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A monoclonal antibody specific for the 200 K polypeptide of the neurofilament triplet

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A mouse monoclonal antibody, designated NF1, was obtained from a cloned hybridoma isolated from a fusion of mouse myeloma Sp2 cells with spleen cells from a BALB/c mouse immunized with a crude neurofilament preparation from porcine spinal cord. NF1 is an IgG1 and recognizes, in immune blotting procedures, only the 200 K neurofilament triplet component. Its neurofilament-specific nature is further revealed by immunofluorescence microscopy studies on frozen tissue sections and various cultured cells. Immunoelectron microscopy studies on cytoskeletons of cultured neurones emphasize the discontinuous display along each neurofilament previously observed with polyclonal antibodies specific for the 200 K component after appropriate but rather cumbersome cross-absorption steps. Use of NF1 on various neuronal cells strongly supports the previous proposal of the existence of certain subpopulations of neurofilament-free neurones and the observation that certain neuronal arrangements, (e.g., those in dendrites of pyramidal cells of the hippocampus), although rich in neurofilaments, probably lack the normal 200 K triplet component. Since NF1 shows a broad cross-species reactivity and is able to react on formaldehyde-fixed tissue, it should be a useful reagent to study differential neurofilament expression and organization in embryonic, adult and pathological tissues.

Key words: brain/immunofluorescence/intermediate filaments/monoclonal antibodies/neurofilaments

Introduction

Neurofilaments are one of the five cell type-specific intermediate filament systems currently recognized in higher vertebrates (for recent reviews see Lazarides, 1980; Osborn *et al.*, 1981). Elegant studies on axonal transport have shown that, in general, neurofilaments contain three major polypeptides (200 K, 145 K, 68 K), the so-called triplet (Hoffman and Lasek, 1975), and later biochemical experiments have fully confirmed this proposal (Schlaepfer and Freeman, 1978; Liem *et al.*, 1978; Mori *et al.*, 1979). Relatively little is known about possible functional or structural relationships between the different members of the triplet. Fingerprinting techniques do not seem to provide clear evidence for sequence homology between the three proteins (e.g., Chiu *et al.*, 1980; Dahl, 1980; Mori *et al.*, 1980) and immunological studies with polyclonal antibodies have led to a rather complex picture of cross-reactivities. Although many reports have documented cross-reactivity between two or all three triplet components, even if antigens were purified by SDS-polyacrylamide gel electrophoresis (Liem *et al.*, 1978; Anderton *et al.*, 1980; Dahl, 1980; Willard and Simon, 1981; Yen and Fields, 1981; Shaw and Weber, 1981), a few antibodies have appeared to be specific for only one triplet protein (Shaw and Weber, 1981). In addition, at least two laboratories have been able to obtain, by suitable antigen cross-absorption experiments, polyclonal antibodies which specifically recognized only one triplet component in immune blotting techniques (Shaw and Weber, 1981) or revealed a greatly reduced cross-reactivity with the other two members of the triplet when assayed by a solid phase-coupled peroxidase assay (Willard and Simon, 1981). Such antibodies have been used to explore neurofilament organization and structure (Sharp *et al.*, 1982; Willard and Simon, 1981; Shaw *et al.*, 1981b).

Given the current difficulties in obtaining triplet component-specific polyclonal antibodies and the general interest in neurofilament structure and expression, interest focusses on the potential use of monoclonal antibodies (Köhler and Milstein, 1975). Here we describe the isolation and properties of a mouse monoclonal IgG1 antibody specifically recognizing the 200 K neurofilament triplet component in higher vertebrates.

Results

Isolation

NF1 was one of several clones isolated following fusion of spleen cells from a BALB/c mouse immunized with a crude neurofilament preparation from porcine spinal cord. The neurofilament preparation contained not only the triplet polypeptides (200 K, 145 K, 68 K), but also various other cytoskeletal polypeptides (Figure 2a). Clones were screened for antibody production on frozen sections of either rat or pig cerebellum using indirect immunofluorescence microscopy with a fluorescent antibody of broad specificity for the different immunoglobulin classes. NF1, which stained neurofilaments strongly, was cloned twice by limiting dilution, again using immunofluorescence microscopy to screen the clones. After being made monoclonal, the supernatant obtained from the NF1 clone stained neurofilaments in tissue sections. The antibody had a wide species specificity since sections from human, rat, and chicken brain were all strongly stained (Figure 1a–c).

Biochemical characterization of NF1

NF1 was identified as an IgG1 by immunodiffusion tests using sera specific for each of the distinct mouse immunoglobulin subclasses. To determine which of the neurofilament polypeptides was recognized by NF1, an immune blotting procedure was used (Towbin *et al.*, 1979). A crude neurofilament preparation from porcine spinal cord or rat sciatic nerve was separated by SDS-polyacrylamide gel electrophoresis, and transferred electrophoretically to nitrocellulose paper. After reaction with NF1, and a peroxidase labelled anti-mouse antibody, the profile shown in Figure 2b was obtained. Although the neurofilament preparation contained not only neurofilaments,but also glial fibrillary acidic protein (GFA), vimentin, tubulin, actin, and other minor but yet not identified polypeptides, the NF1 antibody stained only a

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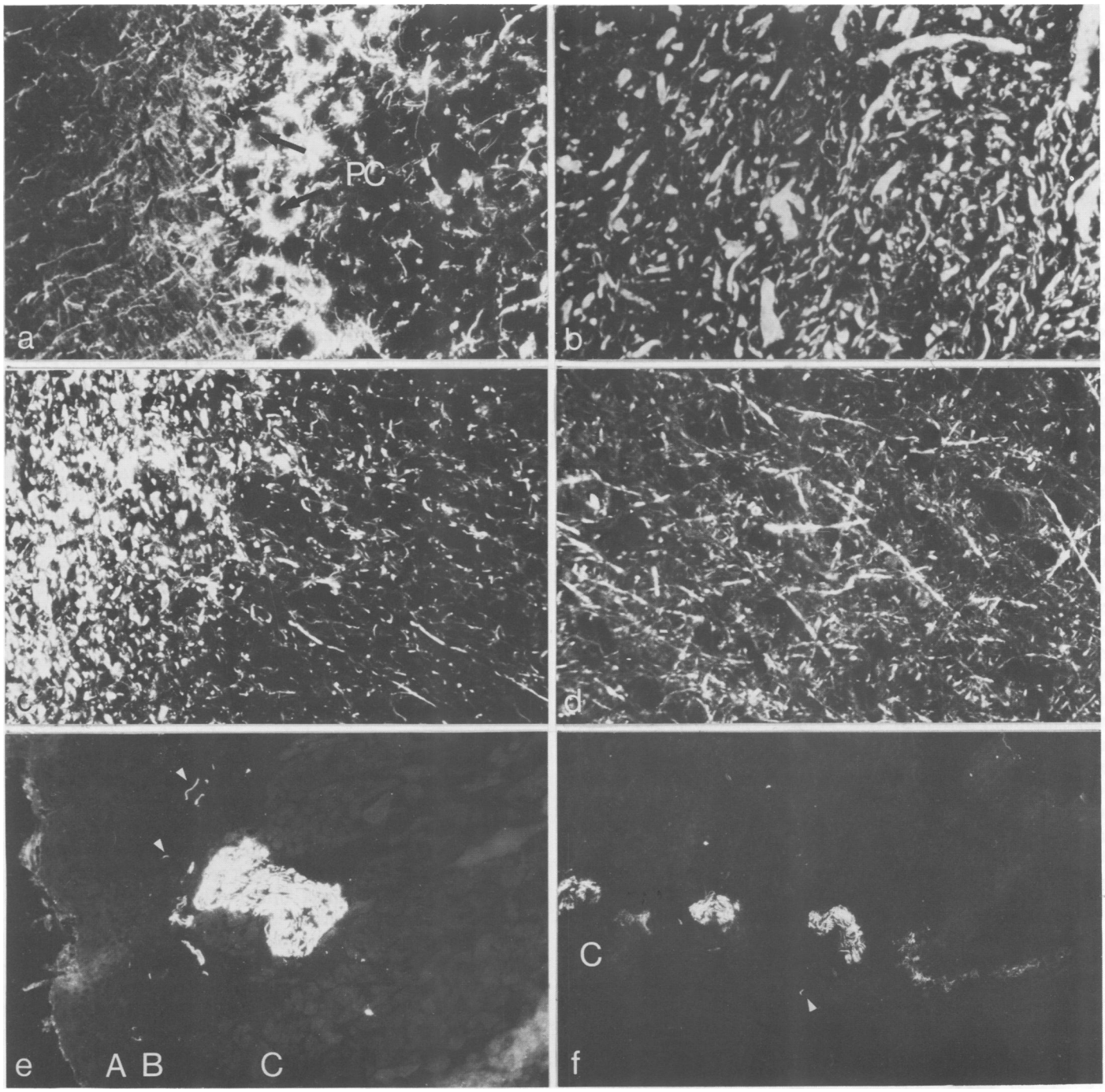

Fig. 1. NF1 staining of **(a,b)** rat cerebellum, **(c)** chick brain, **(d)** cortical region of human brain, **(e)** monkey tongue, **(f)** monkey oesophagus. **(a – c,e,f)** are frozen sections; **(d)** is alcohol-fixed paraffin-embedded material. Note the strong staining of neuronal processes and cell bodies visible in all these specimens. Neuronal processes in the white matter **(b**, left **c)** as well as in the granule cell layer (e.g., right **c)** are strongly positive, as are the basket cell processes surrounding the less strongly labelled Purkinje cells (PC) in **(a)**. In non-neuronal tissues NF1 reveals both bundles of neurofilaments, as well as smaller neuronal processes (arrows) are visualized. Inspection of the figures and particularly of Figure 1e and f shows that neither epithelia (A) nor the cells of the lamina propria (B) nor cells in the muscle layers (C) are labelled by NF1. Thus NF1 staining seems specific for neurofilaments. Magnifications **(a,c,d)** x 300, **(b)** x 450, **(e,f)** x 200.

single band with a molecular weight of ∿200 K (Figure 2b). When a crude rat neurofilament preparation was used, again only a single band at 200 K was stained. The identity of the stained band with the 200 K component of the neurofilament was proven by staining parallel strips with a polyclonal antibody specific for the 200 K component (data not shown).

Characterization of NF1 on cells in culture

The NF1 antibody stained neurofilaments in dorsal root ganglion cells in culture (Figure 3). The pictures resembled those obtained with polyclonal antibodies which recognize each of the three neurofilament polypeptides. Processes were strongly stained (e.g., Figure 3a). In addition, a comparison of fluorescence and phase pictures showed that not all dorsal root ganglion cells with a neuronal morphology were stained with the NF1 antibody (data not shown). This confirms the results with polyclonal antibodies where a subpopulation of neurones which could not be stained with any of the conventional neurofilament antisera was noted and confirmed by

electron microscopy (Shaw *et al.*, 1981a; Shaw and Weber, 1981; Sharp *et al.*, 1982). The NF1 antibody did not stain cells of a non-neuronal morphology in these cultures. In addition, the following cell types were tested and showed no filamentous staining: (a) cells containing prekeratin and vimentin filaments, i.e., HeLa (human), PtK2 (rat kangaroo); (b) cells containing GFA and vimentin filaments, i.e., U333 CG/343 MG (human); (c) cells containing only vimentin filaments, i.e., HUT 14 (human), 3T3 (mouse); (d) cells containing desmin and vimentin filaments, i.e., BHK 21 (hamster). We conclude, therefore, that the NF1 antibody recognizes only neurofilaments and none of the other intermediate filament classes. (Figures 3b, c).

Dorsal root ganglion neurones were also used to investigate the distribution of the NF1 antibody by immunoelectron microscopy. It has previously been shown that an antibody specific for the 200 K polypeptide gave discontinuous decoration of neurofilaments in these cultures, whereas antibodies to the 145 K and 68 K components gave continuous decoration (Sharp *et al.*, 1982). The same experiment was performed with the NF1 antibody and gave the results shown in Figure 3d. Labelling seems to be associated with almost all filaments but is clearly discontinuous. Although it is difficult to measure the separation of the antibody molecules exactly, the minimum separation seems to be ∿1000 Å. Control experiments in which either the NF1 antibody was omitted, or in which cell lines containing the vimentin type of intermediate filaments were substituted for the dorsal root ganglion neurones, showed no labelling of intermediate filaments. This experiment therefore also supports the finding that NF1 is specifically directed against the 200 K neurofilament polypeptide.

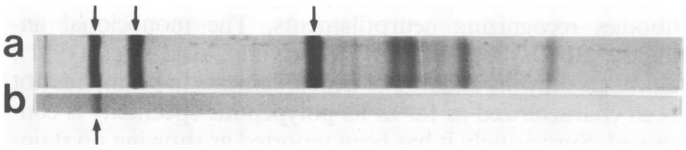

Fig. 2. Immune blot of NF1 supernatant against a crude neurofilament preparation from porcine spinal cord. The gel in **a** was stained with Coomassie blue, and shows not only the neurofilament triplet proteins (arrows) but also a variety of other polypeptides. The immune blot is shown in **b**. Only the 200 K neurofilament polypeptide is stained by the immunoperoxidase reaction (arrow).

Characterization of NF1 antibody on tissue sections

The NF1 antibody stains neurofilament profiles in rat, chicken, human and monkey brain. Representative staining patterns for NF1 on frozen sections of rat cerebellum are shown in Figure 1a and b. Neurofilaments in white matter are strongly stained (Figure 1b), as are neurofilaments in the processes of the basket cells which surround the Purkinje cells (Figure 1a). NF1 also stains neurofilaments in white matter and the granule cell layer in frozen sections of chick brain (Figure 1c) and in alcohol-fixed and paraffin-embedded sec-

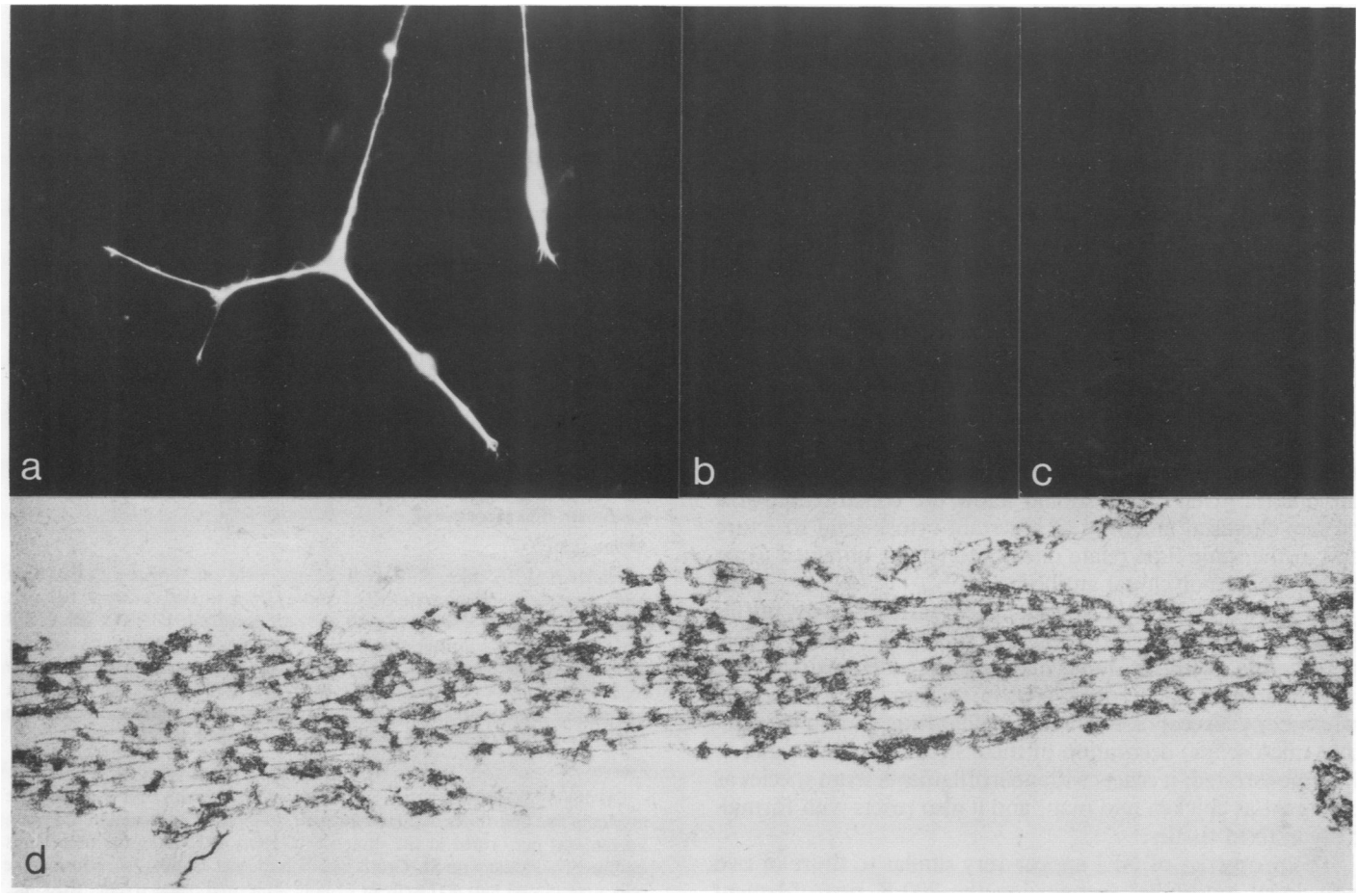

Fig. 3. NF1 staining of (**a**) dorsal root ganglion neurones from rat, (**b**) the human glioma line U333CG/U343MG and (**c**) the human fibroblast line HUT 14. Note the strong staining of the neuronal processes shown in (**a**) and the lack of staining in (**b**) and (**c**). (**d**) Immunoelectron microscopy of cytoskeletons of dorsal root ganglion neurons after treatment with NF1 in the indirect technique, followed by embedding and sectioning. Note the discontinuous distribution of the antibody reaction products along each neurofilament. Magnifications: (**a**) x 550, (**b**,**c**) x 650, (**d**) x 120 000.

tions of the cortical region of human brain (Figure 1d). The same results were obtained if the sections were treated with formaldehyde prior to the acetone step. Reaction of NF1 in different regions of rat brain was investigated in more detail because of the possibility of comparing the results with those obtained with conventional antibodies (Yen and Fields, 1980; Schnitzer *et al.*, 1981; Shaw *et al.*, 1981b). Neurofilaments in the caudate nuclei as well as the corpus callosum were strongly positive. Dendrites of pyramidal cells of the hippocampus stained very poorly, if at all, with NF1, whereas staining of a parallel section with a conventional antibody specific for the 68 K neurofilament polypeptide showed strong staining of these cells (data not shown). The lack of staining of pyramidal cells with NF1 is consistent with it being directed against the 200 K neurofilament polypeptide since a polyclonal antibody specific for the 200 K polypeptide also shows very little staining of these cells (Shaw *et al.*, 1981b).

The specificity of NF1 for neurofilaments is also apparent when tissues other than brain were examined. Thus, for instance Figure 1e shows a large neurofilament bundle as well as a number of smaller processes in monkey tongue lying in the muscle layer of this tissue (for staining of this tissue with conventionally produced antibodies to neurofilaments and other intermediate filament types see Osborn *et al.*, 1981). Figure 1f shows staining by the NF1 antibody on monkey oesophagus. Again the staining is restricted to neurofilaments, and again comparison with profiles obtained with conventionally produced antibodies to prekeratin, vimentin, desmin and neurofilaments shows the specificity of NF1 for neurofilaments.

Discussion

Ideally, monoclonal antibodies for use in studying the distribution of cytoskeletal elements in cells and in tissue sections should fulfil the following criteria: (1) when tested in an immune blotting procedure they should recognize only a single polypeptide; (2) they should recognize only a single identifiable structure in immunofluorescence and immunoelectron microscopy; (3) they should recognize this structure and only this structure in as wide a range of species as possible, including man; (4) for certain applications the ability to react with tissues after routine formaldehyde fixation although not essential in general terms may be helpful for problems in human pathology. Although different features of a monoclonal antibody may be interesting in some studies, the criteria given above should allow the construction of a protein-chemical anatomy of a certain cytoskeletal structure and at the same time relate directly to results obtained using conventional polyclonal antibodies.

As shown above, the monoclonal IgG1 antibody fulfills these criteria. Thus, in the immune blot it recognizes only a polypeptide with a molecular weight identical to the neurofilament 200 K triplet protein; in immunofluorescence microscopy it recognizes only neurofilaments and, in the electron microscope, decoration of these structures can be directly demonstrated; it reacts with neurofilaments from species as divergent as chicken and man; and it also reacts with formaldehyde-fixed tissues.

The properties of NF1 appear very similar to those of two polyclonal antibodies recognizing the 200 K neurofilament polypeptide, in that such antibodies are not a marker for all neurones. NF1 does not decorate all neuronal cells in cultured rat dorsal root ganglion cultures in agreement with previous *in vivo* and *in vitro* studies establishing that a major subpopulation of such neurones lack neurofilament expression (Duce and Keen, 1977; Shaw and Weber, 1981). Furthermore, certain neurone types of the adult rat brain, rich in neurofilaments, as assayed by electron microscopy and immunofluorescence microscopy with polyclonal antibodies against the 145 K and 68 K protein, do not decorate with NF1. Whether the lack of decoration by either NF1 or the polyclonal 200 K antibody is due to the absence of the 200 K polypeptide, to the expression of an immunologically distinct 200 K protein, or perhaps to the activation of a protease specific for the 200 K remains to be determined. The use of NF1 on tissues such as tongue and oesophagus illustrates the potential of neurofilament-specific antibodies to study the innervation of non-neuronal tissue and to map the arrangement of neuronal processes.

Two other laboratories have reported monoclonal antibodies recognizing neurofilaments. The monoclonal antibody BF10 described by Wood and Anderton (1981), although showing staining of neurofilaments in brain, has not been characterized as far as its polypeptide specificity is concerned. Surprisingly it has been reported as showing no staining of heart, liver, and kidney sections where one would expect to see staining of at least some neuronal processes. A monoclonal antibody recognizing not only neurofilaments but also all other classes of intermediate filaments, and in addition other polypeptides, has been reported by Pruss *et al.* (1980).

NF1 should be of use, not only in studies of neurofilament ultrastructure, but also in mapping neurofilament development in brain, to study the innervation of non-neuronal tissue and to map the arrangement of neuronal processes in these tissues. The isolation of NF1 also represents a step towards the development of a set of monoclonal antibodies able to specifically label only one of the five currently known intermediate filament types.

Materials and methods

Antigen preparation

A crude neurofilament preparation was isolated from porcine spinal cord by the modification of the procedure of Delacourte *et al.* (1980) described by Geisler and Weber (1981). Porcine spinal cord (250 g) was homogenized at 4°C in 400 ml of 0.1 M MES containing 1 mM EGTA and 0.05 mM $MgCl_2$ (pH 6.5). Insoluble material was removed by centrifugation first at 29 000 *g* for 60 min and then at 78 000 *g* for 30 min. The supernatant was made 20% in glycerol, incubated for 20 min at 37°C and centrifuged at 20°C for 45 min at 275 000 *g*. This preparation was the generous gift of Dr. N. Geisler and E. Kaufmann (this laboratory).

Immunization

Female BALB/c mice, 6–8 weeks of age, were immunized with the crude neurofilament preparation described above. Prior to immunization one part neurofilament pellet was mixed with one part phosphate buffered saline, and one part Freund's adjuvant and sonicated. Complete adjuvant was used for the first injection; incomplete adjuvant for subsequent injections. Each mouse received 3–4 mg of protein on day 0, 16, 39 and 52. Testing of the mouse sera by indirect immunofluorescence microscopy showed that the sera were positive on rat brain sections at dilutions of at least 1 to 60.

Fusion

On day 55 spleen cells from two mice were fused with cells of the mouse myeloma line Sp2/O-Ag14 (Shulman *et al.*, 1978) in an approximate 1:1 ratio. Fusion was performed in the presence of PEG 4000 using the procedures outlined by Fazekas de St. Groth and Scheidegger (1980). After fusion the cells were placed into 400 wells in 24-hole multi-well plates (Nunc, Roskilde, Denmark), and 2 days later the cells were subjected to HAT medium. The medium was changed on day 8 and twice a week thereafter. The supernatants were assayed by immunofluorescence microscopy on frozen sections of rat cerebellum (see below). Positive hybridomas were cloned at least twice by

limiting dilution. NF1 was also grown as an ascites in BALB/c mice. Approximately 1 x 10^6 cells were injected i.p. into a mouse primed with Pristane (Ega Chemical Co., Steinheim, GFR).

Assays on tissue sections

Supernatants from hybridoma clones were screened on 5 μ frozen sections of rat cerebellum. Sections were fixed with acetone for 10 min at $-10°C$, air-dried and then incubated for 45 min at 37°C with 10 μl supernatant. After washing well with phosphate buffered saline, 10 μl of fluorescently labelled rabbit anti-mouse (IgA + IgM + IgG) (Cappel Laboratories, Cochranville, PA) was then added, and the sections incubated for a further 45 min. After washing in buffer, sections were rinsed in 96% alcohol and mounted in MOWIOL 4-88. NF1 was tested also on frozen sections of chicken brain and on alcohol-fixed and paraffin-embedded sections of human brain (Altmannsberger *et al.*, 1981). Frozen sections of tongue and oesophagus were obtained from material from a 2–3 day old Callithrix monkey (a gift from Dr. H.Rothe).

Assay on dorsal root ganglion cells

Dorsal root ganglion cells were placed in culture (Shaw and Weber, 1981) and used after 1–2 days. For immunoelectron microscopy, cytoskeletons were prepared and processed as described in Sharp *et al.* (1982) using NF1 as the first antibody and rabbit anti-mouse (IgA + IgM + IgG) (Cappell Laboratories) as the second antibody. After reaction with both antibodies the samples were fixed and embedded in Epon. Thin sections parallel to the substratum were then cut.

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References

Altmannsberger,M., Osborn,M., Schauer,A., and Weber,K. (1981) *Lab. Invest.*, **45**, 427-434.

Anderton,B.H., Thorpe,R., Cohen,J., Selvendran,S., and Woodhams,P. (1980) *J. Neurocytol.*, **9**, 835-844.

Chiu,F.-C., Korey,B., and Norton,W.T. (1980) *J. Neurochem.*, **34**, 1149-1159.

Dahl,D. (1980) *FEBS Lett.*, **111**, 152-156.

Delacourte,A., Filliatreau,G., Botteau,F., Biserte,G., and Schrevel,J. (1980) *Biochem. J.*, **191**, 543-546.

Duce,I.R., and Keen,P. (1977) *Cell Tissue Res.*, **185**, 263-277.

Fazekas de St. Groth, and Scheidegger,D. (1980) *J. Immunol. Methods*, **35**, 1-21.

Geisler,N., and Weber,K. (1981) *J. Mol. Biol.*, **151**, 565-571.

Hoffman,P., and Lasek,R.J. (1975) *J. Cell Biol.*, **66**, 351-366.

Köhler,G., and Milstein,C. (1975) *Nature*, **256**, 495-497.

Lazarides,E. (1980) *Nature*, **283**, 249-256.

Liem,K.H., Yen,S.-H., Salomon,G.D., and Shelanski,M.L. (1978) *J. Cell Biol.*, **79**, 637-645.

Mori,H., and Kurokawa,M. (1980) *Biomed. Res.*, **1**, 24-31.

Mori,H., Komiya,Y., and Kurokawa,M. (1979) *J. Cell Biol.*, **82**, 174-184.

Osborn,M., Geisler,N., Shaw,G., and Weber,K. (1981) *Cold Spring Harbor Symp. Quant. Biol.*, **46**, in press.

Pruss,R.M., Mirsky,R., Raff,M.C., Anderton,B., and Thorpe,R. (1980) *J. Cell Biol.*, **87**, 178a.

Schlaepfer,W.W., and Freeman,L.A. (1978) *J. Cell Biol.*, **78**, 653-662.

Schnitzer,J., Franke,W.W., and Schachner,M. (1981) *J. Cell Biol.*, **90**, 435-447.

Sharp,G.A., Shaw,G., and Weber,K. (1982) *Exp. Cell Res.*, in press.

Shaw,G., and Weber,K. (1981) *Exp. Cell Res.*, **136**, 119-125.

Shaw,G., Osborn,M., and Weber,K. (1981a) *Eur. J. Cell Biol.*, **24**, 20-27.

Shaw,G., Osborn,M., and Weber,K. (1981b) *Eur. J. Cell Biol.*, **26**, 68-82.

Shulman,M., Wilde,C.D., and Köhler,G. (1978) *Nature*, **276**, 269-270.

Towbin,H., Staehelin,T., and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.

Willard,M., and Simon,C. (1981) *J. Cell Biol.*, **89**, 198-205.

Wood,J.N., and Anderton,B.H. (1981) *Biosci. Rep.*, **1**, 263-268.

Yen,S.H., and Fields,K.L. (1981) *J. Cell Biol.*, **85**, 115-126.