S.I.M.P. Research Monograph No. 6

IMMUNOLOGICAL STUDIES OF BRAIN CELLS AND FUNCTIONS

Edited by:
MATTEO ADINOLFI, M.D., Ph.D.

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Contents

Introduction

Matteo Adinolfi and Amico Bignami

In recent years the search for a better understanding of the development, structure and function of the nervous system has inspired a variety of approaches in which immunological tools have been employed. To extricate the anatomical and physiopathological complexity of the brain, two basic lines of investigation have been employed. On the one hand, conventional immune sera and monoclonal antibodies have provided the means to identify and characterize the properties of unique types of morphologically identical sets of brain cells (McKay *et al.* 1981, Adinolfi and Brown 1983). On the other hand, the rôle that auto-immune responses play in the pathogenesis of diseases of the nervous system has been investigated with more vigour and success than in the past (Paterson 1979, Leibowitz and Hughes 1983).

It is a measure of the progress of these studies that it is now possible to attribute the expression of specific antigenic markers to different subclasses of brain cells and that, following the pioneer work in which conventional immune sera were used, the introduction of monoclonal antibodies has unravelled the extraordinary molecular complexity of the nervous tissue. Some of these studies are summarized in Chapters 2 and 4 of this volume.

An important point to emerge from these investigations is that monoclonal antibodies can label distinct sub-populations of neurons in different brain regions. An example is a monoclonal antiserum which labels pyramidal cells in the area CA2 but not CA1 of the hippocampus, Purkinje cells but not granule cells of the cerebellum, and stellate cells but not pyramidal cells of the cerebral cortex (Wood *et al.* 1982). This suggests that a single epitope is expressed by one subclass of cells in one region of the nervous system, anatomically separated from another group of cells producing the same antigen. Monoclonal antibodies have also greatly expanded the repertoire of neuro-specific markers. Evidence for molecular heterogeneity among neurons, for example, has been obtained using monoclonal antibodies raised against the grey matter of cat spinal-cord, which can recognize at least five different subsets of neurons (McKay and Hockfield 1982). An interesting aspect of this research is that distinct subclasses of cells were detected in different areas of the brain: furthermore, two monoclonal antibodies could recognize at least two sets of neurons in the cerebellum.

Again using a panel of monoclonal antibodies, subsets of astrocytoma cell-lines have been identified. Two determinants showed mutually exclusive expression on these cells, and one of them was present on GFA-negative cells. Some of the epitopes were expressed preferentially on tumours of neuro-ectodermal origin, but all were expressed in at least one type of normal cell (Cairncross *et al.* 1982).

Monoclonal antibodies have proved to be very useful also in investigating the degree of cross-reactivity of brain antigens in different species. For instance, while

conventional antisera to myelin basic protein react with antigen determinants present in many mammalian species, monoclonal antibodies show a narrow species range and are specific for sub-groups (Franko *et al.* 1982). On the other hand, cross-reactivity between vertebrate species has been documented, using a MCAb raised against human CNS tissue as a primary immunogen (McKenzie *et al.* 1982). This antiserum was found to react against a glycoprotein present in brain tissue from rat, dog and mouse, but not frog or chicken. Several other examples of cross-reactive antigens have been documented, the most interesting being described by Miller and Benzer (1983). A panel of monoclonal antibodies, raised against Drosophila melanogaster tissue as primary immunogen, was tested for cross-reactivity with human CNS. About 69 hybridomas produced antibodies which showed immunofluorescent staining of cells in spinal cord, cerebellum, hippocampus or optic nerve. Many monoclonal antibodies stained comparable structures in all regions, while some were specific to one. This remarkable degree of cross-reactivity between fly and human brain antigens suggests the possibility of an evolutionary conservation of many more CNS molecules than hitherto known.

Two further practical uses of these antibodies can be envisaged: they can be employed to detect antigenic deficiencies in selected human neurological disorders which are similar to those observed in Drosophila mutants; or, in view of their cross-reactivity, they can be used to isolate the MRNA from polysomes, leading to the preparation of CDNA probes and thus the isolation of human genes controlling the synthesis of brain antigens.

Another interesting finding in the field of brain development has been obtained by using monoclonal antibodies. Raff *et al.* (1983) have identified a cell type in the optic nerves of seven-day-old rats which differentiates into fibrous astrocytes if cultured in the presence of fetal calf serum and into oligodendrocytes in the absence of serum. These studies, which provide a striking example of the developmental plasticity of brain cells and the effects of environmental influence in their differentiation, were performed using both conventional and monoclonal antibodies reacting against different cell-markers. Thus they have made it possible to follow the stages of differentiation of the progenitor cells into the different pathways of more mature cells. The close relationship between fibrous astrocytes and oligodendrocytes may explain the occurrence of mixed astrocyte-oligodendrocyte tumours in man, derived from a common progenitor cell which has undergone neoplastic transformation.

At first glance, Chapter 6 may seem to have little relevance to neuroimmunology. However, studies of lectin receptors have proved to be very useful in solving many problems related to cellular recognition, and in recent years a large body of data has been accumulated showing that carbohydrates play a fundamental rôle in the recognition and differentiation of brain cells.

Besides the numerous examples quoted in Chapter 6, it is worth mentioning a recent investigation which confirms the rôle that glycoproteins play in neuron-neuron interaction, and which further illustrates the use of monoclonal antibodies and lectins in unravelling the pathology of the nervous system.

Unique glycoproteins seem to be present on brain cells during fetal life, and judging from the reaction of various lectins to brain cells, some of these glycoproteins are slowly replaced by 'adult' forms later in life (Denis-Donini *et al.* 1978). A few years ago it was shown that wheat-germ lectin agglutinated normal embryonic cells from mouse cerebellum but not adult cells, yet this lectin continued to react with cerebellar cells from adult mutant *staggerer* mice (Hatten and Messer 1978). That postnatal cerebellar cells of *staggerer* mutant mice express immature components on the cell surface was confirmed with the use of conventional antibodies against microbial polysaccharides (Trenkner 1979). A recent investigation using monoclonal antibodies has further elucidated the defect in *staggerer* mice. A unique sialoglycoprotein, termed neural-cell adhesion molecule (N-CAM), is present in one form on embryonic (E) cerebellar cells of normal mice and in an adult (A) form in postnatal brain tissue. Monoclonal antibodies have now clearly shown that in *staggerer* mice the E–A conversion does not take place (Edelman and Chuong 1982). The most marked feature of this mutant is a reduced proliferation of Purkinje cells, and the remaining cells have reduced or absent tertiary dendritic spines; as a consequence, no parallel fibre synapses are made. From this work, the interesting hypothesis emerges that perhaps some inherited brain disorders in humans may also be the result of a failure in the maturation of specific brain-cells.

Another area of intense activity in neuro-immunology is that dealing with the properties of peptides common to the central nervous system and the gut. They seem to have a history extending back well over 500 million years of vertebrate evolution (Barrington 1982), and in this regard it is remarkable that five out of 11 immunoreactive peptides detected in the nerve ganglion of the protocordate *Ciona* should also be present in the gut (Pearce 1980).

The use of immunologically specific protein thus suggests that certain peptides came into use as neuronal messengers very early in evolution and that, partially modified and functionally diversified, they have ultimately produced the complex system seen in mammals.

In Chapter 5, Lotstra and colleagues describe in detail the distribution of cholecystokinin (CCK) in the central nervous system of several vertebrates, and analyze—from pharmacological, behavioural and electrophysiological studies—the rôle that CCK may play in physiological states, as well as in a variety of neurological and psychiatric disorders.

Two other chapters are dedicated to disorders of the nervous system in which auto-antibodies have been detected and are suspected to be responsible for the disease. As pointed out by Kurki and colleagues in Chapter 3, 'In spite of extensive research, the pathogenetic rôle of humoral auto-immunity in spontaneous human neurological disorders is still unclear'. But bold new methods for monitoring immunological molecular events are now available, and with the help of animal models it should be possible soon to elucidate the complex rôles that genetic, environmental and immunological factors play in the aetiology of several brain diseases (Paterson 1979, McFallin and Waksman 1982, Leibowitz and Hughes 1983).

The chapter on myasthenia gravis needs little comment. The demonstration of auto-antibodies in this disease is one of the major successes achieved in neurobiology during the past few years.

Considerable advances have been made in the last decade as to the biochemistry and immunology of glial filaments and neurofilaments. Some of this work is reviewed in Chapter 1.

Staining of glial and nerve fibres traditionally is obtained with histological methods developed at the turn of the century, *e.g.* Weigert stain for astroglia and Cajal's neurofibrillary method. Since these methods are based on the selective decoration of neurofibrils and gliofibrils, that is bundles of filaments at the electron-microscopic level, it was reasonable to assume that the specificity of the stain was based on the specificity of the proteins forming the filaments, as originally proposed by Weigert.

Work conducted in several laboratories has shown the validity of this assumption, not only for brain filaments but also with respect to filaments in non-neural tissues, *i.e.* epithelium, muscle and mesenchyma. Compared with conventional histology, the immunohistochemical methods proved more specific and sensitive, especially in tissue culture, development and tumours.

The last chapter in this book reviews the many attempts to use brain antibodies to affect brain functions in experimental animals. This work started with the demonstration by Levi-Montalcini and her collaborators in 1966 that an 'immunological sympathectomy' could be induced by injecting newborn animals with antibodies against the nerve-growth factor. The permeability of the blood-CSF barrier to antibodies during fetal and perinatal life makes it possible to explore the effect that specific brain antibodies may have during the maturation of the central nervous system, and here, once again, monoclonal antibodies should prove to be useful.

When first asked to edit a volume on the 'immunology of the brain', we hesitated to accept the challenge, aware that the vast literature and the rapid progress of research in this field would have made it impossible to plan a fully comprehensive book. We finally agreed to undertake the task on the understanding that the present volume would contain reviews on topics selected to represent 'models' of brain research involving immunology. We hope that our criteria for selection will further engage the reader to maintain a close interest in this absorbing and rapidly growing area of study.

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1
Intermediate Filament Proteins as Immunocytochemical Markers for Neurons and Glia

Amico Bignami and Doris Dahl

Introduction

The cytoskeleton in eukaryotic cells comprises three types of filamentous structures: microtubules, approximately 25nm in diameter; actin microfilaments, 5 to 7nm in diameter; and 10nm filaments, also referred to as intermediate filaments (IFs) because their width lies between that of microtubules and microfilaments.

IFs are major cytoskeletal components in the nervous system where they form bundles called neurofibrils and glial fibres at the light microscopic level. Glial cells accumulate filaments as a basic reaction to injury (fibrous gliosis), and the same is true for neurons under certain pathological conditions including Alzheimer's senile dementia (neurofibrillary tangles).

Two major neurohistological stains developed at the turn of the century, *i.e.* Weigert's stain for astroglia and Cajal's silver nitrate neurofibrillary method, are based on the selective decoration of glial filaments and neurofilaments respectively; and it was first suggested by Weigert (1895) that the selectivity of the stain was due to the specificity of the 'substance' forming the fibrils.

It is the purpose of this chapter to review work conducted on the intermediate filament during the last decade and leading to the development of immunocytochemical markers for the identification of the cell type in brain and other tissues.

Intermediate filament (IF) proteins

Actins and tubulins, the sub-units of microtubules and microfilaments respectively, are evolutionally conserved proteins and similar if not identical in different cell types. Conversely, IFs in different tissues, although morphologically similar and structurally related (Steinert *et al.* 1980, Rueger *et al.* 1981, Geisler *et al.* 1982), appear biochemically distinct based on immunochemical criteria and polypeptide composition (Lazarides 1980). IF proteins may thus be considered as taxonomic characters allowing the identification of the cell type, especially when the identification is difficult by morphological criteria, *e.g.* in development, *in vitro* and in tumours.

The following IF proteins are currently used as immunocytochemical markers for the identification of the cell type: the neurofilament 'triplet' proteins (neurons); the glial fibrillary acidic (GFA) protein (astroglia); vimentin (mesenchyma); desmin (muscle); and keratins (epithelium). Since keratins have not been identified in the brain, even in early stages of development, they will not be included in this review.

Neurofilament (NF) proteins

Considering the number of laboratories involved in the production of antibodies to NF proteins, it should be emphasized that there are marked regional differences in the distribution of NFs, a fact that is well known to histologists working with silver neurofibrillary methods. A good understanding of these regional differences is essential for the immunohistological screening of the antisera. NFs are numerous in all myelinated axons, certain nonmyelinated axons and axonal terminals (*e.g.* basket axons and their terminals surrounding Purkinje cells of the cerebellar cortex), certain dendrites, and neuronal perikarya (*e.g.* motor neurons in spinal cord and large sensory neurons in posterior root ganglia). Most nerve cells in cerebral grey matter remain unstained both with silver neurofibrillary methods and with NF antisera. Large pyramidal neurons are an important exception. The cerebellar cortex is probably the most convenient tissue for assessing the specificity of the antisera (Dahl and Bignami 1977, Anderton *et al.* 1980) since cells containing different classes of filamentous structures exist in close proximity: Bergmann glia (glial IFs); Purkinje cell baskets and basket axons in the *inner* regions of molecular layer (NFs); and Purkinje cells and dendrites (microtubules).

A triplet of polypeptides at approximately 70K, 150K and 200K daltons constitutes the mammalian NF (Hoffman and Lasek 1975, Schlaepfer and Freeman 1978). According to several laboratories (Dahl 1979, Thorpe *et al.* 1979, Chiu *et al.* 1980, Davison and Jones 1980, Brown *et al.* 1981), peptide mapping shows no major similarity in the primary sequence of the proteins forming the NF triplet. In addition the three NF polypeptides are synthesized in cell-free systems (Czosnek *et al.* 1980, Strocchi *et al.* 1982), a finding suggesting that they are not fragments of a large precursor protein. These observations indicate that NFs are not formed by a single protein (*e.g.* GFA, desmin or vimentin), or by closely related polypeptides (*e.g.* keratins) and that they are different from the other IFs in this respect. Another possibility to be considered on the basis of the pattern of antibody decoration of neurofilaments at the electron microscopic level (Willard and Simon 1981, Sharp *et al.* 1982) is that only one polypeptide constitutes the neurofilament and that the other two polypeptides are NF-associated proteins. Assembly experiments provided support for this hypothesis since only NF 70K formed filaments *in vitro*, while NF 150K and NF 200K appeared unable to form filaments independently (Geisler and Weber 1981, Liem and Hutchison 1982, Zackroff *et al.* 1982). However, it was recently reported that NF 150K is also capable of self-assembly into 10nm filaments indistinguishable in width and appearance from those formed by NF 70K and other IF proteins (Gardner *et al.* 1984). Moreover, all three NF polypeptides share the same general structural model of IF proteins: *i.e.* a basic aminoterminal headpiece, a 40K alpha-helical rod resistant to limited proteolysis, and a carboxyl-terminal tailpiece (Geisler *et al.* 1983). According to this model, the mass of the tailpiece accounts for the molecular weight differences among the NF triplet proteins.

Conflicting results have been reported in the literature concerning the immunological relatedness of the polypeptides forming the NF triplet. Antisera raised in several laboratories to gel-purified NF proteins reacted with other components of the triplet (Liem *et al.* 1978, Dahl 1980, Autilio-Gambetti *et al.*

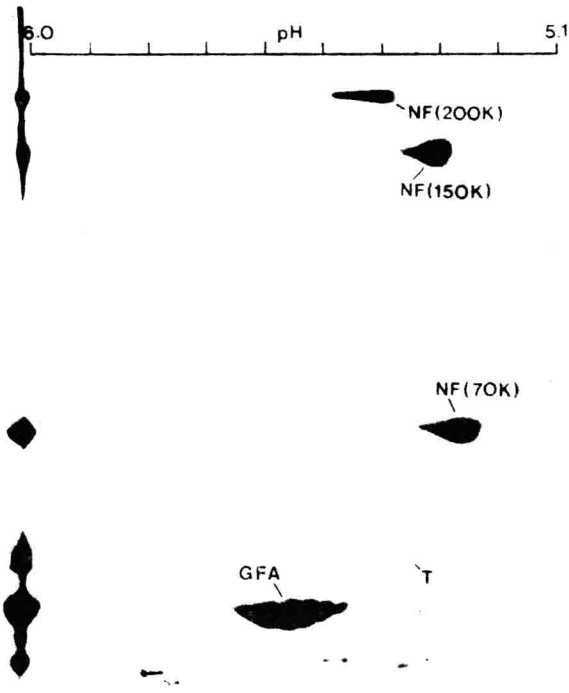

Fig. 1. Two-dimensional gel electrophoresis of the 2M urea spinal cord extract analyzed by anion exchange chromatography. Identified proteins: NF (200K), 200,000 MW neurofilament polypeptide; NF (150K), 150,000 MW neurofilament polypeptide, NF (70K), 70,000 MW neurofilament polypeptide; T, tubulin; GFA protein.

'81, Schlaepfer *et al.* 1981, Shaw *et al.* 1981, Willard and Simon 1981), suggesting that NF proteins may share short amino acid sequences not detectable by peptide mapping.

We recently re-examined the issue using antisera to NF polypeptides isolated by anion exchange chromatography (Dahl 1983), *i.e.* by a procedure allowing the separation of the neurofilament triplet proteins on the basis of charge rather than molecular weight differences (Fig. 1). The neurofilament antisera so obtained appeared specific by double immunodiffusion and by immuno-absorption, *i.e.* they only formed precipitin lines with their own antigen and they were only absorbed by their own antigen at low protein concentration (50 to 100μg protein/ml antiserum). Absorption with the other components of the NF triplet occurred at higher protein concentrations (250μg/ml), but this could be interpreted as nonspecific precipitation due to the tendency of IF proteins to aggregate in solution. However, different results were obtained with the same antisera by the immune blotting procedure. Anti-150K and anti-200K reacted with both 150K and 200K, while anti-70K reacted with all three NF polypeptides. Adsorption of the antisera by affinity chromatography with heterologous NF antigens confirmed the findings from other laboratories as to the presence of unique antigenic determinants (Schlaepfer *et al.* 1981, Shaw and Weber 1981, Willard and Simon 1981).

Immunohistological studies conducted with antisera raised to DEAE-cellulose purified antigens confirmed previous findings in this laboratory as to the different distribution of neurofilament polypeptides in neuronal perikarya and dendrites compared to axons (Dahl *et al.* 1981*a*). All three antisera (anti-70K, anti-150K and anti-200K) stained axons including Purkinje cell baskets in the cerebellar cortex with identical pattern, in agreement with original observations on the polypeptide composition of the mammalian neurofilament as determined by axonal transport studies (Hoffman and Lasek 1975) and by the biochemical analysis of isolated axonal filament (Schlaepfer and Freeman 1978). Conversely, large motor neurons in the spinal cord, posterior root ganglia neurons, and pyramidal neurons in the cerebral cortex, only reacted with anti-70K and anti-200K and remained unstained with anti-150K.

It was previously suggested that the apparent lack of reactivity of perikarya and dendrites with anti-150K could indicate that NF differ in polypeptide composition depending on their location (Dahl *et al.* 1981*a*). However, this hypothesis appears unlikely since in other laboratories monoclonal and polyclonal antibodies to the 150K protein decorated dendritic processes and neuronal perikarya as well as axons (Schlaepfer *et al.* 1981, Shaw *et al.* 1981, Debus *et al.* 1983). Moreover, monoclonal and polyclonal antibodies to 200K reported in one of these laboratories (Shaw *et al.* 1981, Debus *et al.* 1982) failed to stain lower motor neurons and pyramidal neurons. We recently obtained similar results with 150K and 200K monoclonals, *i.e.* neuronal perikarya and dendrites only stained with anti-150K (Dahl *et al.* 1984*a*, Bignami *et al.* 1985). Although it is difficult to account for these contradictory observations, it is possible that 200K and 150K are modified during axonal transport. It would thus be conceivable that some 200K and 150K antibodies mainly recognize axon-specific antigenic sites.

Alzheimer's neurofibrillary tangles

The term describes a characteristic histological change occurring in the brain of many patients with late-onset dementia. The alteration, most frequently observed in the pyramidal neurons of frontal cortex and hippocampus and best demonstrated by silver staining, consists in the accumulation of twisted masses of fibrillary material within the perikaryon and apical dendrite. By electron microscopy, neurofibrillary tangles have a distinctive appearance, different from the neurofilament. They are formed by bundles of filaments, 20nm in diameter, and characterized by constrictions about 10nm in diameter at 80nm intervals (Kidd 1963, Terry 1963). These have been interpreted as constricted microtubules, for which the term 'twisted tubules' was proposed (Terry 1963) or alternatively as paired coiled filaments making a full turn every 160nm (Kidd 1963). The latter interpretation is now more generally accepted (Wisniewski *et al.* 1976) although it was reported that the tangles were selectively decorated with antisera raised against a microtubule preparation isolated from normal human brain by two cycles of the assembly disassembly procedure (Grundke-Iqbal *et al.* 1979).

We have examined four cases of Alzheimer's dementia with neurofilament antisera (Dahl *et al.* 1982*c*). The cerebellum was examined in each case and in

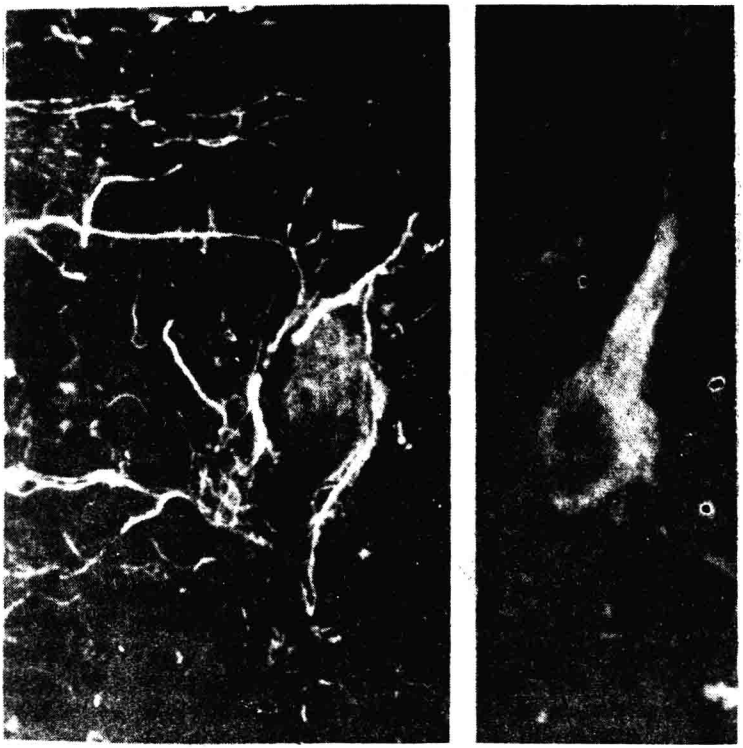

Fig. 2. Staining of Purkinje cell basket in human cerebellar cortex by immunofluorescence with neurofilament antisera. In this figure the antisera were raised to the 200K neurofilament polypeptide, but same results were obtained with antisera to the other components of the 'triplet' (70K and 150K). Note diffuse artefactual staining of Purkinje cell nucleus, perikaryon and dendrite (not observed in murine cerebellum). × 640. *Right:* immunofluorescence staining with antisera to 70K neurofilament polypeptide of neurofibrillary tangle in hippocampus of patient with Alzheimer's dementia. × 640.

non-neurological patients to determine the feasibility of the study in autopsy material. We found that compared to glial (GFA) IFS, NFS are remarkably resistant to postmortem autolysis (Fig. 2). Purkinje cell baskets were selectively decorated in all cases and with all antisera regardless of the time interval between death and autopsy (up to 36 hours postmortem). Conversely, neurofibrillary tangles were only stained by antisera decorating neuronal perikarya and dendrites (Fig. 2b). So our findings, and those reported in other laboratories (Ihara *et al.* 1981, Anderton *et al.* 1982, Gambetti *et al.* 1983), appeared to be compatible with Alzheimer's original hypothesis, that neurofibrillary tangles derive from neurofibrils normally present in neurons (neurofibrils correspond to neurofilaments at the electron microscopic level) (Guillery 1970). As to the nature of the change, it was recently proposed that the filaments in the tangle are cross-linked by nondisulphide covalent bonds into a rigid intracellular polymer (Selkoe *et al.* 1982).

The antibody binding studies did not exclude the existence of a separate

TABLE I
Isolation of GFA protein

Source	*Procedure*	*Purpose*	*Reference*
Human spinal cord	Hydroxyapatite chromatography	Antibody production	Dahl and Bignami 1976
Small laboratory animals	Immuno-affinity chromatography	Isolation of radiolabelled protein	Rueger *et al.* 1978
Slaughterhouse material	Anion exchange chromatography	Protein characterization	Dahl *et al.* 1982*b*

population of tangles unreactive with NF antisera. In fact recent observations appear to support this hypothesis. In a study of isolated perikarya from Alzheimer's cortex it was found that only 30 to 50 per cent of the morphologically identified tangles reacted with anti-NF (Elovaara *et al.* 1983). In this laboratory we have reached the same conclusion by double-labelling Alzheimer's cortex with anti-NF and Congo red (Bignami *et al.* 1984). Tangles showing amyloid-like birefringency through crossed Nicol prisms were NF-negative when viewed through fluorescein optics in the same microscope. Conversely, NF-positive tangles were Congo red-negative.

Glial fibrillary acidic (GFA) protein

Table I summarizes the procedures presently used in this laboratory for the isolation of GFA protein. As indicated in Table I, the main factor in selecting the procedure is the purpose of the experimentation, *i.e.* protein characterization, isolation of radiolabelled products for studies of protein synthesis, or antibody production.

As we shall see, astrocytes contain vimentin and desmin in addition to GFA protein. Antisera should thus be raised in two species (*e.g.* rabbits and mice) to allow double-labelling experiments, *i.e.* to compare the distribution of two antigens in the same cells by indirect immunofluorescence. In this method (Raff *et al.* 1978), the first antibody reacting with the cell (*e.g.* anti-GFA produced in rabbit) is visualized with fluorescein-coupled antirabbit IgG, and the second antibody (*e.g.* antidesmin produced in mice) with rhodamine-coupled antimouse IgG, or vice versa.

Selective decoration of Bergmann radial glia in cryostat sections of murine cerebellum is used in our laboratory as the main assay to screen the antisera for specificity and titre (*i.e.* higher dilutions compatible with bright staining of Bergmann glia). We commonly observe nonspecific staining of fibrous astrocytes in white matter. Strong GFA antisera (immunofluorescence titre 1:160-320) selectively decorate Bergmann glia even at low dilutions (1:5-1:10). With weaker antisera background fluorescence often occurs under these conditions.

Immunohistological localization in CNS

The distribution of astroglia demonstrated with GFA protein antisera in the rat