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Monoclonal antibodies recognize individual neurofilament triplet proteins

(intermediate filaments/unique and shared antigenic determinants)

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ABSTRACT **A series of 12 monoclonal antibodies has been prepared against crude or purified individual bovine neurofilament (NF) triplet proteins (designated as P68, P150, and P200). Six of the antibodies recognized unique determinants on individual NF subunits. The other six recognized shared determinants on at least two NF triplet proteins. Six of the antibodies recognized separated, immobilized individual NF in nitrocellulose paper. By the peroxidase-antiperoxidase technique, all of the antibodies stained neurons and their processes in a manner identical to the well-known distribution of NF in both the central and peripheral nervous system. These results strongly suggest that the NF subunits are distinct but related proteins and that the smaller NF subunits (P68 and P150) are not derived from larger NF proteins.**

Neurofilaments (NF), the intermediate filaments of neurons, are composed of three individual proteins (NF triplet proteins) with molecular weights of 68,000, 150,000, and 200,000 (P68, P150, and P200, respectively). Heterologous antisera against these triplet proteins have been prepared in laboratory animals by various research groups to obtain monospecific antibodies to each of the triplets. However, most of these antisera possess crossreacting activities. For example, Liem (1) reported that antisera to bovine P68 or P150 proteins crossreacted with P200; Anderton *et al.* (2) described antiserum to P150 protein that also recognized P200 on gels; Yen and Fields (3) found that antiserum to P200 protein crossreacted with P150 and P68 proteins in rocket immunoelectrophoresis; and Autilio-Gambetti *et al.* (4) found that antisera to P68, P150, and P200 reacted strongly with its corresponding NF subunit and weakly with the other two subunits. We have raised both rabbit and guinea pig antisera to P68, P150, and P200 proteins and have obtained antisera with different degrees of crossreactivity to other NF triplet proteins (5).

The present study was designed to investigate further the degree of antigenic overlap among the NF subunits. Using the hybridoma technique, we describe the preparation and characterization of a series of monoclonal antibodies and demonstrate unequivocally the presence of unique and shared determinants among the NF triplet proteins.

MATERIALS AND METHODS

Preparation of Highly Purified Triplet Proteins. NF proteins were isolated and purified from bovine spinal cord by a procedure reported by this laboratory (5). NF-rich preparations are usually stored in 8 M urea at −70°C. Further purification of the individual triplet proteins P68, P150, and P200 was carried out by either two-cycle preparative electrophoresis in a 7.5% gel in a Tris/glycine (6) buffer system overnight with two Hoeffer semi-preparative disc gel units (5) or continuous elution preparative electrophoresis with an LKB 7900 Uniphor apparatus (7). The former preparation was used in immunization of rats for fusion with myeloma cells and the latter preparation was used in the enzyme-linked immunosorbent assay (ELISA) (5) for the screening of monoclonal antibodies.

Cell Fusion. Lewis rats were used for immunization with bovine NF proteins. Two fusions were performed. For the first, a Lewis rat was immunized with crude bovine NF proteins in complete Freund adjuvant injected into foot pads and subcutaneous sites. A second immunization was done in the same manner 1 month later except that crushed gels containing P200 protein at 100–200 μg/ml in phosphate-buffered saline (P_i/NaCl) were used. Immunization for the second fusion was performed in a similar fashion except crushed gels containing P150 protein at 100–200 μg were used for both primary and secondary immunization. A small sample of blood was collected 1 wk after the second immunization and the antisera were tested by ELISA for antibody production. Only rats with serum yielding more than 1:1,000 antibody titer as detected by ELISA were selected for fusion. These rats received a third injection of antigen intravenously 3 wk later with 100–200 μg of a Uniphor-purified NF subunit. Five days later the rats were anesthetized and the spleens were removed for fusion. Splenocytes were fused with cultured mouse myeloma cells SP 2/0-Ag14 (Human Genetic Cell Center, Philadelphia) according to the method described by McKearn (8). SP 2/0 is a hypoxanthine phosphoribosyltransferase-negative cell that does not produce immunoglobulin. Hybrid growth was selected in medium containing hypoxanthine/aminopterin/thymidine. After fusion, hybridoma colonies were screened by ELISA for the secretion of antibodies directed against each of the NF subunits. Each positive clone was subcloned at least once in agarose (9) to obtain stable monoclones. Initial characterization of the immunoglobulin classes secreted by the positive clones was performed by [^{35}S]methionine labeling of hybrid cells and analysis of hybrid supernatant by NaDodSO_4/polyacrylamide gel electrophoresis followed by autoradiography (10).

ELISA. Screening of positive clones was conducted by using the ELISA as described (5). Separate solutions of bovine P68, P150, or P200 in aliquots of 50 μl diluted to a concentration of 2 μg/ml were used to coat the 96-well polystyrene Linbro plates. Horse serum (10%) in P_i/NaCl was used to block nonspecific absorption on wells and for dilution of antibodies. Peroxidase-labeled anti-rat IgG (Cappel Laboratories, Cochranville, PA) at a 1:1,000 dilution was used as the second antibody. Reaction product was quantitated by reading optical density at 450 nm on a Multiscan Spectrophotometer.

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Abbreviations: NF, neurofilament(s); ELISA, enzyme-linked immunosorbent assay; P_i/NaCl, phosphate-buffered saline; PAP, peroxidase-antiperoxidase.

Absorption Studies. These experiments were designed to determine whether those monoclonal antibodies that recognized both P150 and P200 reacted with the same or different antigenic determinants on these two NF triplet proteins. Accordingly, the monoclonal antibodies against P150 and P200 were separately absorbed with P68, P150, and P200 by incubating spent supernatant in a series of wells that had been coated with 50 μl of a solution of 2 μg/ml of each of these individual NF subunits. After a 2-hr incubation at room temperature, the spent supernatant was transferred to another well coated with the same concentration of NF subunit. This process was serially repeated until the monoclonal supernatant was devoid of activity against NF subunits in the wells. The anti-NF subunit reactivity of supernatant serially absorbed in this manner was quantitated by the ELISA method as described above.

Localization of NF Proteins by the Peroxidase-Antiperoxidase (PAP) Method. The PAP technique of Sternberger (11) was used on 30-μm-thick vibratome sections of cerebellum from normal human brains that were fixed at autopsy by perfusion with a fixative containing mercuric chloride and formalin. This fixative was prepared by mixing a saturated mercuric chloride solution with stock strength (≈38%) formalin to give a 4% mercuric chloride/8% formaldehyde fixative. Perfused rat cerebellum, spinal cord, and nerve were similarly used for PAP studies. Undiluted culture medium from each clone was used directly on tissue sections. For controls, either spent supernatant from SP2 clones or NF-absorbed hybridoma supernatant was used.

Immunological Detection of NF Proteins on Nitrocellulose Paper. NF triplet proteins were separated on a 5–7.5% NaDodSO$_4$/polyacrylamide gradient gel by using NF preparations from bovine spinal cord. The transfer to nitrocellulose paper was performed as described by Towbin *et al.* (12). The electrode buffer was 25 mM Tris/192 mM glycine/20% (vol/vol) methanol, pH 8.3. The transfer was conducted at room temperature for 3 hr with a current of 150 mA. After transfer, the sheet of nitrocellulose paper was cut into 1-cm strips, left overnight in 10% ethanolamine/0.1 M Tris·HCl, pH 8.0/0.25% bovine serum albumin, and washed in P$_i$/NaCl. The strips were incubated with hybridoma supernatants at room temperature on a shaker for 2 hr, washed with five changes of P$_i$/NaCl for 30 min, and then incubated with 2 ml of a 1:50 dilution of fluorescein-conjugated rabbit anti-rat IgG (Cappel) for 2 hr. Finally, the strips were exhaustively washed in P$_i$/NaCl, air dried, and viewed with a long-wave UV lamp.

RESULTS

Screening of Monoclonal Antibodies Against Individual NF Subunits by ELISA. The spleen cells from two Lewis rats immunized with different preparations of bovine NF were fused with the nonsecreting variant (SP 2/0-Ag14) of mouse myeloma cells for a total of two fusions. For the first fusion, a crude NF preparation was used for the primary immunization but highly purified P200 was used for subsequent challenges. ELISA on serum from this animal before fusion showed recognition for all three NF proteins. Two hybridoma colonies resulted, one being specific for P200 only, the other specific for P68. The antibody classes of these two clones were determined by internally radiolabeling the cells with [^{35}S]methionine and subsequently by analyzing the supernatant on NaDodSO$_4$/polyacrylamide gel electrophoresis. Both hybridomas in this first fusion secreted IgM antibodies. For the second fusion, only purified P150 was used for the immunization. ELISA on serum from this rat showed reactivity for P150 and P200, but not P68 proteins. A total of 10 hybridoma colonies was selected. Four hybridoma colonies were specific for P150 only. The others had varying degrees of crossreactivity with P200. None was found to cross-react with P68. Table 1 summarizes the activities of our monoclonal antibodies against each of the NF triplet proteins. All hybridomas were subcloned at least once and the strongest subclones were selected.

Visualization of Monoclonal Antibody Binding to Individual NF Subunits. The ability of the monoclonal antibodies to recognize antigenic sites on different NF proteins was also examined in preparations of enriched bovine NF that had been chromatographed on a 7.5% polyacrylamide gel and transferred electrophoretically onto nitrocellulose paper. NF proteins recognized by the monoclonal antibodies were revealed by the fluorescein-conjugated goat anti-rat IgG under UV light (Fig. 1). All 12 antibodies listed in Table 1 were tested. Only 6 of these—namely, 1.1A$_3$, 1.4A$_5$, 1.4A$_7$, 1.1A$_9$, 2.4F$_{10}$, and 4.3F$_9$—reacted with protein bands on nitrocellulose paper and were visualized by the fluorescent second antibody. The reactive antibodies were of the IgG class as determined by [^{35}S]methionine metabolic labeling. For unknown reasons, none of the IgM monoclonal antibodies stained NF antigens with the immunoblot method, although both the IgG and IgM monoclonals behaved the same in tissue sections (see below). All reactive monoclonal antibodies showed specificity identical to that detected by ELISA. The binding of 1.1A$_9$, 2.4F$_{10}$, and 4.3F$_9$ to NF subunits is illustrated in Fig. 1. Both 1.1A$_9$ and 4.3F$_9$ recognized shared determinants on P150 and P200, whereas 2.4F$_{10}$ detected determinants only on P150 protein.

In addition, experiments were conducted to determine whether the monoclonal antibodies that recognized both P150 and P200 reacted with the same or different antigenic determinants on these two NF triplet proteins. Accordingly, spent supernatant from such clones was absorbed with P68, P150, and P200 separately. Absorption with P68, even after 12 transfers, did not remove any reactivity to P150 and P200. In contrast, absorption with P150 removed all reactivity after 5 serial transfers on P150-coated wells, whereas absorption with P200 required 12 serial transfers to remove all activities. Furthermore, removal of antigenic activities against P150 also removed reactivity against P200 and removal of activity against P200 also extinguished the activity against P150. This indicates that one antigenic determinant is shared by both of these subunits (data not shown).

Immunocytochemical Localization of NF Antigens in Neural Tissue. The specificity of the monoclonal antibodies was further confirmed by immunocytochemical studies with the PAP tech-

Table 1. NF subunit specificity as detected by rat monoclonal antibodies

Rat monoclonal antibodies	Antibody titer against		
	P68	P150	P200
3.2F$_2$	1:128	–	–
2.1B$_4$	–	–	1:256
1.1A$_3$	–	1:32	1:2
1.1A$_9$	–	1:64	1:2
1.4A$_5$	–	1:16	1:2
1.4A$_7$	–	1:16	1:2
1.3B$_6$	–	1:8	–
1.3G$_{12}$	–	1:32	1:2
2.3F$_6$	–	1:8	–
2.4F$_{10}$	–	1:256	–
3.3E$_{11}$	–	1:256	–
4.3F$_9$	–	1:512	1:128

Antibody titer is given as the final dilution of spent supernatant from rat monoclonal antibodies yielding a 50% colorimetric reaction in ELISA. –, No recognition.

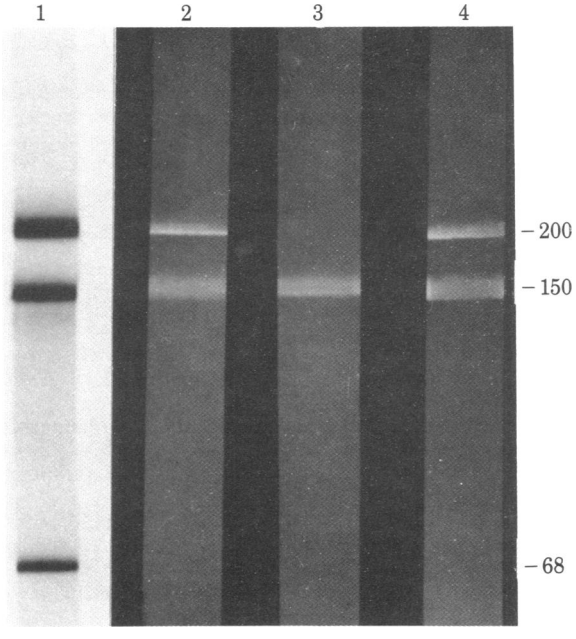

FIG. 1. Detection of individual NF subunits by fluorescein-conjugated antibodies on nitrocellulose paper. Enriched NF preparations were separated and blotted as described. The monoclonal antibodies used were: undiluted culture medium from clone $1.1A_9$ (lane 2), undiluted culture supernatant from clone $2.4F_{10}$ (lane 3), and a 1:10 dilution of culture medium from clone $4.3F_9$ (lane 4). For comparison, a Coomassie blue stain of NF triplet proteins is also illustrated in lane 1. P68, P150, and P200 are indicated by 68, 150, and 200, respectively.

nique. In screening studies that used human cerebellum as well as rat spinal cord, sciatic nerve, and cerebellum, all the monoclonal antibodies showed patterns of neuronal staining that was characteristic for the well-known distribution of NF. For example, in sections of human cerebellum, axons in the cerebellar white matter stained as did neurons in all three layers of the cerebellar cortex (Fig. 2). Glial and vascular elements were negative. The same pattern of NF protein localization was seen with all NF monoclonal antibodies tested, irrespective of their NF subunit specificities. The controls were negative (Fig. 2).

DISCUSSION

This study was undertaken to establish unequivocally the presence of unique and shared determinants among the NF triplet proteins by using the hybridoma technique of Kohler and Milstein (13). To this end, 12 monoclones were isolated. One clone secreted monoclonal antibodies specific to P68, another to P200, and four were found to secrete antibodies specific to P150. Eight others recognized determinants on both P150 and P200 which the absorption experiments indicated were shared by these NF subunits. The successful preparation of these monoclonal antibodies against unique and shared determinants on NF triplet proteins thus settles the controversy over the crossreactivity of heterologous antisera due putatively to contaminants in NF protein immunogens (1–5). In contrast, other anti-NF monoclonal antibodies that have been reported recognize determinants shared by more than one NF subunit or other intermediate filaments (14, 15).

The immunocytochemical localization of NF proteins described here parallels the well-known distribution of NF in neurons and their processes in both the central and peripheral nervous system. All 12 monoclonal antibodies showed similar specificities for NF structures that were indistinguishable from heterologous antisera. However, the intensity of peroxidase

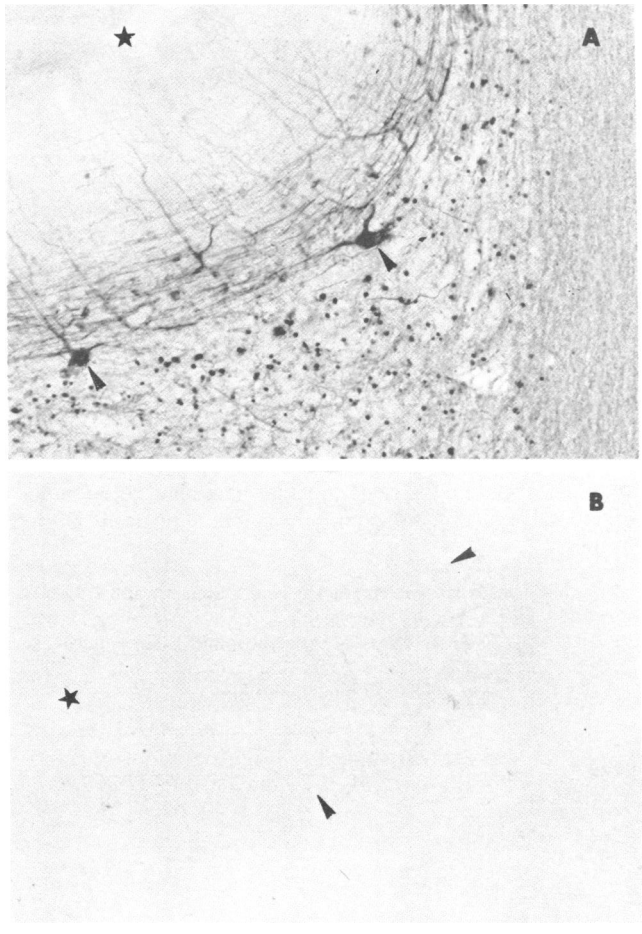

FIG. 2. Photomicrographs of experimental (*A*) and control (*B*) PAP experiments performed on adjacent sections of human cerebellum. The primary antibody in *A* was $4.3F_9$, whereas that in *B* was SP 2/0 supernatant. Otherwise both sections were identically treated with the PAP method. The sections have not been counterstained. The star in each picture indicates the sulcus between adjacent cerebellar folia. Axons in the white matter are stained in *A* (at right in photograph) as are multiple neuronal perikarya and processes in the molecular, Purkinje, and granular cell layers of cerebellum. Two intensely stained Purkinje cells are identified with arrows. In *B* no neuronal or axonal staining is evident. The arrows point to two unstained Purkinje cell bodies in the Purkinje cell layer. (×210.)

staining by the different antibodies did not always correspond to the potency of the antibodies as detected by ELISA. Some of these antibodies—for example, $2.4F_{10}$ and $1.1A_9$—showed high titers in ELISA (Table 1) but stained weakly by the PAP technique. In contrast, $1.4A_7$ and $1.3B_6$ were weak antibodies by ELISA but intensely stained neurofilament proteins in tissue sections. The reason for this discrepancy is not yet known. However, it should be noted that our monoclonal antibodies were made against bovine NF and tested by the PAP technique on rat and human tissue sections. It is well known that NF from mammalian species have slightly different molecular weights and different cross-species specificity (14, 16, 17). It is possible that our monoclonal antibodies are specific for antigenic determinants in bovine NF and variably crossreact with analogous NF determinants in rat and human tissues. Alternatively, fixation parameters significantly affect the visualization of antigens in tissue and these factors may explain the different results seen in the ELISA compared with rat tissue (18, 19).

The present findings together with those of others (14) indicate that there are at least four classes of antigenic determi-

nants in NF. The most specific class of antigenic determinants is restricted only to one NF subunit of a given species and is not common to other NF subunits, other types of intermediate filaments, or the same NF subunit in another species (NF, subunit, and species specific). The second most specific class is restricted also to only one NF subunit but it is present in more than one species (NF and subunit specific). A third class of determinants is present on two or more of the NF triplet proteins but is not shared by other intermediate filaments (NF specific). The fourth class is shared by NF and several others or perhaps all types of intermediate filaments (intermediate-filament specific). From this it is possible to conclude that there are both common and unique antigenic determinants of intermediate filaments.

It can be anticipated that monoclonal antibodies that recognize these different classes of NF antigens will be useful tools for analyzing individual NF subunits at a molecular level in order to better define the structure and function of these important cytoskeletal elements in normal and pathologically altered neurons.

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1. Liem, R. K. H., Yen, S. H., Salomon, G. D. & Shelanski, M. L. (1978) *J. Cell Biol.* **79,** 637–645.
2. Anderton, B. H., Ayers, M. & Thorpe, R. (1978) *FEBS Lett.* **96,** 127–131.
3. Yen, S.-H. & Fields, K. (1981) *J. Cell Biol.* **88,** 115–126.
4. Autilio-Gambetti, L., Velasco, M. E., Sipple, J. & Gambetti, P. (1981) *J. Neurochem.* **37,** 1260–1265.
5. Schlaepfer, W. W., Lee, V. & Wu, H. L. (1981) *Brain Res.* **226,** 259–272.
6. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
7. Hogue-Angeletti, R., Wu, H. L. & Schlaepfer, W. W. (1981) *J. Neurochem.* **38,** 116–120.
8. McKearn, T. J. (1980) in *Monoclonal Antibodies*, eds. Kennett, R. H., McKearn, T. J. & Bechtol, K. B. (Plenum, New York), pp. 368–369.
9. Kennett, R. H. (1980) in *Monoclonal Antibodies*, eds. Kennett, R. H., McKearn, T. J. & Bechtol, K. B. (Plenum, New York), pp. 372–373.
10. Haas, J. B. & Kennett, R. H. (1980) in *Monoclonal Antibodies*, eds. Kennett, R. H., McKearn, T. J. & Bechtol, K. B. (Plenum, New York), pp. 407–411.
11. Sternberger, L. A. (1974) *Immunocytochemistry* (Prentice-Hall, Englewood Cliffs, NJ), pp. 129–171.
12. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
13. Kohler, G. & Milstein, C. (1975) *Nature (London)* **256,** 495–497.
14. Pruss, R. M., Mirsky, R., Raff, M. C., Thorpe, R., Dowding, A. J. & Anderton, B. H. (1981) *Cell* **27,** 419–428.
15. Eng, L. F., Sternberger, N. & Sternberger, L. (1982) *Trans. Am. Soc. Neurochem.* **13,** 108 (abstr.).
16. Franke, W. W., Schmid, E., Winter, S., Osborn, M. & Weber, K. (1979) *Exp. Cell Res.* **123,** 25–46.
17. Lazarides, E. & Boltzer, D. R. (1979) *Cell* **14,** 429–438.
18. Culling, C. F. A., Reid, P. E. & Sinnott, N. M. (1980) *J. Histotechnol.* **3,** 10–19.
19. Berod, A., Hartman, B. K. & Pujol, J. F. (1981) *J. Histochem. Cytochem.* **29,** 844–850.