Localization of the neurofilament protein in neuroblastoma cells by immunofluorescent staining

(calf brain/100 Å filaments/immunofluorescence/tubulin/tropomyosin)

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ABSTRACT Neurofilament protein (54,000–56,000 daltons) has been localized in murine neuroblastoma cells by indirect immunofluorescent staining with antisera to purified calf brain neurofilament protein. In some cells with only short processes, specific staining of fibrous material was present in the perinuclear region while in other cells similar fibers, coiled to varying degrees, were present in other regions of the cytoplasm. In cells with longer processes a stained fiber extended throughout each process. The staining pattern observed followed the distribution of bundles of 100 Å filaments as determined by electron microscopy. The fibers did not stain with antisera to tubulin or tropomyosin. The observations reported strongly indicate (i) that neurofilament protein isolated from calf brain is antigenically related to a component of the bundles of 100 Å filaments in neuroblastoma cells, and (ii) that the neurofilament protein is an integral part of bundles of 100 Å filaments in neuroblastoma cells, while neither tubulin nor tropomyosin is present in these bundles.

Filaments approximately 100 Å in diameter have been described in several cell types (1–7). Similar filaments, referred to as neurofilaments, are commonly found in axons and dendrites of nervous tissue (8) and in cells derived from this tissue, including neuroblastoma cells (9, 10). In neuroblastoma cells the bundles of 100 Å filaments are located in the perinuclear region and extend out into the cell processes, where they are oriented parallel to the long axis of the process (9, 10). Neurofilaments approximately 100 Å in diameter have been isolated and partially characterized from the giant axon of squid (11) and from axons of calf brain (12–14).

Very little is known about the function of these filaments, though it has been suggested that they play a role in axoplasmic transport (15, 16). The major reason for this lack of understanding is that no procedure has been discovered that specifically interferes with the integrity of these filaments. In addition, no specific characteristics that could be used for an assay of the filaments or filament subunits have been identified.

To try to overcome some of these problems and to enable us to study the distribution of these filaments rapidly in a large number of cells under various physiological conditions, we have prepared an antibody to the major protein component (neurofilament protein 54,000–56,000 daltons) of isolated neurofilaments from calf brain axons. In this paper we report the presence of a protein in cultured neuroblastoma cells that can be stained by the indirect fluorescein-labeled antibody technique with antisera against the neurofilament protein. The results presented indicate that the distribution of this protein in neuroblastoma cells corresponds to that of the bundles of 100 Å filaments as determined by electron microscopy.

Abbreviation: NaDodSO₄, sodium dodecyl sulfate.

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MATERIALS AND METHODS

Purification of Neurofilament Protein. Neurofilaments were isolated from the white matter of calf brain (14). A minor modification was introduced by the following substitution. Four repeated centrifugations (18,000 × g for 15 min) of the demyelinated axoplasm suspended in solution B (0.01 M phosphate buffer and 0.01 M mercaptoethanol, pH 6.5) were made through an equal volume of buffer D (0.85 M sucrose, 0.01 M Tris-HCl, pH 0.5, and 0.01 M mercaptoethanol) instead of a centrifugation on a discontinuous sucrose density gradient. To obtain the major protein, the isolated neurofilaments were solubilized in sodium dodecyl sulfate (NaDodSO₄) and their components separated by NaDodSO₄-acrylamide gel electrophoresis (17). The major protein band (54,000–56,000 daltons) was then cut out from the NaDodSO₄-gel (14) and the protein eluted by electrophoresis. This protein will be referred to as the neurofilament protein.

Production of Antisera. Antisera to the neurofilament protein were produced in rabbits by injecting each animal subcutaneously with 500 μg of the electrophoretically purified protein in complete Freund’s adjuvant. Four weeks later an additional 250 μg of the protein in incomplete Freund’s adjuvant was given, and 10 days after that the rabbits were bled from the ear artery. Control sera were obtained from the same rabbits before immunization. The production of antisera to tubulin (18) and tropomyosin (19) has been previously described.

Immunodiffusion Tests. The antisera obtained were analyzed by Ouchterlony’s double diffusion test (20), with 1% agarose in buffer E (0.15 M NaCl, 0.1% NaDodSO₄, and 0.5% Triton X-100) on microscope slides (14) at room temperature for 24–48 hr. In some tests electrophoretically pure neurofilament protein (1 mg/ml) was added to the antiserum well and allowed to diffuse into the agarose for 10 min before application of the antiserum.

Absorption of Antisera. Absorption was done by incubating 0.1 ml of antiserum with 5–15 μg of neurofilament protein (total volume of 0.5 ml) for 30 min at 37° and then overnight at 4°. The resulting immune precipitate was removed by centrifugation and the absorbed antiserum tested by immunofluorescence.

NaDodSO₄-Polyacrylamide Gel Electrophoresis was done with 7.5% acrylamide gels (17). The procedure of Weber and Osborn (21) was used to determine the molecular weight of the purified neurofilament protein.

Neuroblastoma Cells. Murine neuroblastoma cell line N2A-N2A (22), which is a subclone of the N2A cell line isolated from the C-1300 tumor (23), was grown for 48–60 hr in Lab Tek tissue culture chambers (Miles Laboratories) on glass slides in

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The protein used as an antigen for immunization was purified from neurofilaments isolated from calf brain. Electrophoretic analysis of the isolated calf brain filaments on discontinuous NaDodSO4 gels show several bands when 30 μg were applied to the gel (Fig. 1a). The major protein band (neurofilament protein) constitutes about 90% of the total protein, as determined by scanning the gel stained with Fast Green in a spectrophotometer. When the electrophoretic mobility of the neurofilament protein was compared with that of other structural proteins, the protein migrated between the fastest moving tubulin subunit (T1) and actin (Fig. 1c, d, and e), which is in agreement with results reported by Davison and Winslow (13). The molecular weight of the major neurofilament protein was found to be 54,000-56,000 by NaDodSO4-acrylamide gel electrophoresis at neutral pH. This result is very similar to the results previously obtained by others (13, 14).

To obtain pure neurofilament protein for immunization, the neurofilament band (Fig. 1a) was cut out and eluted from the NaDodSO4-acrylamide gel by electrophoresis. When 100 μg of the eluted protein was checked in the same gel system, it migrated as a single band and showed no trace of contamination from adjacent bands (Fig. 1b). The specificities of the antisera to neurofilament protein were determined by immunochemical procedures. The basic premise of these procedures is that if two proteins are sufficiently similar to be indistinguishable by some method of analysis, they will react in a competitive manner in tests performed with antibodies against one of the proteins. The immunological reactivities of the antigens were examined using agar gel diffusion, immunoelectrophoresis, and immunofluorescence.
FIG. 3. Indirect immunofluorescent staining of N2A-A4 mouse neuroblastoma cells with (a) an antiserum against neurofilament protein (1/50) and (b) antiserum against neurofilament protein absorbed with neurofilament protein before staining (1/50) as described in Materials and Methods. ×435. Arrow: a cell with a tightly coiled mass of stained fibrous material. Arrow heads: a cell in which stained fibers are present throughout the two longer processes.

analyzed by Ouchterlony's double-diffusion test (20). When antisera against neurofilament protein were tested against either neurofilament protein or neurofilaments, only one precipitin line was obtained in each case (Fig. 2a). A reaction of fusion between these two lines without observable spurs (Fig. 2a) strongly indicates that the antisera used are specific to the electrophoretically pure neurofilament protein. No precipitin lines were observed when antisera against neurofilament pro-

FIG. 4. Staining patterns observed in the N2A-A4 mouse neuroblastoma cells with an antiserum against neurofilament protein (1/50), using the indirect immunofluorescent technique. Cells with short processes (a–c) showing stained fibrous material extending throughout a considerable portion of the perinuclear region (a). Similar fibers, coiled to varying degrees, in the cytoplasm at various distances from the nucleus (b and c). A cell with processes of intermediate length showing a stained fiber extending from the cell body into the process (d). Note the wavy appearance of the proximal end of the fiber. A cell with a long process showing a stained fiber extending from the cell body throughout the whole length of the process (e).
tein were tested against chick brain tubulin or buffer E. In addition, no precipitin lines were observed when the preimmune control sera were tested against neurofilaments or neurofilament protein (Fig. 2a).

The specificities of the antisera to neurofilament protein were further analyzed by testing the ability of the electrophoretically pure neurofilament protein to block the formation of the precipitin line observed when antisera against neurofilament protein were tested against neurofilaments. The formation of the precipitin line was in each case completely blocked by purified neurofilament protein (Fig. 2b), indicating that the precipitin line observed without blocking was indeed due to the interaction between the electrophoretically pure neurofilament protein and the antibody against neurofilament protein.

The antisera to calf brain neurofilament protein, when used to study the distribution of the neurofilament protein in N2A-A4 murine neuroblastoma cells by the indirect fluorescein-labeled antibody technique, specifically labeled characteristic fibers in the cytoplasm. The staining patterns observed are illustrated in Figs. 3a and 4. In cells with only short processes the stained fibers often extended throughout a considerable portion of the perinuclear region (Fig 4a). Occasionally they encircled the nucleus completely and gave a ringlike staining pattern. In other cells similar fibers, coiled to varying degrees of compactness, were present. In Fig. 3a, a cell with a single tightly coiled mass is illustrated, whereas in Fig. 4b and c masses of more loosely coiled cytoplasmic fibers can be seen. In cells with long processes, a stained fiber extended from the cell body throughout the whole length of the process (Figs. 3a and 4e). Characteristically, in some of the cells with processes of intermediate length, the proximal end of the fiber had a wavy appearance (Fig. 4d).

It is noteworthy that in cells that did not have long processes only a single mass of these fibers was present. Although in cells with two long processes a stained fiber may be present in both (Fig. 3a), these were usually parts of a continuous fiber that extended out into the processes from the cell body. Occasionally, however, the connection between the stained fibers in the two processes of the same cell could not be conclusively demonstrated.

When the neuroblastoma cells were treated with preimmune control sera, no staining was observed. The specific staining of the fibers was almost totally abolished when the antiserum was absorbed with the electrophoretically purified neurofilament protein and then used in the immunofluorescent test (Fig. 3b). Antisera specific for chick brain tubulin and chick skeletal muscle tropomyosin did not label similar fibers in immunofluorescent tests.

Neuroblastoma cells grown in parallel cultures were examined by electron microscopy to determine the distribution of the bundles of 100 Å filaments. Serial sections showed that bundles of 100 Å filaments were located in the perinuclear region of the cytoplasm in some cells (Fig. 5a and b); in others they were located in the cell body farther away from the nucleus. When cells with the longer processes were examined, a bundle of 100 Å filaments running parallel to the long axis of the process was observed (Fig. 5c). These observations are in agreement with previous reports describing the distribution of 100 Å filaments in neuroblastoma cells (9, 10).

**DISCUSSION**

The results indicate that the neurofilament protein isolated from myelinated axons of calf brain is related antigenically to a protein present in the cytoplasm of murine neuroblastoma cells. These results agree with those of Yen et al. (14), who recently reported that neurofilaments isolated from human brains or rabbit sciatic nerve crossreact with antiserum to calf neurofilaments. The distribution of the stained fibers detected by antibodies to calf brain neurofilament protein corresponded to that of bundles of 100 Å filaments as observed in these cells by electron microscopy. However, to verify that the neurofilament protein is indeed an integral component of the 100 Å filament it will be necessary to demonstrate specific binding of the
antibody to the 100 Å filaments at the ultrastructural level using the ferritin-labeled antibody technique. It has been suggested that tubulin is a component of 100 Å filaments (6, 24-28) since these filaments increase in number as the number of microtubules decrease after treatment of cells with drugs such as colchicine and vinblastine. It has also been suggested that tropomyosin might be present in 100 Å filaments in fibroblasts (2). Even though antisera to tropomyosin and to tubulin both give characteristic staining patterns in neuroblastoma cells (unpublished results), neither of the antibodies stained the fibrous structures that were labeled by antisera to neurofilament protein. Since these fibrous structures correspond in distribution to the bundles of 100 Å filaments, our results suggest that neither tropomyosin nor tubulin is a component of the bundles of 100 Å filaments. In addition, these observations suggest that the microfilaments, microtubules, and 100 Å filaments from nervous tissue contain antigenically different proteins and are not different polymeric forms of the same protein. This is in agreement with the results of Yen et al. (14), who showed that antisera to the neurofilament protein cross-react with neither brain tubulin nor actin.

Although there was considerable variation in the distribution of stained fibers in different neuroblastoma cells, the results presented indicate that the distribution of these fibers changed when the cells developed long processes. Our results are consistent with the view that the fiber extends out into the process from the cell body as it develops. Since stained fibers were absent in some of the short processes, it is possible that the fiber migrates into the process after its outgrowth. Another possibility is that the processes lacking stained fibers were retracting, and that the retraction of the fiber preceded the retraction of the process. The use of time-lapse cinematography followed by immunofluorescent staining of the same cells may enable us to study changes in the distribution of the fibrous material during the differentiation of neuroblastoma cells.

Very little is known about the function of the 100 Å neurofilaments. The availability of antisera to the neurofilament protein may now make it possible to investigate the synthesis, assembly, and turnover of this protein, which is abundantly present in mammalian axons. The ability of these antisera to label fibers composed of 100 Å filaments by immunofluorescence should enable us to study changes in their distribution under various physiological conditions. Such studies may contribute to our understanding of the role of these filaments in normal cell processes. In addition, it would be of interest to determine whether or not the proteins in 100 Å filaments from nervous tissues of different mammalian species are antigenically related to proteins in bundles of 100 Å filaments in cell types unrelated to nervous tissues.

It has been reported that large bundles of 100-200 Å filaments accumulate in nerve cells of patients suffering from neurofibrillary degeneration (8), including Wallerian degeneration (29-31), Pick’s disease (32), and Alzheimer’s disease (33). They can also be found in patients undergoing vincristine therapy (34, 35) and can be induced experimentally by aluminum (35). It is possible that the relation between the neurofilament protein and the large bundles of filaments observed in these disorders of the nervous system can be determined by immunocytochemical techniques.

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