-$ neurons attached to the Bergmann glia are visualized with Nuclear yellow (C). Bar, 25 μm.

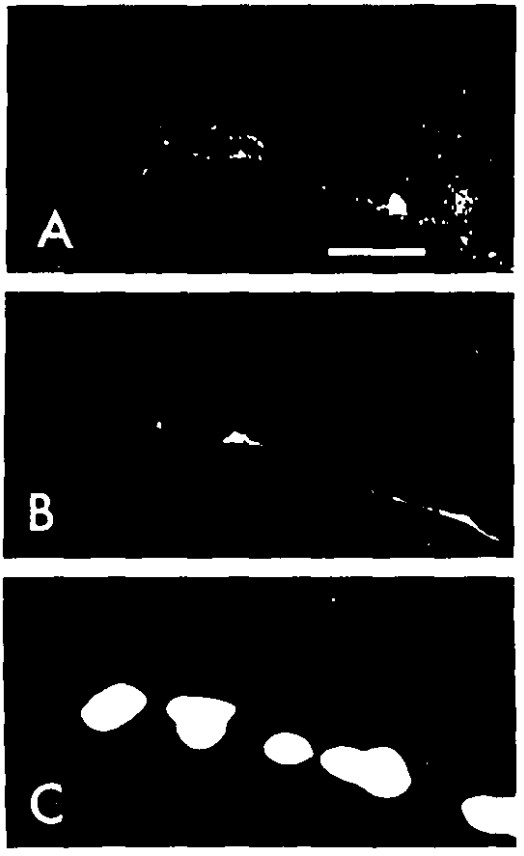
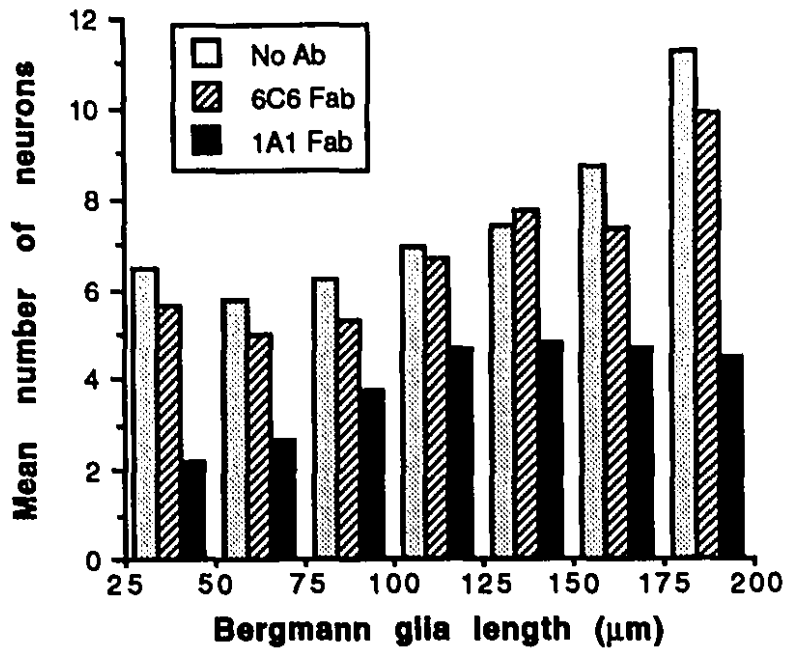

A
B
C

Figure 3.5 (A) The mean number of neurons associated with Bergmann glia of various lengths in dissociated cultures of P7 cerebellar cortex. Cultures were maintained for 24 hr in vitro in the absence or presence of MAb 1A1 or control antibodies (Fab fragments at 500 μg/ml). The histograms include values from 2 independent experiments. A significant inhibition of neuronal adhesion to Bergmann glia by MAb 1A1 is observed with Bergmann glia of all lengths as compared to controls. Friedman's two-way ANOVA was carried out to assess statistical differences ($p < 0.01$). (B) The frequency distribution of Bergmann glia length. Note that in cultures treated with MAb 1A1 (Fab fragments at 500 μg/ml), there is an increase in the number of Bergmann glia shorter than 100 μm in length as compared to control cultures (6C6-treated or untreated). Significance values were assigned using a Chi-squared analysis ($p < 0.001$).

A


No Ab
6C6 Fab
1A1 Fab
Mean number of neurons
0
2
4
6
8
10
12
25
50
75
100
125
150
175
200
Bergmann glia length (μm)

B

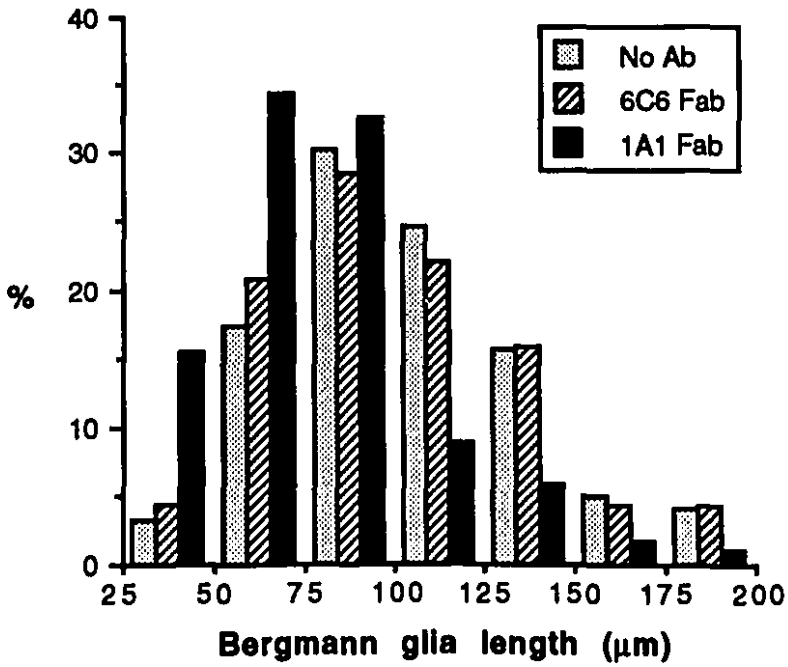

No Ab
6C6 Fab
1A1 Fab
%
0
10
20
30
40
25
50
75
100
125
150
175
200
Bergmann glia length (μm)

Figure 3.6 Double-immunofluorescence labeling of P7 cerebellar microexplant culture after 3.5 days in vitro, with MAb 1A1 (A) and polyclonal anti-GFAP (B). The same field is labeled with Nuclear yellow in (C). The $1A1^+/GFAP^+$ radial Bergmann glial-like cells have extended out of the core of the explant. The $1A1^-/GFAP^-$ neurons, which are visualized here with Nuclear yellow staining (C), have migrated from the core to the periphery of the explant. For each microexplant, cell counts were made from 4 readings obtained at right angles to each other. This explant is smaller than the ones used for quantification. This was chosen so as to illustrate both the core and the periphery of the explant. Bar, 25 μm.

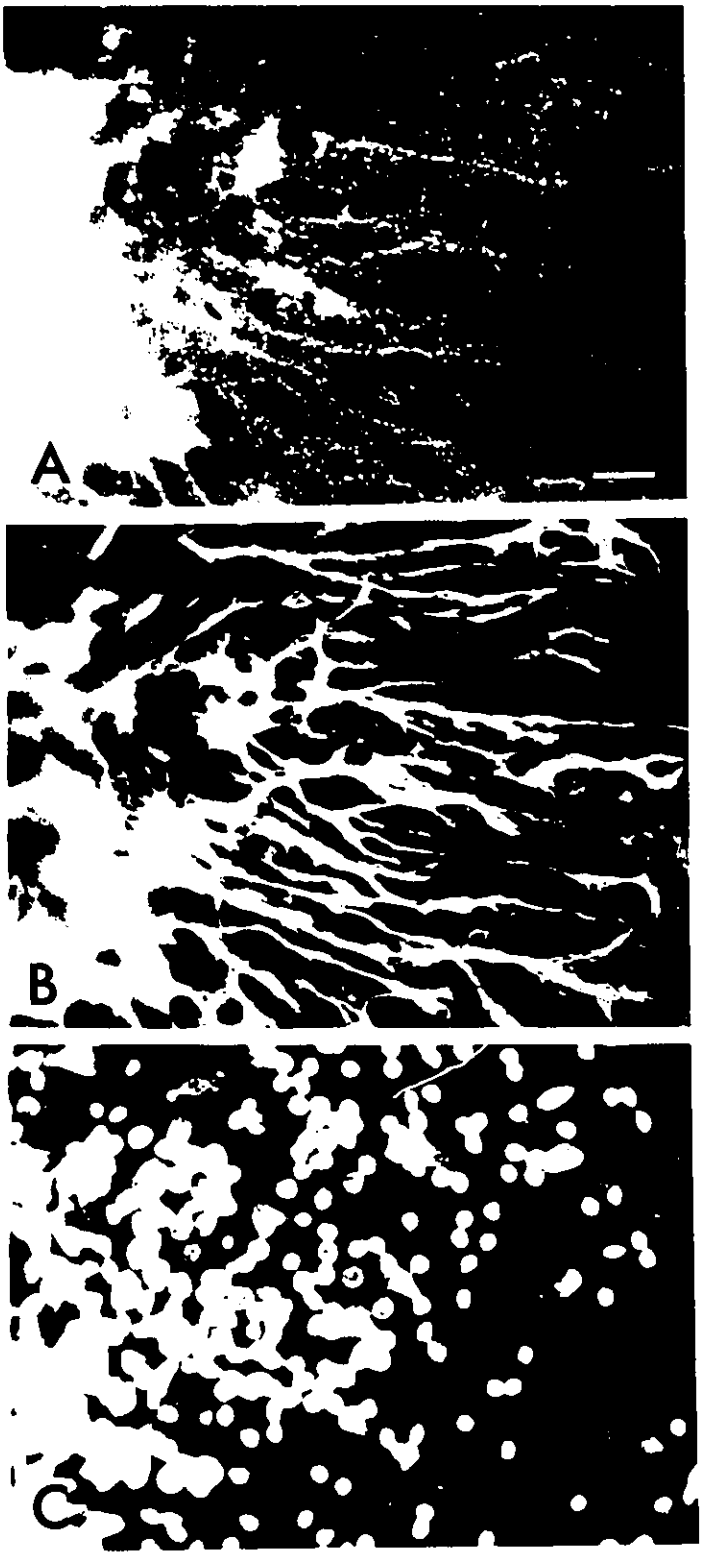

A
B
C

Figure 3.7 The number of neurons that have migrated out to the periphery of the P7 cerebellar microexplants after 3 days in vitro. Antibodies at 500 μg/ml were added 12 hours after plating. The numbers in parenthesis indicate the number of explants evaluated in 3 separate experiments. Each value represents the mean $\pm$ SD. A 60% inhibition in neuronal migration is seen in cultures treated with MAb 1A1 as compared to control cultures treated with MAb 6C6. Student's t-test was used to assign significance levels; * = $p < 0.001$

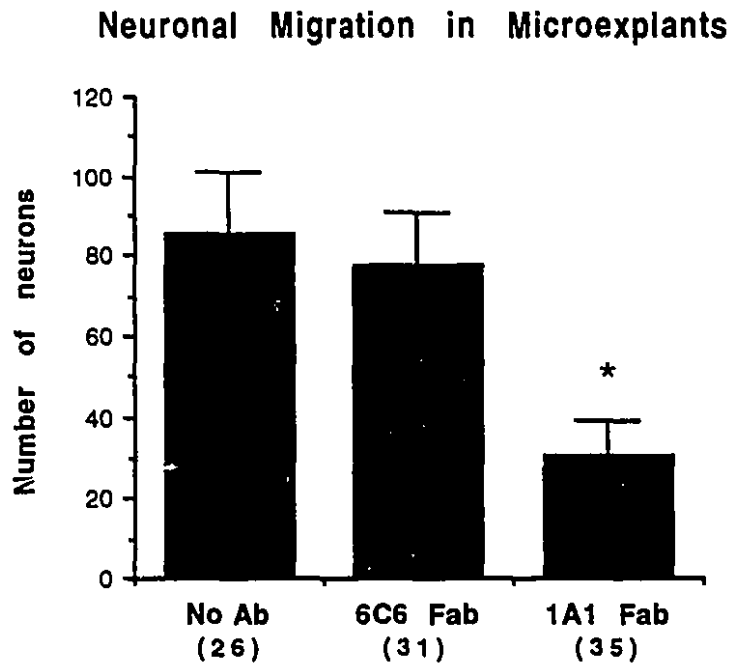

Neuronal Migration in Microexplants
Number of neurons
120
100
80
60
40
20
0
*
No Ab
(26)
6C6 Fab
(31)
1A1 Fab
(35)

Table 3.1 The number of neurons attached per 100 μm length of elongated GFAP$^+$ Bergmann glia was estimated in dissociated cultures of the P7 cerebellar cortex 24 hours after plating. In the presence of MAb 1A1, there is a significant reduction in the attachment of neurons to Bergmann glia as compared to cultures grown in the presence of control antibodies 6C6. Each value represents the mean $\pm$ SD with counts made from duplicate coverslips. The number of experiments is indicated in parenthesis. Student's t-test was used to assign significance levels; a = not significantly different from control cultures, b = $p < 0.05$. Percentage inhibition is the difference in cell attachment compared to 6C6 controls.

NEURON - BERGMANN GLIA BINDING

Antibody (μg/ml)	Number of neurons per 100 μm glia length	Percentage of inhibition
None	7.85 ± 2.15 (3)[a]	
6C6 Fab (500 μg/ml)	7.47 ± 1.67 (3)	
(250 μg/ml)	7.52 ± 1.23 (2)	
(100 μg/ml)	7.48 ± 1.78 (2)	
1A1 Fab (500 μg/ml)	3.90 ± 0.64 (2)[b]	47.8%
(250 μg/ml)	3.94 ± 1.12 (3)[b]	47.6%
(100 μg/ml)	6.60 ± 0.54 (2)[a]	11.8%

Chapter 4: *The involvement of the 1A1 molecule in leptomeningeal cell adhesion and differentiation*

4.1 Introduction

Cell adhesion molecules play an important role in embryonic development and morphogenesis and display unique spatiotemporal patterns of expression. In the nervous system, the expression of 1A1 cell surface molecule with adhesive properties was found to be restricted to subpopulations of astrocytes, namely the flat type-1 astrocytes and Bergmann glia (Chapters 2 and 3). As mentioned earlier in Chapter 2, the 1A1 antigen is also expressed in confluent cultures of non-neural cells of mesenchymal origin (leptomeninges and fibroblasts). Intense $1A1^+$ immunofluorescence labeling was found on neonatal rat leptomeningeal cells only when these cells were cultured to confluency. The expression of 1A1 on leptomeninges therefore differed from that on astrocytes. The latter cells express the 1A1 antigen regardless of the density of the culture. In this study, experiments were done to identify the factors that control the expression and synthesis of 1A1 antigen in cultures of neonatal rat leptomeningeal cells.

Little is known about leptomeningeal histogenesis. Although a subject of some controversy (Gil and Ratto, 1973), very early studies by Farrer (1907) and more recent studies by Angelov and Vasilev (1989) indicate that the leptomeninges develop from an embryonic network of connective tissue-forming cells. The leptomeninges consist of the arachnoid mater and pia mater, which along with the dura mater cover the surface of the CNS. The pia mater forms a membrane that is very closely associated with the surface of the CNS, and is separated from it by a basal lamina (Peters et al., 1991). Under the electron microscope, the leptomeninges are made up of flat,

polygonal cells with varying amounts of collagen and elastic fibers (Peters et al., 1991). Leptomeningeal cells also show characteristic staining for various ECM molecules, such as fibronectin, laminin, and tenascin (Rutka et al., 1986; David, 1988; Esiri and Morris, 1991; Ajemian et al., 1994; Chapter 2). The adhesive interactions that underlie the development of the sheet-like leptomeninges has not been studied previously. I have shown using antibody blocking experiments that the 1A1 molecule mediates neuron-astrocyte and astrocyte-astrocyte adhesion in vitro. Similar functional assays were carried out in order to determine whether the 1A1 molecule also functions in cell adhesion between leptomeningeal cells. In addition, the developmental changes in the expression of 1A1 molecule in vivo and in vitro were examined both by immunocytochemistry and immunoprecipitation. In addition to providing a protective fibrous tissue capsule enclosing the CNS, the proper development and maintenance of the leptomeninges may also be essential for the proper development and foliation of the underlying CNS tissue (Pehlemann et al., 1985; Hartmann et al., 1992).

4.2 Materials and Methods

Antibodies

The following antibodies were used for immunocytochemical analysis. Hybridoma cells secreting the MAb 1A1 were generated as described in Chapter 2. Two cell-type specific rabbit polyclonal antibodies were used to distinguish flat astrocytes from fibroblast-like leptomeningeal cells. Polyclonal anti-GFAP antiserum (diluted 1:1000, obtained from Dr. M.C. Raff, UCL, UK) was used for the identification of astrocytes and anti-FN antiserum (diluted 1:200, obtained from Dr. R.O. Hynes, MIT, Massachusetts) was used to identify leptomeningeal cells. The monoclonal antibody

MRC OX-43 (Serotec) was used to label the surface of endothelial cells.

For the functional assay both MAbs 1A1 and Ran-2 were used, the latter served as a control antibody. Hybridoma cell line producing MAb Ran-2 was obtained from the American Type Culture Collection (ATCC No. TIB 119; Rockville, MD). Ran-2 antibody has previously been shown to bind to viable astrocytes, ependymal cells, Müller cells, leptomeningeal cells and non-myelin-forming Schwann cells (Bartlett et al., 1981, Mirsky and Jessen, 1984). Isolation of IgG from ascites fluid and the production of Fab fragments from 1A1 and Ran-2 antibodies by papain digestion is described in Chapter 2. Fab fragments were dialyzed and filtered through 0.22-μm filters (Millipore) prior to their use in the functional adhesion assays.

The MAb JG22 producing myeloma cells were obtained from Dr. S. Carbonetto and used as a control in immunoprecipitation experiments. JG22 antibody has previously been shown to recognize an epitope on avian integrin β_1 subunit (Horwitz et al., 1984).

Cell Culture

Primary dissociated cultures of leptomeninges were obtained from neonatal and adult Sprague-Dawley rats as described previously (Noble et al., 1984). The leptomeningeal tissue covering the surface of the cerebral cortex was dissected out and incubated for 30 minutes at 37°C in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS) containing 0.125% trypsin (Sigma) or a combination of trypsin (0.125%) and collagenase (0.025%, Sigma) for the dissociation of adult leptomeninges. After neutralizing the trypsin, cells were dissociated in the presence of DNAase (0.04 mg/ml, Sigma) by trituration through a fire polished Pasteur pipette, centrifuged, and plated on 13 mm round glass coverslips or in T-75 tissue culture flasks coated with PLL

(5 μg/ml, Sigma, 300,000 MW). Primary cultures of neonatal leptomeninges were seeded onto coverslips at different densities (0.75 x 10^4 or 3 x 10^4) and labeled with MAb 1A1 after various days in vitro (DIV). Higher plating densities of 7.5 x 10^4 cells were also tried, but accurate quantification of $1A1^+$ leptomeningeal cells was not possible after 4 DIV since these cells had a tendency to form multiple layers at very high cell densities. Therefore, counts from only the low (0.75 x 10^4 cells/cm^2) and high (3 x 10^4 cells/cm^2) density cultures were analyzed. In some experiments, PLL-treated coverslips were coated with various ECM molecules onto which 3 x 10^4 cells/cm^2 were plated. Such cultures were immunostained with MAb 1A1 after 1 DIV. All cultures were maintained in DMEM containing 1% MEM-vitamins, 1% penicillin/streptomycin and 10% FBS (all from Gibco) at 37°C in a humidified atmosphere of 5% CO_2.

For functional adhesion assays, secondary cultures were established from confluent leptomeningeal cultures grown in T-75 flasks. Cells were removed by mild trypsinization and replated onto PLL-coated 96-well plates at a density of 5 x 10^3 cells/well. Cultures were used after 3 DIV when a complete monolayer was formed.

Substrate Preparation

PLL-treated round glass coverslips were coated overnight at 4°C with 50 μg/ml of laminin (BRL), fibronectin (obtained from Dr. S. Carbonetto, McGill University), collagen type I (extracted from adult rat tail) or collagen type IV (Sigma) in Ca^{2+}, Mg^{2+}-free HBSS. Coverslips were subsequently washed 3 times with DMEM. Freshly dissociated leptomeningeal cells from neonatal rats were plated onto these coated coverslips at a density of 3 x 10^4 cells/cm^2 and cultured for 24 hours prior to

indirect immunofluorescence labeling with the MAb 1A1.

Indirect Immunofluorescence

Rat leptomeningeal cultures either in suspension or on coverslips were first incubated with the MAb 1A1 (ascites diluted 1:500) for 30 minutes at room temperature. Following washing, the binding of MAb 1A1 to live cells was visualized by incubating with rhodamine-conjugated goat anti-mouse IgG (Hyclone, 1:200). For staining controls, Sp2/O myeloma ascites fluid (diluted 1:500) was used instead of the primary antibody. Cells were then fixed and permeabilized in 95% ethanol containing 5% acetic acid at -20°C for 30 minutes. The coverslips were then rinsed and incubated with polyclonal antibodies to GFAP or FN. This was followed by incubation with a goat anti-rabbit secondary antibody conjugated to fluorescein (Cappel, 1:400). All antibody incubations were carried out at room temperature for 30 minutes in MEM-H containing 1% NGS.

For immunofluorescence labeling of cells in suspension with the MAb 1A1, washing was done by centrifugation. Prior to fixation, these cells were allowed to adhere to PLL-coated coverslips for 15-20 minutes at 37°C and then subsequently processed for intracellular labeling as described above.

All cultures were labeled with Nuclear yellow (0.001%, Hoechst S769121) for 30 seconds to label nuclei. Coverslips were mounted in bicarbonate buffered glycerol (pH 9.0) containing phenylenediamine (Sigma) onto microscope slides, and viewed with a 40x oil immersion lens on a Leitz Ortholux fluorescence microscope equipped with phase-contrast, and N_2 filter (rhodamine), L_2 filter (fluorescein) and D filter (Nuclear yellow).

Adhesion Assay

The adhesion of dissociated leptomeningeal cells to monolayers of leptomeninges was assayed as described below. Confluent cultures of leptomeninges from neonatal rats growing in T-75 flasks were mildly trypsinized and replated onto PLL-coated 96-well tissue culture plates (5×10^3 cells/well). After culturing these cells for 3 days to obtain a monolayer, leptomeningeal cell attachment to these monolayers was tested in a 90 minute adhesion assay with or without MAb 1A1, or control antibodies. Leptomeningeal cells used as probe cells were labeled with DiI (3×10^{-3} mM) for 1 hour at 37°C while the cells were in T-75 flasks. The cultures were washed extensively with DMEM and cells removed by mild trypsinization in the presence of 0.5 mM $CaCl_2$. After neutralizing the trypsin, cells were triturated to obtain a single cell suspension which was plated onto the monolayers of leptomeninges in 96-well plates at a density of 1000 cells/well. The monolayer cultures were pre-treated with various concentrations of MAb 1A1 or Ran-2 Fab fragments, or no antibodies for 15 minutes at 37°C in a humidified CO_2 incubator prior to adding DiI-labeled probe cells. Following a 90 minute incubation period at 37°C, microwells were washed well and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 10-15 minutes at room temperature. Cultures were washed with PBS and viewed with a Zeiss inverted fluorescence microscope. The number of single DiI-labeled leptomeningeal cells adhering to the leptomeningeal monolayers were counted in the same number of fields for each well. Counts of 500-700 cells were made per experiment from three separate wells. A total of three such experiments were carried out.

Immunoprecipitation

Neonatal and adult rat leptomeninges were dissected and incubated in methionine-free RPMI containing 2% dialyzed FBS for 1 hour, followed by a 7 hour incubation with 50 μCi/ml [^{35}S]methionine (ICN). Subsequently, the cultures were chased with cold HMCF buffer (150 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 5 mM glucose, 10 mM HEPES, pH 7.4) containing 2 mM methionine, followed by washing by centrifugation. Detergent solubilization was then carried out in 2 ml ice-cold solubilization buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.02% NaN_3, pH 7.6) containing protease inhibitors as described in Chapter 2. Immunoprecipitation with MAb 1A1 and control MAb JG22 was carried out using 1 ml aliquots of labeled proteins in samples containing similar amounts of radioactive counts (also see Chapter 3 for procedure). Briefly, NP-40 solubilized samples were incubated (3 hours, 4°C) with pre-swollen Sepharose-protein A beads (Pharmacia), followed by centrifugation to obtain pre-cleared supernatants which were subsequently incubated (2 hours, 4°C) with 15 μg of MAb 1A1 or MAb JG22. This was followed by an overnight incubation at 4°C with 100 μl of Sepharose-protein A beads. All steps were carried out with continuous gentle shaking. The Sepharose-protein A beads were collected, washed 7 times with cold NET buffer (150 mM NaCl, 50 mM Tris, 0.1% NP-40, 0.25% gelatin, 0.02% NaN_3, pH 7.6), and the precipitated proteins eluted (5 minutes, 100°C) in 30 μl SDS-sample buffer (10% glycerol, 50 mM Tris-HCL, 2% SDS, 0.02% bromophenol blue, 5% β-mercaptoethanol, pH 6.8) before separating by PAGE according to Laemmli (1970). Similar volume of protein samples were run on 7.5% acrylamide gel and visualized by autoradiography. High molecular weight standards from Bio-Rad Laboratories were used as marker proteins. To visualize the standard

proteins, gels were stained with a silver-staining procedure as described by Morrissey (1981). This was followed by a 30 minute incubation in a fluorographic reagent "Amplify" (Amersham). Gels were subsequently dried and Kodak X-ray films exposed at -70°C.

4.3 Results

The 1A1 Antigen in Leptomeningeal Cells is Developmentally Regulated

The presence of 1A1 antigen on leptomeninges of neonatal and adult rats was analyzed by immunoprecipitation and immunocytochemistry of dissociated cell suspensions. Western blotting or labeling of frozen sections could not be done with the MAb 1A1 as mentioned in Chapter 2. When neonatal rat leptomeninges covering the surface of the P2 cerebral cortex was metabolically labeled in vitro with [^{35}S]methionine, immunoprecipitation with the MAb 1A1 did not yield any specific band (Figure 4.1, lane 3). However, immunoprecipitation of the adult leptomeninges yielded a 135 kd MW band (Figure 4.1, lane 4). Further evidence of changes in the expression of the 1A1 antigen during development was obtained by immunocytochemical analysis of cell suspensions of freshly dissociated neonatal and adult rat leptomeninges. These cells were initially examined by labeling with MAb 1A1 and Nuclear yellow (Figure 4.2). In contrast to the neonatal leptomeningeal cells which showed no labeling with the MAb 1A1 (Figure 4.2, A and B), the majority of the leptomeningeal cells obtained from adult rats showed intense 1A1 immunoreactivity of the cell surface (Figure 4.2, C and D). Since leptomeningeal cells show intense fibronectin labeling of the surface, this labeling was used to distinguish these cells in suspension from the small number of astrocytes (which are FN$^-$) that may be present in these cultures. The quantification

of the $FN^+/1A1^+$ cells in neonatal and adult leptomeningeal cell suspensions showed that there were about 13% double-labeled cells at P2 and greater than 80% in the adult (Figure 4.3). These results therefore indicated that 1A1 antigen expression on leptomeningeal cells is increased during development in vivo.

Since the adult rat leptomeningeal tissue is highly vascularized, the percentage of $OX\text{-}43^+$ endothelial cells in these cultures was also examined and quantified for 1A1 labeling. The $OX\text{-}43^+$ cells made up approximately 12% of the total cell population in cell suspensions of adult leptomeninges, but less than 2% in suspensions of neonatal leptomeninges. These $OX\text{-}43^+$ endothelial cells were $1A1^-$ and also did not label with the anti-FN antiserum (data not shown).

The 1A1 Antigen is Detected Only in Confluent Cultures of Neonatal Leptomeninges

Differences were reported previously in the regulation of the 1A1 antigen expression in vitro on cells derived from the neuroectoderm versus cells of mesenchymal origin, such as leptomeningeal cells (Chapter 2). The latter cells are $1A1^+$ only when grown to confluency (Figure 4.4, A-D; also Figure 4.1, lane 1). Two factors that could influence 1A1 antigen expression in these experiments are cell density and time in culture. Therefore experiments were carried out to examine the influence of these two parameters on the expression of the 1A1 antigen. To study this, the expression of 1A1 was examined in primary dissociated cell cultures of neonatal rat leptomeninges that were seeded at different densities and cultured for upto 10 DIV. Two different plating densities were used: 0.75×10^4 cells/cm^2 (low density) and 3×10^4 cells/cm^2 (high density). For these experiments which involved long-term cultures, double-labeling was performed with anti-GFAP antibodies instead of antibodies against fibronectin.

This was done because in these long-term cultures the fibronectin labeling does not remain confined to individual cells, but is more diffusely distributed in matrix-like deposits over the entire culture surface.

Primary cultures of leptomeninges obtained from neonatal rats contain two populations of flat, polygonal cells. The majority of the cells (> 90%) are the $GFAP^-$ fibroblast-like leptomeningeal cells (insert of Figure 4.5, A). These $GFAP^-$ cells were also found to be $OX\text{-}43^-$ (data not shown), but FN^+ in sister cultures (Chapter 2; also see Figure 4.4). Astrocytes comprise less than 10% of the cells in these neonatal leptomeningeal cultures. The percentage of astrocytes remained between 2 to 10% throughout the in vitro period regardless of the plating densities (insert of Figure 4.5, B). Differences in the time course of 1A1 antigen expression were observed between the two flat cell populations ($GFAP^-$ leptomeninges and $GFAP^+$ astrocyte populations) in neonatal leptomeningeal cultures. While there was a steady increase in the percentage of astrocytes expressing 1A1 antigen in these primary cultures (Figure 4.5, B), its expression in leptomeningeal cells remained constant at < 5% for upto 4 DIV in both the low and high cell density cultures (Figure 4.5, A). After 6 DIV in high density cultures greater than 92% of the leptomeningeal cells labeled with 1A1 antibodies at which time the cultures were confluent (Figure 4.5, A). Whereas in the low density cultures, only 16% of the leptomeningeal cells expressed the 1A1 antigen at 10 DIV (the longest time period examined). These $1A1^+$ cells in low density cultures were always found in cluster, suggesting that 1A1 antigen expression on leptomeninges might be regulated by the local microenvironment and/or cell-cell contact. In contrast to the $GFAP^-$ leptomeningeal cell population in the two density cultures, the 1A1 expression on the $GFAP^+$ astrocytes in these cultures showed a

different pattern of regulation. Although 100% of 1A1$^+$ labeling was never observed on the leptomeningeal cells, all of the small number of astrocytes in these cultures showed intense 1A1 immunoreactivity after 8 DIV at both plating densities (Figure 4.5, B).

The 1A1 Antigen on Neonatal Leptomeninges is Regulated by ECM Molecules

Since marked increases in the deposition of various ECM molecules is seen in the leptomeninges during development in vivo and in vitro, the influence of some of these matrix molecules in the regulation of 1A1 antigen expression in leptomeningeal cells was examined in vitro. Dissociated neonatal leptomeninges were plated at a density of 3 x 10^4 cells/cm^2 onto substrates of purified laminin, fibronectin, and collagen types I and IV, and the percentage of 1A1$^+$ cells examined 24 hours later. Double-immunofluorescence labeling with anti-GFAP polyclonal antibodies was again performed so that flat astrocytes that make up less than 10% of the cell population in these primary cell cultures could be excluded from the counts. On PLL, only 3-4% of the leptomeningeal cells expressed the 1A1 antigen after 1 DIV. However, there was a significant increase in the percentage of 1A1$^+$ leptomeningeal cells when grown on the various ECM substrata. This increase was in the order of about 3.5-fold for laminin, 4-fold for fibronectin, 5.5-fold for collagen type I and 3-fold for collagen type IV as compared with control cultures grown on PLL alone (Figure 4.6). Furthermore, the 5.5-fold increase in the number of 1A1$^+$ leptomeningeal cells on collagen type I was also significantly different from the increased percentage seen on other ECM molecules that were used in these experiments.

The MAb 1A1 Inhibits Adhesion of Leptomeningeal Cells

In order to determine whether the 1A1 antigen plays an adhesive role in the leptomeninges, confluent cultures of neonatal leptomeninges were used in a short-term adhesion assay. Confluent cultures of neonatal leptomeninges were used since the 1A1 antigen on these cells is expressed upon confluency. Such cultures were pre-treated with different concentrations of Fab fragments of MAb 1A1 or control MAb Ran-2 prior to the addition of DiI-labeled leptomeningeal cell suspension obtained from confluent cultures. The mean number of single DiI-labeled leptomeningeal cells attached to leptomeningeal monolayers decreased by 26% in the presence of 1A1 antibodies as compared to control antibodies (Table 4.1). The blocking effect of MAb 1A1 on the adhesion of leptomeningeal cells to leptomeningeal cells was concentration dependent, with the maximum effect observed at 250 μg/ml of monovalent Fab fragments.

4.4 Discussion

The functional and biochemical characterization of MAb 1A1 has been reported using rat tissue (Chapters 2 and 3). Immunofluorescence analysis showed that the in vitro distribution of the antigen recognized by the MAb 1A1 is restricted to flat type-1 astrocytes and Bergmann glia within the CNS. Neurons and other glia of the CNS were 1A1$^-$. Antibody perturbation experiments in vitro demonstrated that the 1A1 antigen has adhesive properties, and is involved in neurite outgrowth on astrocytes (Chapter 2) and neuronal migration in microexplants of the cerebellar cortex (Chapter 3). Outside the CNS, the 1A1 antigen is expressed in vitro by certain cells of mesenchymal origin, such as leptomeningeal cells and fibroblasts, but only when

they reach confluency (Chapter 2). As mentioned previously, this antibody binds very poorly to tissue sections; hence, the in vivo distribution of this antigen is not known. In this study, the expression of 1A1 antigen and its adhesive function in the leptomeninges that cover the rat cerebral cortex was studied.

Regulation of 1A1 Antigen Expression in Leptomeningeal Cells

It was reported in Chapter 2 that a 135 kd MW band could be immunoprecipitated from confluent cultures of neonatal leptomeninges following metabolic labeling with [^{35}S]methionine. In this chapter, studies were carried out to further examine the level of expression of the 1A1 antigen in vivo in leptomeninges obtained from the neonatal and adult rats. As with confluent leptomeningeal cultures, the MAb 1A1 immunoprecipitated a similar MW band of 135 kd from [^{35}S]methionine-labeled protein extracts of leptomeningeal tissue obtained from the adult rat. This MW band was not seen in immunoprecipitates from leptomeninges of neonatal rats. In addition to the immunoprecipitation data, the immunofluorescence analysis of cell suspensions of neonatal and adult leptomeninges also showed that the percentage of 1A1$^+$ cells increases with development. 1A1$^+$ labeling of leptomeningeal cells increased from 13% at P2 to greater than 80% in the adult. Other experiments also showed that only about 5% of the leptomeningeal cells were 1A1$^+$ at P0. These results therefore indicate that 1A1 antigen expression on leptomeninges increases during postnatal development, suggesting that this antigen is expressed in mature and differentiated leptomeningeal cells in vivo.

Very few neonatal leptomeningeal cells in short-term cultures expressed the 1A1 antigen. However, the majority of these cells became 1A1$^+$ when the cultures were confluent. Both time in culture and cell density might have influenced these in vitro

changes. A differential regulation of the 1A1 antigen expression on neonatal leptomeningeal cells was found as a function of cell density, but only when the cultures were maintained for periods greater than 4 days. In high density cultures there was a rapid increase in the percentage of $1A1^+$ leptomeningeal cells from less than 5% to greater than 90% after 6 DIV by which time the cultures were confluent. However, in the low density cultures the percentage of $1A1^+$ leptomeningeal cells never reached greater than 16% even at 10 DIV which was the longest time period examined. Taken together, these observations suggest that cell density played a significant role in the expression of 1A1 antigen because of the marked increase in $1A1^+$ cells in the higher density cultures. In addition, time in culture was also an important parameter since the number of $1A1^+$ leptomeningeal cells continued to be at about 5% for the first 4 days in culture regardless of the plating densities. A distinct pattern of regulation was observed in the expression of this antigen on the small percentage of astrocytes (2-10%) that could be found in these neonatal leptomeningeal cultures. As compared to 1A1 expression on leptomeningeal cells, the percentage of $1A1^+$ astrocytes continued to increase with time in both low and high density cultures. Therefore the control of 1A1 antigen expression in astrocytes and leptomeningeal cells appears to be different.

The two most likely explanations for 1A1 antigen expression and synthesis by confluent leptomeningeal cultures are: (i) The establishment of cell-cell contacts, and (ii) the effects of maturational differentiation. That cell-cell contact seen in higher density cultures could play a role in the regulation of 1A1 antigen on leptomeninges was further supported by the finding that in the low density culture, the $1A1^+$ leptomeningeal cells at 10 DIV were always in clusters resulting in adjacent cells contacting each other. Density-induced up-regulation of enzymes, cell surface proteins,

and ECM molecules has previously been reported for various cells and (cell lines), such as fibroblasts (3T3), hepatocytes (H4IIEC3) and mammary epithelial cells (HCll) (Hatamochi et al., 1989; Beale et al., 1991; Kornilova et al., 1992; OShima and Simons, 1992). Also, the 1A1 antigen expression could be associated with maturational changes in leptomeningeal cells occurring as a function of time in culture, and thereby be related to the functional and biochemical differentiation of cells in vitro. This possibility was supported by the increase in 1A1 antigen expression seen in the leptomeninges in vivo as discussed earlier. There is evidence that fibroblasts (also derived from the mesenchyme) undergo maturational changes during their in vitro progression in terms of the expression of various differentiation-associated molecules, such as collagen types I and III, and fibronectin (Choi et al., 1992). In this study, an increase and a different pattern of fibronectin staining was observed in confluent neonatal leptomeningeal cultures versus non-confluent cultures. Other results have also indicated an increase in fibronectin expression in human fibroblasts during in vitro cellular senescence (Kumazaki et al., 1991). The increased 1A1 antigen expression in confluent leptomeningeal cultures might therefore be influenced by the composition and quantity of the surrounding ECM.

There is evidence from both in vitro and in vivo studies that leptomeningeal cells, like other mesenchymal-derived cells, are able to secrete several extracellular matrix components of the basal lamina which appear to be deposited in increasing amounts with maturation and differentiation (Rutka et al., 1986; Hernandez et al., 1989; Peters et al., 1991; Ajemian et al., 1994; Chapter 2). In addition, there is a marked deposition of collagen fibers in the adult leptomeninges (Hernandez et al., 1991; Peters et al., 1991). Increased levels of 1A1 antigen expression in confluent

leptomeningeal cultures as well as in the adult leptomeninges in vivo led me to examine whether 1A1 expression in the mature and differentiated leptomeningeal cells could be influenced by the increased deposition of ECM molecules. There have been several reports that ECM molecules can modulate the proliferation, differentiation, and survival of cells by directly or indirectly regulating gene expression (Adamson, 1982; Gospodarowicz et al., 1983; Fridman et al., 1985; Bissell and Barcellos-Hoff, 1987; Davis et al., 1987; Nagano et al., 1993). The percentage of $1A1^+$ leptomeningeal cells in neonatal leptomeningeal cultures increased when plated onto various ECM components (laminin, fibronectin, collagen types I or IV) as compared with those of control cultures on PLL alone. A significant increase of about 5.5-fold was seen on collagen type I and an increase of 3 to 4-fold on collagen type IV, laminin, and fibronectin. This higher percentage of $1A1^+$ leptomeningeal cells observed on collagen type I was significantly different from the other ECM molecules, suggesting that 1A1 antigen expression on leptomeninges is differentially regulated by these ECM molecules in vitro.

The mechanism(s) by which ECM molecules regulate the expression of 1A1 antigen in leptomeningeal cells is not known. It has been reported that individual components of the ECM can regulate gene expression at different levels (reviewed by Bissell et al., 1982). This is a result of direct interaction with receptors of ECM glycoproteins, most of which belong to the superfamily of integrins. The interaction of integrins with their specific ligands in turn induces immediate signal transduction through the intracellular cytoskeletal network resulting in tissue-specific gene expression (Spiegelman and Ginty, 1983; Ben-Ze'ev and Amsterdam, 1986; Streuli et al., 1991). There is now accumulating evidence from in vitro studies suggesting that

cells undergo changes in phenotypic expression of proteins by signals transmitted through receptor binding (reviewed by Juliano and Haskill, 1993). It was shown that certain milk protein gene expression in primary rat mammary cultures can be induced when these cells are plated on the ECM glycoprotein, laminin (Blum et al., 1987). Furthermore, the effect of laminin on the differentiation of mammary epithelial cells may be mediated by the cytoskeleton (Blum and Wicha, 1988) and requires a signal mediated through integrins (Streuli et al., 1991). Similarly, studies with rat hepatocytes showed that cell-matrix interactions determine the differentiated phenotype of these cells in culture as judged by the expression of high levels of liver-specific mRNAs, while synthesizing low levels of DNA and cytoskeletal mRNAs (Ben-Ze'ev et al., 1988). The differentiated phenotype of hepatocytes was also observed when they were seeded at high density and formed aggregates, suggesting that cell-cell interactions are important (Ben-Ze'ev et al., 1988). The expression of the 1A1 antigen in neonatal leptomeningeal cells in culture may also be controlled by both cell-matrix and cell-cell interactions as discussed earlier. Another parameter besides cell-cell contact and cell-matrix contact which might be responsible for the maintenance of 1A1 antigen expression on leptomeninges is that the turnover rate of the 1A1 antigen might be slower in leptomeningeal cells obtained from confluent cultures. This latter hypothesis was not assessed and needs further analysis.

The 1A1 Antigen Mediates Adhesion of Leptomeningeal Cells to Monolayers of Leptomeninges

The MAb 1A1 has been shown to interfere with neuron to astrocyte and astrocyte to astrocyte adhesion, but not with neuron to neuron adhesion (Chapter 2). Since the MAb 1A1 binds to adult leptomeninges and confluent cultures of neonatal

leptomeninges, the involvement of the 1A1 cell surface molecule in mediating leptomeningeal cell adhesion was examined. These experiments were done in a 90 minute adhesion assay using secondary cultures of neonatal leptomeninges which express the 1A1 molecule. Fab fragments of MAb 1A1 at 250 μg/ml inhibited adhesion of the DiI-labeled leptomeningeal cells to the monolayers by 26%. The degree of inhibition by monovalent Fab fragments of 1A1 antibody is comparable to that obtained in neuron to astrocyte and astrocyte to astrocyte adhesion. The MAb 1A1 blocked the adhesion of cerebellar granule cells to monolayers of flat type-1 astrocytes purified from the neonatal rat cerebral cortex by 22% (500 μg/ml), while the adhesion of cerebral cortical astrocytes to monolayers of flat type-1 astrocytes was blocked by 25% with 500 μg/ml of 1A1 Fab fragments (Chapter 2). The results presented in this chapter therefore indicate that the 1A1 antigen might mediate adhesive interactions between leptomeningeal cells.

Not much is known about the molecular basis of leptomeningeal histogenesis. The expression of cell adhesion molecules and matrix proteins in other systems suggest that similar strategies for adhesive interactions may also underlie the formation of leptomeningeal tissue during development. Leptomeningeal cells are derived from the mesenchyme and there is evidence to suggest that they are biochemically and morphologically similar to the mesenchymally-derived fibroblasts (Napolitano et al., 1964; Angelov and Vasilev, 1989; Peters et al., 1991). Fibroblasts express cell adhesion molecules, like N-CAM (Maier et al., 1986; Martini and Schachner, 1988; Gatchalian et al., 1989) and various ECM molecules, such as tenascin, fibronectin, collagen, laminin, and heparan sulfate proteoglycan in culture (Gatchalian et al., 1989; Halfter et al., 1989; Hatamochi et al., 1989; Munaut et al., 1991; Choi et al., 1992).

These ECM molecules are also expressed by leptomeningeal cells (Rutka et al., 1986; David, 1988; Esiri and Morris, 1991; Hernandez et al., 1991; Peters et al., 1991; Ajemian et al., 1994; Chapter 2). The increased 1A1 expression on leptomeninges with maturation in vivo and in vitro suggests that the 1A1 antigen may be a phenotype of differentiated leptomeningeal cells. Given its adhesive properties and expression in the adult animal, the 1A1 antigen could play a role in the maintenance of leptomeninges as a tissue. An increase in 1A1 antigen expression on neonatal leptomeningeal cells in vitro could be induced by ECM components, including laminin, fibronectin, and collagen types I and IV. Interestingly, greater than 90% of the adult leptomeningeal cells in suspension were labeled with antibodies against rat β_1 integrins as compared to only 10-15% in neonatal rats (data not shown). Whether these integrin receptors are functional in vivo in the adult is not known at present. Nevertheless, the increased deposition of ECM molecules and an increase in the expression of their membrane-associated integrin receptors as well as the 1A1 antigen during development suggests that adhesive mechanisms involving both cell-matrix and cell-cell interactions may play an important role in leptomeningeal histogenesis.

The molecular mechanisms mediating leptomeningeal tissue formation is of interest since in vivo studies indicate that the meningeal cells may indirectly influence the normal development of the underlying CNS (reviewed by Sievers et al., 1986; Hartmann et al., 1992). Earlier studies by Sievers and his colleagues showed that there is a positive correlation between the depletion of meningeal cells by 6-OHDA in neonatal hamsters, and the subsequent development of abnormalities in the cerebellum in terms of foliation, lamination, and granule cell number (Pehlemann et al., 1985). They suggested that meningeal cell destruction is associated with the destabilization and

disruption of the basal lamina, and that the production of collagen by meningeal cells might be necessary for its stabilization. Leptomeningeal cells covering the brain might also participate in the development of the underlying cerebral cortex (Abnet et al., 1991).

The results presented in this chapter indicate that the cell surface antigen recognized by the MAb 1A1 may mediate adhesive interactions between leptomeningeal cells. Given its increased expression in the mature animal, this molecule may also play a role in the maintenance of leptomeningeal tissue morphology.

Figure 4.1 Autoradiographs after reducing SDS-PAGE (7.5%) of immunoprecipitates obtained with MAb 1A1 (lanes 1, 3 and 4) and control MAb JG22 (lane 2) from [^{35}S]methionine-labeled protein extracts of confluent cultures of neonatal leptomeninges (lanes 1 and 2), and explants of neonatal (lane 3) and adult (lane 4) leptomeninges dissected and placed in suspension for metabolic labeling. A specific band at approximately 135 kd immunoprecipitated by MAb 1A1 can be seen in confluent cultures of neonatal leptomeninges (lane 1) and the adult leptomeninges (lane 4). Molecular weight markers from top are: 200 and 116 kd.


1
2
3
4

Figure 4.2 Immunofluorescence labeling with MAb 1A1 (A and C) and Nuclear yellow (B and D) in dissociated cell suspension of the newborn (A and B) and adult (C and D) rat leptomeninges. Neonatal leptomeninges (A) are $1A1^{-}$, while the majority of the adult leptomeningeal cells show punctate cell surface labeling with MAb 1A1 (C). Bar, 20 μm.

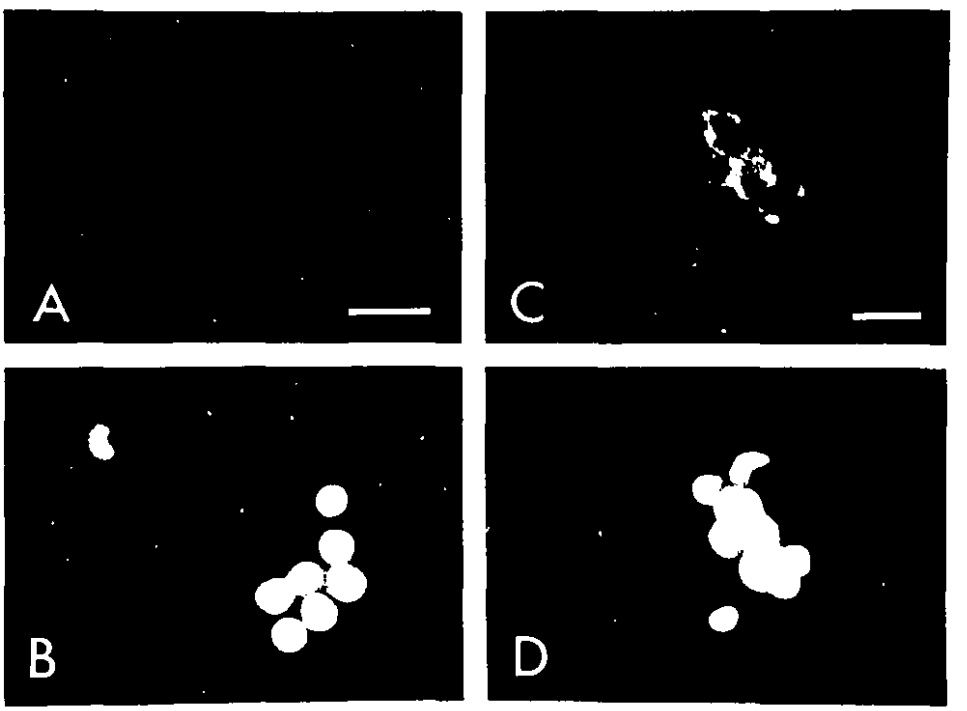

A
C
B
D

Figure 4.3 Percentage of $1A1^+/FN^+$ leptomeningeal cells in dissociated cell suspensions of the P2 and adult rat leptomeninges. Each value represents the mean ± SD of 2 experiments. For each experiment between 500-900 cells were counted on 3-4 coverslips. There is about a 7-fold increase in the number of $1A1^+$ leptomeningeal cells between neonatal and adult.

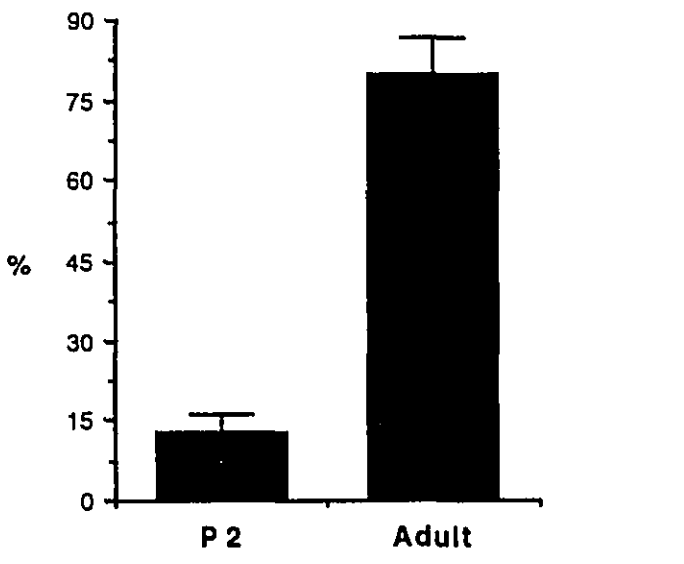

Percentage of 1A1 + Leptomeningeal Cells
90
75
60
%
45
30
15
0
P 2
Adult

Figure 4.4 Double-immunofluorescence labeling with MAb 1A1 (A and C) and polyclonal anti-fibronectin (B and D) in primary non-confluent (A and B) and confluent (C and D) cultures of the neonatal rat leptomeninges. 1A1 immunoreactivity is not seen in non-confluent culture (A), but is intense in confluent culture (C). Note an increase and a difference in the pattern of fibronectin labeling in confluent (D) versus non-confluent (B) cultures. Bar, 35 μm.

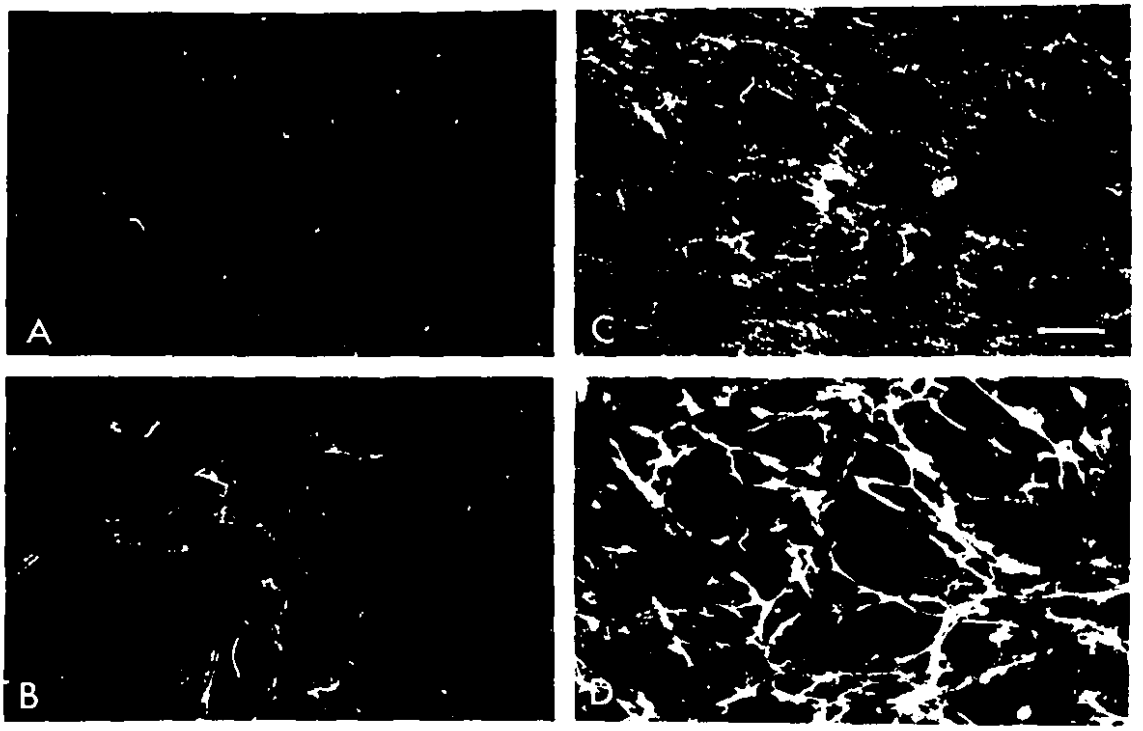
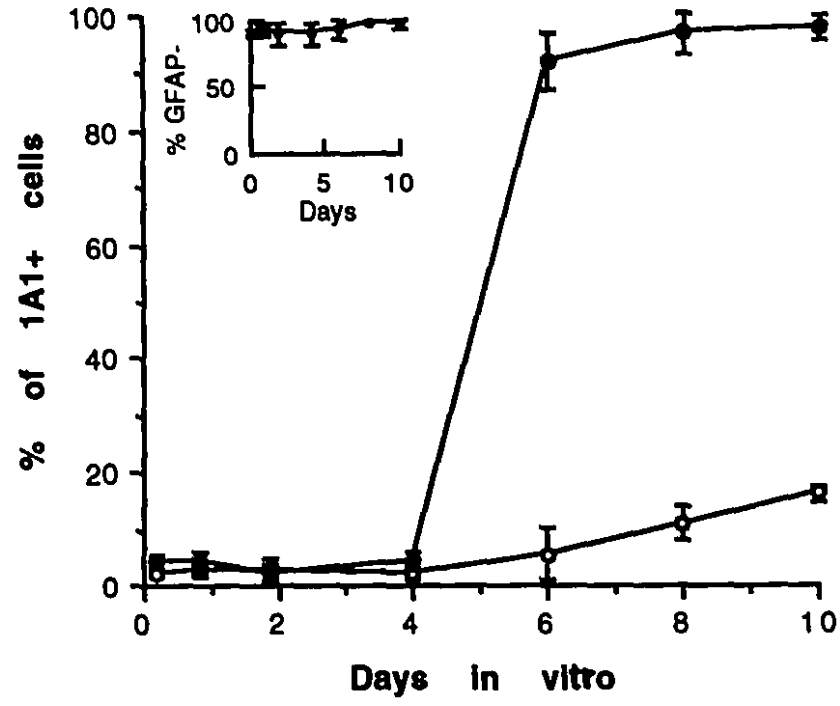

A
C
B
D

Figure 4.5 Changes in 1A1 expression in cultures of neonatal rat leptomeninges plated on PLL-coated substrate at a plating density of 0.75 x 10^4 (○—○) or 3 x 10^4 (●—●) cells/cm^2. In these cultures approximately 90% of the cells are large, flat $GFAP^-$ leptomeningeal cells (see insert in A), and less than 10% are $GFAP^+$ astrocytes (see insert in B). The time course of the changes in 1A1 expression in leptomeningeal cells and astrocytes are shown separately in A and B, respectively. Each value represents the mean ± SD of 2 experiments. For each experiment between 150-750 total cells on 6 coverslips were counted.

A


% of 1A1+ cells
100
80
60
40
20
0
0
2
4
6
8
10
Days in vitro
% GFAP-
100
50
0
0
5
10
Days

B

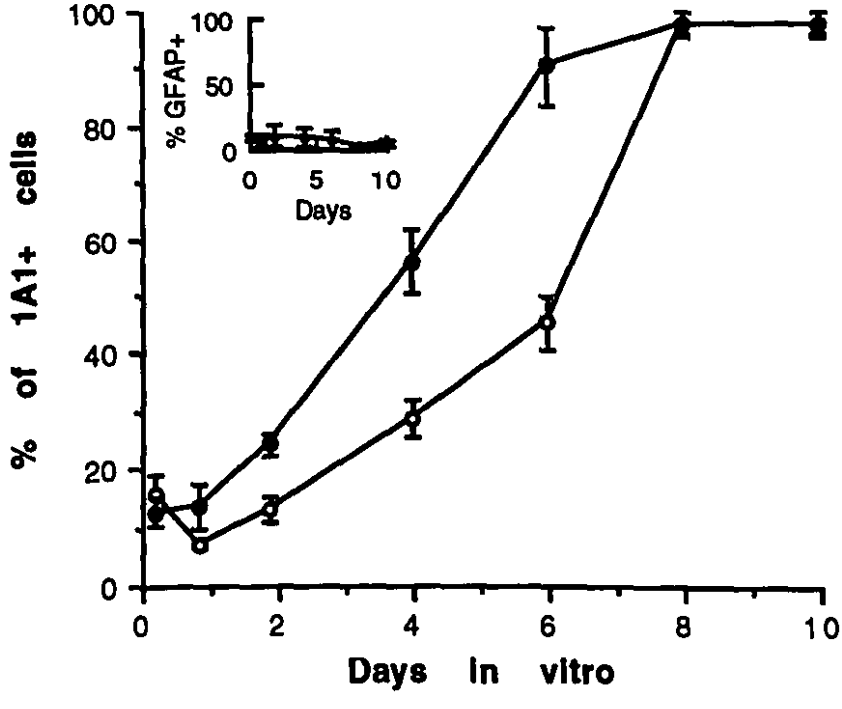

% of 1A1+ cells
100
80
60
40
20
0
0
2
4
6
8
10
Days in vitro
% GFAP+
100
50
0
0
5
10
Days

Figure 4.6 Percentage of $1A1^+$ leptomeningeal cells in dissociated primary cultures of the neonatal rat leptomeninges. Cells were plated at high density (3×10^4 cells/cm^2) on PLL or various ECM substrata and cultured for 24 hours prior to immunofluorescence labeling. Each value represents the mean $\pm$ SD of 3 experiments. 400-800 single $1A1^+/GFAP^-$ cells were counted on duplicate coverslips per experiment. A significant increase in $1A1^+$ cells is observed in all cultures grown on ECM substrata as compared to PLL. Significance levels were determined using a Student's t-test. Fibronectin (FN) and laminin (LN), $p < 0.01$; collagen types I (Col I) and IV (Col IV), $p < 0.001$; * = significantly different from other ECM substrata, $p < 0.05$

Effect of ECM Substrata on 1A1 Expression

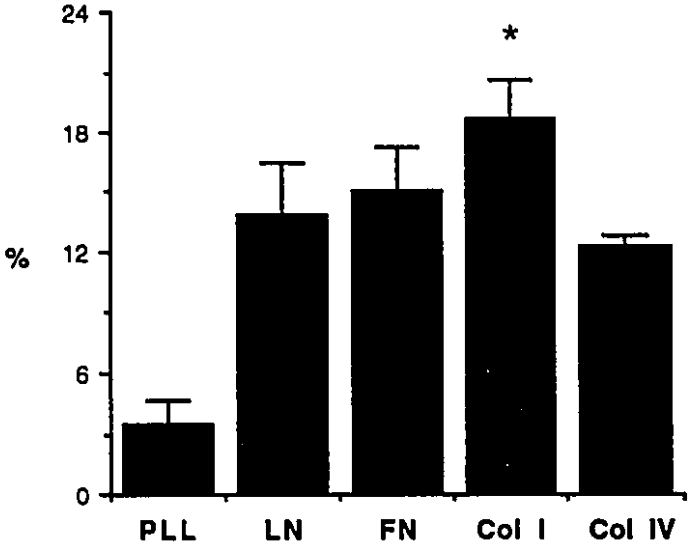


Table 4.1 Blocking effects of the MAb 1A1 on cocultures of DiI-labeled leptomeningeal cell suspension plated onto monolayers of leptomeninges in a 90 minute adhesion assay. The number of single DiI-labeled cells were counted and compared with control cultures treated with MAb Ran-2 or no antibodies. A significant inhibition in leptomeningeal cell adhesion is seen in cultures grown with MAb 1A1. Numbers indicate mean values ± SD. The number of experiments is indicated in parentheses with counts made from duplicate coverslips. Student's t-test was used to assign significance levels; a = not significantly different from antibody treated control cultures, b = $p < 0.01$. Percentage inhibition is the difference in cell attachment compared to Ran-2 controls.

LEPTOMENINGEAL CELL ADHESION

Antibody (μg/ml)	Number of single cells attached in 15 mm^2	Percentage of inhibition
None	230 ± 11 (3)[a]	
Ran-2 Fab (500 μg/ml)	232 ± 21.5 (3)	
(250 μg/ml)	234 ± 13 (3)	
(100 μg/ml)	241 ± 8 (3)	
1A1 Fab (500 μg/ml)	167 ± 2.5 (3)[b]	28%
(250 μg/ml)	174 ± 13 (3)[b]	26%
(100 μg/ml)	234 ± 22 (3)[a]	3%

Chapter 5: *Summary and Future directions*

In this thesis, a potentially novel cell adhesion molecule has been characterized using a monoclonal antibody (MAb 1A1). This MAb 1A1 has its limitations in that it binds very poorly to tissue sections for immunohistochemical studies, nor does it recognize the antigen on Western blots. My work involved using functional in vitro and biochemical approaches and has indicated the following.

1. By indirect immunofluorescence, the 1A1 antigen is found only on flat type-1 astrocytes and Bergmann glia in dissociated cultures of the CNS. Outside the CNS, 1A1 immunoreactivity is also seen in highly confluent cultures of mesenchymally-derived cells, such as leptomeninges and fibroblasts. This suggests that the control of 1A1 antigen expression in neural (i.e. astrocytes) and non-neural cells (i.e. leptomeninges) is different. I have shown that cell-cell contact and ECM molecules (laminin, fibronectin and collagen) play a crucial role in the regulation of 1A1 antigen expression in leptomeninges, but may not be necessary for 1A1 antigen expression in astrocytes.

2. Whole IgG and monovalent Fab fragments of MAb 1A1 inhibit neuron-astrocyte, astrocyte-astrocyte, and leptomeningeal cell adhesion by 27-33%. Neuron-neuron adhesion was unaffected by MAb 1A1, suggesting that the above inhibition by MAb 1A1 is specific. This degree of inhibition is comparable to that obtained with antibodies to other known cell adhesion molecules, such as N-CAM. Unlike N-CAM, the 1A1 antigen likely mediates cell-cell adhesion via a heterophilic-binding mechanism.

3. The MAb 1A1 had no effect on the attachment of astrocytes to various ECM molecules (i.e. laminin, fibronectin, and collagen type I), suggesting that the cellular adhesion by 1A1 antigen is not via these ECM molecules or their receptors.

4. The MAb 1A1 was shown to inhibit neurite outgrowth on astrocytes from E18 hippocampal and P1 cerebellar cortical neurons by about 30%.

5. In dissociated cultures of the developing cerebellum, Fab fragments of the MAb 1A1 inhibit the adhesion of neurons to Bergmann glia by about 50%. In microexplant cultures, the MAb 1A1 blocks the migration of neurons by 60%. These findings suggest that the 1A1 molecule might play a role in glial-guided neuronal migration in the developing cerebellum.

6. The 1A1 antigen is present at the appropriate time to be involved in the migration of granule cells along Bergmann glia in vivo. By immunoprecipitation the level of this antigen increases by about 2-fold between P1 and P20 followed by a decrease to the P1 level at P35. In addition, the number of $1A1^+$ Bergmann glia also increases from 33% to 78% between P1 and P7 in dissociated cell cultures.

7. The 1A1 antigen is a poorly glycosylated molecule that migrates with a MW of about 135 kd on both reduced and non-reduced SDS-PAGE. This suggests that the 1A1 molecule does not contain interchain disulfide bonds.

8. The 1A1 molecule is unlikely to belong to the cadherin family of CAMs, and differs from cadherins in three major properties: the absence of the tripeptide

HAV cell recognition sequence, its binding mechanism, and its insensitivity to trypsin in the absence of calcium.

9. The 1A1 antigen is highly expressed in C6 glioma cells.

Future directions

The results presented in this thesis demonstrate that the 1A1 antigen might be a novel cell adhesion molecule. Future work should be directed at its purification and molecular characterization. For this reason, I had screened various astrocyte cell lines for 1A1 antigen expression. The C6 glioma cell line was found to express high levels of the 1A1 antigen. This cell line is being used presently by others in the laboratory for purification of the 1A1 antigen (Patel et al., 1994). Besides amino-acid sequence determination which will provide information to construct oligonucleotide probes, the purified 1A1 molecule could also be used for generating polyclonal antisera. The generation of polyclonal antibodies will be important since the MAb 1A1 has several limitations, e.g., this MAb cannot be used for Western blotting or on tissue sections. Thus, developmental studies were difficult to carry out, and the in vivo distribution of this molecule could not be determined.

In addition, both the oligonucleotide probes and the polyclonal antibodies can be further used to screen a cDNA expression library for molecular cloning. These probes will be useful in studies of expression and developmental regulation of the 1A1 gene. Molecular cloning of the 1A1 antigen will help establish its relationship to other known cell adhesion molecules.

APPENDIX 1

1A1 Antigen Expression in Various Astrocyte Cell Lines

Cell lines obtained from different investigators		1A1 immunoreactivity
* DI TNC_1	Dr. C.F. Deschepper	positive
** C6 glioma (ATCC No. CCL 107)	Dr. H.D. Durham	positive
A7	Dr. H.M. Geller	negative
TsV1	Dr. H.M. Geller	negative
Human astrocytoma (ATCC No. CRL 1718)	Dr. F. Gervais	negative
rCG U/9-Pϕ2D	Dr. G.M. Smith	negative

* Rahemtulla, N., C.F. Deschepper, J. Maurice, B. Mittal, and S. David (1994) Immunocytochemical and functional characterization of an immortalized type-1 astrocytic cell line. Brain Res. 642:221-227.

** $1A1^+$ C6 gliomas were used to generate large amounts of tumor material by injecting cells subcutaneously into nude rats. Membrane preparations were obtained for use in 1A1 immunoaffinity chromatography for purification purposes. Although this procedure gave positive results, the yields of immunoaffinity-purified 1A1 antigen using the Affi-gel Hydrazide antibody column was insufficient for protein microsequencing.

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