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CHARACTERIZATION OF NOVEL ANTIGENS, DETECTED USING MONOCLONAL ANTIBODIES RAISED AGAINST TISSUE FROM THE CENTRAL NERVOUS SYSTEM OF MULTIPLE SCLEROSIS PATIENTS

BY

DONALD RANDALL WILSON PREDY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

SPRING 1989

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Dr. G. D. Das Signed D. Das

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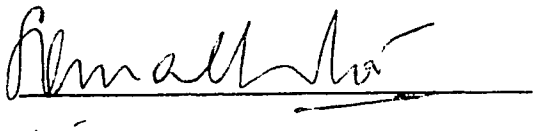
Dr. S. K. Malhotra 

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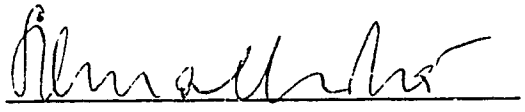
Dr. S. K. Malhotra 

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Date: 13 April 1989

"In fact, I try to think of myself as I would of anyone else. This is the essence of justice."

- J. B. S. Haldane

Clark, Ronald, (1968): J. B. S. The Life and Work of J. B. S. Haldane. Hodder and Stoughton, London, England, p. 60.

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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled "Characterization of Novel Antigens, Detected Using Monoclonal Antibodies Raised Against Tissue From the Central Nervous System of Multiple Sclerosis Patients" submitted by Donald Randall Wilson Predy in partial fulfilment of the requirements for the degree of Master of Science in Experimental Pathology.

Supervisor

Date: April 12, 1989

Dedication

This Master's Thesis is dedicated to Dr. S. K. Malhotra, Dr. T. K. Shnitka, and Dr. A. R. Palmer, who all played important parts in my entry into Graduate School. It is especially dedicated to Dr. Malhotra, as he provided me with an opportunity to do that which I love most. Science.

And to my Poppa, Mr. Sam Thomson, for always impressing upon me the importance of getting an education.

I remember the logarithms!

Abstract:

Investigations have been carried out using mouse monoclonal antibodies (MAbs) raised against autopsy samples of tissue from the central nervous system (CNS) of multiple sclerosis (MS) patients. In sections of CNS tissue, the antigens recognized by MAb J1-31 and MAb G-3-5 are principally localized in astrocytes; the antigen recognized by MAb 6B9 is principally localized in the cell bodies of neurons.

The majority of the studies described herein deal with the expression of J1-31 antigen. Chapter II reports on the localization of J1-31 antigen in astrocytes, retinal Muller cells, tanycytes and ciliated ependymal cells, as well as the expression of this antigen during ontogeny. Chapter III and IV report on enhanced immuno-flourescence staining for J1-31 antigen in reactive astrocytes which arise following spinal cord injury. Chapter V reports on enhanced staining for J1-31 antigen in MS plaques. This is consistent with results from surgical lesions, as MS plaques characteristically show an astroglial reaction (reactive gliosis) which results in the formation of a glial scar. Evidence cited in these chapters suggests that J1-31 antigen is associated with intermediate filaments in astrocytes. Chapter VI reports on the expression of J1-31 antigen by cultured cells. As rat glioma cells (9L) turn-off expression of J1-31 antigen

upon achieving a confluent monolayer, this antigen may be involved in some aspect of cell division and / or growth.

Chapters VII and VIII present results on the expression of G-3-5 antigen and 6B9 antigen in CNS, numerous non-CNS tissues, and during development. G-3-5 antigen is localized in the same types of CNS macroglia as express J1-31 antigen. To date, no central organizing hypothesis has been proposed to account for the distribution of this antigen. The detection of 6B9 antigen in secretory cells, such as plasma cells and exocrine pancreas, suggested the hypothesis that 6B9 antigen is a member of a class of proteins, called reticuloplasmins, which localize to the lumen of the rough endoplasmic reticulum. Although supported by circumstantial evidence, this hypothesis remains to be proven.

Preface

One of the options available to me, as a student registered in the Faculty of Graduate Studies and Research, is to organize my thesis in accordance with the guidelines for Paper-Format Theses. Most of the studies described herein have been published, or accepted for publication (versions of Chapters **II**, **III**, **V** and **VIII** have been published; a version of Chapter **IV** is in the press). Each study addresses a series of related scientific questions.

Sir Peter Medawar said of scientific papers: "The layout of the text that has come to be regarded as conventional is that which perpetuates the *illusion* that scientific research is conducted by the inductive process"[1] (italics mine). Although this may be true of the individual studies presented herein, the order and grouping of these studies accurately reflects the historical course of the research (particularly the studies on J1-31 antigen which are arranged chronologically with respect to completion of the work, which parallels acceptance for publication). Therefore, the Paper-Format preserves the integrity of the individual studies, each of which presents related experimental results.

1. Medawar, P. B., (1979): Advice to a Young Scientist, Harper and Row, New York, p. 67.

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I wish to express my sincere gratitude to Mr. Rakesh Bhatnagar who taught me much about electron microscopy and photography. Dr. R. Singh taught me the basic techniques for immunostaining tissue sections (immunofluorescence microscopy) and ELISA. Dr. D. N. P. Singh taught me the technique for perfusion. Karen Price has provided valuable support in the cell-culture lab and for immunofluorescence microscopy.

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Abreviations

CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
dBcAMP	diButyryl cyclic AdenosineMonoPhosphate
DRG	Dorsal Root Ganglion
FCS	Fetal Calf Serum
FITC	Fluorescein IsoThioCyanate
GFAP	Glial Fibrillary Acidic Protein
IFs	Intermediate Filaments
MAb	Monoclonal Antibody
MS	Multiple Sclerosis
M. W.	Molecular Weight
NMS	Normal Mouse Serum
NRS	Normal Rabbit Serum
PBS	Phosphate Buffered Saline
rER	rough Endoplasmic Reticulum
TRITC	TetramethylRhodamine IsoThioCyanate

Chapter I: Introduction.

Research undertaken in partial fulfilment of the requirements for the Degree of Master of Science (Experimental Pathology) has been an extension of research ongoing in the laboratory of Dr. S. K. Malhotra, as to the nature of certain antigens differentially expressed by the various cell-types in the central nervous system (CNS). These antigens are recognized, and hence operationally defined, by monoclonal antibodies (MAbs) raised against autopsy samples of tissue from the CNS of multiple sclerosis (MS) patients. The MAbs, used in these studies, had been previously raised in attempts to identify unique or characteristic antigens relevant to MS, which is a demyelinating disease of the CNS. It is stated at the outset that the antigens recognized by these MAbs are not specific to MS tissue. However, these MAbs do reveal differences in the molecular composition of CNS cells. One undertakes to study such differences with the hope that the reactive antigens are novel (hitherto unknown), and that they serve important physiological functions. It is emphasized that the aim of this research is to investigate the nature of undefined antigens in CNS tissue, which, initially, are known only through their reactivity with an MAb.

Experimental Approach for Identifying MS Antigens

This project evolved out of research aimed at identifying antigens relevant to MS. For this purpose, Dr. Malhotra raised MAbs against crude homogenate of CNS tissue from MS patients. This experimental approach was made possible through the work of Georges Kohler and Cesar Milstein, who developed a technique for establishing continuous cultures of fused cells secreting antibody of predefined specificity (Kohler and Milstein, 1975). Antibodies are serum proteins secreted by B-cell lymphocytes. These soluble proteins bind to, and hence recognize, antigenic determinants (also called epitopes) on antigens by virtue of complementary shape and charge distribution (Amit et al., 1986; Colman et al., 1987). A central tenet of immunology states that a given B-cell secretes antibodies of uniform structure, all recognizing the same epitope or narrow spectrum of structurally related epitopes. To date, no one has succeeded in cloning B-cells. However, Kohler and Milstein (1975) succeeded in immortalizing such cells by fusing them with myeloma cells, derived from a B-cell tumor (Milstein, 1980). The resultant hybrid cells, or hybridomas, combine the antibody secreting property of the B-cells with the unlimited growth potential of the myeloma cell line. Hybridomas are subcloned by limiting dilution to ensure monoclonality of the antibody source. The progeny, or clone, derived from a single hybridoma cell is a source of structurally uniform

antibody molecules, MAbs.

The principal advantage of this technique lies in the ability to immunize with a mixture of antigens, such as tissue homogenate, and yet generate MAbs which recognize particular epitopes, and hence antigens, in the mixture. In this way, highly specific MAbs can be raised against antigens which are unknown, as was attempted using MS tissue (described herein), or which have proven difficult to purify by conventional biochemical techniques, as was the case of interferon (Milstein, 1980). Such MAbs can then be used in the purification and characterization of the reactive antigens. Traditional methods for antiserum production, requiring purified antigen for immunization, can not be applied in this way to "pull an antigen out of a mixture".

Thus the rational for raising MAbs against CNS tissue from MS patients, is that such MAbs might recognize hitherto unknown antigens which could provide clues as to the causative agent and / or pathological processes underlying this demyelinating disease.

Background on Multiple Sclerosis

Multiple Sclerosis is the leading cause of serious neurological disease affecting young and middle-aged adults in North America and Western Europe (McKhann, 1982). MS is principally a disease of

CNS white matter. The characteristic lesion, called the MS plaque, is a circumscribed region of demyelination, which is associated with the death of myelin forming cells called oligodendrocytes (especially from the centers of plaques; McKhann, 1982; Arnason, 1982). MS is an inflamatory disease, and active MS plaques characteristically show infiltration of large numbers of lymphocytes, plasma cells and macrophages (Arnason, 1982; Waksman, 1982). Such inflamatory reactions suggest that MS may be an autoimmune disease, or alternatively, that it may be caused by an infectious agent (possibly a virus; for reviews see Arnason, 1982; McKhann, 1982; Waksman, 1981; Waksman, 1985; Werkerie et al., 1986). However, the cause of this disease remains a mystery.

MS plaques also show an astroglial reaction (reactive gliosis) which results in the formation of a glial scar (McKhann, 1982; Scheinberg and Raine, 1984). Glial scar formation is not specific to MS plaques, but represents a general response to CNS injury. It has been observed following such diverse types of CNS injury as knife- or laceration-type lesions (Das, 1986; Das, 1987) and focal irradiation (fast-alpha particle; Maxwell and Kruger, 1965), as well as during Wallerian degeneration of severed nerve fiber tracts (Dahl et al., 1981). Astrocytes responding to injury become "reactive": They proliferate (Miller et al., 1986), become enlarged (Fedoroff et al., 1984) and extend numerous cell processes (Bignami and Dahl,

1976). The processes and cell bodies of these "reactive astrocytes" become packed with bundles of intermediate filaments (IFs, 10 nanometer diameter; Perier and Gregoire, 1965; Maxwell and Kruger, 1965) which are chiefly composed of glial fibrillary acidic protein (GFAP, 50 kD; Schactner et al., 1977). These cells comprise a living scar and in the case of invasive injuries (such as stab lesions), these scars appear to isolate the lesion site from adjacent CNS tissue.

In MS and other demyelinating diseases, the neurological deficits result from failure of action potential conduction following demyelination (Arnason, 1982). Myelin is composed of the wrappings of oligodendrocyte cell membranes around axons (Kandel and Schwartz, 1985). A myelin sheath serves to insulate an axon, as it provides a high resistance barrier to the flow of ionic currents. This insulating barrier is not continuous along the entire length of an axon, but is interrupted periodically by nodes (the nodes of Ranvier), where the axonal cell membrane (axolemma) is exposed to the interstitial fluid. The function of myelin sheaths is to increase the conduction velocity of action potentials travelling along axons. Ionic currents can only flow across the axolemma at the nodes of Ranvier and hence, the action potential appears to jump from node to node. This phenomenon is called saltatory conduction.

The action potential results from a rapid influx of sodium ions (into

the axon), and a rapid efflux of potassium ions (out of the axon) (Arnason, 1982). In myelinated axons, sodium ions enter through sodium ion channels which are clustered in the unmyelinalted region of the node itself (Arnason, 1982). Potassium ions, on the other hand, exit through potassium ion channels located in the myelinated paranodal region (Arnason, 1982). Normally, these two types of ion channels are separated by tight junctions between the oligo-dendrocyte cell membrane and the axon (Arnason, 1982). When demyelination disrupts the barrier separating these ion channels, action potential conduction ceases abruptly (Arnason, 1982).

MS plaques can occur anywhere in CNS white matter, and thus the clinical symptoms of this disease reflect slowed or blocked action potential conduction in various nerve fiber tracts (McKhann, 1982; Arnason, 1982). These give rise to sensory and / or motor deficits almost universially associated with fatigue. Characteristically, this disease takes the form of exacerbations, or attacks, which are followed by spontaneous remissions (McKhann, 1982). Remissions constitute a clinically silent stage of the disease which is associated with the restoration of function. However, functional recovery apparently results from the redistribution of sodium ion channels, enabling unmyelinated fibers to conduct action potentials. In MS, remyelination is limited to the plaque margins and does not appear to be a major factor underlying functional recovery during remissions.

As stated previously, the inflamatory characteristics of MS suggest that this disease may be autoimmune, or alternatively, that it may be caused by an infectious agent (possibly a virus). This is supported by a number of other observations:

i) a link with certain histocompatibility antigens,

ii) increased synthesis of immunoglobins within the brain,

iii) decrease in a subset of thymus derived lymphocytes (T-cells), namely suppressor T-cells (T_S-cells), during exacerbations and recovery of this population during remissions,

iv) variation in the prevalence of this disease as a function of latitude (this coupled with migration data),

v) and also, an outbreak, or epidemic, of MS in the Faeroe Islands which followed the stationing of British troops there in 1940.

First, HLA antigens A3 and B7, as well as B-cell antigens DRw2 and DRw3, are over-represented among MS patients in the U.S.A. and Western Europe (Arnason, 1982; McKann, 1982). DRw2 occurs 3-4 times more commonly in MS patients than among the general population. Relatives of MS patients are 15-20 times more likely to develop the disease (incidence highest for siblings, and then for parents, of MS patients). Other diseases which are known to be of autoimmune or viral etiology, similarly exhibit linkages with particular

histocompatibilty antigens.

Second, 70% of MS patients exhibit increased levels of immunoglobins, especially IgG, in thier cerebral spinal fluid (CSF) (McKhann, 1982; Arnason, 1982). In contrast, only 5% of the normal population exhibit increased CSF-IgG. The increase in CSF-IgG is due to synthesis within the brain and not to disruption of the blood brain barrier. In MS, elevated CSF-IgG does not necessarily correlate with attacks, or return to normal during remissions. By electrophoresis, or isoelectric focusing, the CSF-IgG of 80-90% of MS patients can be resolved into "oligoclonal bands" which indicate the production of monoclonal antibodies (McKhann, 1982; Arnason, 1982). However, the IgG eluted from different plaques from the same patient often show different oligoclonal banding patterns, suggesting that the specificities of most or all of these antibodies are not related to MS. It is relevant to point out that oligoclonal banding patterns have been observed in CSF from patients with other chronic inflamatory conditions affecting the CNS. One example is subacute sclerosing panencephalitis (SSPE) which is caused by a persistent measles infection of the brain. In SSPE, the oligoclonal bands can be absorbed out using measles virus. All attempts to absorb out the oligoclonal bands from the CSF of MS patients using candidate viruses, or CNS antigens (which might serve as targets for autoimmune attack), have failed. No MS antigen has been identified.

Third, a number of laboratories have reported that T_S-cells decrease during MS attacks and rebound during remissions (McKhann, 1982; Arnason, 1982). This correlation between MS attacks, which signal the destruction of oligodendrocytes, and the decline in T_S-cells suggests that the disease process may be responsible for both effects. Cell surface antigens shared by T_S-cells and oligodendrocytes might serve as targets for autoimmune attack. In support of this hypothesis, cultured sheep oligodendrocytes were found to react with MAb OKT8 which specifically recognizes the suppressor subpopulation of T-cells (Arnason, 1982). In any given culture, greater than 50% of the oligodendrocytes exhibited reactivity with this MAb. An alternate interpretation for this decrease in T_S-cells is that a "self" determinant may declare itself and thereby trigger an autoimmune attack. Evidence against this hypothesis is that depletion of T_S-cells should give rise to other autoimmune manifestations, and these are not seen in MS (Arnason, 1982).

Fourth, MS is much more prevalent at high latitudes (ex. Denmark: 64 / 100,000) than at low latitudes (ex. Israel: 4 / 100,000) (McKhann, 1982). At the equator, MS is nonexistent or not reported. People migrating from a region of high (low) incidence to a region of low (high) incidence develop MS as frequently as those in their place of origin if they migrate after adolescence. Those migrating before

adolescence develop MS as frequently as those in their adopted country. This evidence has been cited in support of the hypothesis that MS is caused / triggered by exposure to some exogenous agent (possibly a virus) early in life.

Fifth, MS was unknown in the Faeroe Islands prior to the stationing of British troops in 1940 (Waksman, 1981). However, during the period 1943-1960, 24 cases of MS were reported. One additional case was reported in 1970. Similarly, there was a stepwise increase in the number of cases in Iceland following each World War (Waksman, 1981). Again, the introduction of some exogenous agent (possibly a virus) could account for the increased incidence of MS in the Faeroe Islands and Iceland.

All of the observations cited above support the hypothesis that MS is caused by exposure to some exogenous agent, possibly a virus. In a recent study, polymerase chain reaction (PCR) was employed to look for HTLV-1 retroviral sequence in peripheral blood mononuclear cells of MS patients and normal controls (Reddy et al., 1989; briefly reviewed: Waksman, 1989). PCR enables one to amplify minute quanties of specific DNA sequences by repeated rounds of DNA synthesis utilizing the appropriate primer. In this study, HTLV-1 retroviral sequence was detected in all of six MS patients and only one normal control. Thus, HTLV-1 or a related retrovirus may have a direct causative role in MS. It is too early to draw this conclusion.

PCR is subject to a number of flaws, such as the amplification of viral DNA which is present as a contaminant in the samples. However, application of this powerful technique may lead to the identification of a virus which has a causitive role in MS.

Overview of the Research

Thus, the application of MAb techniques for the detection of MS antigens resulted in the production of hybridoma clones secreting MAbs reactive against human brain (homogenate by ELISA, and cryostat sections by immunofluorescence microscopy). The studies described herein have employed three of these MAbs (MAb J1-31, MAb G-3-5 and MAb 6B9) which recognize, and hence define, three antigens (J1-31 antigen, G-3-5 antigen and 6B9 antigen respectively). It is not certain that these MAbs each recognize a single antigen. But the reactive antigens are operationally defined by an MAb and are referred to as a single antigen in the absence of any evidence to the contrary. It is conceivable that these MAbs recognize similar epitopes on otherwise different molecules. As stated previously, these antigens were found to be common to CNS tissue from both MS patients and normal controls. (It is remarked that the MS tissue then available was of poor quality and not suitable for detailed histological study.) Although not specific to MS tissue, these antigens were found

to be differentially expressed by the various cell-types in the CNS. Therefore, these antigens attracted attention as they reveal differences among CNS cells. Both J1-31 antigen and G-3-5 antigen were found to be principally localized in a type of support cell, called an astrocyte (Malhotra et al., 1983; Malhotra et al., 1984a; Malhotra et al., 1984b; Schroder and Malhotra, 1987). 6B9 antigen, on the other hand, was found to be localized in what turned out to be the cell bodies of neurons. (It is safe to say that our histological preparations have shown some improvement over time.)

At the start of this research, J1-31 antigen was the best characterized antigen of the three, and this remains true to the present time. By immunofluorescence microscopy, J1-31 antigen had been localized in astrocytes of both human and rat, as confirmed by double-labeling with antiserum to GFAP which is an accepted marker protein for astrocytes (Malhotra et al., 1984b; Singh et al., 1986). At the electron microscope level, J1-31 antigen had been localized in association with IFs of astrocytes in the rat brain (by peroxidase anti-peroxidase technique: Malhotra et al., 1984b; Singh et al., 1986; and by colloidal gold technique: Malhotra et al., 1986; Malhotra, 1988). These IFs are known to be chiefly made-up of GFAP (Schactner et al., 1977). Yet J1-31 antigen was thought to be distinct from GFAP based on results from blocking experiments where prior incubation with a polyclonal antiserum to GFAP did not appear to inhibit binding of MAb

J1-31 to the antigen (Malhotra et al., 1984; Singh et al., 1986).

Intermediate filaments (IFs) are a poorly understood component of the cytoskeleton (for review see Steinert and Roop, 1988). Based on sequence data, IFs are grouped into five broad classes as follows:

Type I, acidic keratins of epithelia,

Type II, neutral-basic keratins of epithelia,

Type III, vimentin of mesenchymal cells and many cultured cells, desmin of myogenic cells, and glial fibrillary protein of astrocytes,

Type IV, neurofilament proteins of neurons, and

Type V, lamins of the nuclear lamina complex.

All IF core proteins share a central alpha-helical rod domain of conserved secondary structure, which is responsible for their forming cytoplasmic filamentous structures (Types I-IV) which are 10-15 nanometers wide. Although IFs have been postulated to function in mechanical coordination of the cytoskeleton and signal transduction, the significance of these cytoskeletal elements in cell physiology is not known.

J1-31 antigen was shown to be a protein with a molecular weight of 30,000 Daltons as determined by immuno-precipitation with MAb J1-31, followed by SDS gel electrophoresis (reducing conditions) and autoradiography (Singh et al., 1986). This molecular weight determination for J1-31 antigen (Singh et al., 1986) differs from that

reported for GFAP (50 kD; Rueger et al., 1979). In view of the electron microscope localization of J1-31 antigen in association with the IFs of astrocytes, it was tentatively described as a cytoskeletal-associated protein (Malhotra et al., 1984b; Singh et al., 1986; Malhotra et al., 1986).

Subsequently, it was undertaken to investigate J1-31 antigen expression by retinal Muller cells and the ependyma as these cell-types are known to manifest expression of GFAP (see Chapter II). In addition, it was undertaken to investigate the expression of J1-31 antigen during development as such a study could reveal any functional relationship existing between J1-31 antigen and GFAP (see Chapter II). In an early study, enhanced immunofluorescence staining owing to MAb J1-31 was detected in cryostat sections of CNS tissue from MS patients, suggesting the possibility that reactive astrocytes might show enhanced expression of J1-31 antigen. It was undertaken to investigate this hypothesis using a model system for gliosis, namely surgical injury of rat spinal cord (see Chapters III and IV). When MS tissue suitable for histological study subsequently became available, it was then undertaken to investigate J1-31 antigen expression in MS plaques (see Chapter V). The onset of J1-31 antigen expression during development (as detected by immuno-fluorescence microscopy; Chapter II), as well as the enhanced expression of this antigen by astrocytes responding to CNS injury

(Chapters III, IV and V), correlate with the accumulation of GFAP-type IFs by astrocytes. In view of the electron microscope localization in association with IFs in astrocytes, it is conceivable that J1-31 antigen performs some function in the assembly and / or organization of these IFs.

In addition, it was undertaken to investigate J1-31 antigen expression by cultured cells (see Chapter VI). The discovery that rat glioma cells (9L) turn-off expression of J1-31 antigen once they have attained a confluent monolayer state has suggested the possibility that this antigen may play some role in cell division and / or growth. Therefore, this may account for the enhanced expression of J1-31 antigen by astrocytes which exhibit hyperplasia and hypertrophy, as occurs following CNS injury.

The other two studies described herein present results on G-3-5 antigen (Chapter VII) and 6B9 antigen (Chapter VIII). By immunofluorescence microscopy, G-3-5 antigen is localized in the same types of CNS macroglia as express J1-31 antigen. Chapter VII presents immunofluorescence results on the localization of G-3-5 antigen in the CNS, numerous non-CNS tissues, and during development. To date, no central organizing hypothesis has been proposed to account for the distribution of this antigen. Similarly, Chapter VIII presents results on the localization of 6B9 antigen in the CNS, numerous non-CNS tissues, and during development, as well

as preliminary results on the cytochemical characterization of this antigen. The detection 6B9 antigen in numerous secretory cells and tissues suggested the hypothesis that 6B9 antigen is a member of a class of proteins, called reticuloplasmins, which localize to the lumen of the rough endoplasmic reticulum (see Chapter VIII). Although supported by circumstantial evidence, this hypothesis remains to be proven.

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Chapter II: A New Protein (J1-31 Antigen, 30 Kilodaltons) is Expressed by Astrocytes, Muller Glia and Ependyma[1]

This study deals with the cellular and subcellular distribution of a protein antigen (J1-31 antigen, 30KD; Singh et al., 1986) discovered using an MAb (MAb J1-31, isotype IgG 2b; Malhotra et al., 1984). In the CNS of those mammals tested thus far (human, rat and rabbit), MAb J1-31 immunostains those cells which are also stained by antisera to glial fibrillary acidic protein (GFAP, 50 KD; Rueger et al., 1979), as determined by double-label immunofluorescence microscopy (Malhotra et al., 1984; Singh et. al., 1986). Since GFAP is a recognized marker for astrocytes (Bignami and Dahl, 1977), it was tentatively assumed that MAb J1-31 could serve as a marker for astrocytes alternative to GFAP (Singh et al., 1986). By immuno-electron microscopy using peroxidase anti-peroxidase (Malhotra et al., 1984; Singh et al., 1986) and colloidal gold (Malhotra et al.,1986) labeling techniques, J1-31 antigen was localized in association with the intermediate filaments (IFs) of astrocytes in the rat brain; these IFs

1. A version of this chapter has been published.
   Predy, R., Singh, D., Bhatnagar, R., Singh, R. and Malhotra, S.K., (1987): A New Protein (J1-31 Antigen, 30 kD) is Expressed by Astrocytes, Muller Glia and Ependyma, Bioscience Reports **7**: 491-502.

are chiefly composed of GFAP (Schachner et al., 1977). Several lines of evidence suggest that J1-31 antigen and GFAP are distinct proteins. The molecular weight determinations for these proteins differ (Singh et al., 1986). In addition, results from immuno-fluorescence microscopy, and enzyme-linked-immunosorbent assay (ELISA), indicate that prior incubation with a polyclonal antiserum to GFAP does not block binding of MAb J1-31 to the antigen (Singh et al.,1986). Furthermore, a large proportion of J1-31 antigen can be extracted in aqueous buffers (Singh et al., 1986), as compared to GFAP which requires detergent for extraction (Rueger et al., 1979). A small proportion of J1-31 antigen does require detergent (NP 40) for extraction (Singh et al., 1986). In view of the subcellular localization of J1-31 antigen in association with the IFs in astrocytes, it was tentatively described as a cytoskeletal-associated protein (Malhotra et al.,1984; Singh et al., 1986; Malhotra et al., 1986; Malhotra, 1988).

In order to further characterize J1-31 antigen, it was considered desirable to examine various cell-types in the rat CNS, especially Muller cells and the ependyma (which is comprised of ciliated ependymal cells and tanycytes) as they are reported to manifest expression of GFAP (Bjorklund and Dahl, 1985; Bjorklund et al., 1985; Bruni et al., 1985; Roessmann et al., 1980). It was also considered

desirable to examine the spatial and temporal expression of J1-31 antigen and GFAP during development, as such a study could reveal any functional relationship existing between these two proteins. The results presented in this chapter provide further evidence that J1-31 antigen and GFAP are distinct proteins. In addition, these results establish the specificity of MAb J1-31 for use as a cell-type marker for studies on the CNS.

Materials and Methods

Fixation for Immunocytochemistry

MAb J1-31 did not give rise to detectable immunostaining in cryostat sections from glutaraldhyde fixed tissue using peroxidase anti-peroxidase (PAP). The PAP technique was employed as fixation in glutaraldehyde imparts autofluorescence to tissue, thus making it unsuitable for immunofluorescence microscopy (Sternberger, 1986). However, staining (immunofluorescence and PAP) owing to MAb J1-31 was evident in cryostat sections from tissue fixed in 4% para-formaldehyde (0.2 M phosphate buffer, pH 7.2), and therefore, this fixative was used in the immunocytochemical studies for light and electron microscopy. Late fetal (18-21 days gestation), and early postnatal (1, 3, and 5 days old) Sprague-Dawley rats were killed by decapitation. After opening the skull, the brain was fixed *in situ* for 2-3 hr Adult rats were decapitated, or etherized and perfused with 0.9%

NaCl followed by paraformaldehyde. The brain (coronal slice containing the diencephalon), optic nerves and eyes were removed and placed in paraformaldehyde for 2-3 hr.

Light Microscopy

Fixed tissue for light microscopy was washed with phosphate buffered saline (PBS), and cryoprotected in 30% sucrose (12-18 hr, 4C) prior to freezing at -20C. The tissue was embedded in Tissue Tek II O.C.T. Compound and cryostat sections were cut at 8-12 um thickness, mounted on rubber cement-coated coverglasses, and dried at 4C overnight.

Antibodies for Immunofluorescence Microscopy

Staining was carried out using MAb J1-31 ascites fluid (1:500 or 1:1000 dilution); normal mouse serum (NMS; Sigma, St. Louis, MO) served as control. Sections were double-labeled with rabbit antiserum to cow GFAP (Dakopatts, Dimension Labs Inc., Mississauga, Ontario; 1:1000 dilution); normal rabbit serum (NRS; Sigma) served as control. Goat anti-mouse IgG (Sigma) or IgG+IgM (Boehringer-Mannheim, Dorval, Quebec) conjugated to fluorescein isothiocyanate (FITC) was used at a dilution of 1:100. Goat anti-rabbit IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC,

Sigma) was used at dilutions of 1:300 or 1:500. Antibodies and normal sera were used in dilutions made-up in PBS.

Protocol for Immunofluorescence Microscopy

The protocol for immunofluorescence staining was as follows: Sections were washed with PBS, incubated in 30% goat serum (Sigma) for 30 min at 20C (room temperature), and then incubated with primary antibody for 12-18 hr at 4C. Then the sections were washed with PBS and incubated with the appropriate secondary antibody (1hr, 20C). Single-labeled sections were mounted at this point. Double-labeled sections were prepared following a repetition of the procedure using the second set of primary and secondary antibodies. Following washes with PBS and distilled water, sections were mounted in buffered glycerol containing p-phenylenediamine (Johnson and de C. Nogueira Araujo, 1981) and viewed by epifluorescence microscopy.

Protocol for Immunoelectron Microscopy: Pre-embedding

Immunolabeling for electron microscopy was carried out using sections of paraformaldehyde fixed tissue cut on an Oxford Vibratome. A coronal slice of the brain including the diencephalon (fixed by perfusion followed by immersion) was sectioned (20-30 um thick), and sections were incubated in J1-31 ascites fluid or NMS

(1:100 dilution) for 12-18 hr at 4C. Then the sections were washed with PBS, and incubated in a 1:20 dilution of Protein A-Gold (5nm; Janssen Life Sciences, Olen, Belgium) for 3 hrs at 37C on a shaker. The sections were washed with PBS and post-fixed in 2% glutaraldehyde, followed by 2% OsO_4 prior to dehydration and embedding in Epon. Thin sections were examined in the electron microscope with and without staining with heavy metal salts.

Conventional Electron Microscopy

For conventional electron microscopy of the ependyma, an adult rat was etherized and perfused with 0.22 M sucrose followed by 2% glutaraldehyde (0.15 M cacodylate buffer plus 2 mM $CaCl_2$, pH 7.2; Hayat, 1981). The brain was removed, cut into peices, further fixed in glutaraldehyde (12-18 hr, 4C) and post-fixed in 2% OsO_4 (2 hrs) prior to dehydration and embedding in Epon. Thin sections were stained with heavy metal salts before examination in the electron microscope.

Results

The following results from immunofluorescence microscopy on double-labeled sections are consistent with those from single-labeled sections. Therefore the overlapping staining, seen in double-labeled sections, does not appear to be the result of cross-reactivity between

the two sets of primary and secondary antibodies. Control sections incubated with mouse and rabbit sera showed no staining of structures comparable to those delineated by antibodies. However, high background fluorescence was associated with the fluorescein conjugated reagents.

Ependyma

The ependyma consists of ciliated ependymal cells and tanycytes (Bruni et. al., 1985). The latter belong to the category of radially oriented glia and are discussed below.

Ciliated epindymal cells, as visualized by this immuno-fluorescence technique appear as a columnar epithelium which borders on the third ventricle. Staining for J1-31 antigen is evident in ciliated ependymal cells as well as astrocytes in the neuropil (Fig. 1.1 A). However, staining for GFAP is only evident in astrocytes in the neuropil (Fig. 1.1 B). Ciliated ependymal cells do not exhibit staining for GFAP (Fig. 1.1 B). Antiserum to GFAP gives rise to more extensive staining of astrocytic processes than MAb J1-31. This may result from quantitative differences in the antigens found in the finer processes. The polyclonal antiserum to GFAP might be expected to yield a stronger fluorescent signal than MAb J1-31. At the electron microscope level, labeling for J1-31 antigen appears in association with amorphous cytoplasmic material distinct from the perinuclear

bundles of IFs (Fig. 1.2). This amorphous cytoplasmic material is not evident in glutaraldehyde-fixed tissue. The subcellular localization of J1-31 antigen in ciliated ependymal cells, differs from that seen in astrocytes where labeling appears predominately in association with the IFs (Malhotra et al., 1984; Singh et al., 1986; Malhotra et al., 1986; Malhotra, 1988).

Radially Oriented Glia

Muller cells in the preparations appear as radially oriented fibers which span the breadth of the retina. In double-labeled sections, Muller cell profiles exhibit staining for J1-31 antigen and GFAP (Figs. 1.3 A and B). In some preparations however, staining for J1-31 antigen is barely detectable except in those regions adjacent to the iris.

Tanycytes have their cell bodies bordering on the ventricular system, and each cell extends a single process into the neuropil. In these preparations, they appear as fibers which extend radially from the wall of the third ventricle. These cells exhibit staining for J1-31 antigen and GFAP (Figs. 1.3 C and D).

The category of radially oriented glia also includes Bergmann fibers of the cerebellum which are discussed below under Developmental Expression.

Developmental Expression

In the cerebellum of the adult rat, astrocytes in the molecular layer (Bergmann fibers) and granular layer exhibit intense staining for both J1-31 antigen and GFAP (Malhotra et al., 1984; Singh et al., 1986). In the 3 day old rat, astrocytes in the medulla exhibit staining for both J1-31 antigen and GFAP, whereas astrocytes in the cerebellum (immature Bergmann fibers) exhibit staining for GFAP only (Figs. 1.4 A and B). Immunofluorescence staning for J1-31 antigen was not detected in the cerebellum prior to postnatal day 5 when faint staining immature Bergmann fibers was first detected (not shown). Similarly, faint staining for J1-31 antigen in the optic nerve was first detected at postnatal day 5.

Discussion

Identification of astrocytes is commonly based on the use of immunoreagents to GFAP (Bignami and Dahl, 1977; Eng, 1985) which is the principal constituent of IFs in these cells (Schachner et al., 1977). GFAP is also expressed by certain specialized types of glia, such as retinal Muller cells and tanycytes (Bjorklund et al., 1985; Bruni et al., 1985). MAb J1-31 immunostains all of these cell-types and therefore, this monoclonal antibody can be used as a cell-type marker alternative to GFAP. In contrast to immunoreagents for GFAP

(Achtstatter et al., 1986; Fields and Yen, 1985; Gard et al., 1985; Hatfield et al., 1985; Jessen and Mirsky, 1985), MAb J1-31 has not been found to react with cell-types outside the CNS (Malhotra et al., 1984; Singh et al., 1986). It is emphasized that J1-31 antigen appears to be distinct from GFAP based on various criteria outlined in the Introduction. Also, the onset of J1-31 antigen by immature astrocytes appears to lag behind that of GFAP during development of the rat cerebellum and optic nerve (present results). However, in interpreting these results, consideration must be given to the sensitivity of immunofluorescence techniques. A polyclonal antiserum, such as that employed for detection of GFAP, is expected to yield a stronger immunofluorescence signal than MAb J1-31. In a similar immunofluorescence study on the rat retina, researchers were able to demonstrate GFAP in Muller cells using a polyclonal antiserum; yet no GFAP was detected when two MAbs to GFAP were employed (Bjorklund and Dahl, 1985; Bjorklund et al., 1985). These MAbs did give rise to positive staining for GFAP in Muller cells of rats sacrificed four days after the optic nerves had been crushed. Such cells are likely to contain elevated levels of GFAP similar to astrocytes which are known to accumulate GFAP-type IFs in response to CNS injury (Perier and Gregoire, 1965; Maxwell and Kruger, 1965; Schachner et al., 1977).

Further evidence that J1-31 antigen is distinct from GFAP is

provided by the observation that MAb J1-31 intensely stains ciliated ependymal cells which do not express GFAP. These cells express another IF core protein, vimentin (M. W. 57 kD; Dahl et al., 1981; Shaw et al., 1981), which is widely expressed by mesenchymal and non-mesenchymal cells, including astrocytes (Bignami et al., 1982). J1-31 antigen appears to be distinct from vimentin, as the M. W. determinations for these proteins differ. In addition, vimentin is known to be widely expressed during prenatal development of the rat CNS (Bignami et al., 1982), whereas J1-31 antigen is first detected during early postnatal development. Furthermore, there is no evidence that J1-31 antigen is expressed by cells outside the CNS (Malhotra et al., 1984; Singh et al., 1986), such as fibroblasts, which are known to be rich in vimentin (Bignami et al., 1982). Although it is not certain that MAb J1-31 is recognizing the same antigen in ciliated ependymal cells and astrocytes, this seems likely as staining owing to MAb J1-31 appears to be restricted to a few closely related types of CNS macroglia. Ciliated ependymal cells and astrocytes are known to have other antigens in common, such as Ran-2 (Bartlett et al., 1981) and C-1 (Sommer et al., 1981).

In astrocytes, colloidal gold labeling for J1-31 antigen is most evident on the IFs, and some gold particles are seen in association with ill defined material in the cytoplasm (Malhotra, 1988). In mature

ciliated ependymal cells, however, such labeling is evident predominantly on amorphous cytoplasmic material peripheral to the perinuclear bundles of IFs. This difference in the location of J1-31 antigen in astrocytes versus ciliated ependymal cells may reflect differences in the composition of the IFs in the two cell types. J1-31 antigen may preferentially associate with IFs in the astrocytes which are chiefly made-up of GFAP (Schachner et al.,1977). In this respect, J1-31 antigen may be comparable to epinemen (M. W. 50 kD) which associates specifically with IFs composed of vimentin (Lawson, 1984).

In ciliated ependymal cells, the nature of the cytoplasmic material which labels with MAb J1-31 is unknown. This material is not distinguishable in glutaraldehyde fixed tissue. Hence it could be an aggregation of soluble cytoplasmic constituents which occurs during fixation in paraformaldehyde. It is recalled that in homogenates of human brain, there is a large proportion of J1-31 antigen which is soluble and can be extracted with aqueous buffers (Singh et al., 1986). In astrocytes, labeling for J1-31 antigen appears in association with the IFs. Thus far it has not been determined if J1-31 antigen is component of these IFs, or if it is a peptide which is located in those regions of the cytoplasm which contain IFs. Such a peptide may be precipitating onto the IFs during fixation in paraformaldehyde.

The expression of J1-31 antigen in astrocytes may be linked in some way to the assembly of GFAP-type IFs. This possibility is

consistent with the observation that staining for J1-31 antigen, in the developing rat optic nerve, is first detected at a time when astrocytes are accumulating IFs, as was previously determined by electron microscopy (Vaughnn and Peters, 1967). Although GFAP is present in the optic nerve at birth (Dixon and Eng, 1981), intermediate filaments are rare (Vaughn and Peters, 1967). The formation of IFs in astrocytes may require factors in addition to the core protein, that is GFAP. Thus J1-31 antigen may be involved in the assembly and / or organization of GFAP-type IFs, and this could also explain the electron microscope localization of J1-31 antigen in association with the IFs in astrocytes. It is relevant to point out that ciliated ependymal cells have been reported to express GFAP transiently during prenatal development in humans (Roessmann et al., 1980).

Thus through the application of hybridoma technology for the production of MAbs (Kohler and Milstein, 1975), a new protein (J1-31 antigen, 30 kD) has been discovered which is restricted to certain types of CNS macroglia, namely astrocytes (Malhotra et al., 1984; Singh et al., 1986; Schroder and Malhotra, 1987), Muller cells, tanycytes and ciliated ependymal cells (present results). MAb J1-31 can serve as a valuable marker in studies on these cell types. Further studies on the expression of J1-31 antigen during development, and in cultured cells, may provide clues as to its physiological role.

Fig. 1.1 A and B. Cryostat section of rat diencephalon double-labeled with MAb J1-31 (FITC; A) and antiserum to GFAP (TRITC; B). Ciliated ependymal cells (arrow), as well as astrocytes in the neuropil, exhibit staining for J1-31 antigen (A). Ciliated ependymal cells do not exhibit staining for GFAP (B). Staining for GFAP is evident in astrocytes in the neuropil (B).

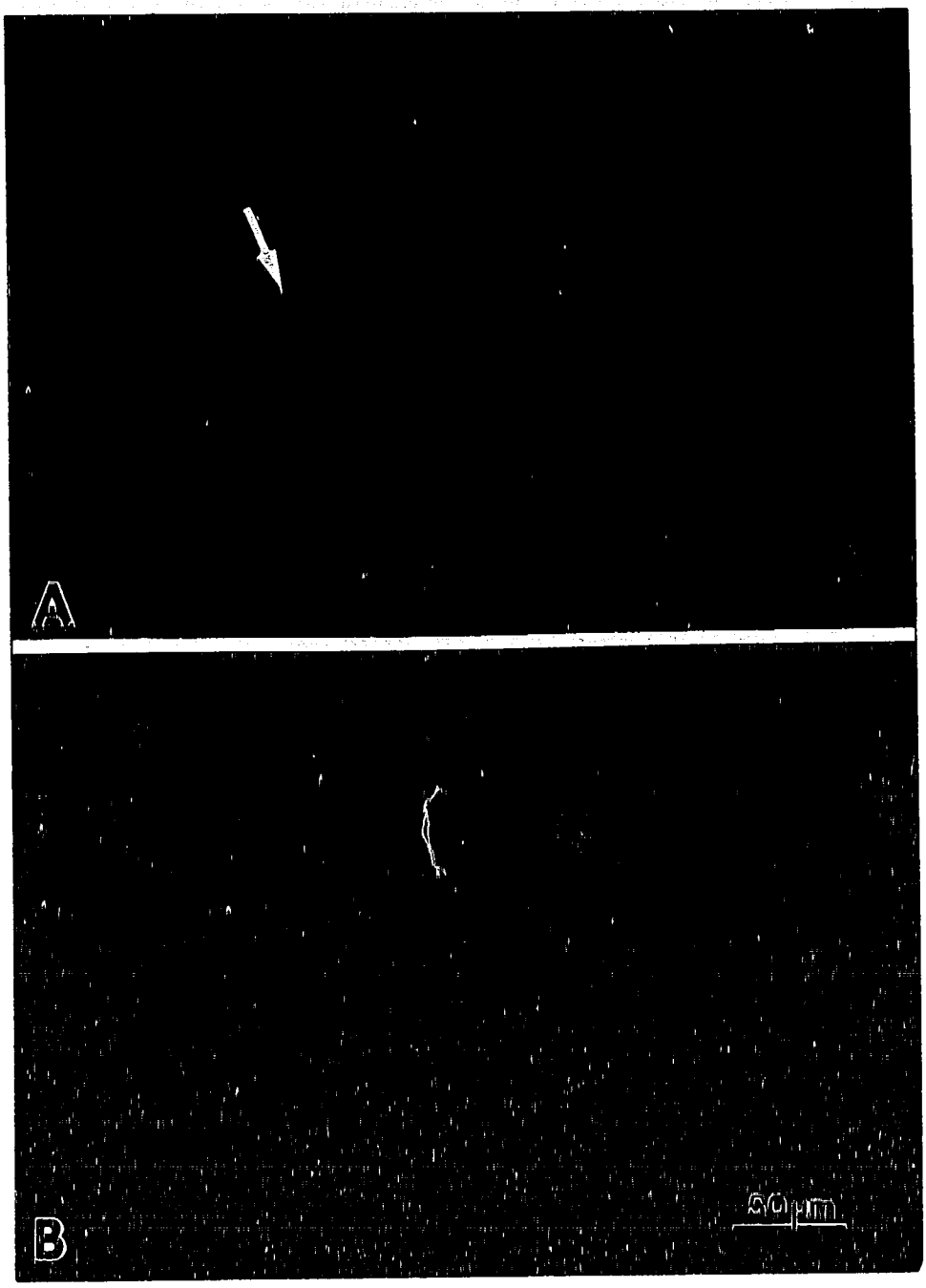

A
B
50 µm

Fig. 1.2. Immunogold (5 nm) labeling for J1-31 antigen is seen predominantly in association with ill-defined cytoplasmic structures (large arrows) which are distinct from the intermediate filaments (asterisks) in ciliated ependymal cells. The thin section was stained with uranyl acetate and lead citrate before examination in the electron microscope. bb, basal body; cp, coated pit; m, mitochondrion; N, nucleus.


200 nm

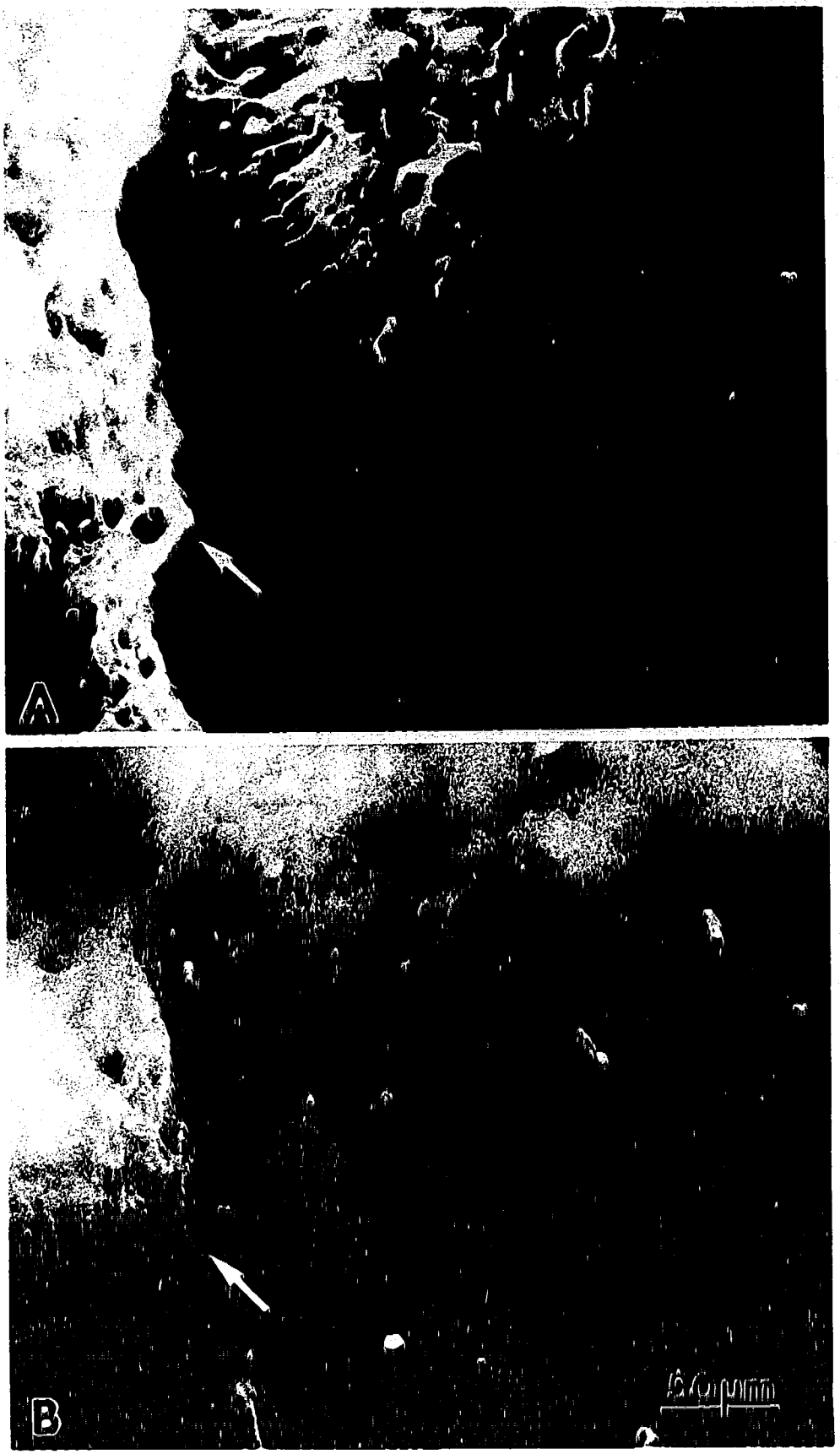
Fig. 1.3 A and B. Cryostat section of rat retina double-labeled with antiserum to GFAP (A; TRITC) and MAb J1-31 (B; FITC). Muller cell profiles exhibit staining for GFAP and J1-31 antigen (arrows).

Fig. 1.3 C. Cryostat section of rat hypothalamus single-labeled with antiserum to GFAP (TRITC). Note tanycytes exhibit staining for GFAP (arrowheads).

Fig. 1.3 D. Cryostat section of rat hypothalamus single-labeled with MAb J1-31 (FITC). Note tanycytes exhibit staining for J1-31 antigen (arrowheads).

A
B
C
D
5x0.1μm

Fig. 1.4 A and B. Cryostat section from the brain of a 3 day old rat double-labeled with antiserum to GFAP (A; TRITC) and MAb J1-31 (B; FITC). Immature Bergmann fibers in the cerebellum show staining for GFAP (A) but not for J1-31 antigen (B). Staining for J1-31 antigen is evident in the medulla and overlaps with staining for GFAP (arrows).


A
B

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Chapter III: Enhanced Expression of a J1-31 Antigen by Reactive Astrocytes in Lacerated Spinal Cord[1]

This study deals with the expression of J1-31 antigen (30 kD protein; Singh et al., 1986) by reactive astrocytes which arise following injury to the central nervous system (CNS). J1-31 antigen is detected using a monoclonal antibody (MAb J1-31; isotype IgG 2b) raised against tissue from the CNS of a multiple sclerosis (MS) patient (autopsy sample; Malhotra et al., 1984). By immuno-fluorescence microscopy, MAb J1-31 stains those cells which are also stained by antiserum to glial fibrillary acidic protein (GFAP), namely astrocytes, retinal Muller cells and tanycytes in the ependyma (Malhotra et al., 1984; Singh et al., 1986; Schroder and Malhotra, 1987; Predy et al., 1987).

In an early study, intense immunofluorescence staining owing to MAb J1-31 was detected in cryostat sections of MS brain tissue, suggesting the possibility that reactive astrocytes might show enhanced expression of J1-31 antigen. MS plaques characteristically

1. A version of this chapter has been published.
   Predy, R., Malhotra, S. K., Das, G. D., (1988): Enhanced Expression of a Protein Antigen (J1-31 Antigen, 30 Kilodaltons) by Reactive Astrocytes in Lacerated Spinal Cord. J. Neuroscience Research **19**: 397-404.

show an astroglial response (reactive gliosis) leading to the formation of a glial scar (McKhann, 1982). However, detailed analysis of MS tissue was not feasible because of poor quality and limited supply of the tissue. Therefore, it was considered desirable to study the expression of J1-31 antigen by reactive astrocytes using an model system for gliosis. Laceration-type surgical lesion of the rat spinal cord constitutes a suitable model system (Das, 1986; Das, 1987). This study reports on a difference between reactive and normal astrocytes revealed by immunostaining with MAb J1-31. An abstract of this study has been presented at the meeting of the Society for Neuroscience (Malhotra et al., 1987).

Materials and Methods

Laceration-type surgical lesions were made in the spinal cords (lumbar level L3-L4) of laboratory bred Long-Evans hooded rats (3-6 months old). This procedure involved cutting of the spinal cord as well as removal of the neural tissue from the site of the lesion, resulting in surgical cavities (dorsally) in the rostro-caudal axis. The lesions extended for one segment in this axis, and 50% in the tranverse plane. These shallow lesions did not induce any paraplegic syndrome, although they did result in some transient autonomic dysfunctions. At the end of the surgery, the incision was sutured and the animals were placed in their cages. The surgical

details and animal care adapted are as previously published (Das, 1986).

Lesioned spinal cords were collected at intervals of 4 days, 16 days, 3.5 months, and 6 months following surgery. These survival periods were chosen to assess the time course of the astrocytic response following surgical trauma. At each of these intervals, two specimens were examined (N=2). The specimens bearing lesions were compared with a sham-operated control collected 3 months following surgery (N=1).

Rats were etherized and perfused with 4% paraformaldehyde (0.2 M phosphate buffer, pH 7.2). The operated spinal cord segments (L3-L4) were removed and further fixed in paraformaldehyde for 24-48 hr during transit from Purdue University (Indiana) to the University of Alberta, Alberta. The tissue was washed with phosphate buffered saline (PBS) and cryoprotected in 30% sucrose (12-18 hr, 4C) prior to freezing at -20C. The tissue was embedded in TIssue Tek II O.C.T. Compound and sagittal sections (cryostat) were cut at 8-12 um thickness, mounted on rubber cement-coated coverglasses, and dried at 4C overnight.

Indirect immunofluorescence staining was carried out using MAb J1-31 ascites fluid (1:250 or 1:500 dilution) or culture supernatant; normal mouse serum (NMS; Sigma, St. Louis, MO) and culture supernatant from NS-1 myeloma cells served as controls. Sections

were double-labeled with rabbit antiserum to cow GFAP (Dakopatts, Dimension Labs Inc., Mississauga, Ontario; 1:1000 dilution); normal rabbit serum (NRS; Sigma) served as control. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC; Sigma), and goat anti-rabbit IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC; Sigma) were used at dilutions of 1:100 and 1:500 respectively. Antibodies and normal sera were used in dilutions made-up in PBS. The protocol for the immunostaining was as described in Chapter II under Materials and Methods.

In order to semiquantitate the immunofluorescence signals, digital image analysis was carried out using a Panasonic Model WV-1550 video camera mounted on the trinocular tube of the microscope. The video signal was processed by an Oculus 200 image digitizing board by Dr. G. Miller according to the protocol published previously (G. Miller et al., 1986). A 50X oil-immersion objective lens was used for this analysis.

Results

In lesioned specimens collected at intervals of 6 months (Fig. 2.1), 3.5 months (Fig. 2.2), and 16 days (Fig. 2.3) following surgery, astrocytes adjacent to the lesion site show intense immuno-fluorescence staining for J1-31 antigen (Figs. 2.1 A, 2.2 A, and 2.3 A).

Astrocytes in adjacent apparently uninjured tissue (nonlesion region) show reduced or even no such staining (Figs. 2.1 B, 2.2 A, 2.4 A and C). Astrocytes exhibiting enhanced staining for J1-31 antigen are presumably "reactive". In these preparations, staining for GFAP was intense in the lesion region (Figs. 2.2 B and 2.3 B) as expected (Bignami and Dahl, 1976) and overlapped with staining for J1-31 antigen (Figs. 2.2 A and 2.3 A). In the nonlesion region, staining of astrocytes by antiserum to GFAP was easily detected (Figs. 2.4 B and D) and served as a guide to locate weak staining owing to MAb J1-31 (Figs. 2.4 A and C). The differential staining between lesion and nonlesion regions (specimen: 3.5 months) is evident in montages showing staining for J1-31 antigen (Fig. 2.2 A) as compared to that for GFAP (Fig. 2.2 B). At higher magnification, overlapping staining for J1-31 antigen (Fig. 2.3 A) and GFAP (Fig. 2.3 B) is apparent in reactive astrocytes adjacent to the lesion site.

Enhanced staining for J1-31 antigen was generally confined to reactive astrocytes adjacent to the lesion. However, some variability in the extent of the glial reaction was observed between specimens (discussed below). In addition, pronounced glial reaction in white matter distant from the lesion site may be in response to degeneration of severed nerve fiber tracts. Axons severed from their cell bodies undergo Wallerian degeneration and this is associated with the death of axonal accessory cells (R.H. Miller et al., 1986). Astrocytes are

known to become reactive in nerve fiber tracts undergoing such degenerative changes (Dahl et al., 1981). Such a glial reaction appears evident in the dorsal white matter of the specimen (3.5 months) shown in Figures 2.2 A and B (arrowheads). Similarly, in a specimen collected 16 days following surgery, intense staining for J1-31 antigen is evident in astrocytic processes in the white matter away from the lesion site (Fig. 2.3 C). This staining shows very good overlap with that for GFAP (Fig. 2.3 D). Yet in another region of this section, staining for J1-31 antigen is weak or absent (not detectable over background) in white matter astrocytes distant from the lesion (Fig. 2.4 A). Staining for GFAP is clearly evident in this region (Fig. 2.4 B). In the gray matter of this specimen, staining for J1-31 antigen is weak or absent distant from the lesion (Fig. 2.4 C), whereas staining for GFAP is clearly evident (Fig. 2.4 D).

It is remarked that the sample size (N=2) is small and variation in results was observed between specimens collected at given intervals following surgery. For example, one of specimens collected 16 days following surgery appeared very gliotic and intense staining for both GFAP and J1-31 antigen was observed along the entire length of the section (approximately 1.5 cm in the midsagittal plane of the spinal cord). Variation was also observed in results from specimens collected 4 days following surgery. One specimen exhibited faint

staining for J1-31 antigen both adjacent as well as distant from the lesion. In this specimen, reactive astrocytes already showed enhanced staining for GFAP adjacent to the lesion site. The other specimen exhibited a more pronounced glial reaction as reactive astrocytes adjacent to the lesion already showed enhanced staining for J1-31 antigen. Such variation in astrocytic reaction, to a large extent, is likely to result from variation in pathological conditions, particularly hemorrhage and edema.

In this study, two types of controls were used to assess the extent of the glial reaction following injury. In lesioned specimens, reactive astrocytes, exhibiting intense staining for J1-31 antigen, were generally confined to regions immediatly adjacent to the lesion site. Therefore, the apparently uninjured regions of these specimens served as internal controls. In addition, the glial reaction in lesioned specimens was also assessed with respect to a sham-operated control collected 3 months following surgery. In this specimen, no effect of the surgical procedure was detected in the spinal cord inferior to site of the operation. This specimen exhibited only faint staining for J1-31 antigen comparable to the uninjured regions of lesion specimens. The controls for the immunoreagents, to assess nonspecific binding, were sections incubated in NMS (or NS-1 supernatant) and NRS. In such sections, no staining of reactive astrocytes or the glial scar was observed compared to sections

incubated with antibodies. However, high background fluorescence was observed in sections incubated with NMS and goat anti-mouse IgG conjugated to FITC, especially in the cavity of the lesion (large arrows, Fig. 2.3 A) infiltrated by blood cells, erythrocytes and leucocytes (inferred from previous studies: Das, 1987). This background may result, in part, from cross-reaction of the FITC reagent with rat IgG. Neutrophils and macrophages, the two major types of phagocytes, have IgG molecules bound to their cell surface via Fc gamma receptors (Kimball, 1986). Concentrated FITC reagent (1:50 dilution) was absorbed against 1 mm cubes of unfixed rat brain (3.5 hr, 4C) in an attempt to reduce nonspecific binding. However, this procedure lowered the titer of the reagent and only very faint staining for J1-31 antigen could be detected in sections incubated with this FITC-conjugated antibody.

In an attempt to semiquantitate the immunofluorescence data, digital image analysis was carried out on sections from a specimen collected 16 days following surgery. Sections double-labeled with MAb J1-31 culture supernatant, and antiserum to GFAP, were examined using a 50X oil-immersion objective. Control sections were incubated with NS-1 supernatant and NRS. MAb J1-31 supernatant yielded lower background fluorescence than ascites fluid in the nervous tissue. In the region of the lesion, the FITC signal was 140%

of control values and the GFAP signal was 220% of control values (100%). In the nonlesion region (most distant from the lesion) these values were 102% and 136%, respectively, of control values. These results essentially confirm the visual observations that expression of J1-31 antigen is enhanced in reactive astrocytes.

Discussion

Astrocytes are operationally defined as GFAP-positive cells in the CNS. MAb J1-31 immunostains GFAP-positive cells in double-labeled sections of various regions of the rat CNS tested thus far-namely cerebellum, medulla oblongata, cerebral cortex, optic nerve, retina, thalmus, hypothalamus, and pineal (Malhotra et al., 1984; Singh et al., 1986; Schroder and Malhotra, 1987; Predy et al., 1987). GFAP-positive astrocytes in the rat spinal cord, particularly in the gray matter, show little or no staining for J1-31 antigen under the conditions employed in this study. However, following laceration of the spinal cord, those astrocytes in the vicinity of the wound stain intensely for J1-31 antigen. Away from the wound, the astrocytes appear normal in that they exhibit reduced or no such staining. Thus, as MAb J1-31 intensely stains reactive astrocytes, the expression of J1-31 antigen appears to increase following traumatic injury. It is recalled that MAb J1-31 was raised using cerebral white matter from an MS patient (autopsy sample; Malhotra et al., 1984). In light of the

present results, such tissue might be expected to contain elevated levels of J1-31 antigen as reactive astrocytes are known to be associated with MS plaques (Maxwell and Kruger, 1965; Perier and Gregoire, 1965).

Another protein whose expression is enhanced following injury to the CNS is the 37 kD protein which is also expressed in the peripheral nervous system (PNS). The 37 kD protein has been reported to be synthesized by macrophages and monocytes and is similar to the apolipoprotein E, a serum protein involved in transport and metabolism of lipid (Ignatius et al., 1986). It is conceivable that the 37 kD protein is also synthesized by astrocytes *in vivo* (unpublished results cited by Ignatius et al., 1986). Whether J1-31 antigen (30 kD protein) is chemically related to this 37 kD protein remains to be determined. However, there is no evidence that J1-31 antigen occurs outside the CNS.

The above-mentioned expression of J1-31 antigen (30 kD protein) and 37 kD protein following CNS injury represent aspects of what is apparently a complex process of wound healing. Astrocytes are also involved in the immunological response as they have been reported to function in antigen presentation to T-cells (Fontana et al., 1984), as well as in the secretion of interleukins (Frei et al., 1985) and interferon (Tedeschi et al., 1986). They have also been implicated in the

inhibition of axonal regeneration in the CNS through activation of an as yet unknown mechanism for terminating axonal growth (Luizzi and Lasek, 1987). This study does not address the question of origin of reactive astrocytes, although studies on optic nerve and corpus callosum have indicated that a particular subtype (Type I; R. H. Miller et al., 1986) gives rise to reactive astrocytes. In addition, this study does not provide answers as to the factor(s) which trigger the astrocytic response to CNS injury, although astrocytes have been reported to respond to factors secreted by microglia (Guilian et al., 1986), as well as myelin basic protein (Bologa et al., 1985).

Nevertheless, these results indicate that reactive astrocytes show enhanced expression of a new protein, which is recognized by MAb J1-31. Therefore, this MAb could serve as a probe for further study of the astrocytic response to CNS injury.

Acknowledgments

I am grateful to Dr. G. Miller (Radiobiology) for digitizing fluorescence data.

Fig. 2.1 A and B. Cryostat section of lesioned spinal cord 6 months following surgery. Reactive astrocytes forming the glial scar exhibit intense staining for J1-31 antigen (A). Distant from the lesion site, little staining for J1-31 antigen is evident (B).
D, dorsal; V, ventral; C, cavity of the lesion.

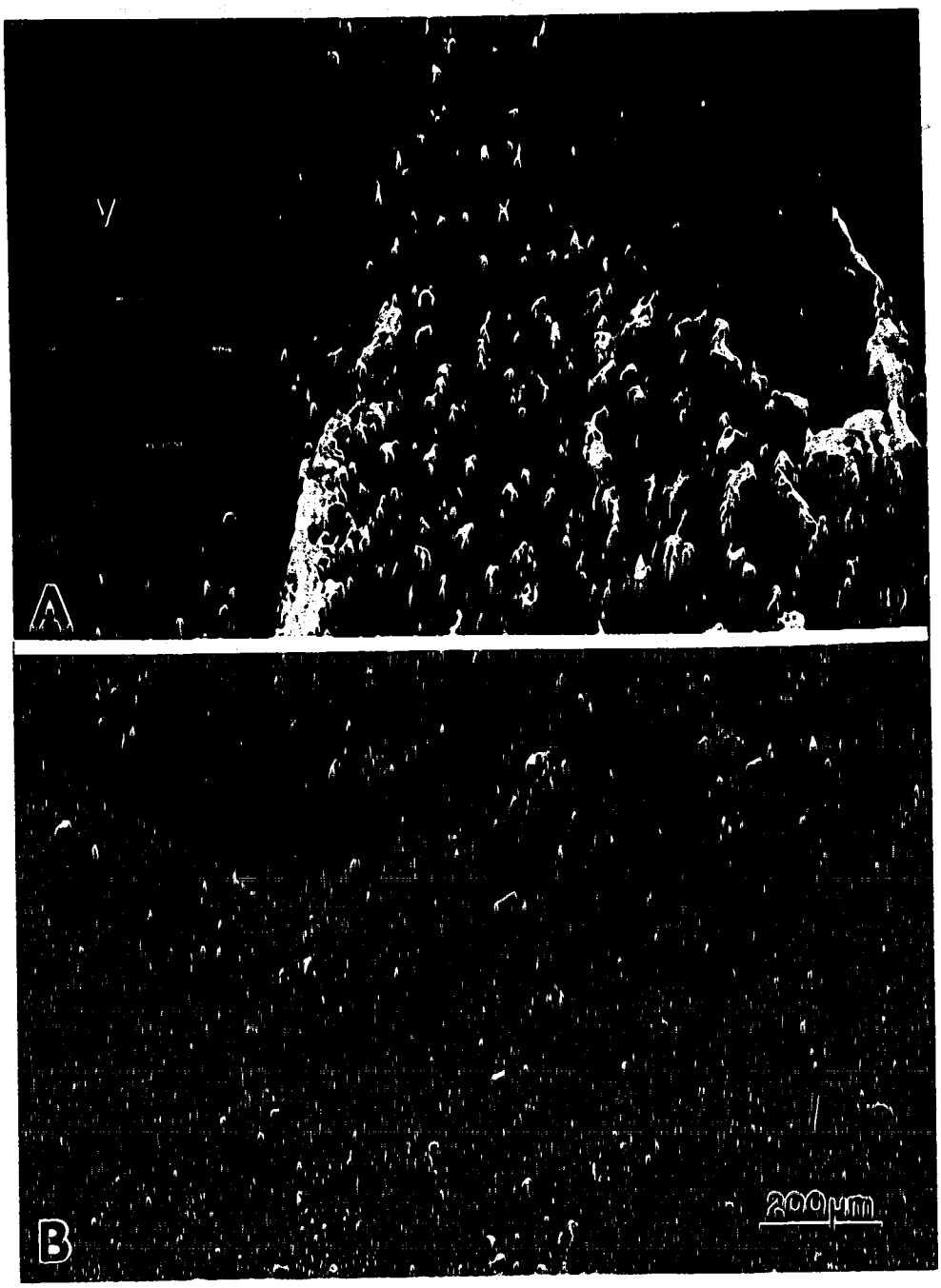

A
B
200μm

Fig. 2.2 A and B. Montages constructed to show the distribution of J1-31 antigen (A; FITC) and GFAP (B; TRITC) in a double-labeled section of lesioned spinal cord 3.5 months following surgery. Intense staining for J1-31 antigen is evident in the glial scar immediately adjacent to the lesion site (A, arrows). Staining for GFAP is evident along the length of the specimen and is especially intense in the glial scar (B, arrows). The dorsal white matter on the right-hand side of these micrographs exhibits a pronounced glial reaction which may be in response to Wallerian degeneration of severed axons and the death of axonal accessory cells (A and B, arrowheads). Asterisks delineate the extent of the lesion on the dorsal surface fo the spinal cord. D, dorsal; V, ventral; C, cavity of the lesion.

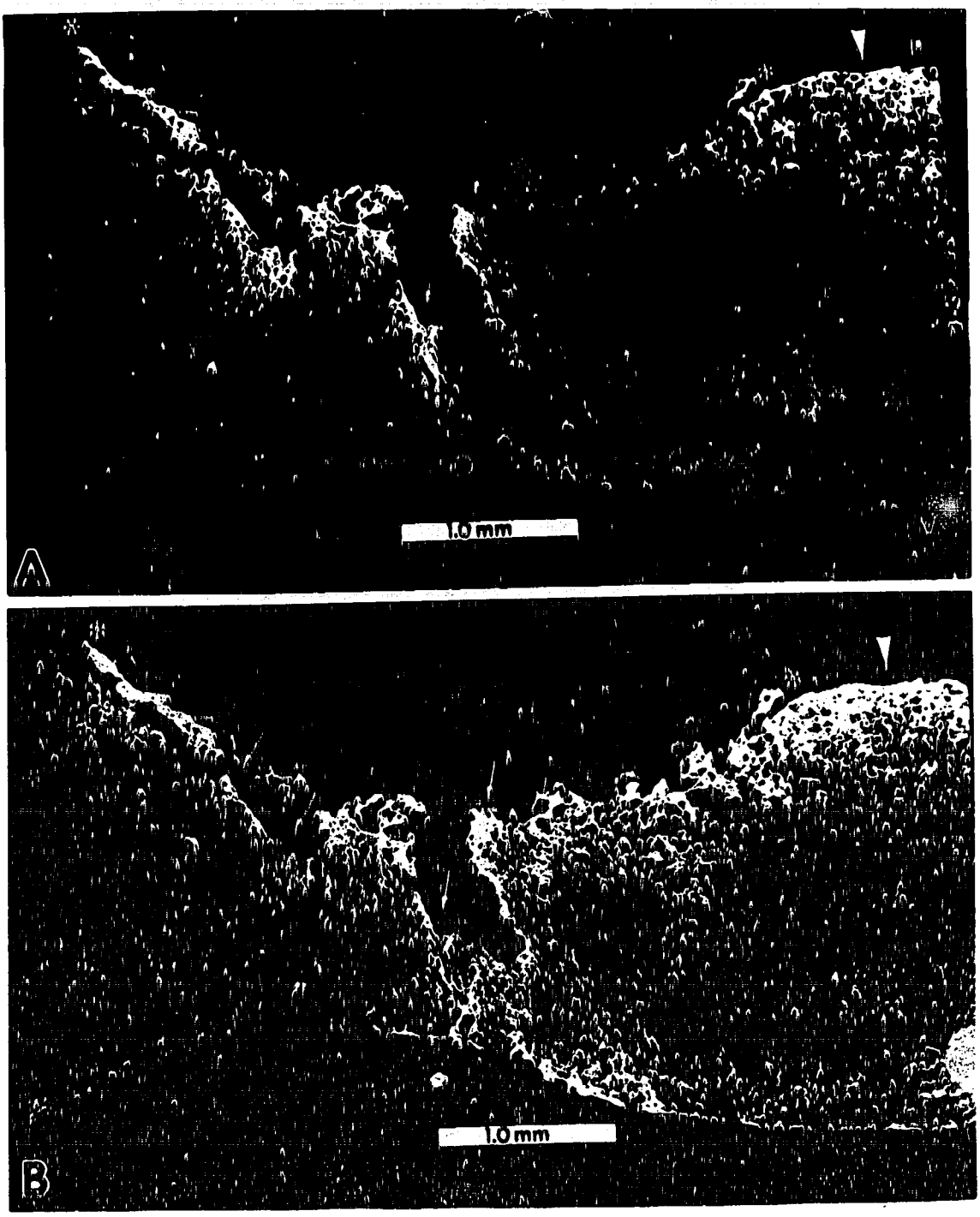

1.0 mm
A
1.0 mm
B

Fig. 2.3 A and B. Cryostat section of lesioned spinal cord 3.5 months following sugery, double-labeled for J1-31 antigen (A; FITC) and GFAP (B; TRITC). Reactive astrocytes adjacent to the lesion site (small arrows) exhibit staining for both J1-31 antigen (A) and GFAP (B). The cavity fo the lesion has been infiltrated by non-nervous tissue (large arrows) which binds the FITC reagent (A) much more than the TRITC reagent (B).

Fig. 2.3 C and D. Cryostat section of lesioned spinal cord 16 days following surgery, double-labeled for J1-31 antigen (C; FITC) and GFAP (D; TRITC). Astrocytic processes in the white matter, away from the lesion site, exhibit intense staining for J1-31 antigen (C). This staining shows very good overlap with that for GFAP (D). This pronounced glial reaction may be in response to Wallerian degeneration of severed nerve fiber tracts. Arrows indicate corresponding points in the two micrographs.

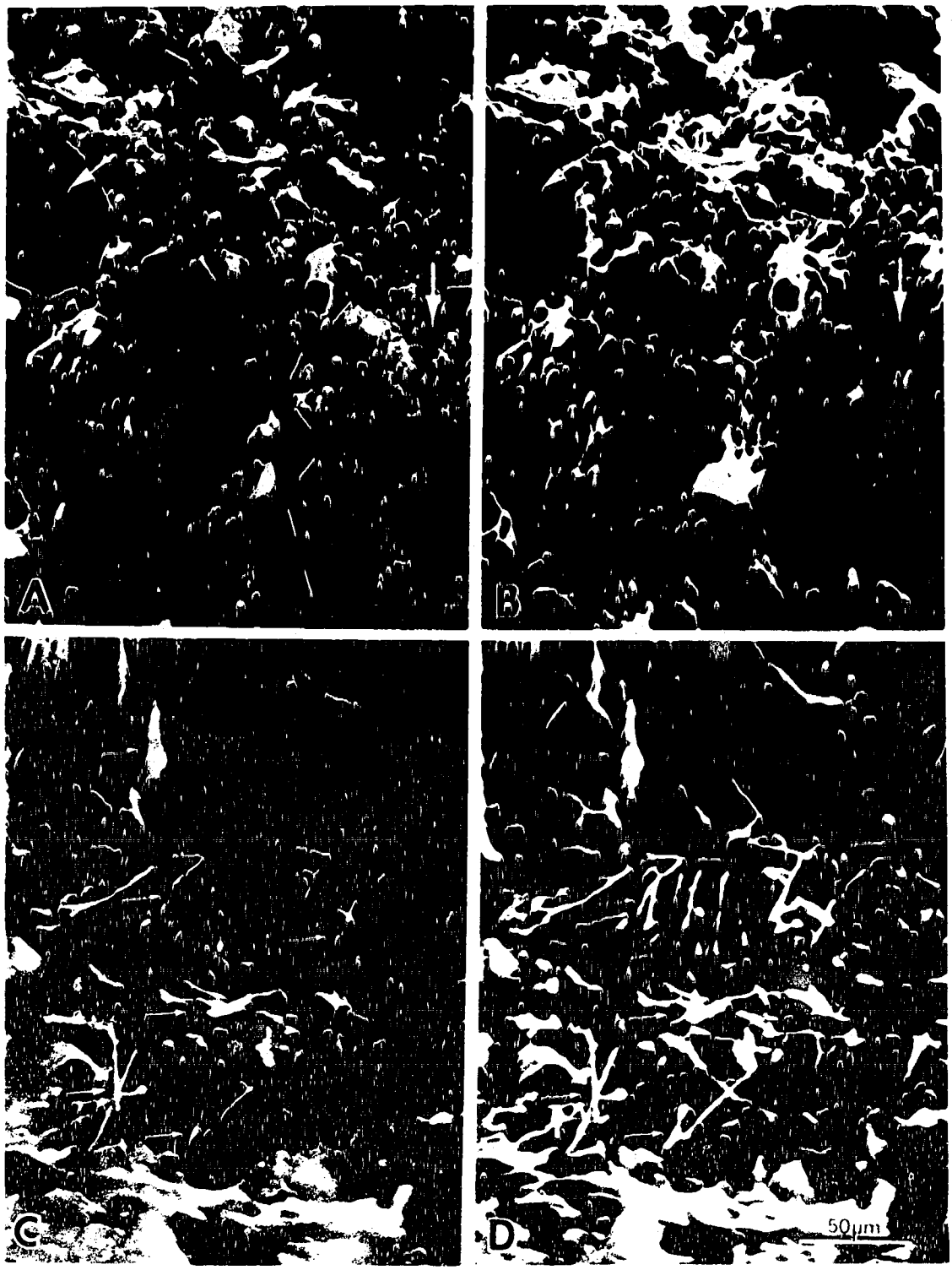
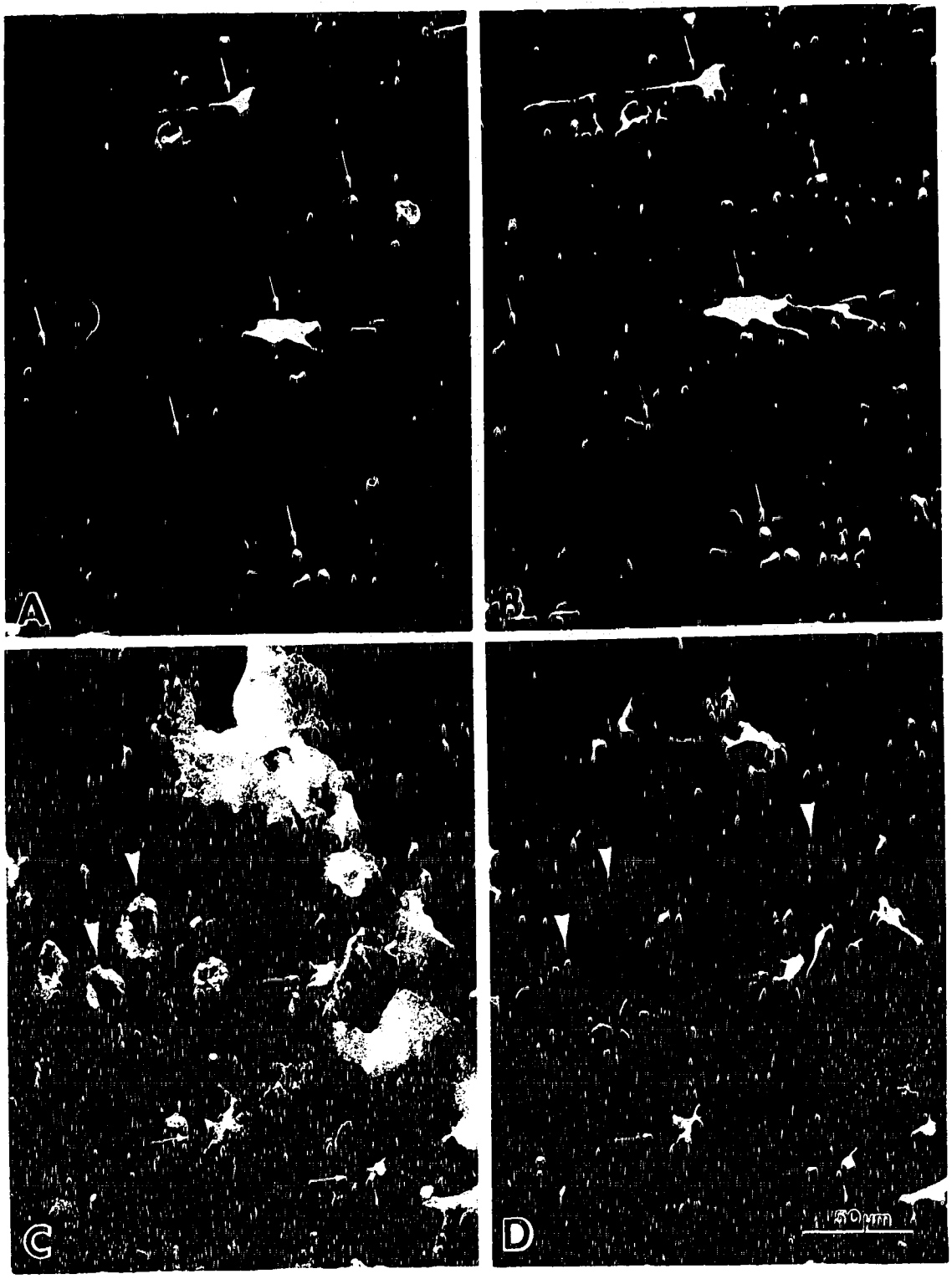

A
B
C
D
50μm

Fig. 2.4 A and B. Same section as that shown in Figs. 2.3 C and D. Away from the lesion site, astrocytes in the white matter exhibit faint staining for J1-31 antigen (A; FITC). However, staining for GFAP is intense in this region (B; TRITC) and serves as a guide to detect weak staining due to MAb J1-31. Arrows indicate corresponding points in the two micrographs.

Fig. 2.4 C and D. Same section as that shown in Figs. 2.3 C and D, and Figs. 2.4 A and B. Away from the lesion site, astrocytes in the gray matter exhibit faint staining for J1-31 antigen (C; FITC). Again, staining for GFAP is intense (D; TRITC) and serves as a guide to detect weak staining owing to MAb J1-31. The large somata of motor neurons (arrowheads) identify this region as the ventral motor horn of the spinal cord. Autofluorescent pigment granules are evident in somata of motor neurons. Arrows indicate corresponding astrocytes in the two micrographs.


A
B
C
D
50μm

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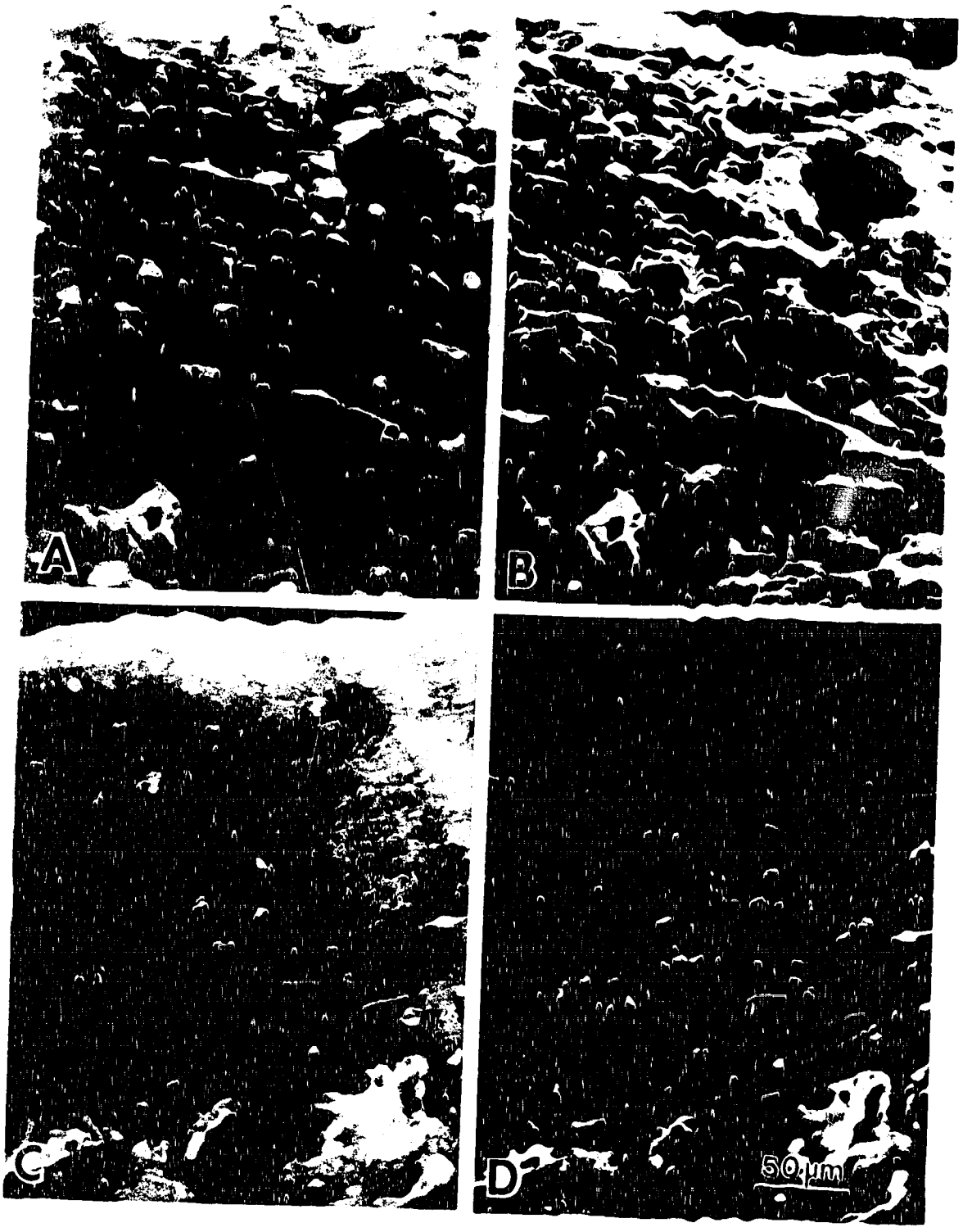
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Chapter IV: Reactive Astrocytes in Lesioned Rat Spinal Cord: Effect of Embryonic Neocortical Transplants[1]

Transplantation of cells or tissue into the adult central nervous system (CNS) is being investigated as a potential treatment strategy for alleviation of neurotransmitter deficits, such as that responsible for Parkinson's disease, as well as for optimization of functional recovery following traumatic injury to spinal cord (Azmitia and Bjorklund, 1987). So far, this strategy has shown only limited success in the treatment of Parkinson's disease (Sladek and Shoulson, 1988). However, neural transplantation has shown promise as a treatment for spinal cord injury. Grafting of embryonic brain tissue into the cavity of a laceration-type surgical lesion, of rat spinal cord, appears to prevent the protracted degeneration of those nerve fiber tracts spared direct injury (Das, 1986; Das, 1987). As CNS neurons exhibit limited capacity for axonal regeneration, functional recovery is restricted to axonal tracts left intact following injury. Prevention of degenerative changes affecting these spared axons is essential for the optimization of functional recovery following injury.

1. A version of this chapter has been accepted for publication. Predy, R., and Malhotra, S. K., (1989): Reactive Astrocytes in Lesioned Rat Spinal Cord: Effect of Neural Transplants. Brain Res. Bulletin (in press).

This article reports on a pilot study undertaken to assess the effect of embryonic neocortical transplants on the astroglial response following a laceration-type surgical lesion of rat spinal cord. This study is based on immunofluorescence microscopy using MAb J1-31, and antiserum to glial fibrillary acidic protein (GFAP, a recognized marker protein for astrocytes; Bignami and Dahl, 1977). MAb J1-31 recognizes an antigen that is common to astrocytes, Muller cells, tanycytes and ciliated ependymal cells (Malhotra et al., 1984; Singh et al., 1986; Schroder and Malhotra, 1987; Predy et al., 1987). This study reports that the appearance of reactive astrocytes (which exhibit enhanced staining for J1-31 antigen) is similar in the presence and absence of embryonic transplants. The astroglial response in the absence of neural transplants has been described previously (Predy et al., 1988). The present results may be of significance in assessing the effectiveness of neural transplantation as a treatment strategy for spinal cord injury, especially in light of work implicating reactive astrocytes in the inhibition of axonal regeneration observed in the CNS (Liuzzi and Lasek, 1987).

Materials and Methods

Laceration-type surgical lesions were made in the spinal cords (lumbar level L3-L4) of laboratory bred Long-Evans hooded rats (3-6 months old). This procedure involved cutting of the spinal cord as well as

removal of the neural tissue from the site of the lesion, resulting in surgical cavities in the rostro-caudal axis. The lesions extended for one segment in the longitudinal plane, and 50% in the transverse plane. These shallow lesions did not induce any paraplegic syndrome, although they did result in some transient autonomic dysfunctions. Neocortical tissue, from 14.5 day old rat embryos, was grafted into the cavity of the lesion. At the end of the surgery, the incision was sutured and the animals were placed in their cages. The surgical details and animal care adapted are as previously published (Das, 1986).

Specimens of lesioned spinal cords, bearing transplants, have been collected at intervals of 6 days, 15 days, 6 weeks, and 3 months following surgery. These survival periods were chosen to assess the time course of the astrocytic response following surgical trauma. At each of these intervals, one specimen was examined (N=1).

Rats were etherized and perfused with 4% paraformaldehyde (0.2 M phosphate buffer; pH 7.2). The operated spinal cord segments (L3-L4) were removed and further fixed in paraformaldehyde for 48-72 hr during transit from Purdue University (Indiana) to the University fo Alberta, Alberta. The tissue was washed with phosphate buffered saline (PBS) and cryoprotected in 30% sucrose (12-18 hr, 4C) prior to freezing at -20C. The tissue was embedded in Tissue Tek II O.C.T. compound and sagittal sections were cut on a cryostat at 8-12 um thickness, mounted on rubber cement-coated coverglasses, and dried at 4C overnight.

Indirect immunofluorescence staining was carried out using MAb J1-31 culture supernatant; supernatant from NS-1 myeloma cells served as control. Sections were double-labeled with rabbit antiserum to cow GFAP (Dakopatts, Dimension Labs Inc., Mississauga, Ontario; 1:1000 dilution); normal rabbit serum (NRS; Sigma, St. Louis, MO) served as control. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC; Sigma) and goat anti-rabbit IgG conjugated to tetramethyl-rhodamine isothiocyanate (TRITC; Sigma) were used at dilutions of 1:100 and 1:500 respectively. Antibodies and normal sera were used in dilutions made-up in PBS. The protocol for the immunostaining was as outlined in Chapter II under Materials and Methods.

Results

In transplant specimens collected 3 months (Fig. 1), 6 weeks and 15 days following surgery, astrocytes adjacent to the lesion show intense immunofluorescence staining for J1-31 antigen (Fig. 1 A) and GFAP (Fig. 1B). In adjacent apparently uninjured tissue (non-lesion region), astrocytes show reduced staining for J1-31 antigen (Fig. 1 C) and GFAP (Fig. 1 D). The situation in transplant specimens is comparable to that observed in lesioned specimens in the absence of neural transplants, where staining for J1-31 antigen and GFAP is intense adjacent to the lesion, and much reduced in the non-lesion region (Predy et al., 1988).

In the transplant specimen collected 6 days following surgery, staining for J1-31 antigen adjacent to the lesion was not appreciably greater than that observed in the non-lesion region. Staining for GFAP was intense in the region adjacent to the lesion and served as a guide to detect weak staining for J1-31 antigen. In control sections incubated with culture supernatant from NS-1 myeloma cells and NRS, there was no staining of reactive astrocytes comparable to that observed in sections incubated with antibodies.

Discussion

Enhanced staining for J1-31 antigen has been observed in reactive astrocytes which arise following surgical injury to rat spinal cord (Predy et al., 1988) and cerebral cortex (unpublished), as well as in association with multiple sclerosis plaques (Malhotra et al., 1989). Therefore, enhanced staining for J1-31 antigen reveals a new aspect of the astroglial response to injury. Consequently, MAb J1-31 can be employed to probe the appearance of reactive astrocytes. In this study it is reported that the astroglial response following surgical lesion of rat spinal cord (laceration-type) does not appear to be affected by neural transplantation to the lesion site. The astroglial response in the absence of such transplants has been described previously (Predy et al., 1988). The assessment of this response is based on immunofluorescence staining for J1-31 antigen and GFAP.

It has been suggested that reactive astrocytes are responsible for the inhibition of axonal regeneration observed in the CNS (Liuzzi and Lasek, 1987). In light of results presented in this paper, grafts of embryonic neocortical tissue do not inhibit the formation of reactive astrocytes following a spinal cord injury. However, such transplants do appear to prevent the protracted degeneration of those fiber tracts spared direct injury, possibly by preventing the infiltration of loose connective tissue and other foreign materials into the lesion site (Das, 1986; Das, 1987).

In summary, the astroglial reaction following injury presumably results from trauma to the spinal cord. This effect of trauma does not appear to be inhibited by the transplantation of embryonic neocortical tissue to the lesion site. These observations may be relevant to the assessment of strategies for treatment of spinal cord injury.

Fig. 3.1 Cryostat section of lacerated rat spinal cord bearing embryonic neocortical transplant, 3 months following surgery. In the dorsal white matter immediately adjacent to the lesion, astrocytes exhibit intense immuno-fluorescence staining for J1-31 antigen (A, FITC) and GFAP (B, TRITC). In the dorsal white matter distant from the lesion, staining for J1-31 antigen (C) and GFAP (D) is much reduced.

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Chapter V: Enhanced Expression of J1-31 Antigen by Astrocytes in Multiple Sclerosis Plaques[1]

This study deals with the expression of J1-31 antigen (30 kD protein, Singh et al., 1986) by astrocytes in multiple sclerosis (MS) plaques. J1-31 antigen is detected using monoclonal antibody J1-31 (MAb J1-31; isotype IgG 2b) which was raised against tissue from the central nervous system (CNS) of an MS patient (Malhotra et al., 1984). By immunofluorescence microscopy, MAb J1-31 stains astrocytes, Muller glia, tanycytes and ciliated ependymal cells (Malhotra et al., 1984; Singh et al., 1986; Schroder and Malhotra, 1987; Predy et al., 1987).

In an early study, intense immunofluorescence staining owing to MAb J1-31 was detected in cryostat sections of MS brain tissue, suggesting the possibility that reactive astrocytes might show enhanced expression of J1-31 antigen. However, this tissue was in poor condition and not suitable for detailed study. Consequently, it was undertaken to investigate J1-31 antigen expression by reactive astrocytes which arise following surgical injury (laceration-type) of

1. A version of this chapter has been published.
   Malhotra, S. K., Predy, R., Johnson, E. S., Singh, R., Leeuw, K., (1989): A Novel Astrocytic Protein in Multiple Sclerosis Plaques. J. Neuroscience Research **22**: 36-49.

the rat spinal cord. Using this experimental system, it was shown that astrocytes in the vicinity of the lesion (presumably reactive) do indeed show greatly enhanced staining for J1-31 antigen (Predy et al., 1988; Predy and Malhotra, 1989). When MS tissue suitable for histological study subsequently became available, it was considered desirable to follow-up the original observation by investigating J1-31 antigen expression in MS plaques. This chapter reports that staining for J1-31 antigen is enhanced in MS plaques as compared to adjacent "apparently normal" white matter. Thus MS tissue appears to represent a rich source of J1-31 antigen, and this may have been a major factor in the generation of MAb J1-31 following immunization of a BALB / c mouse with MS brain homogenate (Malhotra et al., 1984). Additional evidence that J1-31 antigen is distinct from glial fibrillary acidic protein (GFAP) is provided in this Chapter.

Materials and Methods

Human MS brain samples (8 hr postmortem) were shipped on dry ice from the Brain Bank (VA Wadsworth Medical Center, Los Angeles, California). Tissue was thawed and fixed in 4% paraformaldehyde (phosphate buffered, pH 7.2) for 20 hr at 4C. Then the tissue was cryoprotected in 30% sucrose (20 hr, 4C), prior to freezing at -70C.

A 3 month old Sprague-Dawley rat (male) was etherized and

perfused with 0.9% NaCl followed by 4% paraformaldehyde. The cerebellum and a coronal slice of the brain, containing the diencephalon, were removed and immersed in fixative for 1-2 hr. Then the tissue was washed with phosphate buffered saline (PBS) and cryoprotected in 30% sucrose (12-18 hr, 4C), prior to freezing at -20C.

Tissue was embedded in Tissue Tek II O.C.T. compound and cryostat sections were cut at 8-12 um thickness, mounted on rubber cement-coated coverglasses, and dried at 4C overnight. The description of the histology of MS plaques is based on cryostat sections stained by haematoxylin and eosin, as well as by Darrow-Red-o (Augulis and Sepinwall, 1969) and gallocyanin (Augulis and Sepinwall, 1971). It is pointed out that Darrow-Red stains nuclei orange, and that gallocyanin stains myelin dark purple.

Indirect immunofluorescence staining was carried out using MAb J1-31 culture supernatant; culture supernatant from NS-1 myeloma cells served as control. Sections were double-labeled with rabbit antiserum to cow GFAP (Dakopatts, Dimension Labs Inc., Mississauga, Ontario, Canada; 1:1000 dilution); normal rabbit serum (NRS; Sigma, St. Louis, MO) served as control. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC; Sigma), and goat anti-rabbit IgG conjugated to tetramethylrhodamine isothiocyanate

(TRITC; Sigma) were used at dilutions of 1:100 and 1:500 respectively. Antibodies and normal sera were used in dilutions made up in PBS. The protocol for the immunostaining was as described in Chapter II under Materials and Methods.

Immunofluorescence Blocking Experiments

Experiments were carried out to see whether prior incubation with a polyclonal antiserum to GFAP (anti-GFAP) would inhibit staining for J1-31 antigen. Cryostat sections of rat cerebellum were incubated with various concentrations of anti-GFAP or NRS (dilutions of 1:1000, 1:100 or 1:10), prior to staining for J1-31 antigen. The secondary antibody (goat anti-rabbit IgG-TRITC) was not applied to those preparations receiving anti-GFAP (or NRS) diluted 1:100 or 1:10. At these concentrations of primary antibody, the staining would have been so intense as to penetrate the fluorescein filter used for epifluorescence microscopy. The staining for J1-31 antigen in these preparations was compared to that in single-labeled sections. Sections single-labeled with anti-GFAP diluted 1:10 served as positive controls for this antibody.

ELISA Blocking Experiments

Experiments were carried out to see if prior incubation with anti-GFAP would inhibit binding of MAb J1-31 to brain homogenate. The

ELISA protocol was as follows: Human brain (Alzheimer's patients, autopsy samples) was homogenized in Tris-HCl buffer (pH 7.2). 250 ul of this homogenate was diluted to 5 ml and used to coat a 96-well microtitre plate (50 ul / well). The plates were incubated for 17-26 hr at 4C to allow the antigens to bind. The wells were washed with PBS containing Tween 20 detergent (PBS-T; 3X, 200 ul / well) and incubated with blocking antibodies (anti-GFAP, or NRS control, serially diluted 1:10, 1:20, ..., 1:1280) for 4-6 hr at 20C. Then the plates were washed with PBS-T and incubated with the primary antibodies (1:500 dilution of MAb J1-31 ascites fluid or MAb G-3-5 ascites fluid, with NMS serving as control) for 4-8 hr at 20C. Then the wells were washed PBS-T and incubated with the secondary antibodies (goat anti-mouse IgG (Boehringer-Mannheim, Dorval, Quebec), or goat anti-mouse IgM (Sigma), conjugated to alkaline phosphatase; diluted 1:500 or 1:400) for 2 hr at 20C. Then the wells were washed with PBS-T and incubated with enzyme substrate: 5 mg tablet of paranitrophenylphosphate (disodium; Sigma) dissolved in 5 ml of diethanolamine buffer (pH 9.8). The reaction was stopped by the addition of 4 M NaOH and the plates were quantitated using an ELISA Reader.

Results

J1-31 Antigen Expression in MS Plaques

In cryostat sections of MS plaques, intense staining for J1-31 antigen (Fig. 4.1 A) and GFAP (Fig. 4.1 B) is evident. In control sections incubated with culture supernatant from NS-1 myeloma cells and NRS, no staining was detected in the MS plaque as compared to that observed in sections incubated with antibodies. The intense staining observed within the MS plaque contrasts markedly with the situation in adjacent "apparently normal" white matter. Under the conditions employed in this study, little or no staining for J1-31 antigen (Fig. 4.1 C) or GFAP (Fig. 4.1 D) was detected in apparently normal white matter adjacent to the plaque. It is emphasized that these observations have been made on single sections which contain regions of MS plaque, as well as apparently normal white matter. Therefore these regions, in single sections, are directly comparable with respect to the treatment for immunostaining.

It is remarked that staining owing to MAb J1-31 is likely to be more intense in unfixed tissue. However, in view of the possible viral etiology for MS, tissue samples for immunostaining and histology were fixed in 4% paraformaldehyde as this treatment should inactivate most viral particles.

Semi-quantitative analysis, of the immunofluorescence preparations, was carried out by Dr. G. Miller using image digitalizing

equipment as described previously (G. Miller et al., 1986; Predy et al., 1988). However, this analysis was confounded by autofluorescent pigment granules which are numerous in human CNS tissue (most evident in Figs. 4.1 C and D, arrows).

A haematoxylin and eosin stained cryostat section (of the plaque shown in Fig. 4.1) is shown in Figures 4.2 A and B. The boundary between the darkly staining glial scar and adjacent white matter is well delineated (arrow, Fig. 4.2 A). The fibrous nature of the glial scar is clearly evident at higher magnification (Fig. 4.2 B).

A Darrow-red-o and gallocyanin stained section (cryostat) from an MS plaque is shown in Figures 4.2 C and D. This is a chronic inactive plaque with a periventricular focus. Active plaques characteristically show vascular infiltration of lymphocytes (McKhann, 1982). This plaque, however, does not show either perivascular cuffing by lymphocytes, or infiltration of the plaque or plaque margins by lymphocyes. The plaque exhibits hypercellularity and reactive gliosis. Gallocyanin stains myelin dark purple and consequently, the plaque is lightly stained owing to the destruction of myelin. It is pointed out that in the haematoxylin / eosin stained section, the plaque appears darkly stained; whereas in the Darrow-red / gallocyanin stained section, the plaque appears lightly stained. As the MS tissue was cut into pieces prior to fixation in paraformaldehyde, it is not certain that

the plaque shown in Figures 4.2 A and B is distinct from that shown in Figures 4.2 C and D.

J1-31 Antigen as a Protein Distinct From GFAP

The results on enhanced expression of J1-31 antigen by reactive astrocytes (Chapters III - V) are consistent with the possibility that MAb J1-31 recognizes an antigenic determinant on GFAP. Therefore, it is important to provide evidence that these two proteins, J1-31 antigen and GFAP, are distinct. Although the molecular weight (M. W.) determination for J1-31 antigen (30 kD; Singh et al., 1986) differs from that reported for GFAP (50 kD; Rueger et al., 1979), this does not rule out the possibility that MAb J1-31 is recognizing a fragment of GFAP with a M. W. of 30 kD.

Previously it was reported that by immunofluorescence microscopy and enzyme-linked-immunosorbent assay (ELISA), prior incubation with a polyclonal antiserum to GFAP does not block binding of MAb J1-31 to the antigen (Singh et al., 1986). These experiments were carried out using antiserum to GFAP (anti-GFAP) at a dilution of 1:1000. However, when the immunofluorescence blocking experiments were repeated using very high concentrations of anti-GFAP (dilutions of 1:100 and 1:10), it was found that staining owing to MAb J1-31 was inhibited, as compared to controls incubated with NRS. These results are shown in Figure 4.3 A-F. These figures

show staining in corresponding regions (adjacent sections) of white matter in the rat cerebellum.

As reported previously (Singh et al., 1986), staining for J1-31 antigen (Fig. 4.3 A was not abolished by prior staining for GFAP (anti-GFAP diluted 1:1000; Fig. 4.3 B). In this double-labeled section, staining for J1-31 antigen (Fig. 4.3 A) shows good overlap with that for GFAP (Fig. 4.3 B). However, prior incubation with anti-GFAP diluted 1:100 did give rise to detectable inhibition of staining for J1-31 antigen in astrocytes (Fig. 4.3 C), as compared to prior incubation with NRS (Fig. 4.3 D). This inhibition in staining for J1-31 antigen is even more pronounced when anti-GFAP is employed at a dilution of 1:10. Staining for J1-31 antigen in astrocytes is essentially abolished under these conditions (Fig. 4.3 E), as compared to prior incubation with NRS (Fig. 4.3 F). Therefore prior incubation with a polyclonal antiserum to GFAP, at very high concentrations, does appear to inhibit immunofluorescence staining owing to MAb J1-31.

Similarly by ELISA, prior incubation with very high concentrations of anti-GFAP does appear to block binding of MAb J1-31 to the brain homogenate. Data from two representative ELISA experiments are shown in Tables I and II. When experimental values (E, wells incubated with NRS or anti-GFAP followed by MAb J1-31) are adjusted by subtracting control values (C, wells incubated with NRS

or anti-GFAP followed by NMS; presumably a measure of nonspecific binding (background)), the ELISA reaction owing to MAb J1-31 following incubation with NRS appears greater than that following incubation with anti-GFAP. This difference between the adjusted ELISA values, (A) - (B), has been used to calculate a percent inhibition. In these representative experiments, the maximum calculated inhibition was 72% (Table I) and 85% (Table II). In other experiments, the maximum calculated inhibition ranged from 59% to 91%.

It was then undertaken to investigate whether prior incubation with anti-GFAP would inhibit binding of another of the MAbs (MAb G-3-5, isotype IgM; Malhotra et al., 1983; Malhotra et al., 1984) which also stains astrocytes in cryostat sections. However, the antigen recognized by this MAb (G-3-5 antigen) is obviously distinct from GFAP based on its resistance to digestion by pronase, as well as its wide distribution in fetal tissues and numerous non-CNS tissues in the adult (see Chapter VII). Similarly, when experimental ELISA values are adjusted by subtraction of control values (E - C), prior incubation with anti-GFAP appears to block binding of MAb G-3-5 to the brain homogenate. In these experiments, the maximum calculated inhibition ranged from 40% to 50%. Therefore, prior incubation with a polyclonal antiserum to GFAP appears to inhibit binding of MAb G-3-5 to its antigen which is totally unrelated to GFAP.

Although the calculated inhibition in the G-3-5 experiments (40%-50%) is apparently lower than that calculated for the J1-31 experiments (59%-91%), this difference may stem from the fact that MAb J1-31 is isotype IgG 2b, whereas MAb G-3-5 is isotype IgM. It is pointed out that the secondary antibodies employed in these experiments, goat anti-mouse IgG versus goat anti-mouse IgM (alkaline phosphatase conjugates), gave rise to different levels of background reaction. In the MAb J1-31 experiments, there was high background in wells incubated with high concentrations of anti-GFAP. It is believed that this high background stems, in part, from cross-reactivity between the goat anti-mouse IgG (alkaline phosphatase conjugate) and the rabbit immunoglobins (anti-GFAP; principally IgG molecules) bound to GFAP molecules in the wells.

In light of other results, the inhibition of binding of MAb J1-31 to the antigen (seen in immunofluorescence and ELISA blocking experiments) is interpreted as an effect of steric hinderance (or some other form of nonspecific inhibition) limiting the accessibility of the antigenic determinant recognized by MAb J1-31. It is pointed out that J1-31 antigen has been localized, at the electron microscope level, in association with the IFs of astrocytes in the rat brain (by peroxidase anti-peroxidase technique: Malhotra et al., 1984; Singh et al., 1986; and by colloidal gold technique: Malhotra et al., 1986; Predy et al.,

1987). These IFs are known to be chiefly composed of GFAP (Schachner et al., 1977). Consequently, J1-31 antigen was tentatively described as a cytoskeletal-associated protein (Malhotra et al., 1984; Singh et al., 1986; Malhotra et al., 1986). Along these lines, the results from the blocking experiments are consistent with the hypothesis that J1-31 antigen is a distinct protein closely associated with GFAP. Evidence that J1-31 antigen is distinct from GFAP is provided by the observation that MAb J1-31 intensely stains ciliated ependymal cells (Fig. 4.4 A, large arrow) which do not express GFAP (Fig. 4.4 B, large arrow). In sections incubated with supernatant from NS-1 myeloma cells (Fig. 4.4 C) and NRS (Fig. 4.4 D), there is no staining of structures comparable to those delineated by antibodies. Similar results have been obtained using MAb J1-31 ascites fluid (dilutions of 1:1000 and 1:500) with NMS serving as control (Predy et al., 1987). It can be argued that NMS constitutes a safer control than NS-1 supernatant which does not contain mouse immunoglobins.

Obviously, the polyclonal antiserum to GFAP can not be recognizing the specific antigenic determinant recognized by MAb J1-31. Otherwise ciliated ependymal cells should stain for GFAP in these preparations. It is reported that in the immunofluorescence blocking experiments, prior incubation with high concentrations of anti-GFAP (dilutions of 1:100 and 1:10) did not inhibit staining owing to MAb J1-31 in ciliated ependymal cells. Therefore the detection of

J1-31 antigen in a cell-type not expressing GFAP argues strongly that these two proteins are distinct. Additional evidence has been provided previously, and is recapped in the Discussion.

Discussion

In human brain, J1-31 antigen has a M. W. of 30 000 Daltons (30 kD) as determined by immunoprecipitation with MAb J1-31, followed by SDS gel electrophoresis (reducing conditions) and autoradiography (Singh et al., 1986). MAb J1-31 recognizes a similar antigen in rat CNS and thus the cellular and subcellular localization of J1-31 antigen was initially determined using rat tissue, as suitable human tissue is in limited supply. By immunofluorescence microscopy, MAb J1-31 stains those cells which are also stained by antiserum to GFAP, namely astrocytes, retinal Muller cells and tanycytes in the ependyma (Malhotra et al., 1984; Singh et al., 1986; Schroder and Malhotra, 1987; Predy et al., 1987). In addition to these cell-types, MAb J1-31 also stains ciliated ependymal cells (Predy et al., 1987). These cells do not express GFAP in the mature rat (Predy et al., 1987), but have been reported to transiently express GFAP during prenatal development in humans (Roessmann et al., 1980). This result provides strong evidence that J1-31 antigen is distinct from GFAP (Predy et al., 1987). This is further supported by the different M. W.

determinations for these proteins (Singh et al., 1986). In addition, a large proportion of J1-31 antigen can be extracted in aqueous buffers (Singh et al., 1986), in contrast to GFAP which requires detergent for extraction (Rueger et al., 1979). A small proportion of J1-31 antigen does require detergent (NP-40) for extraction (Singh et al., 1986). Furthermore, the onset of J1-31 antigen expression during development of the rat CNS appears to lag behind that of GFAP, as detected by immunofluorescence microscopy (Predy et al., 1987). Although J1-31 antigen is distinct from GFAP which forms IFs in astrocytes (Schachner et al., 1977), it may be associated with these IFs as determined by immunoelectron microscopy (Malhotra et al., 1984; Singh et al., 1986; Malhotra et al., 1986; Predy et al., 1987).

Thus through application of hybridoma technology for the production of MAbs (Kohler and Milstein, 1975), a novel astrocytic protein has been discovered, whose expression is enhanced during transformation to the reactive state; MS plaques represent one such instance. MAb J1-31 resulted from a fusion which used homogenized cerebral white matter from an MS patient (autopsy sample) as immunogen in a BALB / c mouse (Malhotra et al., 1984). In light of results presented in this article, such tissue represents a rich source of J1-31 antigen. Further characterization of J1-31 antigen and the changes in its expression which follow CNS injury should provide information of interest to clinicians and basic scientists working on MS.

Acknowledgments

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Legend for Tables I and II

ELISA reaction owing to MAb J1-31 following incubation with blocking antibodies (anti-GFAP, or NRS control, serially diluted 1:10, 1:20, ..., 1:1280). Experimental wells (E), incubated with NRS / MAb J1-31, are compared to control wells (C), incubated with NRS / NMS. Similarly, experimental wells (E), incubated with anti-GFAP / MAb J1-31, are compared to control wells (C), incubated with anti-GFAP / NMS. Control values (C) are subtracted from experimental values (E) to yield adjusted ELISA values (E - C). The adjusted values following incubation with NRS (E - C, Column: A) appear greater than the adjusted values following incubation with anti-GFAP (E - C, Column: B). This difference between the adjusted ELISA values, Column: (A) - (B), has been used to calculate a percent inhibition. Based on this analysis, it appears that prior incubation with very high concentrations of anti-GFAP blocks binding of MAb J1-31 to the brain homogenate. NC, not calculated.

Table I: Blocking of Binding of MAb J1-31 to the Brain Homogenate (Exp't 1).

Dilution of Blocking Abs		ELISA Reaction: Following NRS (A)	Following Anti-GFAP (B)	(A) - (B)	Percent Inhibition
1:10	E	>2.000	1.831		
	C	0.479	1.131		
	(E - C)	>1.521	0.700	>0.821	NC
1:20		1.921	1.812		
		0.453	0.888		
		1.468	0.924	0.544	37%
1:40		1.731	1.501		
		0.389	1.120		
		1.342	0.381	0.961	72%
1:80		1.864	1.451		
		0.362	0.768		
		1.502	0.683	0.819	55%
1:160		1.817	1.401		
		0.400	0.908		
		1.417	0.493	0.924	65%
1:320		1.747	1.615		
		0.385	0.873		
		1.362	0.742	0.620	46%
1:640		1.704	1.594		
		0.359	0.629		
		1.345	0.965	0.380	28%
1:1280		1.690	1.649		
		0.278	0.510		
		1.412	1.139	0.273	19%

Table II: Blocking of Binding of MAb J1-31 to the Brain Homogenate (Exp't 2).

Dilution of Blocking Abs		ELISA Reaction: Following NRS (A)	Following Anti-GFAP (B)	(A) - (B)	Percent Inhibition
1:10	E	1.830	1.293		
	C	0.772	0.986		
	(E - C)	1.058	0.307	0.751	71%
1:20		1.756	0.894		
		0.538	0.996		
		1.218	(-) 0.102	1.320	NC
1:40		1.878	1.109		
		0.779	0.941		
		1.099	0.168	0.931	85%
1:80		1.767	1.154		
		0.418	0.648		
		1.349	0.506	0.843	63%
1:160		1.802	1.232		
		0.515	0.653		
		1.287	0.579	0.708	55%
1:320		1.653	1.125		
		0.415	0.509		
		1.238	0.616	0.622	50%
1:640		1.599	1.112		
		0.481	0.460		
		1.118	0.652	0.466	42%
1:1280		1.547	1.281		
		0.408	0.390		
		1.139	0.891	0.248	22%

Fig. 4.1. Cryostat section of an MS plaque, double-labeled with MAb J1-31 (FITC) and antiserum to GFAP (TRITC). Intense staining for J1-31 antigen (A) and GFAP (B) is evident in astrocytic processses which constitute the glial scar. (Arrows indicate corresponding astrocytic processes.) In "apparently normal" white matter adjacent to the plaque, little or no staining for J1-31 antigen (C) and GFAP (D) is evident. (Arrows indicate corresponding autofluorescent pigment granules.)

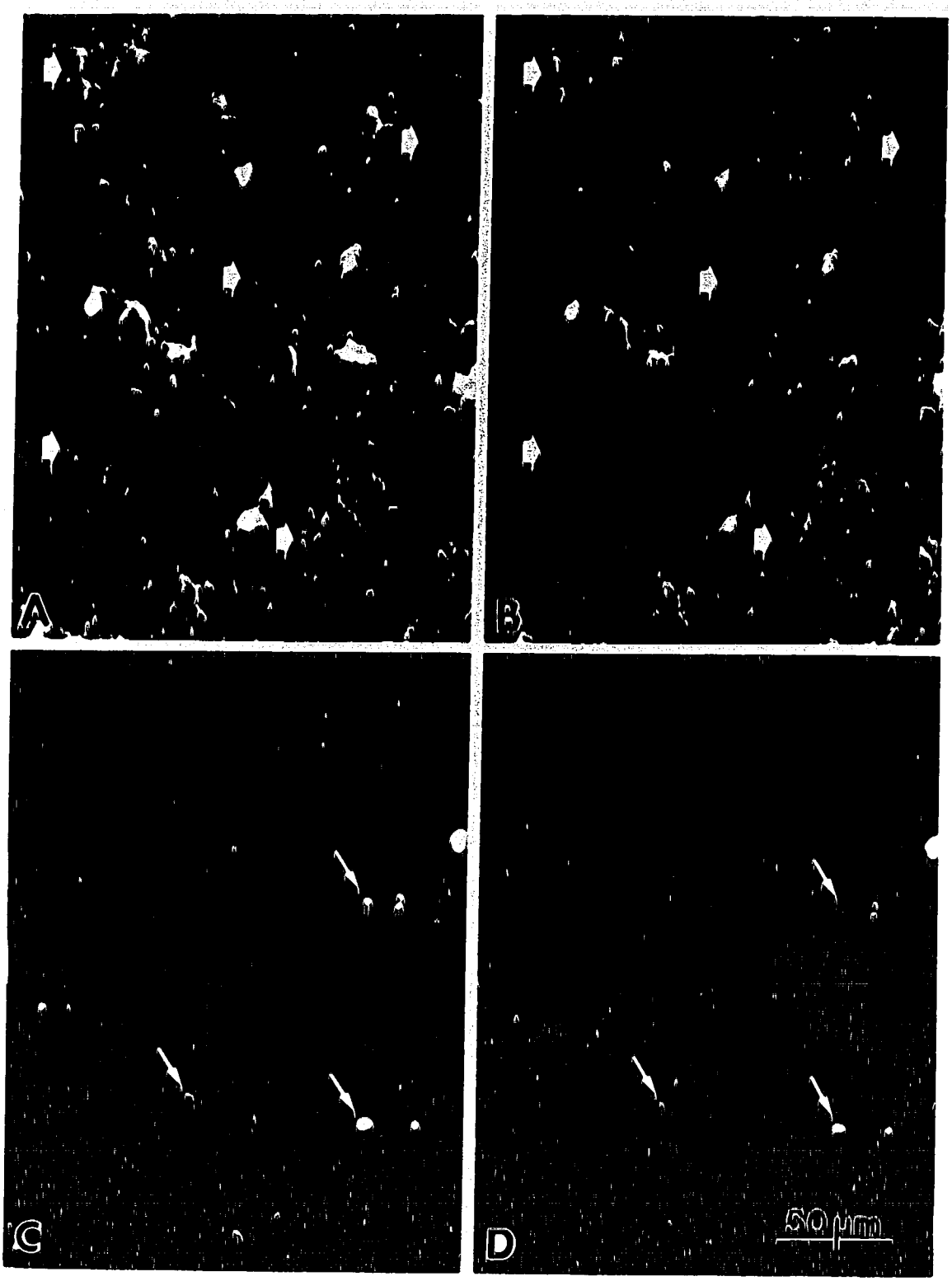

A
B
C
D
50 µm

Fig. 4.2 A and B. Haematoxylin and eosin stained section (cryostat) from the same MS plaque as shown in Fig. 4.1. In this preparation, the plaque (P, Fig. 4.2 A) stains darkly. The boundary between the darkly staining glial scar and the lightly staining adjacent white matter is well delineated (arrow). The fibrous nature of the glial scar is clearly evident at higher magnification (B). Photographed through a green filter.

Fig. 4.2 C and D. A Darrow-red-o and gallocyanin stained section (cryostat) from an MS plaque. This is a chronic inactive plaque with a periventricular focus. In this preparation, the plaque (P, Fig. 4.2 C) stains lightly. This plaque exhibits hypercellularity and reactive gliosis. At higher magnification, numerous darkly staining nuclei (predominantly of astrocytes) are evident within the plaque (D). V, ventricle.

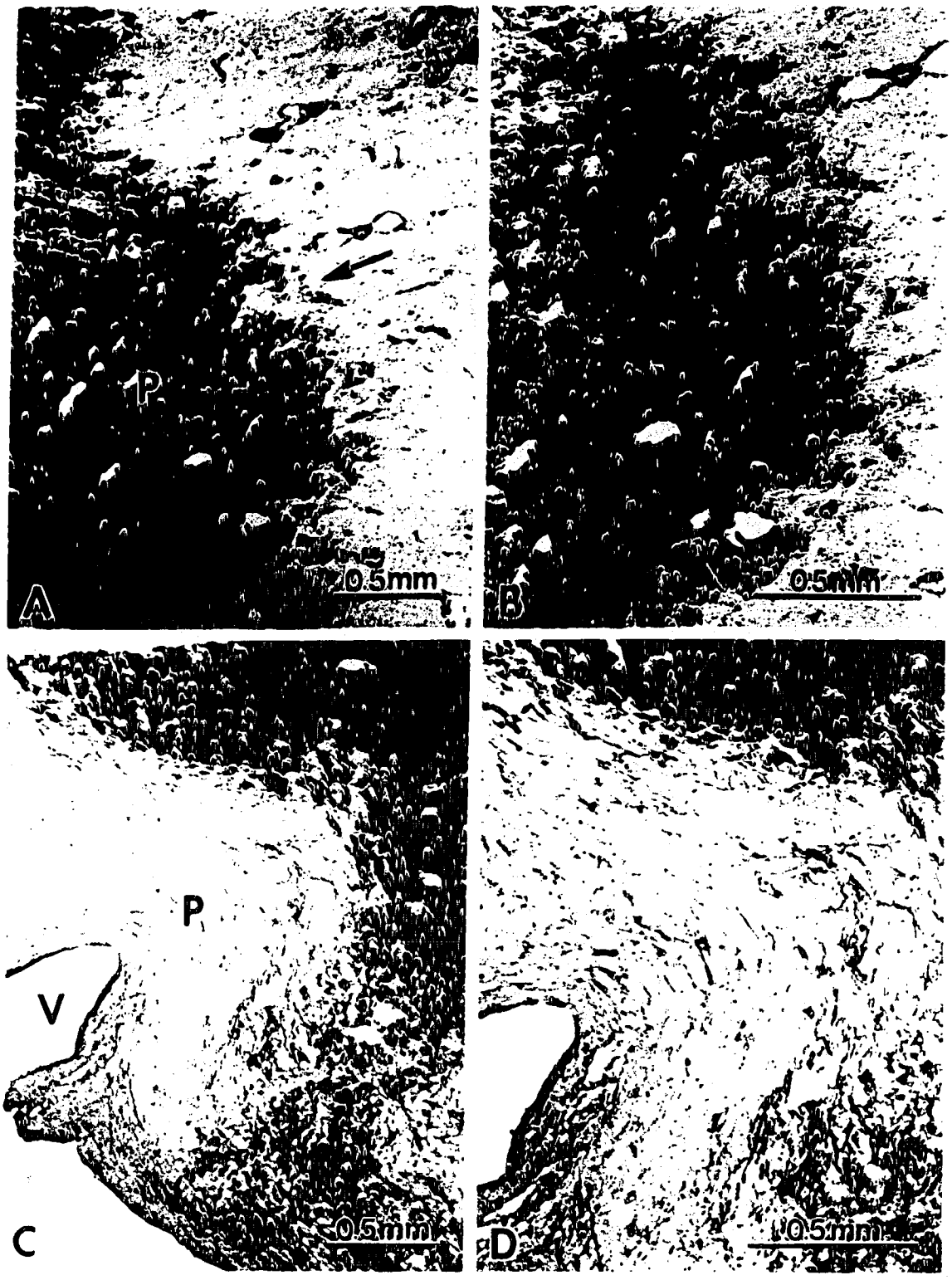

P
0.5mm
A
0.5mm
B
P
V
0.5mm
C
0.5mm
D

Fig. 4.3. Results from immunofluorescence blocking experiments. Cryostat sections of rat cerebellum incubated with anti-GFAP or NRS, prior to staining for J1-31 antigen. Astrocytes in the white matter show intense staining for J1-31 antigen (A, FITC) following staining for GFAP (B, TRITC; anti-GFAP diluted 1:1000). However, prior incubation with anti-GFAP diluted 1:100 inhibits staining for J1-31 antigen (C), as compared to controls incubated with NRS (D). Prior incubation with anti-GFAP diluted 1:10 essentially abolishes staining for J1-31 antigen (E), as compared to controls incubated with NRS (F). Sections incubated with NRS diluted 1:10 (F) show some nonspecific inhibition of staining for J1-31 antigen (c.f. D).

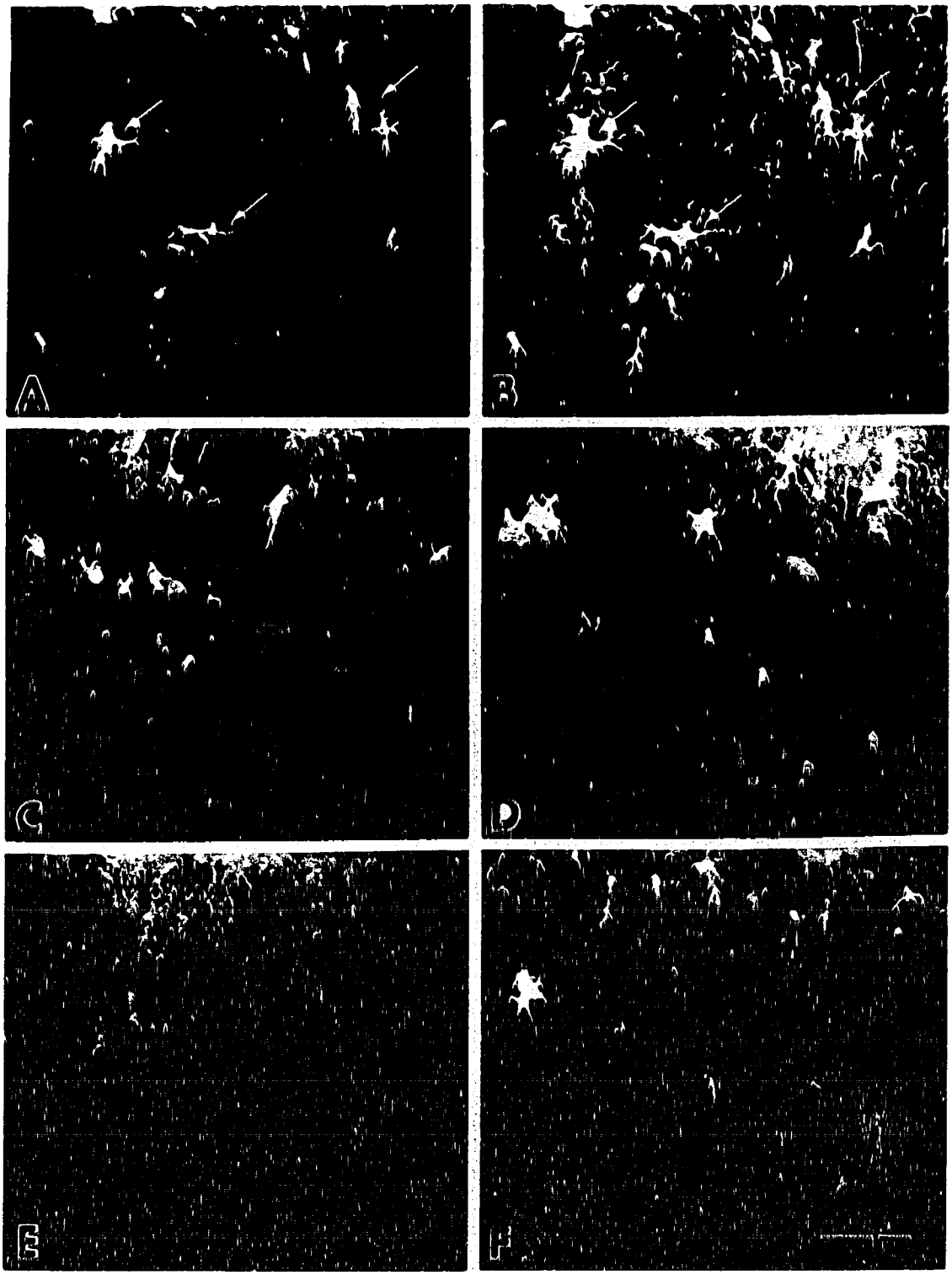
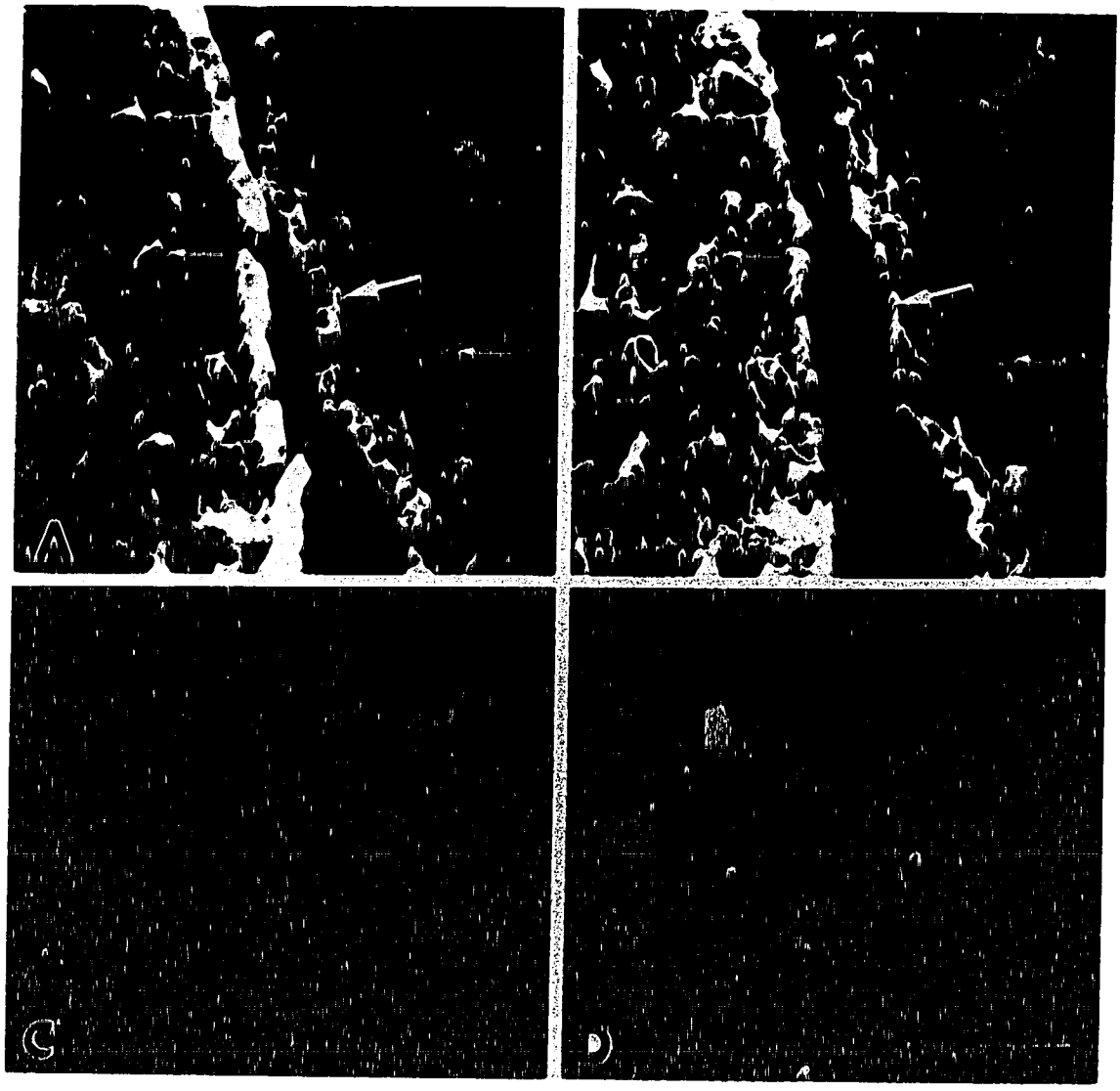

A
B
C
D
E
F

Fig. 4.4. Cryostat secton of rat diencephalon double-labeled with MAb J1-31 and antiserum to GFAP. Ciliated ependymal cells (large arrow) lining Ventricle III (V), as well as astrocytes in the neuropil, exhibit staining for J1-31 antigen (A, FITC). Ciliated ependymal cells do not stain for GFAP (large arrow; Fig. 4.4 B, TRITC). Staining for GFAP is exhibited by astrocytes, and small arrows delineate corresponding astrocytes in the two micrographs (A and B). In control sections incubated with NS-1 supernatant (C, FITC) and NRS (D, TRITC), there is no staining of structures comparable to those delineated by antibodies. However, ciliated ependymal cells show nonspecific staining following incubation with NRS (arrow; Fig. 4.4 D).


A
B
C
D

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Chapter VI: Expression of J1-31 Antigen by cells *in vitro*

This study deals with the expression of J1-31 antigen (30 kD protein; Singh et al., 1986) by cells grown in culture. As astrocytes in association with CNS lesions (surgical lesions: see Chapters III and IV; multiple sclerosis plaques: see Chapter V) exhibit enhanced immunofluorescence staining for J1-31 antigen, there must be some signal which leads to the induction of this antigen following injury. However, the identification of such a signal using *in vivo* model systems is not readily accomplished. Cell-culture offers a promising alternative for the elucidation of such cause-effect relationships as the environment can be manipulated in a controlled manner. Preliminary to such studies, it is necessary to determine if J1-31 antigen is expressed by cells grown in culture. In this study, the drug dibutyryl cyclic adenosine monophosphate (dBcAMP) has been employed as it has been reported to induce the formation of cells resembling reactive astrocytes (Fedoroff et al., 1984). In addition, it is conceivable that the greater resolution provided by cell-culture systems will yield information on the spatial and temporal relationship existing between J1-31 antigen and glial fibrillary acidic protein (GFAP).

Materials and Methods

Primary Cultures

Whole brains were removed from neonatal (<24 hr old) Sprague-Dawley rats and teased apart in cell-culture medium (Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 20% fetal calf serum (FCS), penicillin, streptomycin, sodium pyruvate, oxalic acid and glutamine. The suspension of tissue fragments were then passed through a Nitex mesh (75 um pore diameter) and seeded onto poly-(L)-lysine coated coverglasses in small Petri-plates or 6 well tissue culture plates. The cells were grown in 37C incubators in an atmosphere of 5% CO_2 and high humidity. Cells were fed at 2-4 day intervals by removing approximately half of the spent medium and replacing it with fresh medium.

Treatment of Primary Cultures with dBcAMP

According to the protocol provided by Fedoroff et al. (1984), some primary cultures were fed with medium containing 25 uM dibutyryl cyclic adenosine monophosphate (dBcAMP; Sigma, St. Louis, MO) which is a lipophilic derivative of cyclic adenosine monophosphate (cAMP).

Astrocytes treated with dBcAMP resemble reactive astrocytes which arise in vivo (Fedoroff et al., 1984).

Rat Glioma Cells (9L)

Rat glioma cells (9L; Weizsaecker et al., 1981; Barker et al., 1973) were grown on poly-(L)-lysine coated cover-glasses in cell culture medium as described above.

Fixation and Immunofluorescence Staining

Cover-glasses bearing cells were immersed in 4% para-formaldehyde (0.2 M phosphate buffer, pH 7.2) for 30-60 minutes at 20C (room temperature), and then washed with phosphate buffered saline (PBS). Indirect immuno-fluorescence staining was carried out using MAb J1-31 culture supernatant; culture supernatant from NS-1 myeloma cells served as control. Sections were double-labeled with rabbit antiserum to cow GFAP (Dakopatts, Dimension Labs Inc., Mississauga, Ontario; 1:1000 dilution); normal rabbit serum (NRS; Sigma) served as control. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC; Sigma), and goat anti-rabbit IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC; Sigma) were used at dilutions of 1:100 and 1:500 respectively.

Antibodies and normal sera were used in dilutions made-up in PBS. The protocol for immunostaining was as described in Chapter II under Materials and Methods.

Results and Discussion

Effects of dBcAMP

Cultures treated with dBcAMP contained cells with large cell bodies and numerous slender processes, which exhibited staining for both J1-31 antigen (Figs. 5.1 A and C, Fig. 5.2 A) and GFAP (Figs. 5.1 B and D, Fig. 5.2 B). Based on their morphology and immunofluorescence staining characteristics, these cells were judged to be astrocytes. It is pointed out that primary cultures contain a mixed population of cells. Staining for J1-31 antigen and GFAP, in dBcAMP treated astrocytes, was limited to the cell processes as little or no staining was detected in the cell bodies (large arrows, Figs. 5.1 A and B). It is pointed out that a subset of the processes were stained by antiserum to GFAP, but not by MAb J1-31 (see sector demarcated with a star in Figs. 5.1 C and D) providing further evidence that these two proteins, J1-31 antigen and GFAP, are distinct. In addition, the staining patterns for these two proteins differed in appearance. The staining for J1-31 antigen is

punctate (large arrow, Fig. 5.2 A) whereas the staining for GFAP is clearly filamentous (large arrow, Fig. 5.2 B). Staining for these proteins was not uniform along the length of the astrocytic processes, but was confined to particular regions (small arrows, Figs. 5.1 A-D, Figs. 5.2 A and B). Therefore, in the majority of the processes, J1-31 antigen did colocalize with GFAP (consistent with the results from electron microscopy where labeling for J1-31 antigen appears in association with GFAP-type intermediate filaments in astrocytes (Malhotra et al., 1984; Singh et al., 1986).

9L Cells

Cultured 9L cells were stained by MAb J1-31 (Figs. 5.2 C and D), but not by antiserum to GFAP. Again the staining for J1-31 antigen is punctate in appearance. However, 9L cells which had attained a confluent monolayer state exhibited little or no staining for J1-31 antigen (Fig. 5.2 C), except at the limit of the monolayer which stained intensely (Fig. 5.2 C and D). Therefore, 9L cells appear to turn-off expression of J1-31 antigen upon attaining a confluent monolayer, possibly due to some form of contact inhibition. This result

suggested the possibility that J1-31 antigen may serve some function in astrocytes which are undergoing division and / or growth. This could explain the results on enhanced expression of J1-31 antigen by astrocytes responding to spinal cord injury (see Chapters III and IV), as well as astroctyes in association with multiple sclerosis plaques (see Chapter V), as such cells exhibit hyperplasia and hypertrophy. In addition, this result provided an explanation for results on the expression of J1-31 antigen in primary cultures which are not treated with dBcAMP. In cultures fixed and stained 7-10 days after seeding, there are large numbers of cells which exhibit staining for J1-31 antigen, but not GFAP (asterisk, Figs. 5.3 A and B; hollow arrows, Figs. 5.3 C and D). This result was inconsistent with results from tissue sections where staining for J1-31 antigen (in astrocytes) is always accompanied by staining for GFAP. Yet if J1-31 antigen serves some role in those astrocytes which are dividing and / or growing, then it is conceivable that during the period immediately following seeding, astrocytes (and prospective astrocytes) may express J1-31 antigen in the absence of GFAP, which may be responsible for the development of complex morphology during cellular differentiation.

Fig. 5.1 A and B. Cultured rat brain cells treated with dBcAMP, double-labeled with MAb J1-31 (A, FITC) and GFAP (B, TRITC). Intense staining is evident in regions along the astrocytic processes (small arrows, A and B). Little or no staining is evident in the cell bodies of these astrocytes (large arrows, A and B).

Fig. 5.1 C and D. Cultured rat brain cells treated with dBcAMP, double-labeled with MAb J1-31 (C, FITC) and GFAP (D, TRITC). Intense staining is evident in regions along the majority of the astrocytic processes (arrows, C and D). However, some processes exhibit staining for GFAP, but not J1-31 antigen (sector demarcated with a star, C and D).

A
B
C
D

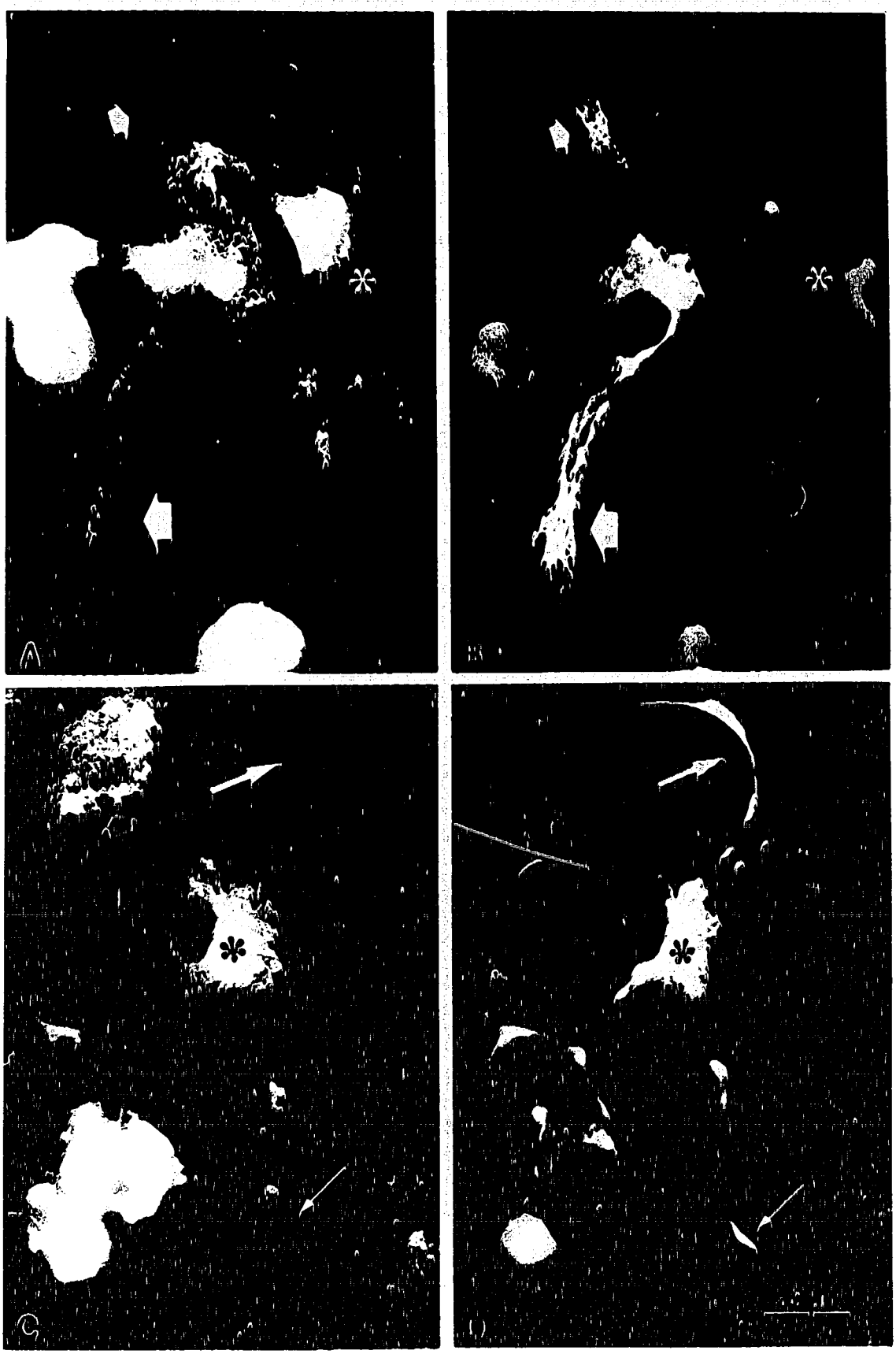
Fig. 5.2 A and B. Cultured rat brain cells treated with dBcAMP, double-labeled with MAb J1-31 (A, FITC) and GFAP (B, TRITC). Intense staining is evident in regions along the astrocytic processes (small arrows, A and B). In the cell body, the staining for J1-31 antigen appears punctate (large arrow, A) whereas the staining pattern for GFAP (large arrow, B) appears filamentous.

Fig. 5.2 C and D. Rat glioma cells (9L) stained with MAb J1-31. Staining is evident in cells at the edge of a confluent monolayer (large arrow, C; fat arrow, D). Most cells within the confluent monolayer do not stain for J1-31 antigen (asterisk, C); however, some of these cells do stain for J1-31 antigen (small arrow, D). The staining pattern in 9L cells is punctate in appearance, as is evident in cells which have migrated beyond the border of the monolayer (skinny arrow, D).

A
B
C
D
25μm

Fig. 5.3 A and B. Rat brain cells in primary culture, double-labeled for J1-31 antigen (A, FITC) and GFAP (B, TRITC). The staining for J1-31 antigen is punctate (arrows, A) whereas the staining for GFAP is filamentous (arrows, B). In these cultures, MAb stains some cells (asterisk, A) which are not stained by antiserum to GFAP (asterisk, B).

Fig. 5.3 C and D. Rat brain cells in primary culture, double-labeled for J1-31 antigen (A, FITC) and GFAP (B, TRITC). Some cells (asterisk, C and D) exhibit staining for both J1-31 antigen (C) and GFAP (D). Within these cells however, there are regions which show staining for GFAP, but not J1-31 antigen (solid arrows, C and D). In these cultures, there are also cells which exhibit staining for J1-31 antigen, but not GFAP (hollow arrows, C and D).



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Chapter VII: Fetal Antigen Retained by Mature Astrocytes, Muller Glia and Ependyma Studied With a Monoclonal Antibody (G-3-5)

This Chapter presents results on the localization of an antigen (G-3-5 antigen) which is recognized by an MAb (MAb G-3-5) raised against autopsy samples of tissue from the CNS of MS patients. By immunofluorescence microscopy, MAb G-3-5 stains most or all cells in fetal rats. In the mature CNS, however, staining owing to MAb G-3-5 is primarily restricted to astrocytes, Muller glia, and the ependyma. (Work carried out by Dr. D. N. P. Singh has indicated that some neurons also exhibit staining for G-3-5 antigen.)

In many respects, G-3-5 antigen remains the least well characterized of the three antigens studied. This chapter presents results on the expression of G-3-5 antigen in CNS tissue, and a number of non-CNS tissues. Unlike J1-31 antigen and 6B9 antigen, no central organizing hypothesis has been proposed to account for the distribution of this antigen. Therefore, this chapter is of necessity a collection of seemingly disconnected results on the localization of G-3-5 antigen in the various tissues surveyed. However,

inclusion of this study in the thesis serves two purposes:

i) comparing and contrasting the distribution of G-3-5 antigen with J1-31 antigen and 6B9 antigen, suggests relationships among various cell-types based on the expression of what are obviously different molecules,

ii) some future researcher may discern the underlying thread which ties these results together (Well done!).

Materials and Methods

Fixation for Immunocytochemistry

MAb G-3-5 did not give rise to detectable immunostaining in cryostat sections from glutaraldehyde fixed tissue using peroxidase anti-peroxidase (PAP). However, strong staining (immunofluorescence and PAP) owing to MAb G-3-5 was evident in cryostat sections from tissue fixed in 4% paraformaldehyde (0.2 M phosphate buffer, pH 7.2) and therefore, this fixative was used in the immunocytochemical studies. Adult Sprague-Dawley rats were killed by decapitation, or etherized and perfused with 0.9% NaCl followed by paraformaldehyde. Tissue (including fetuses from pregnant rats) was removed and immersed in fixative for 1-3 hr.

Immunofluorescence Microscopy

Fixed tissue was washed with phosphate buffered saline (PBS) and cryoprotected in 30% sucrose (12-18 hr, 4C) prior to freezing at -20C. Tissue was embedded in Tissue Tek II O. C. T. Compound and cryostat sections were cut at 8-12 um thickness, mounted on rubber cement-coated coverglasses, and dried at 4C overnight.

Primary cultures of rat brain cells were grown on poly-(L)-lysine coated coverglasses as described in Chapter VI under Materials and Methods. Similarly, human plasmacytoma cells (HNY-2) were grown on poly-(L)-lysine coated coverslips in RPMI medium (Gibco, Grand Island, NY) supplemented with 20% fetal calf serum, penicillin, streptomycin, sodium pyruvate, oxalic acid and glutamine. Coverslips bearing cells were immersed in 4% paraformaldehyde for 30-60 minutes, and then washed with PBS prior to staining for immunofluorescence microscopy.

Indirect immunofluorescence staining was carried out using MAb G-3-5 ascites fluid (1:1000 dilution) or culture supernatant; normal mouse serum (NMS; Sigma, St. Louis, MO) and culture supernatant from NS-1 myeloma cells served as controls. Some of the sections were double-

labeled with rabbit antiserum to cow GFAP (Dakopatts, Dimension Labs Inc., Mississauga, Ontario; 1:1000 dilution); normal rabbit serum (NRS; Sigma) served as control. Goat anti-mouse IgM (mu-chain specific) conjugated to fluorescein isothiocyanate (FITC; Sigma) and goat anti-rabbit IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC; Sigma) were used at dilutions of 1:100 and 1:500 respectively. Dilutions were made-up in PBS. The protocol for immunofluorescence staining was as described in Chapter II under Materials and Methods.

Results and Discussion

Results from immunofluorescence microscopy described below have been ascertained using several batches of MAb G-3-5 ascites fluid and culture supernatant, and no staining has been detected in control sections incubated with normal mouse serum or NS-1 supernatant. It is remarked that the goat anti-mouse IgM (FITC conjugate) gave rise to minimal background fluorescence as compared to the high background associated with the goat anti-mouse IgG (FITC conjugate) used for localization of J1-31 antigen (Chapters II-VI).

Distribution of G-3-5 Antigen in the Rat CNS

In the rat CNS, staining owing to MAb G-3-5 is localized in those types of macroglial cells which also stain for J1-31 antigen (see Chapter II). MAb G-3-5 intensely stains astrocytes (Figs. 6.1 A, B and D), retinal Muller cells (Fig. 6.1 B), tanycytes in the ependyma (Fig. 6.1 C), and ciliated ependymal cells (Fig. 6.1 D).

Distribution of G-3-5 Antigen in Non-CNS Tissues

Muscle cells. In cryostat sections of rat skeletal (Fig. 6.2 A) and cardiac (Fig. 6.2 B) muscle, MAb G-3-5 gives rise to staining which has the typical banded appearance of striated muscle. It is not known if this banding is an artifact of the ultrastructure, reflecting the organization of the contractile filaments, or if the antigen itself is localized in association with some component of these filament assemblies.

Intestine and Kidney. In cryostat sections of rat intestine, MAb G-3-5 intensely stains a subset of the cells in intestinal villi (Fig. 6.2 C). The significance of this differential staining is not known. These stained cells may

correspond to a particular cell-type (mucus secreting gland cell; p. 119 of Bloom and Fawcett, 1975), or alternatively they may correspond to cells in a given physiological state. In cryostat sections of rat kidney, MAb G-3-5 intensely stains some of the tubules (fat arrow, Fig. 6.2 D). The stained tubules are immediately adjacent to tubules exhibiting little or no such staining (skinny arrow, Fig. 6.2 D). Again, the significance of this differential staining is not known.

Testis and Ovary. In cryostat sections of rat seminiferous tubules, MAb G-3-5 stains cells at the extreme periphery (Fig. 6.3 A). Little or no staining is evident in cells located towards the center of the tubule (Fig. 6.3 A). This observation may be of significance, as prospective sperm cells differentiate as they progress toward the center of the tubule (Hopper and Hart, 1980). Consequently, this differential staining may reflect the loss of some cell organelle. In cryostat sections of rat ovary, MAb G-3-5 intensely stains the germinal epithelium (arrow) which covers the surface of this organ (Fig. 6.3 B). Some cells within the ovary itself are also stained by MAb G-3-5 (Fig. 6.3 B).

Pancreas. In cryostat sections of rat pancreas, MAb G-3-5 intensely stains cells of the Islets of Langerhans (endocrine pancreas, Fig. 6.3 C). No staining owing to MAb G-3-5 is evident in pancreatic acinar cells (exocrine pancreas; asterisk, Fig. 6.4 C and D). In some preparations, intense staining owing to MAb G-3-5 is evident only in cells in the periphery of the Islets (arrow, Fig. 6.3 D). This may reflect a fixation artifact or alternatively, it may reflect preferential staining of a given cell-type or of cells in a particular physiological state.

Distribution of G-3-5 Antigen in Fetal Tissues

In the rat fetus (crown-rump length 12 mm), MAb G-3-5 stains most or all of the cells (Fig. 6.4 A and B). However, the staining is not uniform across all cells at this stage. Such differential staining is evident in the snout of the developiing fetus (Fig. 6.4 A), where staining is most intense in the outer layer of the developing skin (ectoderm: epidermis) and is much reduced in the inner layer (mesoderm: dermis). In a cross-section of the developing midgut, staining for G-3-5 is evident in most of all of the cells (Fig. 6.4 B).

Differential staining for G-3-5 antigen is pronounced in the rat fetus late in gestation (18-21 days, just prior to birth on day 21). At this stage, staining for G-3-5 antigen is evident in what appear to be radial glial cells in the developing brain (arrows, Figs. 6.4 C and D). In the lens of the eye, staining is evident in the lens fibers (LF), whereas no staining is evident in the lens epithelium (arrow) from which the lens fibers arise (Fig. 6.5 A). Similar to the situation observed in the 12 mm rat embryo, intense staining is evident in the outer layer, epidermis (arrows), of the skin (eyelid, Fig. 6.5 B; skin on forepaw, Fig. 6.5 C), whereas little or no staining is evident in the mesodermal component of these structures (asterisks, Figs. 6.5 B and C). However, intense staining for G-3-5 antigen was detected in other mesodermal derivatives such as chondrocytes in the forepaw (Fig. 6.5 D).

Distribution of G-3-5 Antigen in Cultured Cells

In primary cultures of rat brain cells, MAb G-3-5 gives rise to punctate staining pattern which suggests that this antigen may be associated with some form of microbody (Figs. 6.6 A and B). In some of the cells, the staining appears to be arranged in rows (Fig. 6.6 A). Hence, G-3-5

antigen may be associated with some element of the cytoskeleton, such as microtubules. It is possible that G-3-5 antigen may be associated with microbodies which are themselves aligned, or even moving, on microtubules. This could account for both the punctate appearance of the staining and its organization in linear arrays.

In human plasmacytoma cells (HNY-2; Fig. 6.6 C), MAb G-3-5 again gives rise to punctate staining in the cytoplasm. As these cells are continuously dividing (round cells), it is not expected that they would have a cytoskeletal organization comparable to that in rat brain cells.

Summary

An antigen has been identified, which is widely expressed in rat embryos and exhibits differential expression by various cell-types during development. This study has yielded information on the distribution of G-3-5 antigen in the CNS and numerous non-CNS tissues in the adult, as well as changes in its distribution which occur during development. Results on the expression of G-3-5 antigen by cells in culture have indicated that this antigen is localized in the cytoplasm, possibly in

association with some form of microbody, and its organization in linear arrays suggests that it may be associated with some component of the cytoskeleton.

Fig. 6.1 A. Cryostat section of rat cerebellum and medulla stained with MAb G-3-5. Staining is evident in Bergmann fibers of the cerebellum (skinny arrows), as well as ependymal cells lining the surface of the medulla (fat arrow). Staining is also evident in astrocytes in the medulla.

Fig. 6.1 B. Cryostat section of rat retina stained with MAb G-3-5. Staining is evident in astrocytes in the nerve fiber layer (fat arrow), as well as Muller cells which span the breadth of the retina (arrowheads).

Fig. 6.1 C. Cryostat section of rat diencephalon stained with MAb G-3-5. Staining is evident in tanycytes which extend radially from the wall of the ventricle (arrow).

Fig. 6.1 D. Cryostat section of rat diencephalon stained with MAb G-3-5. Staining is evident in ciliated ependymal cells lining the wall of ventricle III (large arrow), as well as astrocytes in the neuropil (small arrows).

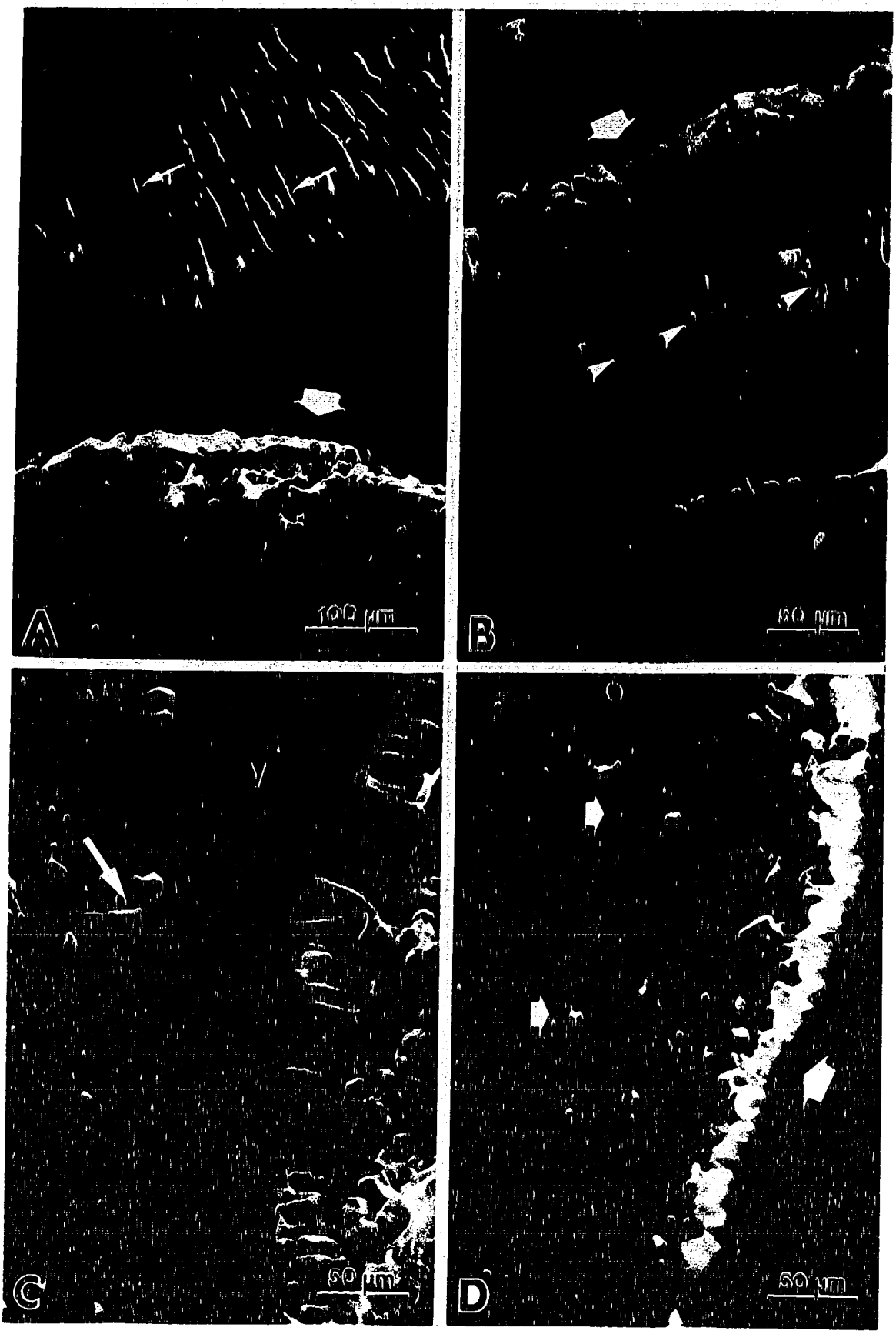

A
100 µm
B
50 µm
C
50 µm
D
50 µm

Fig. 6.2 A. Cryostat section of rat skeletal muscle stained with MAb G-3-5. Note the banded appearance of the staining (arrow).

Fig. 6.2 B. Cryostat section of rat cardiac muscle stained with MAb G-3-5. Note the banded appearance of the staining (arrow).

Fig. 6.2 C. Cryostat section of rat intestine stained with MAb G-3-5. Some cells in the intestinal villus exhibit staining for G-3-5 antigen (arrows).

Fig. 6.2 D. Cryostat section of rat kidney stained with MAb G-3-5. Some tubules exhibit staining for G-3-5 antigen (fat arrow). Adjacent tubules exhibit little or no such staining (skinny arrow).

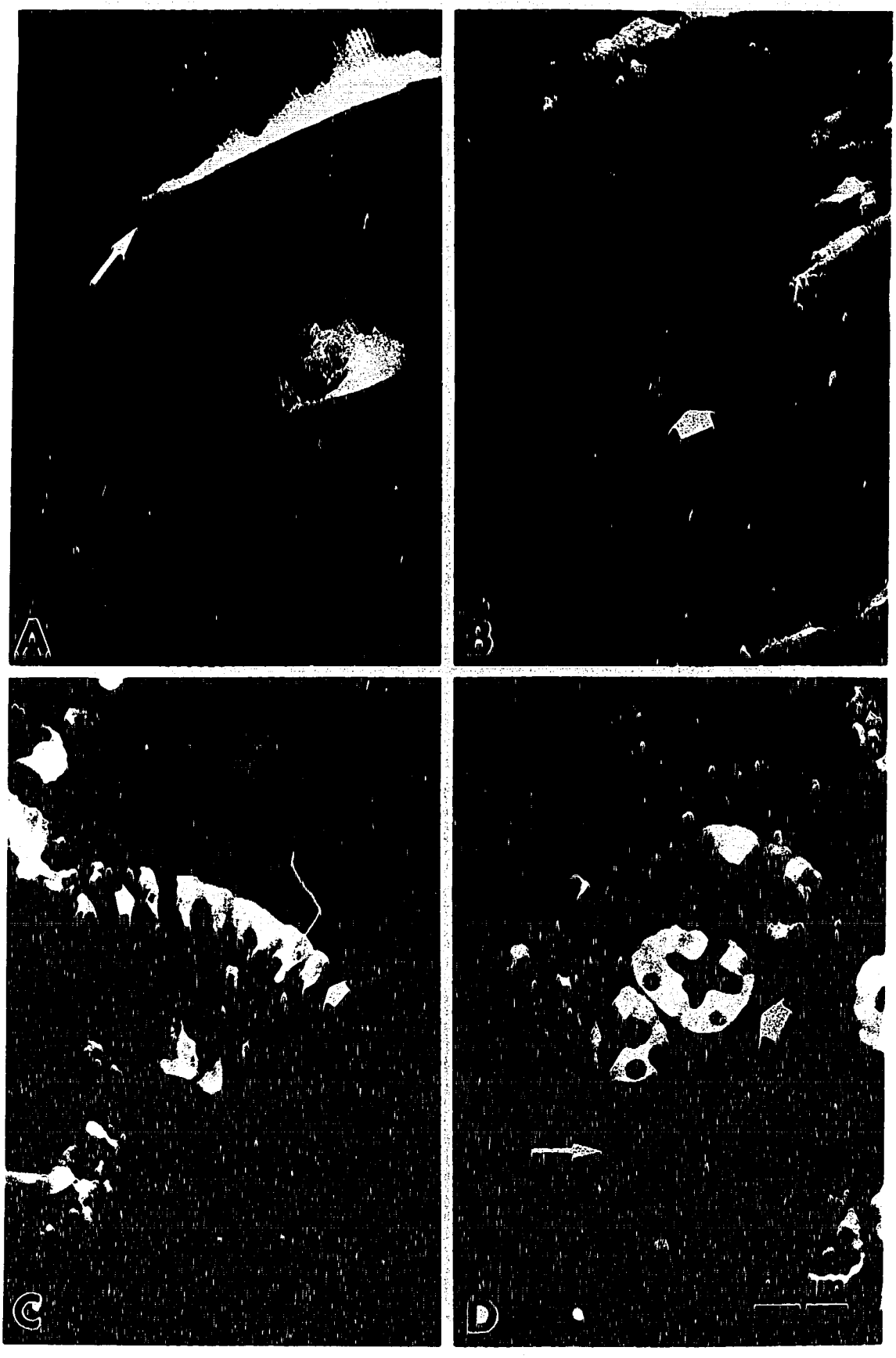

A
B
C
D

Fig. 6.3 A. Cryostat section of rat testis stained with MAb G-3-5. Cells in the periphery of the seminiferous tubule exhibit staining for G-3-5 antigen (arrow). Essentially no staining is evident near the lumen of the tubule (asterisk).

Fig. 6.3 B. Cryostat section of rat ovary stained with MAb G-3-5. Staining is exhibited by cells of the germinal epithelium (arrow) as well as by cells within the organ.

Fig. 6.3 C. Cryostat section of rat pancreas stained with MAb G-3-5. Pancreatic Islets (endocrine pancreas) exhibit staining for G-3-5 antigen. No staining is evident in the exocrine pancreas (asterisk).

Fig. 6.3 D. Cryostat section of rat pancreas stained with MAb G-3-5. Intense staining for G-3-5 antigen is exhibited by only those cells in the periphery of the Islet (arrow). Again, no staining is evident in the exocrine pancreas (asterisk).

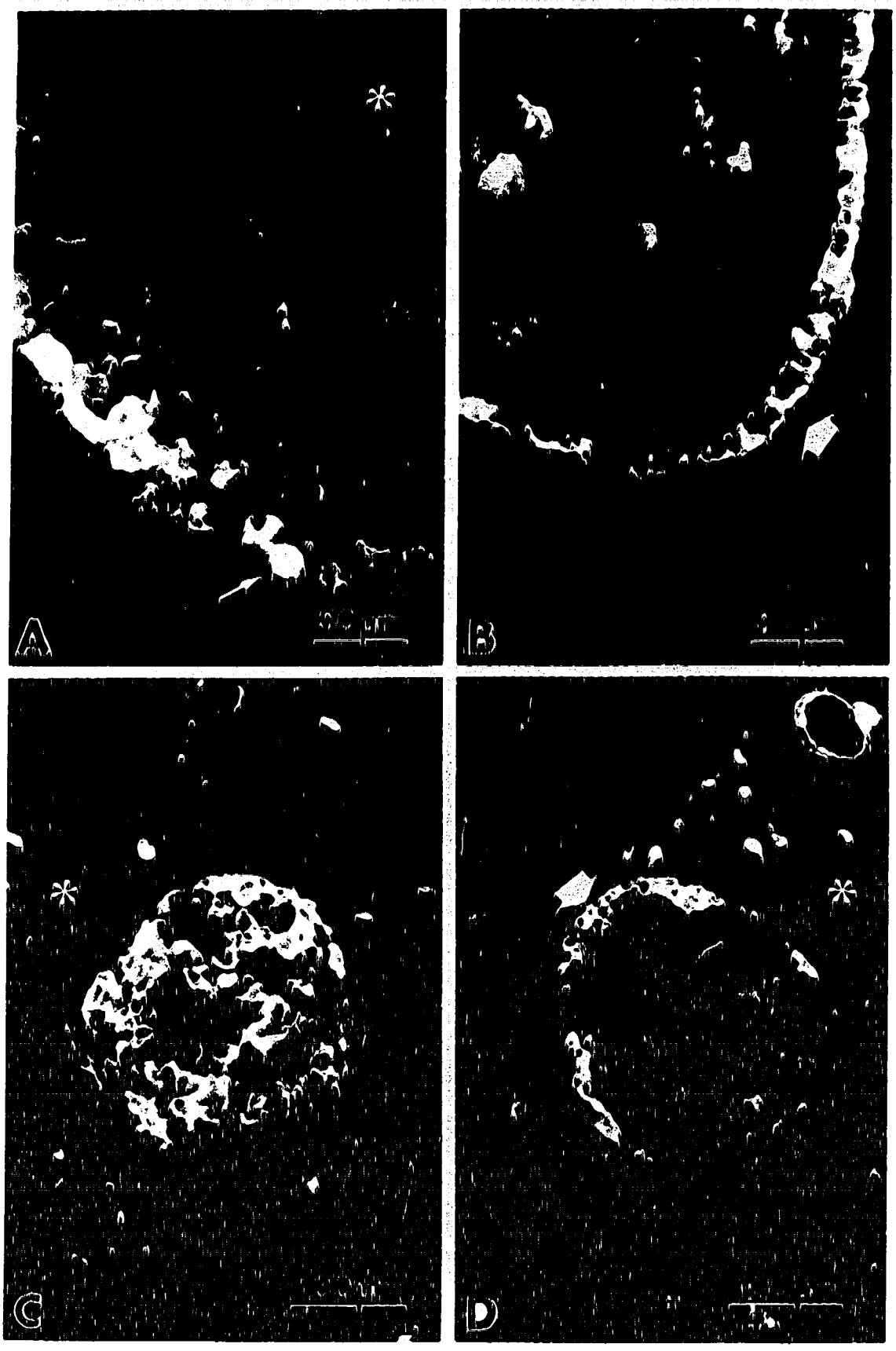

A
B
C
D

Fig. 6.4 A. Cryostat section of a rat fetus (crown-rump length 12 mm) stained with MAb G-3-5. Differential staining is evident in the various cells in this region (anterior, snout).

Fig. 6.4 B. Cryostat section of a rat fetus (crown-rump length 12 mm) stained with MAb G-3-5. Staining is evident in most or all of the cells in this cross-section of the intestine. L, lumen.

Fig. 6.4 C and D. Cryostat section of fetal rat brain (18-21 days gestation) stained with MAb G-3-5. Staining is evident in what appear to be radially oriented glial cells (arrows).

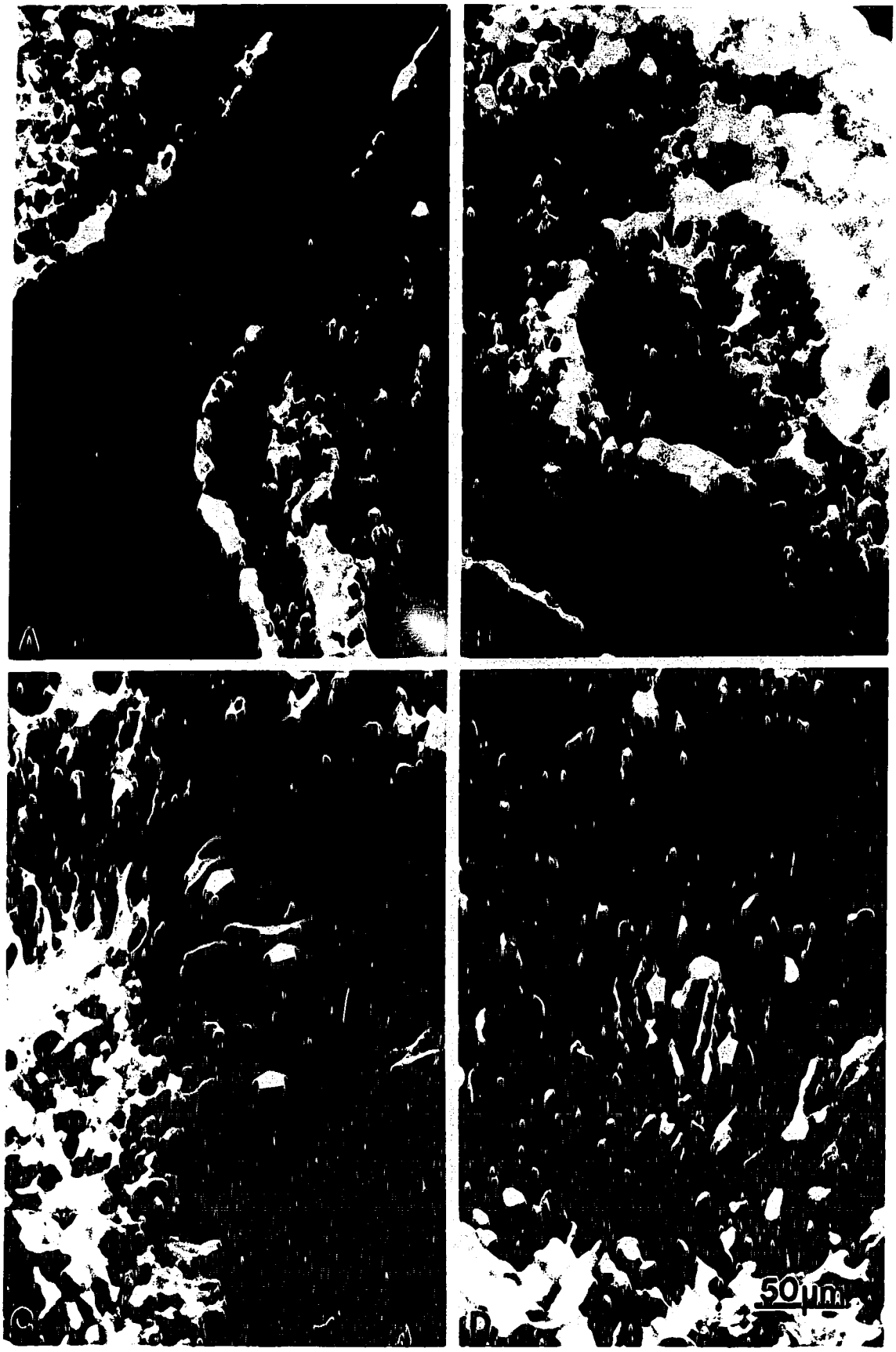
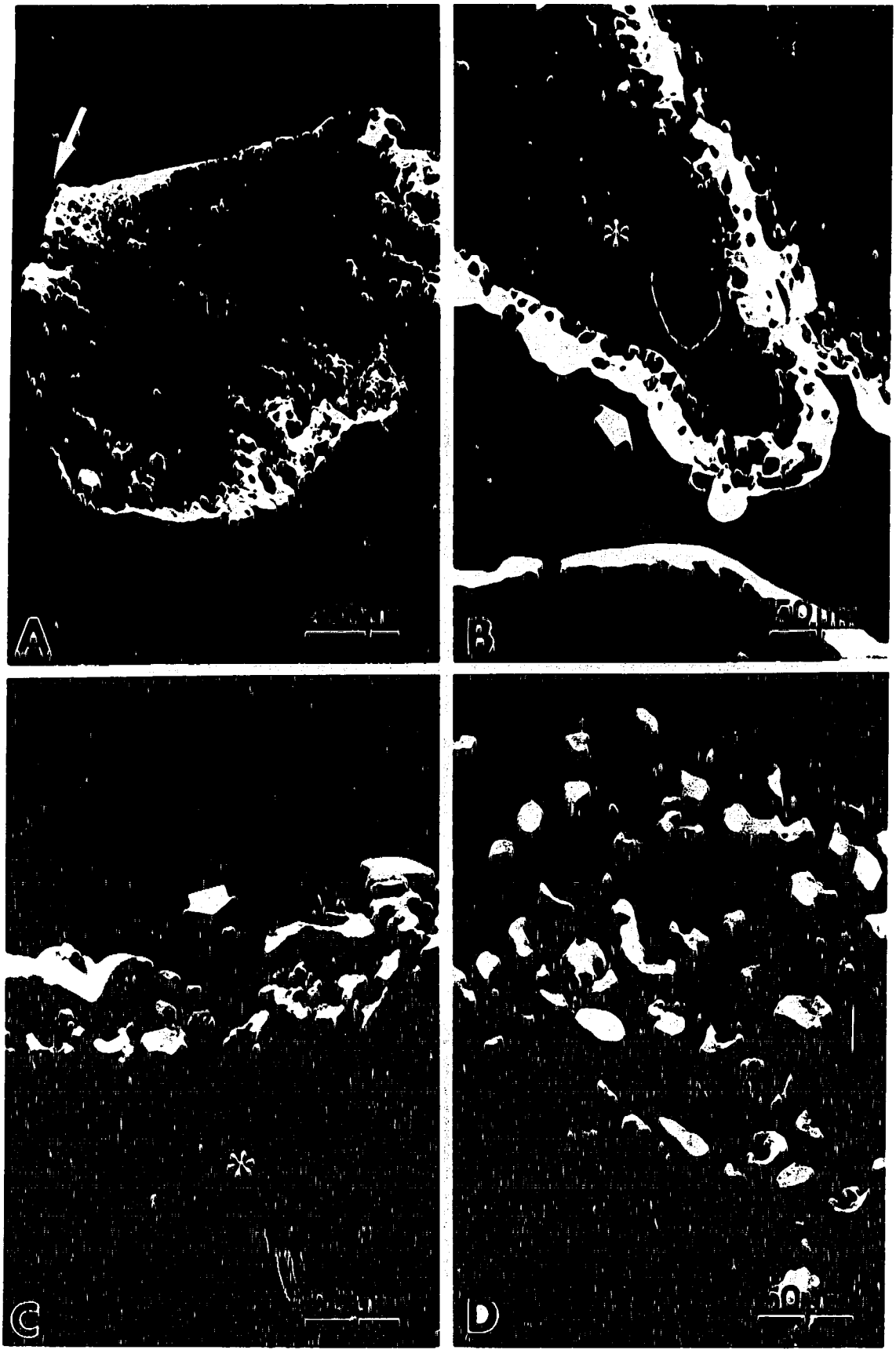

A
B
C
D
50µm

Fig. 6.5 A. Cryostat section of a fetal rat eye (18-21 days gestation) stained with MAb G-3-5. Intense staining is evident in the lens fibers (LF), but no staining is evident in the lens epithelium (arrow) from which the lens fibers arise.
R, retina.

Fig. 6.5 B. Cryostat section of a fetal rat eye (18-21 days gestation) stained with MAb G-3-5. Intense staining is evident in ectodermal cells (epidermis; arrow) of the developing eyelid. Staining is much reduced in the mesodermal cells within the eyelid (asterisk).

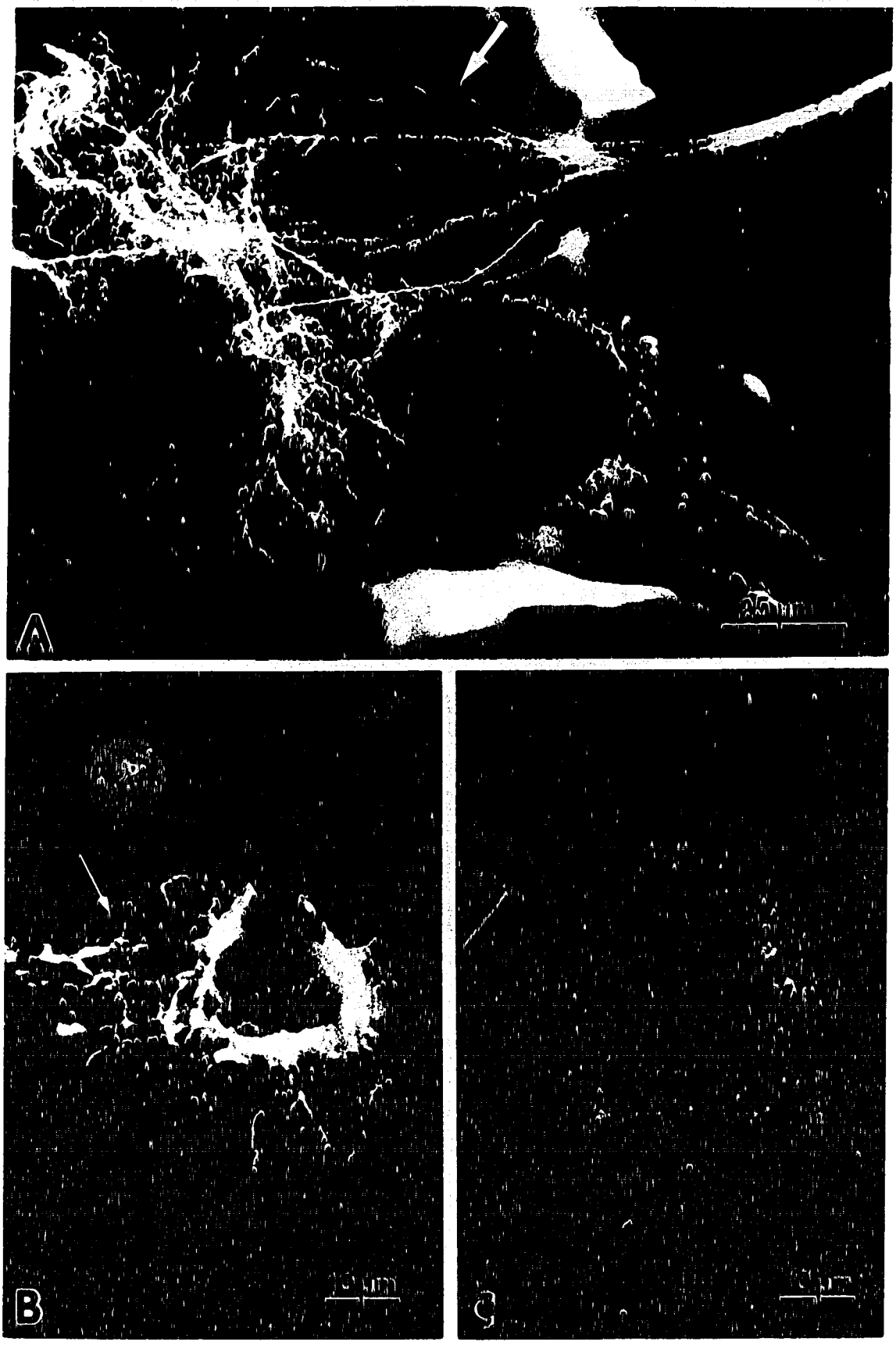
Fig. 6.5 C. Cryostat section of a fetal rat paw (18-21 days gestation) stained with MAb G-3-5. Intense staining is evident in the epidermis of the skin (arrow). Little or no staining is evident in the dermis of the skin (asterisk).

Fig. 6.5 D. Cryostat section of a fetal rat paw (18-21 days gestation) stained with MAb G-3-5. Intense staining is evident in chondrocytes of a developing bone.


A
B
C
D

Figs. 6.6 A and B. Rat brain cells, grown in culture, stained with MAb G-3-5. The staining is cytoplasmic and punctate in appearance. In some cells, the G-3-5 antigen appears to be organized in linear arrays (arrow, A) suggesting an association with some component of the cytoskeleton. In other cells, the staining has the appearance of a reticulum (arrow, B).

Fig. 6.6 C. Human plasmacytoma cell (HNY-2) stained with MAb G-3-5. In these continuously dividing cells, the staining has the appearance of small vesicles.


A
B
C

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Chapter VIII: Fetal Antigen Retained by Mature Neurons and Ependyma Studied With a Monoclonal Antibody (6B9)[1]

This Chapter presents results on the localization of an antigen (6B9 antigen) which is recognized by an MAb (MAb 6B9) raised against autopsy samples of tissue from the CNS of MS patients. By immunofluorescence microscopy, MAb 6B9 stains most or all cells in cryostat sections of fetal rats. In the mature CNS, however, staining owing to MAb 6B9 is restricted to neuronal somata and the ependyma. Upon examination of paraformaldehyde fixed blood smears, it was observed that MAb 6B9 intensely stains plasma cells which function to secrete antibodies. This observation suggested the possibility that 6B9 antigen may be a member of a class of molecules, termed reticuloplasmins, which are localized in the lumen of the rough endoplasmic reticulum (rER). The rER is well developed in neurons and plasma cells. Although supported by circumstantial evidence, this hypothesis remains to be proven.

1. A version of this chapter has been published.
   Malhotra, S. K., Predy, R., Singh, R., Gupta, B. L., Bhatnagar, R., Leeuw, K., (1988): Fetal Antigen Retained by Mature Neurons and Ependyma Studied With a Monoclonal Antibody (6B9). J. Neuroscience Research **19**: 420-427.

Materials and Methods

Fixation for Immunocytochemistry

MAb 6B9 did not give rise to detectable immunostaining in cryostat sections from glutaraldehyde fixed tissue using peroxidase anti-peroxidase (PAP). However, strong staining (immuno-fluorescence and PAP) owing to MAb 6B9 was evident in cryostat sections from tissue fixed in 4% paraformaldehyde (0.2 M phosphate buffer, pH 7.2), and therefore this fixative was used in the immunocytochemical studies. Adult Sprague-Dawley rats were killed by decapitation, or etherized and perfused with 0.9 % NaCl followed by paraformaldehyde. Tissue (including fetuses from pregnant rats) was removed and immersed in fixative for 1-3 hr. Tissue from young rats (6 and 12 days old, killed by decapitation), rabbits (killed by lethal injection), frogs (pithed), *Drosophila* and *Lumbricus*, as well as human tissue samples received at autopsy were all fixed by immersion (1-3 hr, 20C).

Primary cultures of rat brain cells were grown on poly-(L)-lysine coated coverglasses as described in Chapter VI under Materials and Methods. Coverglasses bearing cells were immersed in 4% paraformaldehyde for 30-60 minutes, washed with PBS, and then stained for immunofluorescence microscopy.

Immunofluorescence Microscopy

Cryostat sections. Fixed tissue was washed with phosphate buffered saline (PBS) and cryoprotected in 30% sucrose (12-18 hr, 4C) prior to freezing at -20C. Tissue was embedded in Tissue Tek II O.C.T. Compound and cryostat sections were cut at 8-12 um thickness, mounted on rubber cement-coated coverglasses, and dried at 4C overnight.

Indirect immunofluorescence staining was carried out using MAb 6B9 ascites fluid (1:1000 dilution) or culture supernatant; normal mouse serum (NMS; Sigma, St. Louis, MO) and culture supernatant from NS-1 myeloma cells served as controls. Some of the sections were double-labeled with rabbit antiserum to cow GFAP (Dakopatts, Dimension Labs Inc., Mississauga, Ontario; 1:1000 dilution); normal rabbit serum (NRS; Sigma) served as control. Dilutions were made-up in PBS. GFAP is recognized marker for certain types of neuroglia including tanycytes (de Vitry et al., 1981) and retinal Muller cells (Bjorklund et al., 1985) and therefore, antiserum to GFAP has been used to visualize these cells in double-labeled sections.

Semithin Sections. Fixed tissue was trimmed in PBS and cryoprotected in 10% sucrose (12-18 hr, 4C; Tokuyasu, 1973). The tissue was transferred to 2.3 M sucrose (4 hr, 4C), and then frozen on

pins in liquid nitrogen. Sections were cut at 0.5 - 1 um thickness on a Reichert-Jung Cryo-ultramicrotome and mounted on poly-L-lysine coated coverglasses. Shortly after sectioning, immuno-fluorescence staining was carried out using MAb 6B9 ascites fluid (1:300 dilution) and purified rabbit IgG to neurofilament (NF) proteins (Advanced Immunochemicals, Los Angeles, CA; 1:100 dilution). Normal sera from mouse and rabbit served as the respective controls.

Protocol for Immunofluorescence Staining

The protocol for immunofluorescence staining was as described in Chapter II under Materials and Methods. Goat anti-mouse IgM (mu-chain specific) conjugated to fluorescein isothiocyanate (FITC; Sigma), and goat anti-rabbit IgG conjugated to tetramethylrhodamine isothiocyante (TRITC; Sigma) were used at dilutions of 1:100 and 1:500 respectively.

Immunoelectron Microscopy

Ultrathin cryo-sections were cut from blocks prepared as outlined for semithin sections. Sections were mounted on copper-paladium grids, and immunogold staining was carried out according to the protocol outlined in ffrench-Constant et al. (1986). Sections were incubated in 6B9 ascites fluid (1:50 and 1:100 dilutions) and culture supernatant; NMS and NS-1 supernatant served as controls. Goat

antimouse IgM gold conjugate (5nm; Janssen Life Sciences, Olen, Belgium) was employed at a dilution of 1:30. Sections were examined in the electron microscope with and without staining with uranyl acetate.

Results and Discussion

Results from immunofluorescence microscopy described below have been ascertained using several batches of MAb 6B9 ascites fluid and culture supernatant, and no staining has been detected in control sections incubated with normal mouse serum or NS-1 myeloma supernatant.

Distribution of 6B9 Antigen in the Rat

In mature rats, MAb 6B9 immunostains neuronal somata (Figs. 7.1 A and B; Figs. 7.2 A and B), ciliated ependymal cells (Fig. 7.2 C), tanycytes (Fig. 7.3 A), and chromaffin cells of the adrenal medulla (not shown). MAb 6B9 stains somata of sensory neurons in dorsal root ganglia (DRGs) which are a component of the peripheral nervous system (PNS; Fig. 7.1 A). In DRG, both neuronal somata and nerve fibers exhibit staining for NF proteins (Fig. 7.1 A); however, these nerve fibers are not stained by MAb 6B9 (Fig. 7.1 A). MAb 6B9 stains various and diverse types of neurons in the CNS, such as motor

neurons in the ventral horn of the spinal cord (Fig. 7.1 B) as well as ganglion and bipolar cells of the neural retina (Fig. 7.1 D and Fig. 7.2 A). Purkinje cells of the cerebellar cortex often exhibit variability in the intensity of staining within a given animal. Those Purkinje cells located near the tips of folia stain most intensely (Fig. 7.2 B), whereas those near the bases of folia exhibit little or no such staining. Staining due to MAb 6B9 in neurons appears to be limited to somata and large-diameter dendrites; axons do not exhibit staining. The cytoplasmic staining owing to MAb 6B9 in neurons, ependyma, and chromaffin cells is punctate in appearance as is evident in semithin sections of the DRG (Fig. 7.1 A).

Ependyma consists of ciliated ependymal cells and tanycytes (Bruni et al., 1985). Ciliated ependymal cells form a cuboidal or columnar epithelium which is intensely stained by MAb 6B9 (Fig. 7.2 C). Tanycytes have their cell bodies bordering on the ventricular system, and each cell extends a single process into the neuropil. MAb 6B9 stains cell bodies of tanycytes (Fig. 7.3 A), but little staining is evident in their processes, which are visualized double-labeled sections using antiserum to GFAP (Fig. 7.3 B).

In the rat fetus (crown-rump length 12 mm), MAb 6B9 stains most or all of the cells. In the preparations (sagittal sections), the only structures not stained were nerve fibers in the spinal nerve roots (Fig. 7.2 D). This result is consistent with the observation that MAb 6B9

does not stain small-diameter neuronal processes in mature rats. MAb 6B9 differentially stains various cell types at later stages of development. In the adult rat CNS, MAb 6B9 does not stain astrocytes or oligodendrocytes. Staining in liver, muscle (cardiac, skeletal, intestinal smooth), renal cortex and adrenal cortex was much reduced compared to fetal tissue or absent. Upon examination of sections of heart from fetal and 6-day-old rats, it was observed that erythrocytes (RBCs) are variably stained by MAb 6B9. Some RBCs were intensely stained, whereas others exhibited little or no such staining. RBCs from mature human and rat were not stained by MAb 6B9.

6B9 Antigen in Developing Retina

The expression of 6B9 antigen has been followed in the developing neural retina of the rat (Weidman and Kuwabara, 1969) by immunofluorescence microscopy. In the mature retina, MAb 6B9 stains the ganglion cell layer and the inner nuclear layer which contains the somata of bipolar, horizontal, and amacrine cells (Fig. 7.2 A). No staining was detected in Muller cells which were visualized in double-labeled sections using antiserum to GFAP. In the 20-day fetus, staining is evident across the entire breadth of the retina (Fig. 7.1 C). The inner plexiform layer is already evident at this

stage. At postnatal day 6, staining is limited to the ganglion cell layer and a broad layer which includes prospective cells of the inner nuclear layer and photoreceptors (Fig. 7.3 C). The nerve fiber layer (axons of ganglion cells) and inner plexiform layer are devoid of staining. The outer plexiform layer is not well delineated at this stage. Photoreceptors lose this antigen during differentiation and a gradient in the intensity of staining is apparent (Fig. 7.3 C). Staining is most intense in the developing bipolar cell layer and fades away toward the periphery of the neural retina. At postnatal day 12, the situation is comparable to that seen at maturity where staining is limited to the ganglion cell and inner nuclear layers (Fig. 7.1 D). The nerve fiber layer, inner and outer plexiform layers, and photoreceptors are devoid of staining. The staining pattern, which arises during differentiation of the neural retina, supports the observation that neuronal processes, with the exception of large-diameter dendrites, are not stained by MAb 6B9 in the mature rat.

6B9 Antigen in Species Other Than Rat

MAb 6B9 intensely stains neurons from human, rabbit and frog studied thus far. In sections of human brain, MAb 6B9 stained some but not all of the ciliated ependymal cells. This may reflect the preservation of human tissue where there is a 12 hr or longer delay in postmortem prior to the start of fixation. (The ciliated ependyma of

one mature rat showed a similar phenomenon, where there was large variability in the intensity of staining among adjacent cells. However, the ependymal layer in this animal appeared to be drawn into folds and may have been in a pathological state.)

MAb 6B9 extensively stains sections of human fetal brain similar to that seen in rat. Extensive staining has also been observed in sections of *Xenopus* embryos (eary cleavage through gastrulation). The intensity of staining was not uniform across all cells of the *Xenopus* embryo, and this may reflect differential expression of 6B9 antigen by various cell lineages.

The 6B9 reactive epitope is not restricted to vertebrates as staining owing to MAb 6B9 has been detected in sections of *Drosophila* (heads) and *Lumbricus*. In *Drosophila*, MAb 6B9 stains neuronal somata in the brain but not neuronal processes (neuropil). In *Lumbricus*, staining owing to MAb 6B9 is evident in what appear to be mucus-secreting gland cells in the epidermis (Barnes, 1980) as well as globular structures and extra-cellular matrix material in the body wall musculature (Fig. 7.3 D). Staining was not detected in neurons of *Lumbricus*.

Cytochemical Characterization

In semithin sections of rat DRG treated with protease (Type XXI,

Sigma; 1 mg / ml, 1 min, 20C) prior to immunostaining, the staining owing to MAb 6B9 is greatly enhanced compared to the untreated controls. Similarly, treatment of cryostat sections of human frontal cortex with hyaluronidase (Boehringer-Mannheim, Dorval, Quebec; 5 mg / ml, 30 min, 37C) resulted in greatly enhanced staining owing to MAb 6B9. Therefore protease and hyaluronidase both appear to unmask antigen. In addition, treatment of cryostat sections of rat spinal cord with ribonuclease (Type 1-A, Sigma; 5 mg / ml, 30 min, 37C) did not reduce staining owing to MAb 6B9. Treatment of cryostat and semithin sections with periodic acid, prior to immunostaining, abolishes staining owing to MAb 6B9. Under the most severe conditions employed (1% periodic acid, distilled water, 1hr, 37C), staining for 6B9 antigen in cryostat sections of rat DRG was barely detectable. Therefore, the 6B9 reactive epitope could be a carbohydrate as periodic acid cleaves 1,2-diol groups (Pearse, 1985); immunoprecipitation and transblots have thus far failed to provide any evidence that 6B9 antigen is a protein (work carried out previously).

Subcellular Localization

Studies on semithin sections show that the immunofluorescence staining caused by MAb 6B9 is intracellular. This result has been further ascertained by staining cultured neuroblastoma cells (N115), as no stainng has been detected in unfixed cells, whereas staining is

evident following fixation in paraformaldehyde. (It should be mentioned that fixation in 5% acetic acid in ethanol for 10 min a -20C results in greatly reduced staining.)

In electron micrographs of neurons (rat DRG), colloidal gold labeling for 6B9 antigen appears in the the cytoplasm distinct from any identifiable organelle. The cytoplasm in paraformaldehyde-fixed tissue appears discontinuous, and this may represent the precipitation of soluble cytoplasmic constituents. It is emphasized that no labeling appeared in association with the Golgi complex.

An Organizing Hypothesis

Upon examination of paraformaldehyde fixed blood smears (mine), it was observed that MAb 6B9 intensely stains plasma cells which function to secrete antibodies (Fig. 7.4 A). These cells bear a striking resemblance to plasmacytoma cells which have been immunofluorescently stained with antibodies to a known protein, endoplasmin (Koch et al., 1987). Endoplasmin appears to be a member of a class of proteins, called reticuloplasmins, which are localized in the lumen of the rough endoplasmic reticulum (rER) (for short review see Koch, 1987). Data on the localization of endoplasmin, provided in a previous paper (Koch et al., 1985), correlates well with that of 6B9 antigen. These molecules,

endoplasmin and 6B9 antigen, are coexpressed by human brain, rat brain, *Xenopus* oocytes, and human cultured neuroblastoid cells. Neither endoplasmin (Koch et al., 1985) nor 6B9 antigen was detected in human RBCs. It is pointed out that endoplasmin was detected in chicken RBCs (Koch et al., 1985) which resemble the RBCs in fetal rats in that these cells are nucleated. (The RBCs of most mature mammals are enucleate cells.) It is recalled that 6B9 antigen was detected in RBCs of fetal rats. In another paper (Koch et al. 1986), data was provided on the expression of endoplasmin in secretory tissues and cells. High levels of endoplasmin were detected in pancreas and plasmacytoma cells. Consequently, it was undertaken to investigate the expression of 6B9 antigen in such cells. As expected, both pancreas (Fig. 7.4 B) and human plasmacytoma cells (HNY-2; not shown, similar to the human plasma cell shown in Fig. 7.4 A) were intensely stained by MAb 6B9. Consistent with the hypothesis that 6B9 antigen may be a reticuloplasmin, even possibly endoplasmin itself, is that staining owing to MAb 6B9 was also detected in cells of intestinal villi (Fig. 7.4 C) and the epithelial cells lining the rat vesicular gland (Fig. 7.4 D), which are expected to have well developed rER. Essentially no staining was detected in the smooth muscle cells comprizing the wall of the vesicular gland (Fig. 7.4 D). These are not secretory cells, and are not expected to have well developed rER. It is recalled that MAb 6B9 intensely stains

neurons, which have well developed rER (Peters et al., 1970). The localization of 6B9 antigen in neurons is restricted to the cell body and large-diameter dendrites, as axons and small-diameter dendrites do not stain. These small diameter processes are essentially devoid of rER. In sections of *Lumbricus,* MAb 6B9 intensely stains what are apparently mucus-secreting gland cells in the epidermis (Fig. 6.3 D). The staining in these cells is concentrated near the basal surface, reminiscent of the localization of rER in the goblet cells of intestinal villi which are specialized to perform the same function, namely the secretion of mucus (see p. 119 of Bloom and Fawcett, 1975).

The lipophilic, cationic dye $DiOC_6$ (Terasaki et al., 1984) is employed to stain membranous organelles, such as mitochondria and endoplasmic reticulum. Cultured rat brain cells stained with MAb 6B9 culture supernatant and goat anti-mouse IgM (mu-chain specific: Cappel, Belgium) conjugated to rhodamine were incubated for 30 seconds with a 1:200 dilution of $DiOC_6$ and mounted in buffered glycerol. In such preparations, the $DiOC_6$ stained the mitochondria bright green (Fig. 7.5 A and Fig. 7.6 B). They appear as little sausage-shaped organelles. In the background however, staining is evident in an extensive region of the cytoplasm, which likely corresponds to the endoplasmic reticulum (Fig. 7.5 A and Fig. 7.6 B). Staining for 6B9 antigen, in these preparations, is evident throughout

large regions of the cytoplasm (Fig. 7.5 B and Figs. 7.6 A and C). The appearance of the 6B9 staining is consistent with the possibility that 6B9 antigen is associated with endoplasmic reticulum, which is clustered around the nucleus in these cells. Cells which were freeze-thawed (twice at -20C), prior to staining for 6B9 antigen, exhibit punctate cytoplasmic staining, which possibly corresponds to vesiculated (freeze damaged) rER. Therefore, results from immunofluorescence microscopy, employing $DiOC_6$ as a stain for endoplasmic reticulum, have not ruled out the possibility that 6B9 antigen is associated with this organelle.

Futhermore, endoplasmin is a glycoprotein and the carbohydrate moiety is completely suseptable to digestion with endoglycosidase H (Koch et al., 1985; Koch et al., 1986). It is recalled that the 6B9 epitope is sensitive to periodic acid suggesting that it is a carbohydrate. However, when human plasmacytoma cells (HNY-2; pelleted, fixed with paraformaldehyde, and embedded for cryostat sectioning) were treated with endogycosidase H (from *Streptomyces griseus,* Sigma; 0.02 units in a 10 ul volume, 55 min, 37C) prior to staining for 6B9 antigen, immunofluorescence staining was enhanced as compared to untreated controls. Therefore, this treatment appears to unmask antigen. This result called into question whether enzyme digests are valid when carried out on fixed tissue.

Previous work on 6B9 antigen has indicated that fixation in para-

formaldhye enhances reactivity with MAb 6B9 (i.e. reduced or even no immunofluorescence staining in unfixed tissue). It is not known if this effect is the result of increased permeability of the cells following fixation, and hence greater accessibility of the 6B9 epitope. This seems unlikely as frozen / thawed cells should be permeable to antibodies. Alternatively, fixation may serve to preserve the conformation of a sensitive epitope or formaldehyde molecules may actually constitute part of the epitope itself.

It is recalled that 6B9 antigen was localized, at the electron microscope level, in the ground cytoplasm of sensory neurons, and was not associated with any identifiable organelle. This may appear inconsistent with the hypothesis that 6B9 antigen is a reticuloplasmin and therefore should localize to the lumen of the rER. However, ultrastructural preservation is relatively poor in paraformaldehyde fixed tissue. It is likely that the rER ruptures and vesiculates under such conditions. Although fixation with glutaraldehyde would give rise to better preservation of the ultrastructure, MAb 6B9 does not show reactivity with glutaraldehyde fixed tissue. Confirmation of the hypothesis that 6B9 antigen is a reticuloplasmin may have to await the development of a polyclonal antiserum to 6B9 antigen, exhibiting reactivity with glutaraldehyde fixed cells. HNY-2 cells appear to be a rich source of the antigen. Therefore, MAb 6B9 could be used to purify 6B9 antigen, by affinity chromatography, for immunization

leading to the production of a polyclonal antiserum.

Conclusions

An antigen (6B9 antigen) has been identified, which is widely expressed in embryos (rat and *Xenopus*) and exhibits differential expression by various cell-types during development. This study is centered on the nervous system, and it is reported that certain cells of neuroectodermal origin (neurons, ependymal cells, and adrenal chromaffin cells) express 6B9 antigen at maturity, while other cells (astrocytes and oligodendrocytes) do not. The localization of 6B9 antigen in neurons is restricted to the cell body and large-diameter dendrites, as axons and small-diameter dendrites do not stain.

6B9 antigen has been found in vertebrates (human, rat, rabbit and frog) as well as invertebrates (*Drosophila* and *Lumbricus*) tested thus far. This staining of various cell-types from diverse species may result from MAb 6B9's recognizing similar epitopes on otherwise different molecules. The 6B9-reactive epitope is insensitive to treatment with protease, ribonuclease, and hyaluronidase. However, the sensitivity of the epitope to periodic acid suggests that it may be a carbohydrate.

Results from immunoelectron microscopy have localized 6B9 antigen in the cytoplasm of sensory neurons, distinct from any

identifiable organelle. It is emphasized that no colloidal gold labeling for 6B9 antigen was associated with the Golgi Complex.

The detection of 6B9 antigen in human plasma cells suggested the hypothesis that this antigen may be associated with the rER. Consistent with this hypothesis is the detection of 6B9 antigen in numerous secretory cells (neurons, adrenal chromaffin cells, plasma and plasmacytoma cells, endocrine and exocrine pacreatic cells, cells of intestinal villi, epithelial cells lining the vesicular gland, and mucous secreting gland cells in the epidermis of *Lumbricus*), which have well developed rER. Although this hypothesis is not consistent with the electron microscope localization of 6B9 antigen in the ground cytoplasm, ultra-structural preservation in paraformaldehyde fixed tissue is relatively poor. It is likely that the rER ruptures and vesiculates under these conditions. This could easily explain the cytoplasmic localization of 6B9 antigen. Confirmation of the hypothesis that 6B9 antigen is associated with the rER may have to await the development of a polyclonal antiserum, exhibiting reactivity with glutaraldehye fixed tissue.

The staining owing to MAb 6B9 in the retina is similar to that reported for MAb A2B5 (Eisenbarth et al., 1979). However, MAb A2B5 reacts with GQ ganglioside in the plasma membrane of neurons (Eisenbarth et al., 1979) and astrocytes (Miller and Raff, 1984). The epitopes recognized by thse two MAbs appear distinct, as labeling

owing MAb 6B9 in neurons and ependymal cells is intracellular, and no staining of astrocytes has been detected. 6B9 antigen resembles 5E10 antigen (filamin-like protein) as both antigens are expressed in all neuroepithelial cells early in development. Although both antigens become restricted during development, 5E10 antigen differs from 6B9 antigen as it is expressed by Muller cells (Lemon, 1986).

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Fig. 7.1 A. Semithin section of rat DRG double-labeled with MAb 6B9 (FITC) and antibodies to NF proteins (TRITC). Neuronal somata exhibit labeling for 6B9 antigen and NF proteins. Nerve fibers, however, exhibit labeling for NF proteins only.

Fig. 7.1 B. Cryostat section of rat spinal cord (cross section) labeled with MAb 6B9. Large motor neurons in the ventral horn exhibit staining for 6B9 antigen.

Fig. 7.1 C. Cryostat section of 20-day-old fetal rat retina labeled with MAb 6B9; staining for 6B9 antigen is evident across the breadth of the retina. IP, inner plexiform layer; G, ganglion cell layer.

Fig. 7.1 D. Cryostat section of 12-day-old rat retina labeled with MAb 6B9. Staining for 6B9 antigen is restricted to the ganglion cell (G) and inner nuclear (IN) layers. IP, inner plexiform layer; arrow, outer plexiform layer; P, photoreceptors.

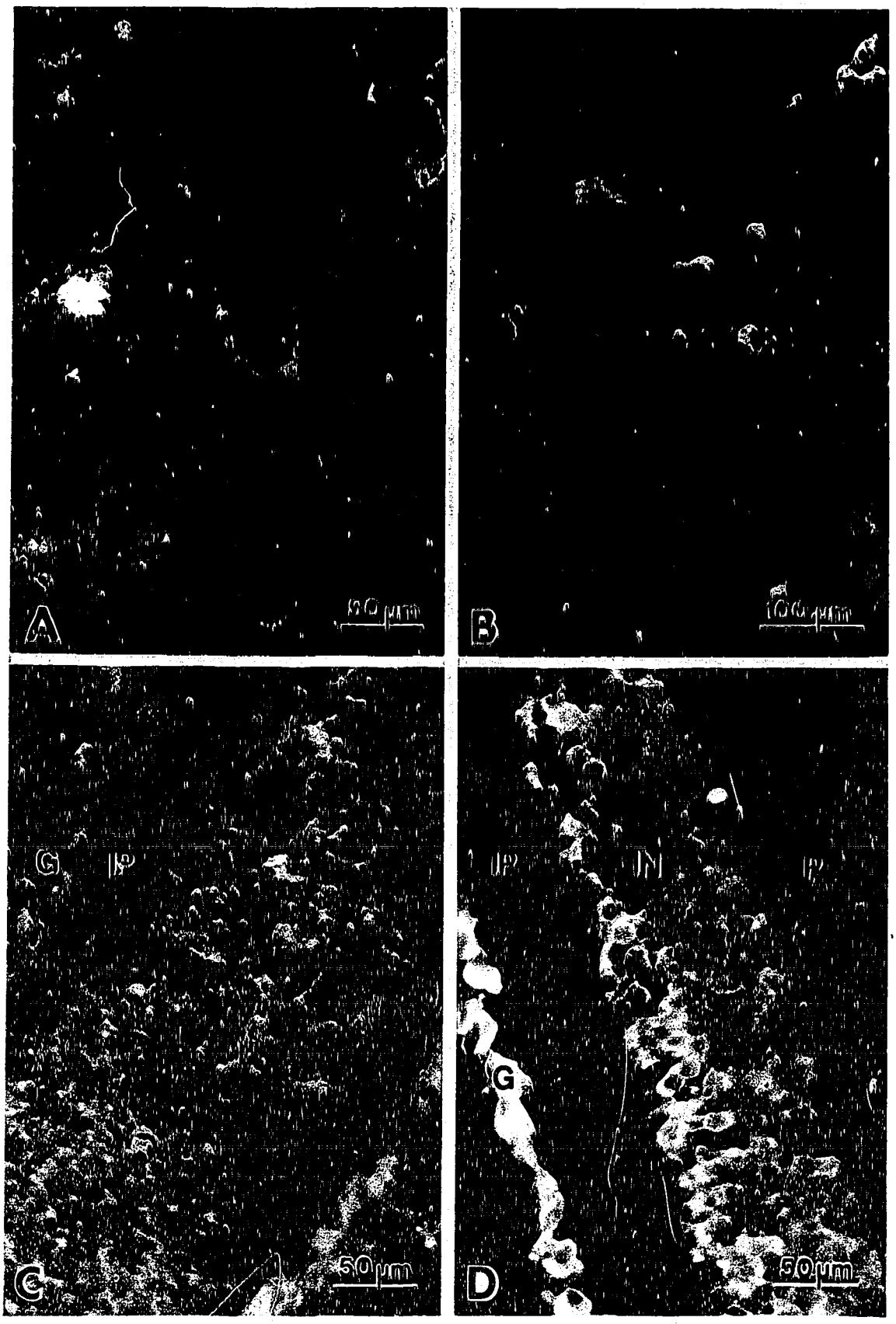
A
50 µm
B
100 µm
G
IP
IN
IP
C
50 µm
IP
IN
IP
G
D
50 µm

Fig. 7.2 A. Cryostat section of adult rat retina labeled with MAb 6B9 (FITC). The gangion cell (G) and inner nuclear (IN) layers exhibit staining for 6B9 antigen.

Fig. 7.2 B. Cryostat section of adult rat cerebellum labeled with MAb 6B9 (FITC). Purkinje neurons (P), located near the tip of a folium, exhibit intense staining for 6B9 antigen. Weak staining is evident in cells of the granular cell layer (g). m, molecular layer.

Fig. 7.2 C. Cryostat section of adult rat diencephalon labeled with MAb 6B9 (FITC). Staining for 6B9 antigen is evident in ciliated ependymal cells (arrow) as well as neuronal somata in the parenchyma. V, Ventricle III.

Fig. 7.2 D. Cryostat section of rat fetus labeled with MAb 6B9 (FITC). Nerve processes in the spinal nerve root are devoid of staining for 6B9 antigen (large arrow). However, intense staining is evident in chondrocytes of the developing vertebra (small arrow).

A
B
C
D