Antibodies to Neurofilament, Glial Filament, and Fibroblast Intermediate Filament Proteins Bind to Different Cell Types of the Nervous System

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ABSTRACT Antisera were raised to the 210,000-dalton and the 49,000-dalton proteins of a fraction enriched in intermediate (10 nm) filaments from human brain. Proteins of the filament preparation were separated by SDS-polyacrylamide gel electrophoresis and used for immunization and subsequent analysis of the reactions of the sera by rocket immunoelectrophoresis. Anti-210,000-dalton serum precipitated proteins of molecular weights 210,000, 160,000, and 68,000, and, thus, reacted with all the neurofilament triplet components. Anti-49,000-dalton serum did not react with the triplet proteins but precipitated the 49,000-dalton protein.

By immunofluorescence on tissue sections, anti-210,000-dalton serum bound to neuronal axons in sciatic nerve and cerebellum. In dissociated cell cultures, rat dorsal root ganglion cells and their processes bound the serum, whereas nonneuronal cells did not. Some cultured cerebellar neurons were also positive, whereas astrocytes were not. At the ultrastructural level, anti-210,000-dalton serum bound to intermediate filaments inside axonal processes.

Anti-49,000-dalton serum bound to astrocytes in sections of the cerebellum, and cultured astrocytes had filaments that stained, whereas other cell types did not. In sciatic nerve sections, elements stained with this serum, but cultured cells from newborn sciatic nerve were negative.

An antiserum against the 58,000-dalton protein of the cytoskeleton of NIL-8 fibroblasts strongly stained sciatic nerve sections, binding to Schwann cells but not to axons or to myelin. In cerebellar sections, astrocytes were positive, as were blood vessels and cells in the pia. In cell cultures, anti-58,000-dalton serum stained filaments inside Schwann cells, fibroblasts, and astrocytes, but neurons were negative. Cells in the cultures and tissue sections of the nervous system failed to react with antiserum to the 58,000-dalton protein of skin intermediate filaments. In these studies, astrocytes in vivo and in culture were the only cells which had antigens related to two classes of intermediate filaments.

Using radioactive labeling techniques in vivo, Hoffman and Lasek (30) observed a triplet of proteins as components of slow axonal transport, which they suggested could be the subunits of neurofilaments. These proteins have molecular weights of ~210,000, 160,000, and 68,000 on SDS-polyacrylamide gels. Similar proteins were found in filament preparations from peripheral nerves, which had 10-nm diameter filaments identified by electron microscopy (35, 47, 48). An antiserum against this peripheral nervous system filament preparation had strong neuronal specificity at the light microscope level in the central and peripheral nervous systems (CNS and PNS) (50) and bound to the 10-nm filaments (47). Although small amounts of triplet proteins were present in filament preparations isolated by an axon-flotation method from CNS white matter, the major component was a 51,000 polypeptide (19, 21, 52, 54, 65), and it was then thought to be the subunit of CNS neurofilaments. However, this protein was immunologically related to glial fibrillary acidic protein (GFAP), a soluble protein that was isolated from gliosed plaques of multiple sclerosis patients or from normal brain (13, 22). Anti-GFAP sera stained fibrous astrocytes in tissue sections (5, 36, 45) and filaments in cultured glia (1), and rabbit antisera to the bovine 51,000-dalton protein

were similar (35). Isolated human glial filaments were composed of a 50,000-dalton polypeptide similar biochemically and immunologically to the bovine 51,000-dalton brain filament component (27), which, it now seems clear, came primarily from glial filaments of the astrocytes in white matter (40).

When the axon-flotation method was modified, filament preparations from CNS white matter were relatively enriched in polypeptides similar to the triplet proteins in peripheral nerve, although there was still a major band at ~50,000 daltons (11, 35, 62). Antiserum to the bovine CNS 160,000-dalton protein band stained neuronal axons and resembled antiserum to PNS neurofilaments (14, 35, 50). However, one anti-160,000-dalton serum gave cross-reactions detected by radioimmunoassay with 210,000 dalton, 68,000 dalton, and some of the protein(s) at 51,000 daltons (35) and another could be adsorbed by 200,000-dalton or 160,000-dalton bands but not by 68,000-dalton protein (14). Other sera with neuronal or mixed specificity have been raised to filament preparations without separation of component proteins (39, 46, 47), to extracts with several bands (3, 16) or to 54,000-dalton protein likely to include proteolytic fragments (46).

Antisera to the largest 210,000-dalton triplet protein was sought primarily to test whether this antigen was associated only with neuronal filaments. We have tested for cross-reactions with smaller triplet proteins and with the glial-filament protein by rocket immunoelectrophoresis using SDS-polyacrylamide gel-purified protein bands from filament preparations from human autopsy brain.

Previous antisera that react with glial filaments have been raised to mixtures of 51,000-dalton protein from glia and degraded triplet protein from neurons (35, 46) or to GFAP preparations (5, 15, 22, 46). We have raised an antiserum to the 49,000-dalton protein(s) from the human filament preparation and compare it here to anti-GFAP sera and to the anti-210,000-dalton serum.

For these studies, we have used tissue sections of cerebellum and peripheral nerve, and cultures of cerebellum, sciatic nerve, and dorsal root ganglion. Cultures allow good documentation of fibrous staining patterns, and it could be tested whether the cell type specificity shown in cultures was in agreement with tissue sections. Cell type identification can be a problem in cultures, whereas in tissue sections the cytoarchitecture is preserved, and the identification and location of classes of cells has been documented at the ultrastructural level. Use of tissue sections has also allowed direct comparison with previous studies of brain filament proteins.

Cell types other than neurons and astrocytes are known to contain 10-nm filaments. Filaments from muscle, epithelial tissue, and the cytoskeleton of fibroblasts have been purified and characterized (3, 9, 12, 24, 25, 31, 33, 55–57, 59, 66). Their subunit proteins have molecular weights in the range of 50,000–60,000. Antisera to these different proteins have shown substantial cell-type specificity, although the fibroblast 58,000-dalton protein antigen is found in chondroblasts, muscle, pigment cells (3), and even in cultured epithelial cells (25; for review, see reference 34). Using the antiserum of Hynes and Destree (31) to the 58,000-dalton fibroblast protein, we report here the distribution and cell-type specificity of this antigen in the nervous system.

MATERIALS AND METHODS

Filament Preparation and Characterization

Normal human brains were obtained 10 h postmortem and usually stored at −70°C for 2 wk. Brain filaments were isolated according to the procedure of Liem et al. (35). A portion of the final filament pellet was fixed for electron microscope examination with 2.5% glutaraldehyde in Sorensen's phosphate buffer, pH 7.4, and processed for electron microscopy. The rest of the pellets were dissolved in a solution containing 1% SDS, 0.12 M mercaptoethanol, 0.1% EDTA and 0.35 M Tris-HCl, pH 8.3. After electrophoresis on slab gels (35) and brief staining, the polypeptides with molecular weights of 210,000, 160,000, 68,000, and 49,000 were excised. Each band was emulsified with Freund's complete adjuvant and injected into rabbits. Each animal received 300 μg of protein at multiple subcutaneous sites on day 1. Further injections of 300 μg in Freund's incomplete adjuvant were given at days 28 and 35. Animals were bled 1 wk later.

Excised bands were reelectrophoresed without elution from the first gel. The gel strip was equilibrated with sample buffer and laid on top of the stacking gel, and electrophoresis carried out as usual.

Precipitin reactions between sera and antigens were detected by use of a modified rocket electrophoresis method (37). Stained bands were excised from slab gels, embedded in agarose on small glass plates, and electrophoresed through a strip of agarose containing 0.5% Berol (or Triton X-100) into upper gels containing 10% immune serum, 1% agarose, 0.1% Berol, and Tris-glycine-Veronal buffer, pH 8.7 (37). Precipitin bands were observed after staining with Coomassie Blue.

Other Antisera

Antiserum against 58,000-dalton protein of Triton-treated NIL 8 fibroblasts was kindly provided by Drs. R. Hynes and A. Destree, who have described the staining of intermediate filaments by this serum (31). Guinea pig serum directed against a 58,000-dalton epidermal filament protein has been previously described (66). Rabbit antiserum specific for actin was made by Dr. F. Frankel and has been described previously (63). Rabbit antitubulin was a gift of Dr. K. Fujiwara (26). Rabbit anti-rat type I collagen was a gift of Dr. S. Akazawa. Rabbit antibasement membrane from liver was a gift of Dr. S. Heinemann. Rabbit antigalactocerebroside was a gift of Dr. C. Raine, and rabbit antifibronectin was a gift of Dr. R. Adler. Mouse alloantiserum against Thy-1.1 surface antigen was a gift of Dr. P. Lake and was used as previously described (42). Rabbit anti-GFAP sera prepared against antigen from human CNS tissue (15) were provided by Drs. D. Dahl and A. Bignami and by Dr. R. Pruss. Rabbit anti-GFAP made against SDS gel-purified protein from multiple sclerosis plaques was a gift of Dr. L. Eng. Rabbit anti-51,000-dalton protein of bovine brain filaments has been described previously (65).

Antisera to the filament proteins were adsorbed with human brain-filament pellets. Filaments were resuspended in phosphate-buffered saline (PBS) and washed twice by centrifugation at 128,000 *g*. 1 vol of pelleted filaments was resuspended in 10 vol of undiluted antiserum, incubated at room temperature for 1 h, and centrifuged, and the adsorption was repeated with the supernate.

Dissociated Cell Cultures

Cerebella from newborn Wistar rats were dissociated using trypsin, trypsin inhibitor, and DNase as previously described (42). 3×10^5 cells were added to 14-mm diameter wells containing poly-L-lysine coated glass cover slips. The cells were grown in MEM medium containing 10% fetal calf serum, 0.6% glucose, and 25 mM KCl. In addition, 8×10^{-5} flurodeoxyuridine (FUdR) was added starting at the 2nd or 3rd d of culture. The cultures were generally used for indirect immunofluorescence after 5–10 d in culture; occasionally, older cultures (14–22 d in culture) were examined.

Primary cultures containing 80–90% astrocytes (10) were prepared by the same procedure from cerebral hemispheres of newborn rats, plated at 4×10^6 cells/60-mm dish, and grown without added FUdR for 3 wk to confluence. Confluent cultures of Rat-1 fibroblasts, a cell line obtained from Dr. P. Canary, were grown in DMEM plus 10% fetal calf serum. Equal volumes of Triton-insoluble cytoskeleton material (31) of each culture were used to adsorb 10 vol of anti-49,000-dalton serum, as described above for brain filaments.

Secondary cultures of cerebellar cells were prepared from cells grown for 25 d without FUdR, trypsinized, plated on fresh cover slips, and grown for 3 d in the absence, 32 d in the presence, of FUdR. Colchicine (Sigma Chemical Co., St. Louis, Mo.) was added (10 μg/ml) 18 h before staining.

Dorsal root ganglia were dissociated with trypsin and collagenase as previously described (23), and 2×10^4 cells were added to each glass cover slip. The cells, a mixture of Schwann cells, fibroblasts, and sensory neurons, were grown in DMEM plus 10% fetal calf serum and used for staining on the 2nd or 3rd d in culture.

Sciatic nerves of newborn to 3-d-old rats were dissociated with trypsin and collagenase as described (7). The cells were plated at $3–10 \times 10^4$ per plain glass cover slip. Most Schwann cells are readily identifiable by their elongated shape and narrow nucleus compared with accompanying fibroblasts, and the two cell types have different surface antigens (7). The primary cultures were used within 3–10 d.

Cryostat sections were cut of frozen, unfixed adult rat cerebellum or sciatic nerve at 10 μm, placed on egg-albumin-coated slides, air-dried, and stored at −20°C.

Immunofluorescent Staining

Cells on cover slips were rinsed briefly in PBS with added 0.9 mM $CaCl_2$ and 0.5 mM $MgSO_4$ and fixed with 100% methanol for 7 min at −20°C. Fixation of cultured cells or tissue sections with 3.7% formalin followed by methanol gave staining patterns similar to those obtained with methanol alone. Anti-49,000, anti-58,000, and anti-210,000-dalton sera were used at dilutions of 1:50, 1:75, and 1:100, respectively (unless otherwise noted on the figures). Immune, preimmune, and normal rabbit sera and serum absorbed with brain filaments were compared at the same dilutions.

Cells or tissue sections were exposed to rabbit sera for 30–60 min at room temperature and washed well, and bound immunoglobulin was detected with goat anti-rabbit immunoglobulin (GARIG)-fluorescein (Antibodies, Inc., Davis, Calif.; diluted 1:60) or pepsin-digested GARIG-rhodamine (Nordic, San Clemente, Calif.; diluted 1:150).

Stained cover slips or sections were mounted in 50% glycerol in PBS, or a polyvinyl alcohol mounting medium (43), sealed with nail varnish, and examined with a Zeiss standard microscope equipped with epifluorescent UV illumination, selective filters for fluorescein or rhodamine, and alternate phase-contrast illumination.

For electron microscope immunolocalization, rats were perfused with 4% *p*-formaldehyde in PBS, pH 7.4, and the cerebrum was removed and cut into 50-μm slices with a tissue chopper. After washing, slices were incubated with anti-210,000-dalton serum or preimmune serum at 1:200 dilution in PBS plus 10% normal goat serum. Subsequent incubations were performed with GARIG (1:20) and then rabbit peroxidase-antiperoxidase complex (PAP) (1:50; Cappel Laboratories, Inc., Cochranville, Pa.) (58). After reaction with diaminobenzidine and osmium tetroxide, the tissue was embedded in Epon. Thin sections were examined in a Siemens 101 microscope.

RESULTS

Characterization of the Filament Preparation

Filament preparations from human autopsy brain, when examined in the electron microscope, mainly consist of loose bundles of 10-nm filaments (Fig. 1), which are comparable to those seen in bovine filament preparations (54). There are dense structures among the loose bundles, in some of which a filamentous profile is detected. These may be an altered form of the tight bundles of filaments present in bovine preparations. Small amounts of myelin, membrane vesicles, and nuclei are seen as contaminants. When the preparations are analyzed on SDS-polyacrylamide gels, there are many obvious bands, including proteins with molecular weights of 210,000, 160,000, 68,000, 49,000, and 17,000 (Fig. 2*a*). Compared with calf brain filaments prepared by the same procedure (35), there are more strong protein bands, especially at 105,000 daltons, at 55–60,000 daltons, and several between 49,000 and 17,000 daltons. Preparations from human brains that were not frozen have a less complex gel pattern; the bands at 210,000, 160,000, 68,000, 49,000, and 17,000 daltons remain strong, whereas most of the other bands are weaker (Fig. 2*c*).

Reelectrophoresis of the 210,000, 160,000, 68,000, and 49,000-dalton bands shows that most of each protein remains as a single band; a small amount of protein derived from the excised 210,000-dalton band is detected as a band near 160,000 daltons (Fig. 2*b*).

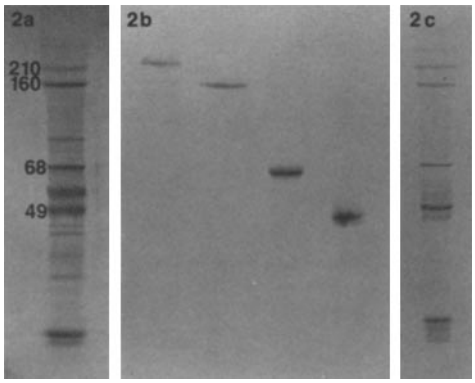

FIGURE 2 Gel electrophoresis of human filament proteins. *a*, Filament preparation from frozen brain; *b*, reelectrophoresis of excised bands with molecular weights of 210,000, 160,000, 68,000, and 49,000; *c*, filament preparation from fresh brain. Molecular weights were assigned using ferritin (220,000), β-galactosidase (130,000), phosphorylase *b* (94,000), bovine serum albumin (68,000), catalase (60,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400) as standards.

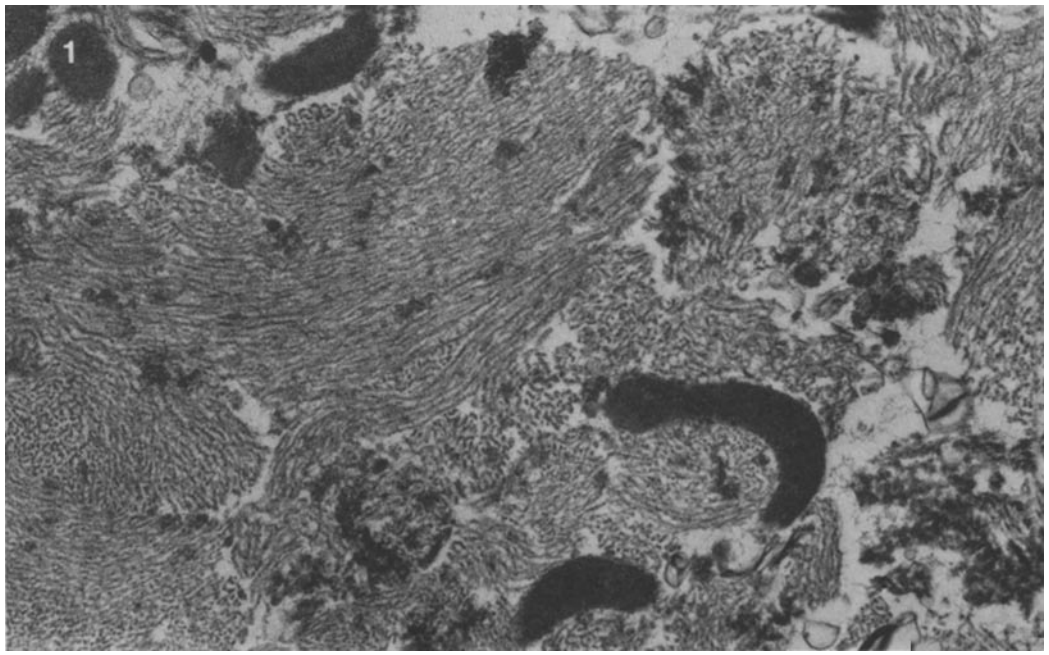

FIGURE 1 Electron micrograph of isolated human brain filaments. The filament pellet was fixed with 2.5% glutaraldehyde and postfixed with 2% OsO_4. Thin sections were stained with uranylacetate and lead citrate. × 18,000.

Immunization of rabbits with gel slices containing 210,000-dalton protein or 49,000-dalton protein gives strong antisera which can be characterized by rocket immunoelectrophoresis. The anti-49,000-dalton serum gives a precipitin line with 49,000-dalton antigen from SDS gels, but not with 210,000, 160,000, or 68,000-dalton proteins (Fig. 3*a*). Anti-210,000-dalton serum forms precipitin lines with 210,000, 160,000, and 68,000-dalton proteins, but not with 49,000-dalton protein (Fig. 3*b*). Neither anti-49,000-dalton nor anti-210,000-dalton serum gives reactions with the protein bands at 60–55,000 daltons or 17,000 daltons. The proteins at 60–55,000 daltons, which are prominent in the preparations from frozen brain, did not react with antitubulin serum.

Neuronal Staining by Anti-210,000-dalton Serum

Anti-210,000-dalton serum binds to sections of sciatic nerve as shown in Fig. 4*a* and *b*. The pattern seen is of solid cylinders of uniform diameter of positive stain extending considerable distances in longitudinal sections. The myelin sheath is not stained, nor are the Schwann cells or the connective tissue sheath around the nerve. This pattern is consistent with exclusive axoplasmic staining of the large myelinated axons that dominate this nerve. Absorption with isolated filaments removes all the staining.

The staining of neuronal axons by anti-210,000 dalton serum could also be demonstrated in cultures of rat dorsal root ganglia and attached nerves. The sensory neurons have long processes and large (15–20 μm) diameter cell bodies, characterized by distinctive, usually eccentric nuclei and a prominent, single nucleolus. 80% of these neurons are stained intensely by anti-210,000-dalton serum (Fig. 5*a*). The stain is in the form of a poorly defined meshwork in the cytoplasm of the cell body (Fig. 5*b*) and is also intense in the cell processes (Fig. 5*c*). Staining of Schwann cells and fibroblasts with anti-210,000-dalton serum is low and equal to preimmune or normal rabbit serum controls, even though the cells contain filaments readily visualized with different antisera (see below).

Anti-210,000-dalton serum also stains elements in tissue sections of the cerebellum. Fiber tracts stain strongly, and positive processes are seen in the granular layer (Fig. 6). Staining is seen in the Purkinje cell layer, but Purkinje cell bodies do not stain more than with preimmune or adsorbed serum. The Bergmann glial fibers, which are seen clearly with other antisera, are not stained with anti-210,000-dalton serum nor are other glia, blood vessels, or the pia.

In cerebellar cultures, most neurons are small granule cells although there are a few larger neurons. Some of the neuronal cell bodies and processes stain with anti-210,000-dalton serum (Fig. 7). The background cells of the cultures are astrocytes and fibroblasts (42), most of which (>90%) show no staining

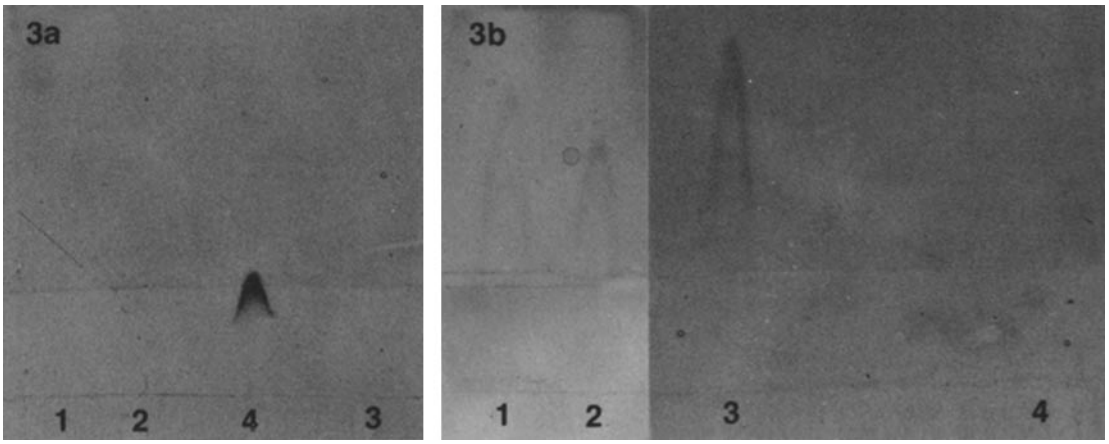

FIGURE 3 Rocket immunoelectrophoresis of brain filament proteins. Precipitin bands formed by (*a*) rabbit anti-49,000-dalton serum (1:10), or (*b*) rabbit anti-210,000-dalton serum (1:10). Antigen *1* is 210,000-dalton protein; *2* is 160,000-dalton protein, *3* is 68,000-dalton protein, and *4* is 49,000-dalton protein. Antigens (*2*–*6* μg/sample) were excised protein bands from gradient gels (Fig. 2 *a*) embedded at the indicated site in agarose and electrophoresed upward into the layers containing immune serum and nonionic detergent (37).

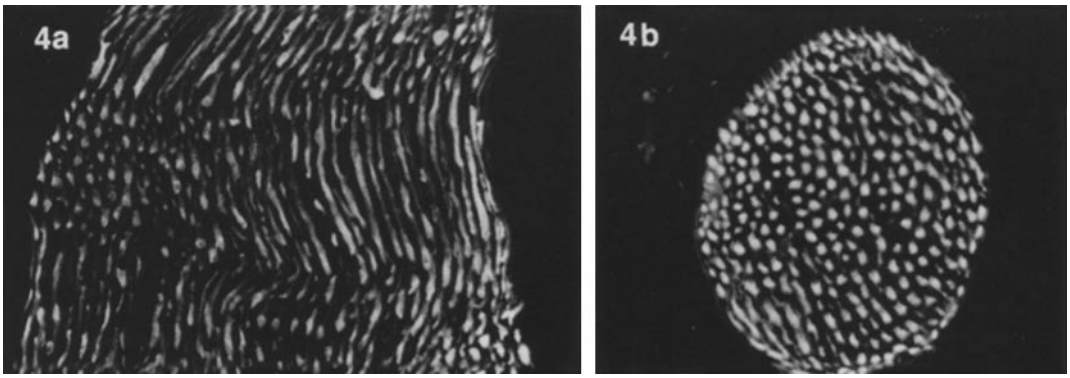

FIGURE 4 Immunofluorescent staining of rat sciatic nerve sections by anti-210,000-dalton serum. Methanol-fixed cryostat sections were stained with serum diluted 1:50, and bound antibody was detected with goat anti-rabbit immunoglobulin-fluorescein (1:60). *a*, Longitudinal section; *b*, cross section. × 153.

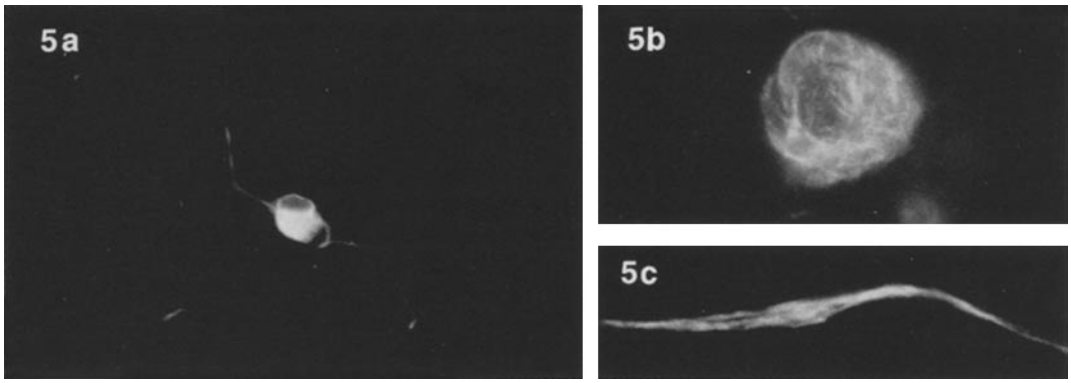

FIGURE 5 Immunofluorescent staining of dissociated neurons from the dorsal root ganglion with anti-210,000-dalton serum. (*a*) Neuron is stained strongly in cell body and processes, using serum diluted 1:300. Four Schwann cells are present in this field but are not stained. × 354. (*b*) Focus on antigen staining in neuronal cell body. Serum diluted 1:100. × 1,010. (*c*) Antigen inside neuronal process. Serum diluted 1:100. × 1,760. For staining of cultured cells rhodamine labeled goat anti-rabbit immunoglobulin was used.

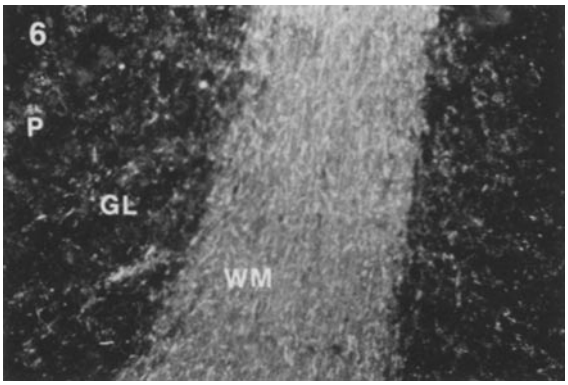

FIGURE 6 Immunofluorescent staining of cerebellar tissue section with anti-210,000-dalton serum (1:50). The white matter (*WM*) tract is stained brightly, and scattered processes in the granular layer (*GL*) are positive. Purkinje cells (*P*) are not stained, nor are Bergmann glial fibers, which are barely included in this section. × 153.

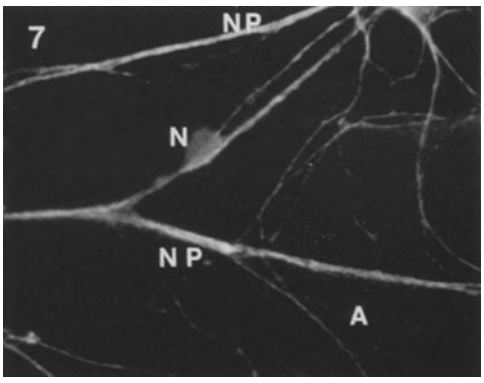

FIGURE 7 Immunofluorescent staining of cerebellar cells in culture with anti-210,000-dalton serum. Cells were cultured for 8 d (with FUdR); serum was diluted 1:100. Small neuronal cell body (*N*) is not stained, but neuronal processes (*NP*) stain. Astrocytes (*A*) or other nonneuronal cells are not specifically stained above preimmune or normal rabbit serum. × 580.

above the preimmune control. A minority (7%) of them stain with anti-210,000-dalton serum and look like astrocytes, but this staining is not intense and does not seem to be specific because a similar pattern is seen with several hyperimmune control sera.

Immunoperoxidase Localization in Brain Tissue of Anti-210,000-dalton Binding

Using the unlabeled antibody method (58), a strong peroxidase reaction is found within axons upon incubation of brain tissue with anti-210,000-dalton serum and examination by electron microscopy. Filamentous elements inside the axons were specifically stained (Fig. 8). The elements and associated reaction product have a diameter of 250 Å. The density of positive elements in cross sections of axons is 270 ± 30/μm^2. Axonal plasma membrane and axonal mitochondrial membranes are also stained. Postsynaptic dense structures are not visualized, and filaments, membranes, or mitochondria of glial cells are devoid of stain.

Specific Staining of Sciatic Nerve Sections by Anti-49,000-dalton Serum

In sciatic nerve sections, the prominent axon cylinders seen with anti-210,000-dalton serum are not stained specifically by anti-49,000-dalton serum. Instead, infrequent, irregularly spaced elements are strongly stained (Fig. 9*a*). These elements are long and narrow in longitudinal sections and are stained with roughly equal intensity as Bergmann glial fibers in the cerebellum sections with dilutions of anti-49,000-dalton serum between 1:50 and 1:500. In cross sections of sciatic nerve, the Schwann cells immediately adjacent to each myelin sheath are not stained (Fig. 9*b* and *c*). Instead, a few positive elements are stained, corresponding in frequency and diameter to the long elements seen in the longitudinal sections (Fig. 9*a* and *c*). These long narrow elements are not stained by normal rabbit sera, preimmune serum, or other hyperimmune sera. The epineurium and perineurium of the nerve are not stained by anti-49,000-dalton serum. Some blood vessels in the epineurium and the endoneurium (Fig. 9*b*) are stained in a thin, delicate pattern (Fig. 9*c*) at serum dilution of 1:50, but at higher dilutions these are not seen by the indirect fluorescence technique.

Sciatic nerve staining by anti-49,000-dalton serum can be adsorbed by the brain filament preparation (Fig. 9*d*) or by Triton-insoluble cytoskeletal proteins from cultured astrocytes. Cytoskeletal proteins from cultured Rat-1 fibroblasts fail to adsorb the stain.

Patterns of fluorescence identical to Fig. 9*a–c* are found with three other rabbit anti-GFAP sera. With each one, sciatic nerve

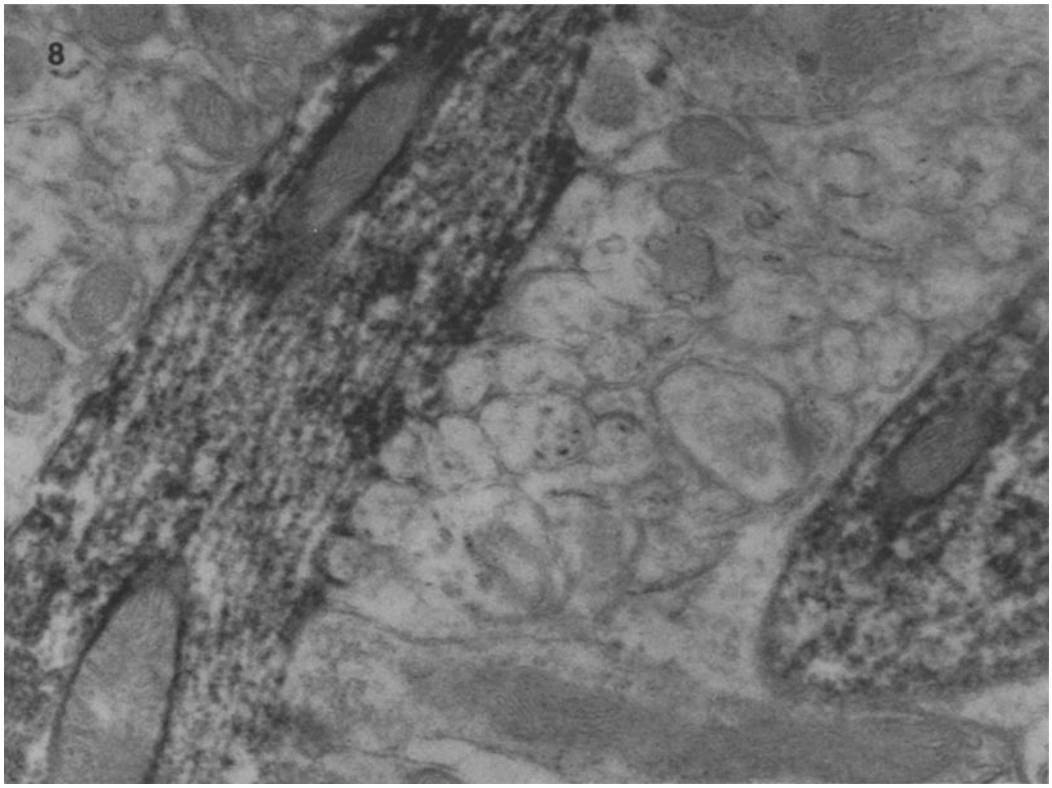

FIGURE 8 Immunoperoxidase labeling of cerebral cortex slices with anti-210,000-dalton serum. Binding of serum (diluted 1:200) is detected by unlabeled antibody method using peroxidase-antiperoxidase complex (55). Stain is associated with filamentous structures inside axons, adjacent plasma membrane, and mitochondrial membranes. × 32,400.

staining is seen at the same dilutions which strongly stain filaments in cultured astrocytes, or processes of astrocytes in tissue sections (see below). No anti-GFAP serum has failed to demonstrate a strong cross-reaction with the sciatic nerve elements.

Antisera against rat type I collagen, galactocerebroside, basement membrane, and fibronectin give staining patterns on sciatic nerve sections which are obviously different from that of anti-49,000-dalton serum.

Astrocyte-specific Staining by Anti-49,000-dalton Serum

Neurons, Schwann cells, or other nonneuronal cell types in the dorsal root ganglion cultures do not stain with anti-49,000-dalton serum, nor do Schwann cells or fibroblasts from neonatal sciatic nerve.

In cerebellar tissue sections, anti-49,000-dalton serum strongly stains Bergmann glial fibers and astroglial cells in white matter fiber tracts and in the granular layer (Fig. 10*a*). Astroglial end feet around the blood vessels (Fig. 10*b*) and at the pial surface are also stained. This staining pattern is identical to that reported for anti-GFAP (5, 36). There is little or no staining of the leptomeninges or pia, and blood vessels of the pia-arachnoid membrane are usually unstained (Fig. 10*c*).

In cerebellar cell cultures, the neurons and extensive network of neuronal processes are not stained at all by anti-49,000-dalton serum. Most of the nonneuronal cells are strongly stained and may be roughly grouped into three types. At one extreme is a cell shaped like a classical fibrous astrocyte, having a cell body 15–25 μm in diameter and several radiating branched processes (Fig. 11*b*). Bundles of filaments that stain with anti-49,000-dalton serum extend from the perinuclear region out into all the processes (Fig. 11*b*) and are composed of fine filaments (Fig. 11*c*). A second morphological cell type is very flat, has few processes, and also contains numerous filaments that stain with the antiserum (Fig. 11*a*). Many positive staining cells have intermediate morphologies. Finally, there are fibroblasts in these cultures, which may be recognized by thick stress fibers that are visible with phase contrast microscopy and are positive in immunofluorescent staining with antiactin sera. These cells also bind anti-Thy-1 serum (42), whereas the astrocytes do not stain at 1 wk in culture. The fibroblasts do not stain with anti-49,000-dalton serum, and constitute 5% of all the nonneuronal cells in the cultures.

The staining of elements in sciatic nerve, glial cells and processes in cerebellum, and cultured astrocytes has all been observed with five sera: anti-49,000-dalton serum, anti-bovine 51,000-dalton serum, (65) and three antisera to GFAP preparations. Absorption of anti-49,000-dalton serum with the human filament preparation, or with a Triton-insoluble cytoskeletal preparation (10, 31) from cultured astrocytes removes all the staining including that of sciatic nerve, whereas fibroblast cytoskeletal proteins do not adsorb any of it.

Distribution of 58,000-dalton Antigen in Brain

Hynes and Destree (31) raised an antiserum against a 58,000-dalton, Triton-insoluble, SDS gel-purified cytoskeletal protein of hamster NIL-8 fibroblasts. Sciatic nerve sections are positively stained by this anti-58,000-dalton serum (Fig. 12), and the pattern is very different from that seen with anti-210,000 (Fig. 4) or anti-49,000-dalton sera (Fig. 9). Inside the nerve, numerous elements that are wavy and long stain in longitudinal

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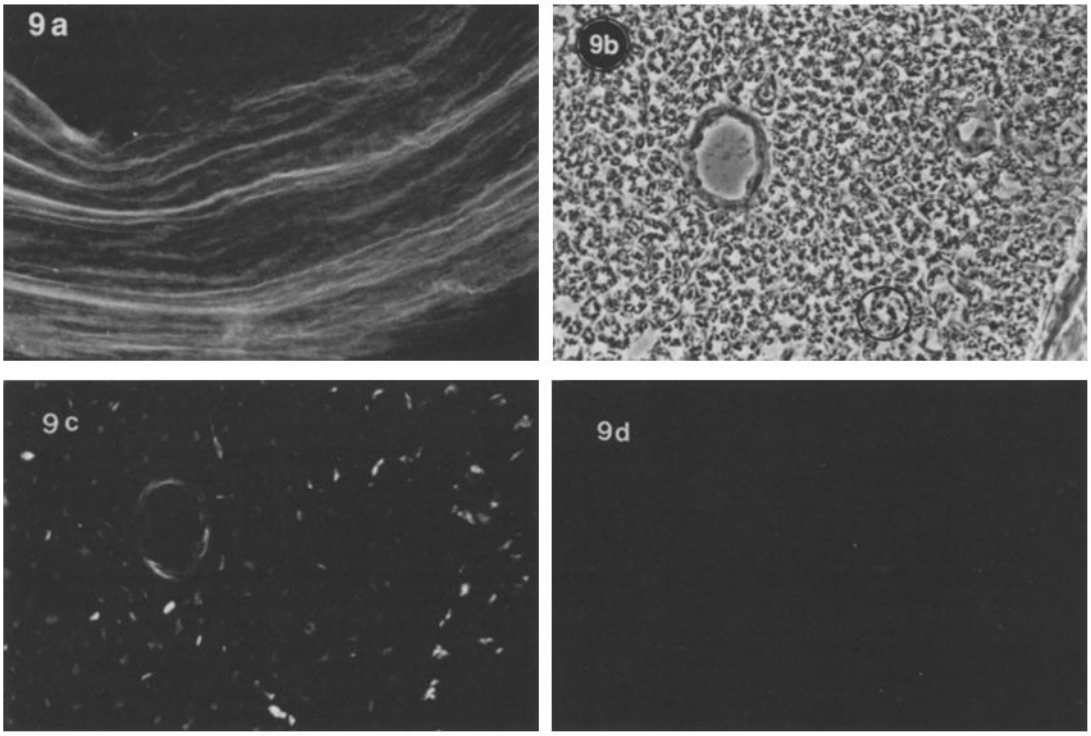

FIGURE 9 Indirect immunofluorescent staining of cryostat sections of sciatic nerve. (*a*) Longitudinal section fixed with methanol, stained with anti-49,000-dalton serum, diluted 1:50. × 153. (*b*) Phase contrast image of cross section fixed for 5 min with 3.7% *p*-formaldehyde in PBS. Note perineurium at lower right, and two endoneurial blood vessels. A myelin sheath is circled. × 320. (*c*) Same field as *b*, viewed with optics for fluorescein fluorescence. Serum is anti-49,000 dalton absorbed with rat liver powder (1 mg/ml) and diluted 1:50. Note staining elements at large blood vessel, and strong staining of elements in the endoneurium. × 320. (*d*) Immunofluorescent staining of sciatic nerve section with anti-49,000-dalton serum, adsorbed twice with the brain filament preparation, and diluted 1:50. Section, fixation, staining, photography, and printing are parallel to *c*. Staining has been adsorbed effectively by brain filaments down to levels seen with normal rabbit serum. Absorption with cytoskeletal proteins from cultured astrocytes results in low levels of fluorescence, equivalent to that shown in this photograph. × 320.

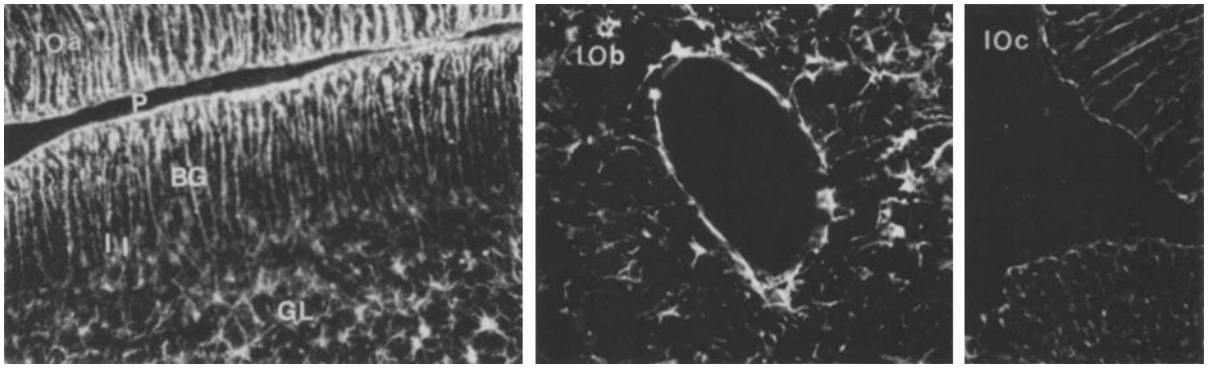

FIGURE 10 Immunofluorescent staining of cerebellar tissue section with anti-49,000-dalton serum. Serum dilution was 1:50. (*a*) Bergmann glial fibers (*BG*) in the molecular layer, fibrous astrocytes in the granular layer (*GL*), and white matter (not shown in this photograph) are stained. The pial membrane is not stained except for glial end feet at the inner surface (*P*). Methanol fixation. × 153. (*b*) Strong staining of glial elements is found at large blood vessels in the white matter. Fixation with acid alcohol (5 s) after antibody incubations. × 320. (*c*) Region at edge of cerebellum section, methanol fixation. Meninges and blood vessels are nearly always unstained. × 136.

sections (Fig. 12*a*). The elements that stain strongly and specifically are located outside the rings of myelin, as seen clearly in transverse sections (Fig. 12*b*). There is faint staining of axons, which is greater than with most normal rabbit sera, but it is not specific because the preimmune serum reacts with axons to the same extent as the immune anti-58,000-dalton serum. The specifically stained elements do not resemble axons, and they are much more frequent than the elements that are positive with anti-49,000-dalton serum. The pattern corresponds to the distribution of Schwann cells inside the nerve.

Schwann cells in dissociated sciatic nerve or dorsal root ganglion cultures stain strongly with anti-58,000-dalton serum. Most Schwann cells are elongated, bipolar or tripolar cells; occasionally the cells are much flatter or have one flat region and one process (Fig. 13). In the flat portion, the staining can be clearly seen to be filamentous. In the processes, individual filaments cannot be resolved, but often at the end of processes filaments are seen, which merge at the narrow portion. All the larger, flat fibroblasts in sciatic nerve and ganglion cultures also stain in the filamentous pattern described by Hynes and

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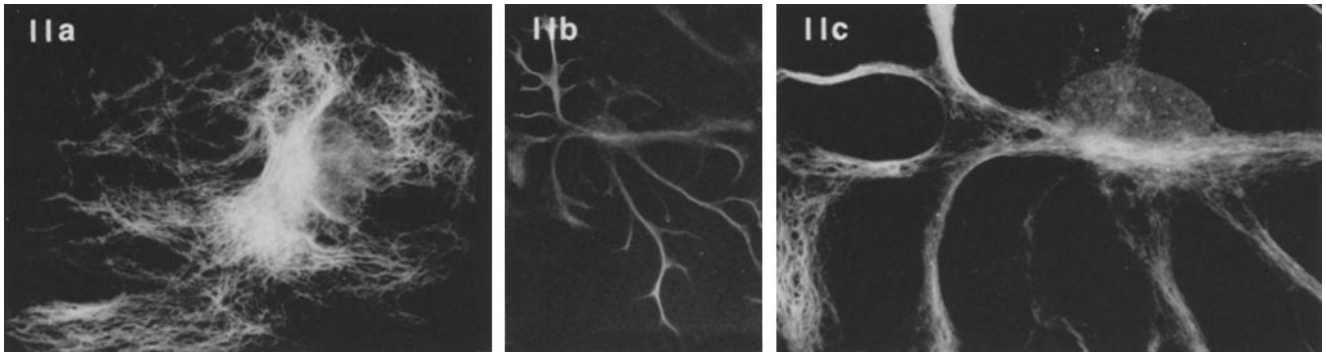
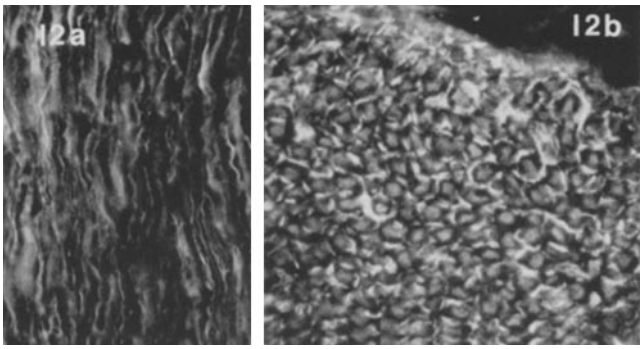


FIGURE 11 Immunofluorescent staining of cerebellar cells in culture with anti-49,000-dalton serum. Cells were cultured for 23 d and stained with serum diluted 1:50. (*a*) Flat astrocyte stains in a filamentous pattern. × 595. (*b*) Astrocyte with processes also stains. × 158. (*c*) Same cell at higher magnification to show filaments. × 595.



nerve with anti-58,000-dalton serum. Serum dilution was 1:75. *a*, Longitudinal section; *b*, cross section. × 360.

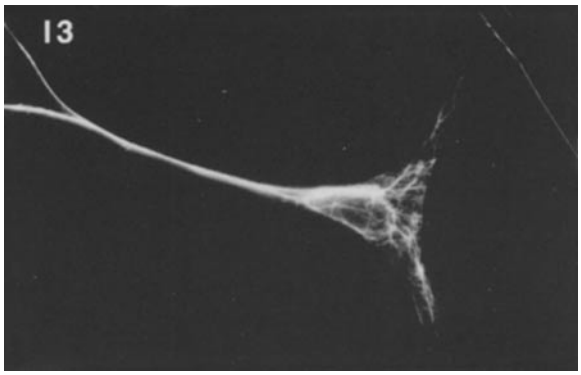


FIGURE 13 Immunofluorescent staining of sciatic nerve cultures with anti-58,000-dalton serum. Serum (diluted 1:75) shows a strong filamentous staining pattern in elongated Schwann cells. × 620.

Destree (31). In dorsal root ganglion cultures, the neuronal cells are not specifically stained by anti-58,000-dalton serum compared with normal or preimmune sera.

In cerebellar tissue sections, anti-58,000-dalton serum stains blood vessels and the pia (Fig. 14*a* and *b*). This staining of blood vessels is different from the staining around vessels caused by adjacent astrocytic end feet as observed with anti-GFAP (36) or anti-49,000-dalton sera (Fig. 10*b*). Bergmann glial fibers are positive (Fig. 14*b*), as are cells shaped like fibrous astrocytes in the white matter (Fig. 14*c*). Anti-49,000 and anti-58,000-dalton sera differ in that fibrous astrocytes in the granular layer, which are positively stained with anti-49,000-dalton serum, are not stained with anti-58,000-dalton serum (Fig. 14*c*).

In cerebellum cultures, anti-58,000-dalton serum does not stain the neuronal cell bodies or processes. Almost all the flat cells, both fibroblasts and astrocytes, have positively stained fibrous elements (Fig. 15*a*). Some process-bearing cells are not stained or are weakly stained, whereas others are strongly stained (Fig. 15*b*).

Individual Astrocytes Contain Both 49,000 and 58,000-dalton Antigens

Variation in the intensity of staining of astrocytes in primary cultures makes the scoring of positive cells quite subjective. Cells in longer term cultures appear to be less variable and to have more filaments, which makes it possible to test whether individual cells have both types of filament antigen. Secondary cultures of cerebellar cells contain flat cells and no neurons. Colchicine alters the distribution of both 49,000 and 58,000-dalton antigens; the filaments are condensed near the cell body and the intensity of staining is increased. All the cells in the cultures stain strongly with anti-58,000-dalton serum. With anti-49,000-dalton serum, 21% of the cells are not stained or are weakly stained, and 79% stain strongly (Fig. 16). Thus, the majority of cells have both filament antigens.

Epidermal Filaments

Antiserum to the 58,000-dalton protein of rat epidermal filaments stains sections of rat skin but not sciatic nerve or cerebellum. Cultures of the dorsal root ganglion or cerebellum contain no positively staining cells.

DISCUSSION

Antisera to Human Filament Proteins

Human brain filaments, prepared by a method that avoids prolonged exposure to low ionic strength buffers, contain substantial amounts of a triplet of proteins thought to comprise mammalian neuronal filaments, as well as a band at 49,000 daltons suspected of originating from astrocytes. By ultrastructural criteria, the human filament preparation is comparable to preparations from bovine brain (54) and is dominated by bundles of intermediate filaments. Immunization with the protein bands at 210,000 and 49,000 daltons, separated and cut from SDS gels, results in strong antisera. In addition to reacting with rat tissue, these sera stain filaments in human tissue sections (S.-H. Yen, unpublished results), and may be optimal

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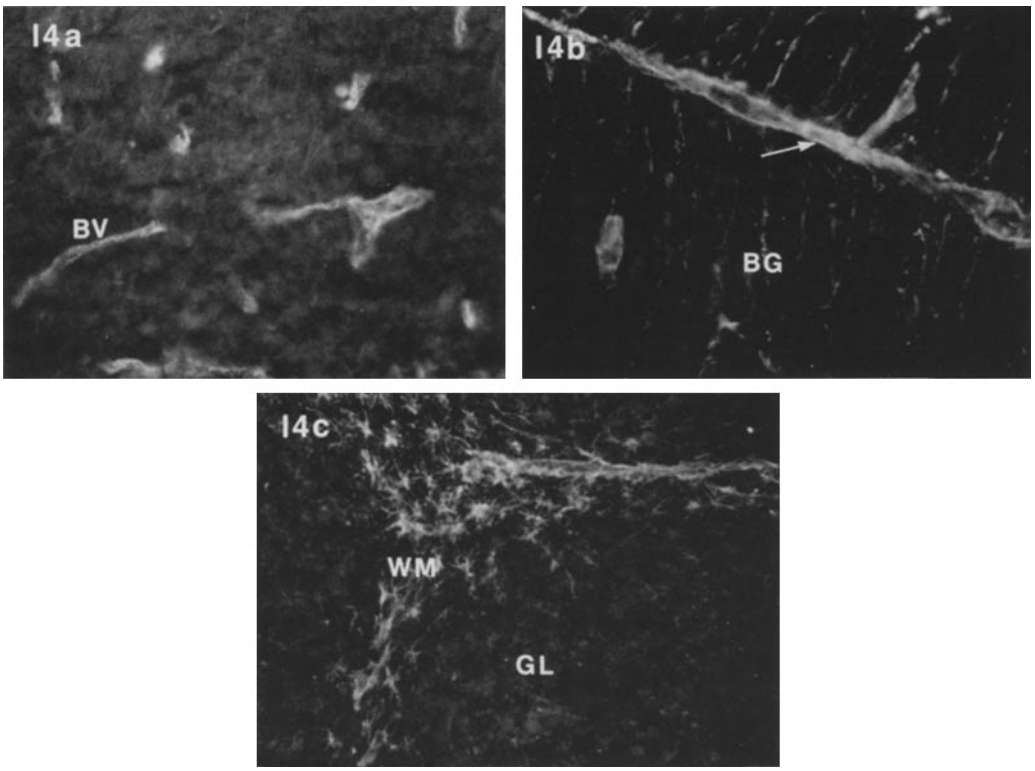

FIGURE 14 Immunofluorescent staining of cerebellar tissue sections with anti-58,000-dalton serum. (*a*) Positive staining is seen of blood vessels (*BV*). (*b*) Bergmann glial fibers (*BG*), the pia (arrow), and (*c*) fibrous astrocytes in the white matter (*WM*) tract, were stained with anti-58,000-dalton serum diluted 1:50. Astrocytes in the granular layer (*GL*) are not visible with this serum. *a*, × 380; *b*, × 380; *c*, × 153.

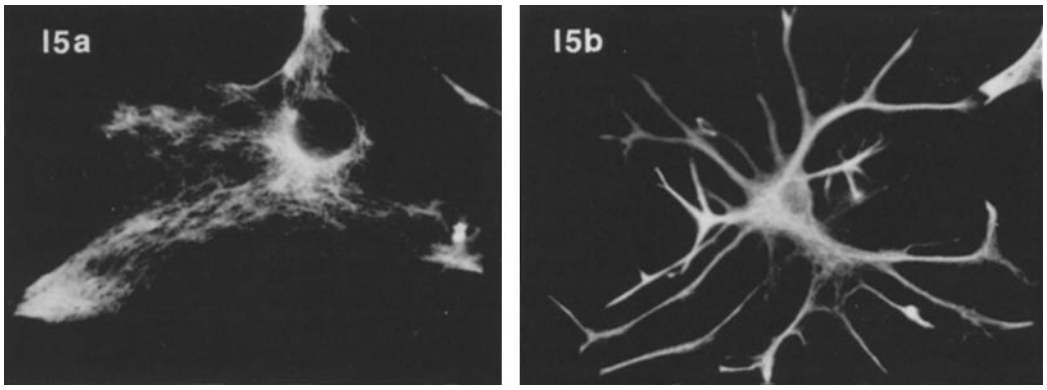

FIGURE 15 Immunofluorescent staining of cerebellar cell culture by anti-58,000-dalton serum. Filamentous network can be seen in flat astrocyte (*a*) and process-bearing astrocyte (*b*). Serum was diluted 1:75. *a*, × 580; *b*, × 380.

for the study of brain filament proteins in human pathological material.

Specificity of Anti-210,000-dalton Serum for Neurons

Although the characterization is preliminary, the pattern observed at the ultrastructural level strongly suggests that this antiserum binds to 100-Å neurofilaments. Peroxidase reaction product is associated with densely packed elements arranged longitudinally inside myelinated axons or processes that are identified as axons by their relatively uniform diameter, round cross section, or associated synapses. Glial processes are not stained. In cross section, the reaction product has a diameter of 250 Å, which is consistent with an underlying structure of 100 Å. Immunoperoxidase stain associated with microtubules, on the other hand, has a diameter of 1,000 Å (20). The number of positive elements per square micrometer is also consistent with intermediate filaments in axons (40). Staining of mitochondrial and plasma membranes of axons, but not glia, is observed, and has been described, along with a staining of filaments, by others using antisera against whole brain filament preparations (39, 46) or 54,000-dalton bovine filament protein (46).

At the light level, the antiserum against the 210,000-dalton triplet protein binds specifically to axons and neuronal cell bodies. The pattern of immunofluorescent staining of tissue sections by anti-210,000-dalton serum resembles the patterns reported by Liem et al. (35) and others (14) for sera raised against 160,000-dalton protein from bovine brain. Similar patterns were also obtained by Schlaepfer and Lynch (50) with antiserum raised against a peripheral nerve filament preparation (47). These staining patterns constitute the best available

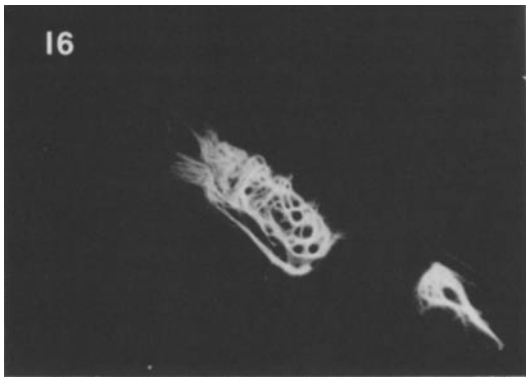

FIGURE 16 Immunofluorescent staining of colchicine-treated cerebellar cells. Secondary cultures of cerebellar cells were treated with colchicine for 18 h and stained with anti-49,000-dalton serum diluted 1:50. Coiled fibers are visible in the majority of cells. × 407. Similar coiled fibers are visible in all cells (not shown) when stained with anti-58,000-dalton serum, diluted 1:75.

immunological evidence that 210,000, 160,000, and 68,000-dalton proteins are three components of neuronal filaments.

Although the staining pattern is consistent with this interpretation, the strong cross-reaction of the anti-210,000-dalton serum with proteins at 160,000 and 68,000 daltons makes it an unsuitable reagent for discriminating between the triplet proteins. Thus, if there were filaments made up entirely of 68,000-dalton protein, they would also be expected to stain with this anti-210,000-dalton serum. Anti-160,000-dalton sera also show cross-reactions among the triplet proteins (14, 35). Recently, three other laboratories have obtained similar results with antisera to individual triplet proteins (2, 64).[1] Localization studies of a particular triplet protein with any of these sera will require extensive adsorption with the other cross-reacting proteins or equivalent blocking experiments (64).

A number of chemical agents result in the pathological accumulation of 100 Å filaments inside neurons (40). The demonstration of a selective increase of 210,000, 160,000, and 68,000-dalton polypeptides in isolated neurons from rabbits treated with aluminum is supportive biochemical evidence for the association of all three triplet proteins with neurofilaments (53). Similarly, radioactively labeled triplet proteins accumulate in β, β'-iminodipropionitrile intoxication (29).

Although the triplet proteins represent a variable proportion of all the protein in the filament preparation, human tissue is a good source of the 210,000-dalton protein. Quantitative analysis of filament preparations from different species shows that human material yields three to four times more 210,000-dalton protein, relative to the other triplet and 49,000-dalton proteins, than bovine or rat brain (11).

Specificity of Anti-49,000-dalton Serum

The anti-49,000-dalton serum stains astrocytes well in cerebellar tissue sections and in cultures. The pattern is indistinguishable from that reported for anti-GFAP (1, 5, 36, 42) and resembles that of antisera to bovine brain 51,000-dalton protein tested on tissue sections (35).

The cell-type-specific staining shows that the 49,000-dalton antigen is antigenically distinct from fibroblast or neuronal filaments. However, the filamentous pattern and the formation of coils in response to colchicine treatment are similar for glial and fibroblast filaments. The distinctive separation of astrocyte, fibroblast, and neuronal reactivities of our sera differs somewhat from a naturally occurring autoantibody (28) and a serum against a GFAP preparation (8).

Presence of 49,000 dalton Cross-reactive Antigen in Peripheral Nerve

Morphological studies of peripheral nerve have shown that astrocytes extend only a short distance into spinal nerve roots (4, 60). It is surprising, then, to find definite staining of elements in sciatic nerve with the anti-49,000-dalton serum. This staining is eliminated by adsorption with CNS filaments or astrocyte cytoskeletal proteins, which shows, along with the usual normal and preimmune serum controls, that the staining is antigen-specific rather than attributable to nonspecific artifacts. Also, all other rabbit antisera to GFAP preparations give an identical pattern on sciatic nerve sections. The methods of isolating the antigen and the purity of such preparations differ significantly, but the strong cross-reaction with sciatic nerve is constant with these sera despite the variations. Each serum has sciatic nerve staining equal to that of the anti-GFAP staining of cerebellar sections. Other groups have observed sciatic nerve staining.[2]

Others have found no GFAP antigen in distal regions of human spinal nerves (49). We have confirmed their finding with human nerve roots of autopsy specimens with the anti-49,000-dalton serum described here (S.-H. Yen, unpublished results). In short, we find no differences in the staining pattern of the anti-49,000-dalton serum and that of other rabbit anti-GFAP sera. One contradictory finding that is unexplained is the report that mouse anti-GFAP sera failed to stain mouse sciatic nerve sections (46).

The staining elements appear to be long and thin. We have considered whether the stain is consistent with a type of collagen fiber. Antibody to rat type I collagen stains the nerve sections in a totally different pattern from that of anti-49,000-dalton serum. The anticollagen antibody binds strongly to the epineurium and perineurium (areas that are not stained by anti-49,000-dalton serum), and the pattern in the endoneurium is diffusely reticular. No elements like those of Fig. 9 are seen. In addition, rat tail collagen fails to remove the staining antibody from anti-49,000-dalton serum in adsorption tests. Thus, we have no evidence in favor of a cross-reaction with collagen.

The pattern of stain does not resemble that published for sera against PNS filaments (50), triplet proteins (35), or other neuronal proteins (16, 46). If the antigen were in a subclass of neuronal axons, then, in distal portions of transected nerves, antigen would disappear with axonal degeneration. On the contrary, preliminary experiments of Yen and Spencer (unpublished observations) show an increased number of elements staining with anti-49,000-dalton serum in cut nerves compared with intact control nerves, at 2, 4, and 8 wk after transection. This finding increases the possible importance and interest of the 49,000-dalton protein-GFAP cross-reactive antigen.

One consequence of nerve transection is the proliferation of Schwann cells and formation of bands of Büngner in the

[1] Anderton, B., University of London. Personal communication. Willard, M., Washington University. Personal communication.

[2] Kalnins, V., University of Toronto. Personal communication. Bignami, A., and D. Dahl, Harvard Medical School. Personal communication. Mirsky, R., University College, London. Personal communication. Davison, P., Boston Biomedical Research Institute. Personal communication.

nerves. However, collagen increases as well, and other cells, such as fibroblasts, proliferate. Abundant intermediate filaments have been reported in the Schwann cells (of the bands) (6, 51). We suspect that some of these cells have made filaments antigenically related to the 49,000-dalton protein, which is abundant in reactive astrocytes in the CNS. However, it will require examination at the ultrastructural level to determine whether the antigen is intracellular and filamentous, and whether the cell involved resembles a Schwann cell, astrocyte, or fibroblast.

The biochemical nature of the antigen in the sciatic nerve is still undetermined. The adsorption of staining by brain filaments is consistent with the antigen being a major protein of that preparation. Astrocyte cytoskeletal proteins also adsorb, and there are fewer major proteins in the cytoskeleton preparation than in the brain filaments. One of the few that is present in astrocytes and missing from fibroblasts is a protein at 50,000 daltons, which is identical by cyanogen bromide mapping to the rat brain 50,000-dalton protein of filaments (10). However, the molecular weight of the cross-reacting antigen in sciatic nerve has not been determined. The elements that stain are sparse in normal nerve. Thus, when nerve homogenates fail to adsorb staining of nerve or brain, we think it is because the antigen is present in low amounts. In any case, the extent of cross-reaction of the sciatic nerve antigen and the CNS-antigen is not yet determined.

There is some biochemical evidence for the presence of 50,000-dalton polypeptides in the peripheral nervous system. Davison and co-workers have found that different cranial nerves (18) as well as sciatic nerve (17) have variable amounts of 50,000-dalton protein in guanidine hydrochloride eluates, but it has not been shown that this 50,000-dalton protein reacts with anti-49,000-dalton serum or anti-GFAP antibodies, or that it corresponds to the staining elements in peripheral nerve. In PNS filament preparations, little protein in the region of 50,000 daltons is seen on SDS gels (35, 48), but a rise in a protein doublet of 49–51,000 daltons, as well as other proteins, has been noted in transected nerves (51).

It is unlikely that the 49,000-dalton protein band used for our immunization was homogeneous because two-dimensional gels of the 50,000 dalton region of bovine CNS filaments show considerable heterogeneity (62). A second independent antigen seems unlikely because all anti-GFAP antisera exhibit the staining patterns in sciatic nerve and cerebellum with equal intensity. A cross-reactive antigen in the sciatic nerve is a much simpler explanation than invoking a second astrocyte-related, cytoskeletal antigen and equal titers of antibodies in five independent sera. Monoclonal antibodies may be one way to settle the question of two different antigens. A collection of independent monoclonal antibodies against different domains of the 49,000-dalton protein or GFAP will also serve to define the extent of cross-reactivity of the two antigens in these two regions of the nervous system.

We have no firm evidence that the antigen in the peripheral nerve is present as filaments. However, Jessen and Mirsky (32) have observed a GFAP-cross-reactive antigen in rat enteric ganglia, and in cultured enteric ganglia some nonneuronal cells stain in a filamentous pattern with anti-GFAP sera. It is possible that the nonneuronal cell type which is common in the enteric ganglia is represented sparsely in normal sciatic nerve, and the same cell type may proliferate in response to nerve damage.

Fibroblast-type Filaments in the Nervous System

Antisera against fibroblast 55–58,000-dalton cytoskeletal proteins bind to filaments in fibroblasts and other cell types (3, 24, 31). In tissue sections, as expected, the anti-58,000-dalton serum reacts with connective tissue elements in nerve sheath, pia, and blood vessels, as well as with rat fibroblasts in the various cultures. In addition, there is a strong reaction with cultured Schwann cells and a pattern of staining of sciatic nerve consistent with positive cytoplasmic elements in mature Schwann cells. Our results suggest that most Schwann cells in peripheral nerve have only fibroblast-type filaments. The presence of intermediate filaments in normal, mature Schwann cells has been noted in ultrastructural studies of nerve in vivo (61), and abundant intermediate filaments have been noted in developing rat sciatic nerve (38) and in isolated Schwann cells in culture (44). Filament preparations from rabbit intradural nerve roots have a 60,000-dalton protein (35) that may come from the Schwann cell and correspond to the sciatic nerve antigen detected with anti-58,000-dalton serum.

Fibrous astrocytes have very prominent bundles of tightly packed 8 to 10-nm filaments, and staining by anti-GFAP of such cells (5, 36) and filaments at the ultrastructural level has been reported (45). It is surprising to find that radial elements resembling Bergmann glial fibers and cells looking like fibrous astrocytes in white matter are positive with anti-58,000-dalton serum. Astrocytes in the granular layer, which stain with anti-49,000-dalton serum, are not stained with anti-58,000-dalton serum; thus, the distributions of the two antigens overlap but do not coincide.

In secondary cultures of cerebellar nonneuronal cells, it is clear that the astrocytes have abundant filaments of two classes inside the same cells. Their existence as separate proteins is supported by isolation of both 49,000- and 58,000-dalton bands in cytoskeletal preparations (10). It has been observed that epithelial cells in culture have both fibroblast-type and keratin-type filaments (25), and that fibroblast and smooth muscle–type filaments are in muscle fibers (see reference 34 for review). Coexistence of fibroblast-type filaments and GFAP has recently been demonstrated in some cells of a human astocytoma cell line (41). Whereas filaments of the fibroblast-type are observed in tumor cells or in inmature cultured cells, it is unusual in tissue sections to find the two classes of filaments in the same cell type (25). From the work presented here, astrocytes are an exception, for even in the adult our staining of tissue sections shows both fibroblast and glial filament antigens.

Although the intermediate filaments of different cell types of the nervous system are similar in diameter, it is striking that they do not share similar-sized subunit proteins or exhibit cross-reacting antigenic specificities. The 210,000-dalton antigen is completely specific, by immunofluorescent methods, for neuronal elements. The 49,000-dalton antigen has astrocyte specificity, as well as a representation or cross-reacting antigen in peripheral nerve described here for the first time. The 58,000-dalton fibroblast filament antigen is absent from neurons, but is shared by astrocytes, Schwann cells, and connective tissue cells, which clearly indicates that the fibroblast antigen has the broadest cell type distribution of the intermediate filament subunit proteins.

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