A.C.H. Yu, L. Hertz, M.D. Norenberg, E. Syková and S.G. Waxman (Eds.)
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CHAPTER 30

Astrocytic response to injury

Lawrence F. Eng, Albert C.H. Yu and Yuen Ling Lee

Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, and Department of Veterans Affairs Medical Center, Palo Alto, CA 94304, U.S.A.

Introduction

The role of neurons, oligodendroglia, microglia, and infiltrating inflammatory cells (monocytes and neutrophils) as well as cytokines, growth factors, protease inhibitors, myelin membrane proteins, extracellular matrix or adhesion receptor systems in central nervous system (CNS) regeneration are under intensive study in many laboratories (for review, see Hatten et al., 1991). Our research is focused on the role of the astrocyte in CNS injury and disease. Astrocytes comprise as much as 25% of the cells and 35% of the total mass of the CNS. Astrocytes form barriers around blood vessels and connections between nerve cells. Numerous functions have been assigned to the astrocyte depending on its stage of maturation, location in the CNS, and response to CNS insult. These functions for the most part attempt to maintain and support the normal function of the CNS. In some instances, however, these beneficial responses may compete and inhibit the regeneration response of the oligodendrocytes and neurons. There are several reasons why astrocytes may not support CNS regeneration (Reier and Houlé, 1988). (1) Astrocytes may not provide the necessary tropic and/or trophic support for axonal regeneration. (2) Rapid astrogliosis forms a physical barrier of astrocytes which rapidly occupies the space caused by the injury and thus prevents remyelination and axonal migration. (3) Glial scars may lack the appropriate cell surface properties for neuronal interactions. (4) Glia in the mature CNS may not synthesize the extracellular matrix molecules that are conducive to axonal outgrowth. (5) Astrocytes and/or other constituents of glial scars may not have the appropriate proteolytic mechanisms. (6) Astrocytes induce the formation of pre-synaptic terminals. (7) Astrocytes may produce inhibitors of axonal extension or neuronal migration.

Of current interest are the reports that a membrane protein of CNS myelin inhibits neurite growth in vitro (Schwab and Caroni, 1988; Schwab, 1990) and that inhibitory molecules have been isolated from oligodendrocytes (Pesheva et al., 1989). Bovolenta et al. (this volume) have also identified inhibitory molecules from cellular membranes isolated from glial scar tissue. The failure of axons to penetrate scars is likely, at least in part, to result from inhibitory factors derived from oligodendrocytes, astrocytes, microglia, or immune cells present at the site of injury. Experiments suggesting that the astrocytic scar may not form a barrier to axon regrowth come from in vivo studies (Silver and Sidman, 1980; Kliot et al., 1990) and in vitro studies which provide evidence that astrocytes support axonal growth (Lindsay, 1979; Noble et al., 1984; Fallon, 1985; Gage et al., 1988; Neugebauer et al., 1988; Hatten, 1989). More recent studies suggest that astrocytes that are not favorable for axonal growth, are substrates for neurite growth (Lindsay, 1979; Baehr and Bunge, 1990). Evidence that axons grow well on glial scars has recently been reported (David et al., 1990). In another type of in vitro

study, Rudge and Silver (1990) demonstrated that the glial scar, at best, stimulates only minimal neurite outgrowth over its surface as compared to the immature environment explanted in the same manner. Presently the in vivo evidence that the glial scar supports regeneration presents a mixed impression. The in vitro evidence, while quite extensive, is a two-dimensional model which agrees well with three-dimensional in vivo observations which show that axons do not penetrate the glial barrier but grow along the surface of the scar. It is evident that regeneration at the site of a CNS lesion is complex and involves factors which promote and inhibit axon regrowth. It has been suggested that too much attention has been focused on astrocytes and glial filaments (Hatten et al., 1991), however, the glial barrier hypothesis remains one of the important problems in CNS regeneration. Therefore, methods which inhibit or delay the astrogliotic response merit continued investigation.

Reactive astrocytes

Any type of insult to the CNS can induce astrogliosis whether it results from physical trauma such as a liquid nitrogen lesion (Amaducci et al., 1981), light-induced photoreceptor degeneration in the rat retina (Eisenfeld et al., 1984) or spinal cord transection (Reier, 1986), immunologic cellular insult such as experimental allergic encephalomyelitis (EAE) (Smith et al., 1983), Creutzfeldt-Jakob disease (CJD) infection (Manuelidis et al., 1987) or biochemical alteration due to a genetic defect (Eisenfeld et al., 1984). When the blood-brain barrier is disrupted, numerous factors can activate the astrocytes: (1) anoxia may occur due to interruption of the blood supply; (2) dilution of inhibitory "chalones" around the site of edema may occur; (3) blood-born substances such as complement components can enter the CNS; (4) cell-derived substances from infiltrating cells can be released; macrophages and activated microglia express mediators such as interleukin 1, tumor necrosis factor, prostaglandins, leukotrienes, neutral proteinases such as plasminogen activator and other myelin degrading enzymes, and cytotoxic agents such as oxidative radicals; (5) mitogenic and non-mitogenic substances from degenerating neurons and fibers can be released; and (6) astrocytes can be released from contact inhibition due to increase in space.

The reactive astrocyte undergoes numerous cytological, histochemical and biochemical features, including: increases in nuclear diameter (Hortega and Penfield, 1927; Cavanagh, 1970), elevated DNA levels (Lapham and Johnstone, 1964), accumulation of intermediate filaments (Nathaniel and Nathaniel, 1977), elevated oxidoreductive enzyme activity (Öhmichen, 1980) and increased synthesis of glial fibrillary acidic protein (GFAP) (Bignami and Dahl, 1976; Amaducci et al., 1981), vimentin (Dahl, 1981; Dahl et al., 1981, 1982), glutamine synthetase (Norenberg, 1983), and glycogen (Nathaniel and Nathaniel, 1981).

Some signals which regulate gene expression in development and response to astrocyte injury are: growth factors, prion protein from Scrapies, neural and immunological adhesion molecules such as NCAM, LFA-1, gangliosides, low density lipoproteins, cytokines from T-cells, macrophages and other glia, neurotransmitters and neuropeptides such as catecholamines, monoamines, glutamate, ATP, substance P, and antigen-antibody complexes. Astrocytic responses to these signals include: (1) proliferation, movement and differentiation; (2) changes in shape, cell volume, cytoskeletal organization, endocytic activity, lysosomal fragility and enzyme content; (3) buffering capacity for K^+, glutamate and GABA; (4) expression of nerve growth factor, tumor necrosis factor, interferon α and β, interleukin 1 and 6, colony stimulating factor-1, fibroblast growth factor, neurotropic factors, neurite promoting agents, MHC class I and II histocompatibility antigens, amyloid protein, GD3 ganglioside, ICAM-1, Na^+ channel protein, GFAP, crystallin, vimentin and heat shock proteins.

Astrogliosis

Reactive astrogliosis is a stereotypic reaction of

astrocytes within and adjacent to the site of injury. It also occurs in CNS demyelination such as multiple sclerosis (MS) and the degenerative diseases – Alzheimer's disease, CJD, and Huntington's disease. Astrogliosis is characterized by astrocyte proliferation and extensive hypertrophy of the cell body and cytoplasmic processes. Astrogliosis may participate in the healing phase following CNS injury by actively monitoring and controlling the molecular and ionic contents of the extracellular space of the CNS. They can wall off areas of the CNS that are exposed to non-CNS tissue environments following trauma. On the other hand, astrogliosis may have pathological effects by interfering with the function of residual neuronal circuits, by preventing remyelination, or by inhibiting axonal regeneration (Eng et al., 1987; Reier and Houlé, 1988).

GFAP is the intermediate filament in differentiated astrocytes. Extensive use of mono- and polyclonal antibodies to GFAP in neurobiology has established GFAP as a prototype brain antigen in CNS immunocytochemistry and as a standard astrocyte marker for neuroscience research (Eng, 1985; Eng and Shiurba, 1988). Astrogliosis is characterized by extensive synthesis of GFAP intermediate filaments and by hypertrophy of the astrocytic cytoplasmic processes. The functional significance for this increase in intermediate filaments (IFs) is not known. Evidence from studies with rat optic nerve astrocyte cultures suggests that the content and subcellular distribution of IFs are important for cytoplasmic process formation and for structural stability of astrocytes. The relatively slow metabolic turnover rate for GFAP is consistent with such a structural role (DeArmond et al., 1983, 1986; Smith et al., 1984b). Ultrastructural and immunocytochemical studies of astrocytic differentiation in vitro show that the flat, polygonal astroblast contains abundant microtubules and actin stress fibers; however, these elements progressively decrease while GFAP increases during the change in shape of this astroblast to a stellate cell having slender, unbranched processes (Ciesielski-Treska et al., 1982a,b; Trimmer et al., 1982; Fedoroff, 1985). A recent study provides further evidence for a structural role for GFAP. Weinstein et al. (1991) permanently transfected a human astroglioma cell line with an antisense GFAP DNA construct and showed that this cell could no longer synthesize GFAP or form processes in response to neurons in culture.

Increased protein content or immunostaining of GFAP have been found in experimental models involving gliosis, such as the cryogenic lesion (Amaducci et al., 1981), stab wounds (Latov et al., 1979; Mathewson and Berry, 1985; Takamiya et al., 1986, 1988; Jeneczko, 1988; Miyake et al., 1988; Topp et al., 1989; Hozumi et al., 1990; Vijayan et al., 1990), toxic lesions (Brock and O'Callaghan, 1987; Reinhard et al., 1988; Rataboul et al., 1989), and EAE (Smith et al., 1983, 1984a; Goldmuntz et al., 1986; Aquino et al., 1988a,b). Increases in mRNA to GFAP have been reported in normal rat brain development (Tardy et al., 1989; Landry et al., 1990), as well as mechanical rat brain injury (Condorelli et al., 1990; Steward et al., 1990), toxic lesions (Rataboul et al., 1989) and in EAE (Aquino et al., 1990) consistent with de novo synthesis of GFAP protein being involved in these responses to injury.

GFAP and the glial barrier hypothesis

Our current investigations are directed toward characterization of the normal functions and properties of the astrocyte and the specific factors which induce its reactive response to injury in cultured astrocytes and injured rat spinal cord. The rapid formation of an astrocytic scar at the site of spinal cord injury may form a physical barrier which prevents regeneration. The most prominent feature of astrocytic gliosis is the accumulation of GFAP, the intermediate filament of differentiated astrocytes. Our working hypothesis is that control of astrocyte proliferation, differentiation, and astrogliosis may be linked to GFAP synthesis. One might be able to modulate astrogliosis to promote healing and functional recovery of neuronal pathways. For example, inhibition of GFAP synthesis immediately following injury might delay astrogliosis. A delay in astrogliosis may allow neurons the opportunity to

regenerate and oligodendrocytes to proliferate and remyelinate.

Alternatively even if gliosis were delayed, the environment might not be conducive for regeneration. Astrocytes in immature neural tissue have been shown to migrate and are thought to have a restorative role when they are introduced into the glial scar (Smith and Miller, 1991). It is possible that such enhanced CNS restoration may be due to factors produced in embryonic neural tissue which are no longer produced in significant amounts in mature, fully differentiated neural tissue. Astrocytes in culture have been shown to produce growth factors such as β nerve growth factor (βNGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor β (PDGF β). We are combining molecular biological approaches and immunochemical and biochemical detection systems with high resolution nuclear magnetic resonance microimaging (NMRI) of lesions in vitro and in situ to gain new perspectives on neural regeneration.

Experimental allergic encephalomyelitis

Experimental allergic encephalomyelitis (EAE), a cell-mediated autoimmune disease, has been the principal experimental model for studying the etiology and pathogenesis of multiple sclerosis (MS) (Smith et al., 1984a, 1987, 1988; Eng et al., 1986a; Smith and Eng, 1988). Astrocyte proliferation and hypertrophy of cell processes appear very early in this model, coincidentally with the first inflammatory foci and is apparent by an increase in immunostaining of the astrocytes with GFAP antibodies without an increase in GFAP content (Smith et al., 1983; Goldmuntz et al., 1986). A more recent report has shown that the increase in GFAP immunoreactivity of the astrocytes without a corresponding increase in GFAP content observed in EAE during the first 13 – 18 days post-inoculation is not due to an increase in GFAP epitopes resulting from limited proteolysis, glial filament dissociation yielding an aqueous soluble fraction, or to differences in the avidity of a number of different antibody preparations tested (Aquino et al., 1988a,b). In acute EAE, the increase in GFAP immunostaining of the astrocytes is wide spread and not confined to lesion sites (Smith et al., 1983). With chronic relapsing EAE in the mouse, astrocyte activation and gliotic scar formation appears in areas at the vicinity of the inflammatory lesion (Smith and Eng, 1987). The reason for astrocyte hypertrophy and increased immunostaining for GFAP without a demonstrable increase in GFAP content is unknown. The onset of edema due to disruption of the blood-brain barrier (BBB) and leakage of blood-borne substances into the CNS (Levine et al., 1966; Cutler et al., 1967; Juhler et al., 1984) may contribute to these phenomena. Swollen astrocytic processes filled with disrupted bundles of glial filaments and glycogen particles have been shown by electron microscopy in edematous brain tissue (Kimelberg et al., 1982).

Recently we examined the early EAE lesion by electron microscopy and obtained results similar to that reported by Kimelberg et al. (1982). The astroglial processes contained many glycogen particles. The glial filaments were arranged in small bundles or loose thin filaments adjacent to the bundles. The glial filaments that normally appear as tight bundles expanded and appeared less dense. The general picture indicates that the first stages of EAE are pathogenetically related to an abnormal BBB permeability. The "watery" cytoplasm of astrocytes at this early stage of EAE is most likely expressing a "partial" breakdown of the BBB which is characterized by the absence of anatomical abnormalities of the vascular walls and the presence of intracellular (astrocytic) edematous fluid (Lee, 1982; Miquel et al., 1982). "Leaky" vascular walls together with the injury of astroglial membranes may induce metabolic disturbances through the influx of edema fluid which may contain potassium, biogenic amines, prostaglandins and their immediate precursor arachidonic acid, free radicals and glutamate among other components (Bourke et al., 1980; Chan and Fishman, 1985; Halliwell and Gutteridge, 1985; Wahl et al., 1988). We have suggested that the increase in GFAP immunostaining without an increase in GFAP content is due primari-

ly to the disruption of the blood-brain barrier. The resulting edema allows the tight bundles of glial filaments to dissociate and thus expose more antigenic sites (epitopes) to GFAP antibodies (Eng et al., 1988, 1989). The ensuing migration of cells with the ability to release cytokines (Waksman and Reynolds, 1984) contributes to the continuation of this process in the astrocytes.

As a result of our immunohistochemical, light-microscopic and ultrastructural study of the EAE rat spinal cord (D'Amelio et al., 1989), we have suggested that the pathogenesis of EAE involves the following sequence of events. (1) Edema induced by partial breakdown of the BBB that affect predominantly astrocytes which exhibit increased GFAP immunoreactivity due to dissociation of glial filaments and consequent exposure of more antigenic sites. (2) With further disruption of the BBB, "activated" lymphocytes and blood-borne macrophages penetrate the vascular endothelium "en route" to the neuropil. (3) Myelin breakdown is initiated by macrophagic activity upon oligodendroglia and myelin sheaths. Macrophages in the tissue are derived from the blood, pericytes and microglia. (4) Demyelination is enhanced by the ability of microglial cells/macrophages to express Ia-antigens induced by the secretion of factors (INF-γ) from "activated" lymphocytes. (5) Finally, the demyelinated areas become filled with fibrous astrocytes which form the glial scar.

Antisense oligonucleotides

Synthetic oligonucleotides have been used successfully to inhibit DNA replication, retroviral replication, pre-mRNA processing and protein synthesis with high specificity. Different mechanisms for the inhibitor effects have been proposed (Chrisey, 1990). The antisense oligonucleotides are complementary to the selected RNA target and are generally between 15 and 30 nucleotides in length, which is sufficient to define a unique sequence. Synthetic oligomers may be actively transported into cells and lead to irrevisible antisense effects. They also allow a control on the number of antisense molecules that enter the cell (Cohen, 1989; Chrisey, 1990). Not all oligonucleotides are suitable for antisense studies due to their inherent instability, inefficient uptake, and degradation by exo- and endonucleases. Phosphorothioate (Agrawal et al., 1989a,b; Cazenave et al., 1989; Mori et al., 1989; Paules et al., 1989), methylphosphonates (Miller et al., 1985a,b; Kean et al., 1988; Agrawal et al., 1989a,b; Brown et al., 1989) , O-alkylphosphotriesters (Moody et al., 1989a,b) and α-anomeric DNA (Gagnor et al., 1989; Lavigon et al., 1989) are chemically modified oligonucleotides with substantial improvement in stability, uptake and nuclease resistance (Chrisey, 1990). In general, phosphorothioate DNA oligomers are more potent inhibitors than phosphodiester oligomer α-anomeric DNA and methylphosphonates (Agrawal et al., 1989a,b; Cazenave et al., 1989; Mori et al., 1989; Paules et al., 1989). Addition of "reactive" molecules (intercalator, photochemically activated cross-linking or cleaving agents, alkylating agents, or redox active nucleic acid cleaving groups) (Asseline et al., 1985; Helene, 1987; Vlassov et al., 1988a,b; Amirkhanov and Zarytova, 1989; Boutorin et al., 1989; Pieles and Englisch, 1989) will either stabilize the hybrids and/or permit damage of the target nucleic acid. Other modifications include the attachment of poly-L-lysine (Chu and Orgel, 1988) or lipophilic moieties (Boutorin et al., 1989; Letsinger et al., 1989; Kabanov et al., 1990) to increase hydrophobicity and promote uptake. Another approach has been to encapsulate the oligonucleotides in liposomes which protect them from attack by nucleases and facilitate cellular uptake (Loke et al., 1988; Leonetti et al., 1990; Eng et al., 1991; Yu et al., 1991). Other potential antisense agents include synthetic RNA and ribozymes (Haseloff and Gerlach, 1988; Cameron and Jennings, 1989) 2'-O-methylribose RNA is resistant to RNases. Ribozymes can be designed to irreversibly damage a specific substrate RNA molecule, and thus represent an exciting new class of antisense molecules.

Introduction of DNA into primary cultures of astrocytes with Lipofectin™

Primary astrocytes grown in 35 mm plastic dishes were fed deoxyribonucleic acid complexed with a commercial cationic liposome preparation, Lipofectin™ reagent (LF). Astrocytes exposed to 11 and 17 μg LF for 24 h excluded trypan blue indicating that the cells were intact. Astrocytes treated with 34 μg LF showed a loss of cell integrity and many of them were stained with trypan blue. Similar cultures of astrocytes exposed to 11 and 17 μg LF for 30 h released 7 – 10% of the intracellular lactate dehydrogenase (LDH). Four hours of exposure to 34 μg LF induced a 2% LDH release which was increased to 15 – 20% after 8 h. A non-toxic dose of 11 μg was chosen to mediate the uptake of nucleic acids. After 30 min exposure to LF/photo-biotinylated λDNA, astrocytes were positively immunostained with antibody to biotin. Uptake of LF/^{3}H-λDNA in astrocytes was rapid and reached an equilibrium (3.25 ng/mg protein) within 30 min. λDNA alone entered astrocytes slowly with an equilibrium 1/6 of the LF/DNA complex. The uptake of LF/DNA for glial fibrillary acidic protein and LF/DNA for neurofilament (NF) were comparable to λDNA. Astrocytes transfected with LF/NF-DNA showed a detectable level of NF by enzyme-linked immunosorbent assay. This observation indicated a transient expression of the transfected NF-DNA in cultured astrocytes (Eng et al., 1990).

Inhibition of GFAP synthesis with antisense RNA in cultured astrocytes

Antisense GFAP mRNA uptake by cultured astrocytes

Astrocytes cultured in 35 mm plastic dishes with 2 ml of medium and exposed to biotinylated antisense GFAP RNA showed positive reaction with antibody to biotin by immunohistology. The staining was observed as early as 30 min of exposure to the complex. No staining was observed in cultures exposed to LF or biotinylated antisense RNA alone demonstrating that LF facilitated the entry of antisense GFAP RNA into astrocytes. ^{3}H-Antisense RNA alone entered astrocytes even in the absence of LF. Between 2.5 and 6 h of exposure, astrocytes took up 7 – 8.8 ηg antisense RNA per mg protein, with an uptake efficiency of 3.4%. The uptake was accelerated and enhanced when antisense RNA was complexed with LF. In Dulbecco's Modified Eagle Medium (DMEM), the time for astrocytes to take up a similar amount of antisense RNA in LF as compared to antisense alone was shortened to 10 min. The total uptake was also elevated to 14 ηg/mg protein in a 6 h experimental period, within an uptake efficiency of 5.6%. When the LF complex was incubated in Hepes buffered saline (HBS), the amount of antisense RNA that entered the astrocytes almost doubled (> 20 ηg/mg protein) in 10 min of transfection, and the uptake efficiency was increased to almost 10%.

The uptake of antisense RNA was increased when the dose of antisense RNA in the LF complex was elevated. The uptake of antisense RNA alone after 1 h incubation was low with an uptake efficiency of about 1.5%, despite the increased doses. With LF as a mediator, the uptake was higher than antisense RNA alone. In HBS, the LF complex uptake efficiency averaged 7.6%, higher than 2.9% in DMEM. The uptake efficiency remained the same at doses up to 15 μg. The initial uptake rate in HBS reached an optimal efficiency within 10 min and was always higher than the complex in DMEM which in turn was higher than for antisense RNA alone. At least 6 h were required to achieve optimal efficiency. The data also showed that the 11 μg of LF in the complex could introduce a large amount of antisense RNA into astrocytes.

Effects of antisense RNA on GFAP synthesis in dBcAMP treated culture

Cultures of astrocytes treated with 0.25 mM of dibutyryl adenosine 3′,5′-cyclic monophosphate (dBcAMP) showed an increase in GFAP content over an 8 day experimental period. The GFAP content in astrocytes at day 0 (i.e., immediately before transfection) was used as the control and the changes were expressed as percent increase or de-

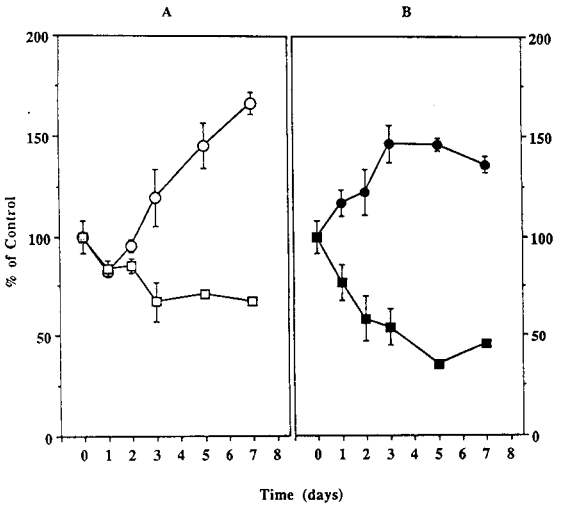

Fig. 1. Effect of LF/antisense GFAP RNA transfection on the GFAP content of astrocytes in cultures. Cultures were incubated in DMEM contained 0.25 mM dBcAMP and 10% serum for 2 days before transfected for 3 h in HBS. Then all cultures were incubated in DMEM contained dBcAMP supplemented with (*A*) or without (*B*) serum. GFAP content was quantitated by ELISA. The GFAP contents in transfected (□; ■) and non-transfected (○; ●) cultures were expressed as the percentage of the value at day 0. One milliliter of complex contained 6 μg antisense RNA and 11 μg of LF. Each point was average of four measurements and S.E.M. values are shown by vertical bars if they extend beyond the symbols. (Taken from Yu et al., 1991.)

crease with respect to this value (Fig. 1). In serum-free medium, a rapid increase in GFAP content induced by dBcAMP was observed. The increase reached about 160% on day 3, then remained at this level for the rest of the experiment. In serum-containing medium, the stimulation by dBcAMP was delayed and a gradual increase was observed after 2 days. The GFAP content employing serum-containing medium also reached a level greater than 160% of the control at the end of the experiment.

Although dBcAMP was in the culture medium, all transfected cultures showed a decrease in GFAP content, the decrease being more pronounced in cultures maintained in serum-free medium. One day after transfection, a 30% decrease in GFAP content was achieved. The content was further decreased to less than 50% of its original level after the third day of transfection. In serum-containing medium, a significant decrease in GFAP content of 30% was observed 3 days after transfection. The GFAP content began to return gradually to the day 0 level 5 days after transfection in some cultures but never reached a level comparable to the corresponding non-transfected sister cultures. A similar result was observed in cultures of astrocytes exposed to a chemically defined medium, a condition also known to increase the GFAP content of astrocytes in culture (Morrison et al., 1985). Antisense RNA alone and complexes with lower doses of antisense RNA (< 3 μg per culture) did not induce any observable effect on the GFAP content. The inhibition was further confirmed by separating the total cellular protein with SDS-PAGE, transblotting to nitrocellulose and estimating the changes in GFAP content by immunoperoxidase staining with antibody to GFAP. Results indicated that the GFAP content was clearly decreased in cultures 3 days after transfection. The content gradually returned to the day 0 level but remained lower than comparable non-transfected cultures.

Inhibition was not detected in cultures transfected with antisense RNA alone, nor with low doses (< 3 μg) of antisense RNA in LF complex. Weak inhibition was observed in some cultures transfected with a complex which contained 3 μg antisense RNA. Only with complexes containing 6 μg of antisense RNA was the inhibition of GFAP synthesis significant and reproducible. With a 7.6% uptake efficiency, 450 ηg of antisense RNA entered the culture. Based on our previous findings (Eng et al., 1990), at least 1/6 of this antisense RNA (75 ηg) would be available to hybridize with the sense GFAP mRNA (Eng et al., 1991; Yu et al., 1991).

Discussion

The method for controlling gene expression with antisense nucleic acid has already provided a powerful tool for identifying genes, characterizing gene functions, controlling virus and parasite infections, and manipulating metabolic pathways (Agris et al., 1986; Smith et al., 1986; Morvan et al., 1987; Thuong et al., 1987; Le Doan et al., 1989). The pres-

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