Neurofibrillary Tangles in Senile Dementia of the Alzheimer Type Share an Antigenic Determinant With Intermediate Filaments of the Vimentin Class

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A monoclonal antibody produced by a hybridoma between a plasmacytoma cell and a spleen cell from a mouse immunized with human brain microtubule fraction was demonstrated to stain neurofibrillary tangles of senile dementia of the Alzheimer type (SDAT). The antibody recognized at least 50% of the tangles in neuronal perikarya isolated from SDAT brains and stained a filamentous network in HeLa cells, fibroblasts, and astrocytes. It did not stain skin epithelial cells or neurons isolated from normal brains but reacted with Z bands in skeletal muscle. The monoclonal antibody stained coils in colchicine or colcemid-treated cultured cells in a pattern characteristic of 10-nm intermediate-sized filaments. Immunoblotting of Triton-insoluble cytoskeletal proteins of cultured cells electrophoresed in SDS polyacrylamide gels showed that the antigenic determinant is present in proteins of molecular weight 58,000 which comigrates with vimentin. Thus, it appears that the neurofibrillary tangles in SDAT share an antigenic determinant with vimentin. (Am J Pathol 1983, 113:373–381)

IN ALZHEIMER'S DISEASE and senile dementia of the Alzheimer type (SDAT), one of the most striking histopathologic lesions is the presence of intraneuronal argentophilic fibrillary tangles composed primarily of paired helical filaments[1] as well as 15-nm straight filaments.[2–4] Immunologic methods have been used to study the molecular nature of neurofibrillary tangles. Antibodies raised against normal human microtubule fractions have been shown to bind to neurofibrillary tangles in SDAT brain[5,6] and to tangles found in postencephalitic Parkinsonism and progressive supranuclear palsy.[7] These results suggest that abnormal neurofibrous elements found in these pathologic conditions are immunologically related to proteins present in normal brain. Microtubule fractions used in these studies were prepared by two cycles of assembly and disassembly; and they contained tubulin, ferritin, microtubule-associated proteins, neurofilament triplet proteins, and other minor polypeptides.[6] Previous studies showed that polyclonal antibodies against this microtubule preparation had multiple specificities and that the anti-tangle activities were not removed by adsorption with purified tubulin, ferritin, or neurofilament triplet proteins. In order to determine which minor component in the microtubule fraction might be responsible for eliciting the anti-tangle antibodies, we used hybridoma technology to make monoclonal antibodies to a normal human brain microtubule preparation. One hybridoma clone recognized tangles of SDAT. It also stained intermediate filaments in cultured cells.

Materials and Methods

Preparation of Monoclonal Antibodies

The microtubule fraction was prepared from a neurologically normal human brain obtained in autopsy of a 79-year-old individual by two cycles of assembly and disassembly according to the method of Shelanski et al[8] with minor modifications.[6] A female BALB/c mouse was immunized intraperitoneally

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with 150 μg of microtubule fraction in phosphate-buffered saline (PBS) and 4 mg alum. The second immunization was performed in the same manner 3 weeks later without alum. Six further injections were carried out at biweekly intervals. Cell fusions were done according to the method described by Kennett.[9] Indirect immunofluorescence was used for screening for positive clones whose supernatants stained neurofibrillary tangles or HeLa cells. Positive hybridomas were cloned in soft agar, and the selected clones were recloned again with rat fibroblasts as a feeder layer. The hybridoma cells were injected into BALB/c mice pretreated with pristane for generation of ascitic fluid.

Immunocytochemistry

Neurons were isolated from SDAT brains by serial sieving through meshes of decreasing pore size and

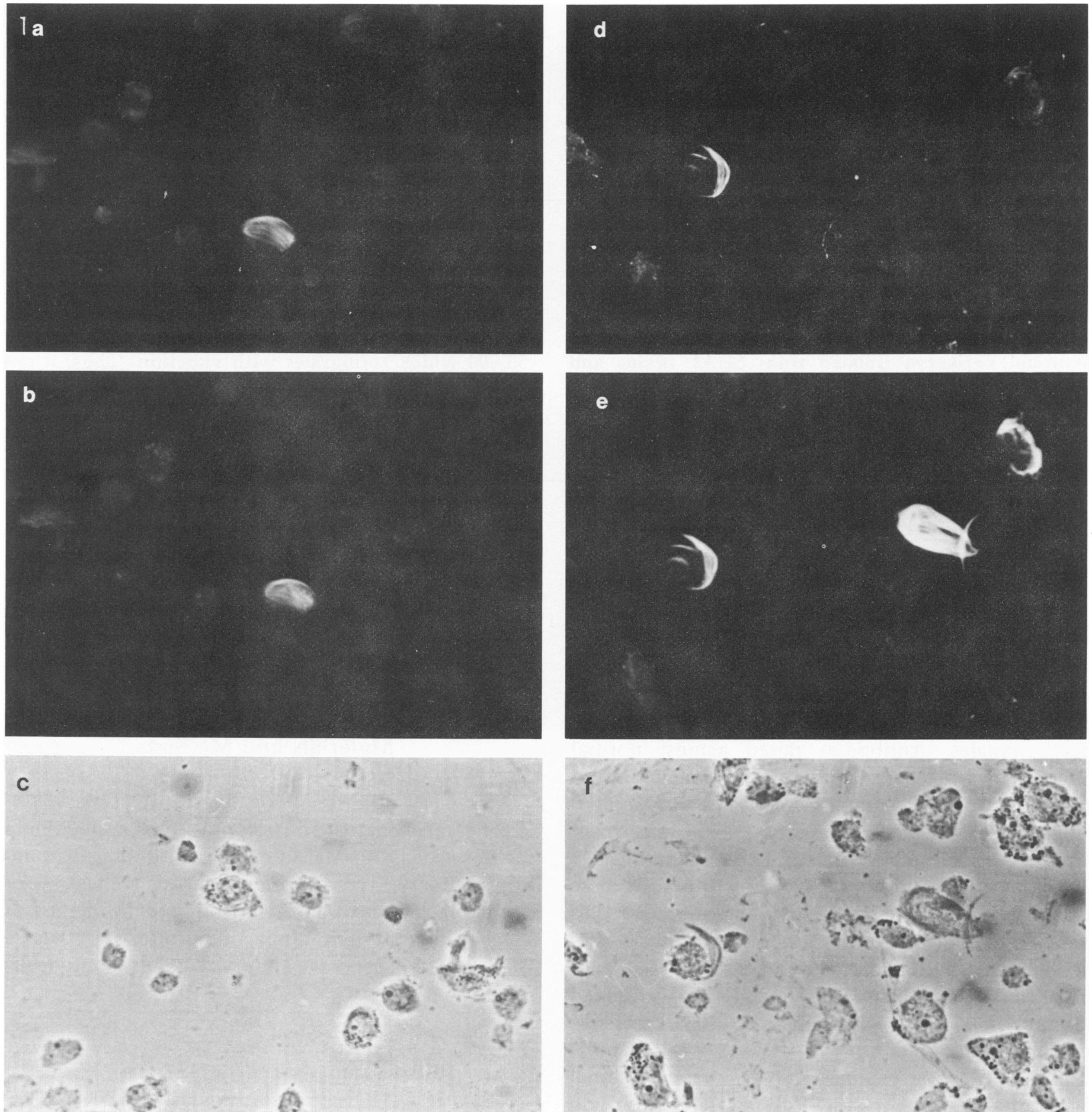

Figure 1—Double labeling of neuronal perikarya isolated from SDAT brains with the monoclonal antibody and thioflavin-S reagent. The binding of (**a** and **d**) immunoglobulins was with rhodamine-conjugated goat anti-mouse immunoglobulin and (**b** and **e**) thioflavin with green fluorescence. **c** and **f**)—Phase-contrast image of the same field as **a** and **d**, respectively. Some thioflavin-positive tangles were not stained by the monoclonal antibody as shown in **d**. (×420) (With a photographic reduction of 6%)

fractionation in density gradients.[6] The isolated neurons, suspended in hexose phosphate buffer, were smeared on microscopic glass slides coated with egg albumin. The location of the smear was marked by a diamond pencil. HeLa cells, initially a gift from Dr. Lola Reid, were maintained in culture, split, and plated on coverslips in DME medium containing 10% fetal calf serum for 1 or 2 days. Some coverslips were then treated overnight with colchicine or colcemid (10 μg/ml).

The isolated neurons or HeLa cells were fixed with anhydrous methanol for 7 minutes at −20 C. After washing with PBS, the cells were incubated with undiluted, 1:10 diluted culture medium from each clone or 1:100 to 1:200 diluted ascitic fluid from each clone for 45 minutes at room temperature, and the excess medium was washed off. The bound immunoglobulin was detected with rhodamine-conjugated goat anti-mouse immunoglobulin or rabbit anti-mouse immunoglobulin. The isolated neurons were further treated with 0.0001% thioflavin S in 10% formalin. The peroxidase-antiperoxidase (PAP) method was also used for the study of monoclonal antibody binding in neurons isolated from SDAT and normal brain tissue and HeLa cells.

The tangle-positive antibody was further characterized on cryostat sections of rat spinal cord, cerebellum, sciatic nerve, skeletal muscle, skin, and on cultured rat cerebrum cells and human fibroblasts. Details for growing cerebral cells and fibroblasts have been described previously.[10] The indirect immunofluorescence method again was used for detecting the bound immunoglobulin.

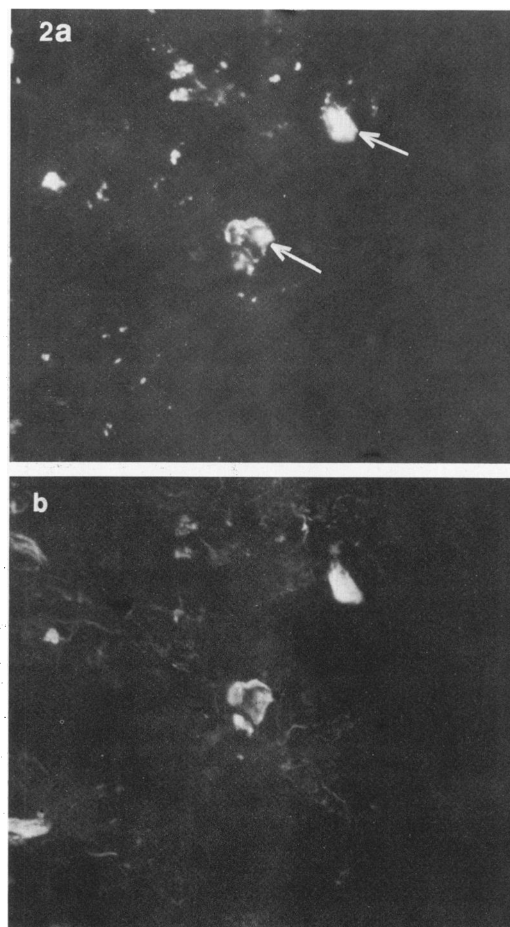

Figure 2—Double labeling of SDAT brain section with the monoclonal antibody (**a**) and thioflavin-S reagent (**b**). (×400) Out of four tangles, two (indicated by *arrows*) were positive with immunofluorescence. The small granular fluorescent immages were due to the autofluorescence of lipofuscin.

Immunologic Detection of Antigens by Protein Blotting

Four types of preparations were used in protein blotting experiments. They included human brain microtubule fraction, rat brain filament fraction, and cytoskeletal proteins of HeLa cells and of cultured cerebral cells. The cytoskeleton of cultured astrocytes contained three major polypeptides, one of which comigrated with vimentin and had peptide maps identical to those of vimentin.[11] The brain filament fraction was prepared by the axonal flotation method, and cytoskeletal proteins were prepared according to the procedure of Chiu and Norton.[12] The polypeptides in each preparation were separated on a 7.5% SDS polyacrylamide gel and transferred to nitrocellulose paper.[13] The transfer was conducted in 2.5 mM Tris, 19.2 mM glycine buffer, pH 8.3, and 20% methanol at room temperature for 3 hours with a constant current of 125 mM. Following the transfer, the nitrocellulose paper was incubated with 5% bovine serum albumin (BSA) in PBS for 90 minutes. The paper were then incubated with 1:200 diluted ascitic fluid in 5% BSA with PBS for 2 hours at room temperature, washed with PBS, incubated with biotinated labeled horse anti-mouse immunoglobulin followed by washing and incubation with avidin-biotin-peroxidase complex (Vector). The substrate for peroxidase enzymes contained 3 mg DAB and 2 μl 30% H_2O_2 in 10 ml 0.1 M Tris buffer, pH 7.6.

Adsorption Studies and Immunodiffusion

Fibroblasts (5×10^5) were homogenized in phosphate buffer and centrifuged at 12,000 g for 10 minutes. The pellet was resuspended in 500 μl of ascitic fluid diluted 10 times, incubated at room temperature for 1 hour, and centrifuged. The supernatant was diluted 20 times before use. The antibody was also incubated with two cycles of purified human brain

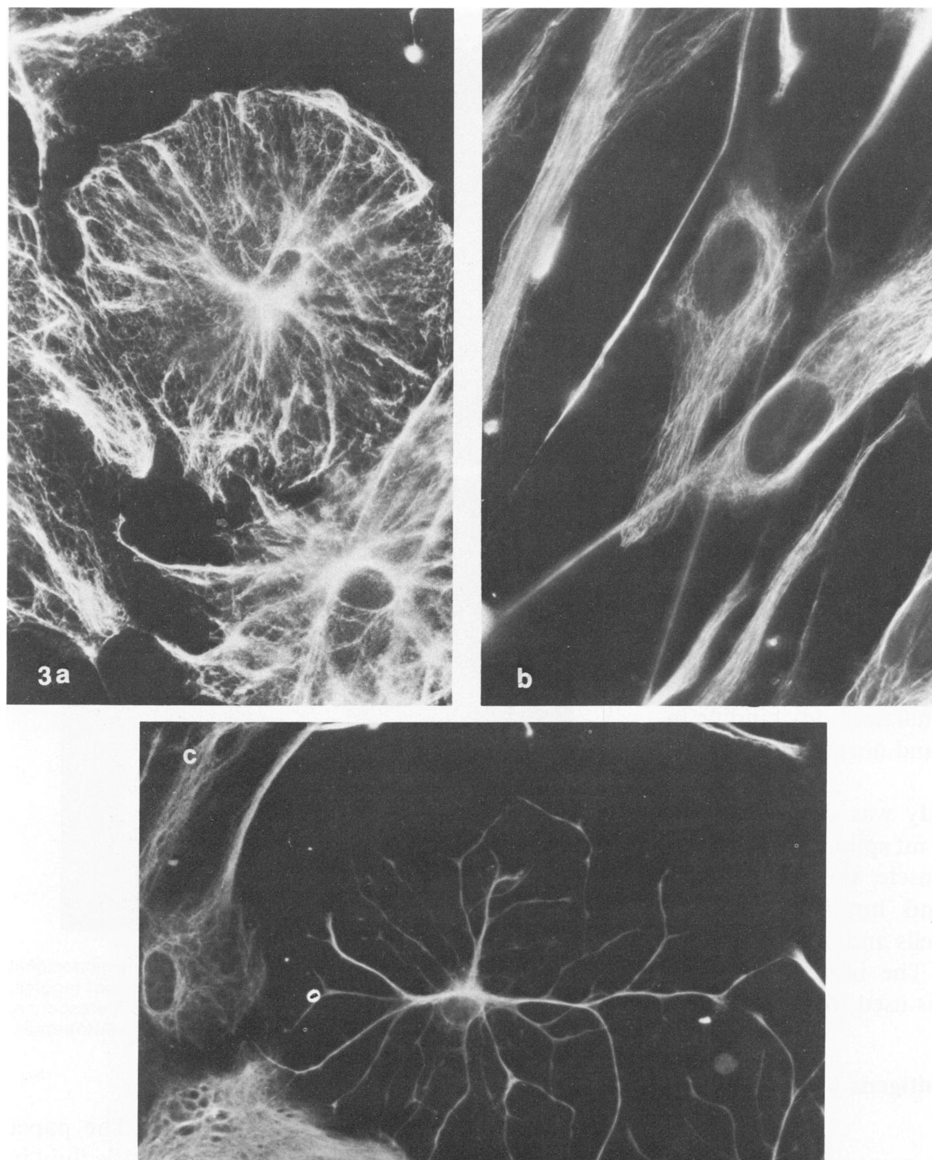

Figure 3—Immunofluorescent staining of HeLa cells (a), fibroblasts (b) and cerebral cells (c) in cultures with a monoclonal antibody which binds SDAT tangles. (×700)

microtubule fraction (14 μg proteins/μl antibodies) or vimentin (10 μg/μl antibodies) eluted electrophoretically from SDS polyacrylamide gels. After 2 hours at room temperature the mixtures were centrifuged, and the supernatants were used for immunostaining.

The hybridoma medium and ascitic fluid were tested on an Ouchterlony plate against rabbit anti-mouse immunoglobulins. After overnight incubation at room temperature, the agarose plate was pressed, dried, dialyzed against PBS, and stained with Coomassie brilliant blue.

Results

A mouse was immunized repeatedly with a human microtubule fraction, and its serum was found to stain neurofibrillary tangles of SDAT neurons. Its spleen cells were fused with SP2/0 plasmacytoma cells. The supernatants of 310 hybridomas were screened by indirect immunofluorescence. One of these stained normal neurons but not HeLa cells or neurofibrillary tangles. Twenty-three stained HeLa cells. One of these 23 stained fibrous elements in some neuronal perikarya isolated from SDAT brains. This hybridoma was of special interest and was cloned twice on soft agar. One of the selected clones with similar specificity was used in the subsequent studies. The monoclonal antibody, by immunodiffusion test, was found to be of the IgM class.

In a previous study,[14] it has been shown that thioflavin bound neurofibrillary tangles of SDAT and emitted green fluorescence under ultraviolet illumina-

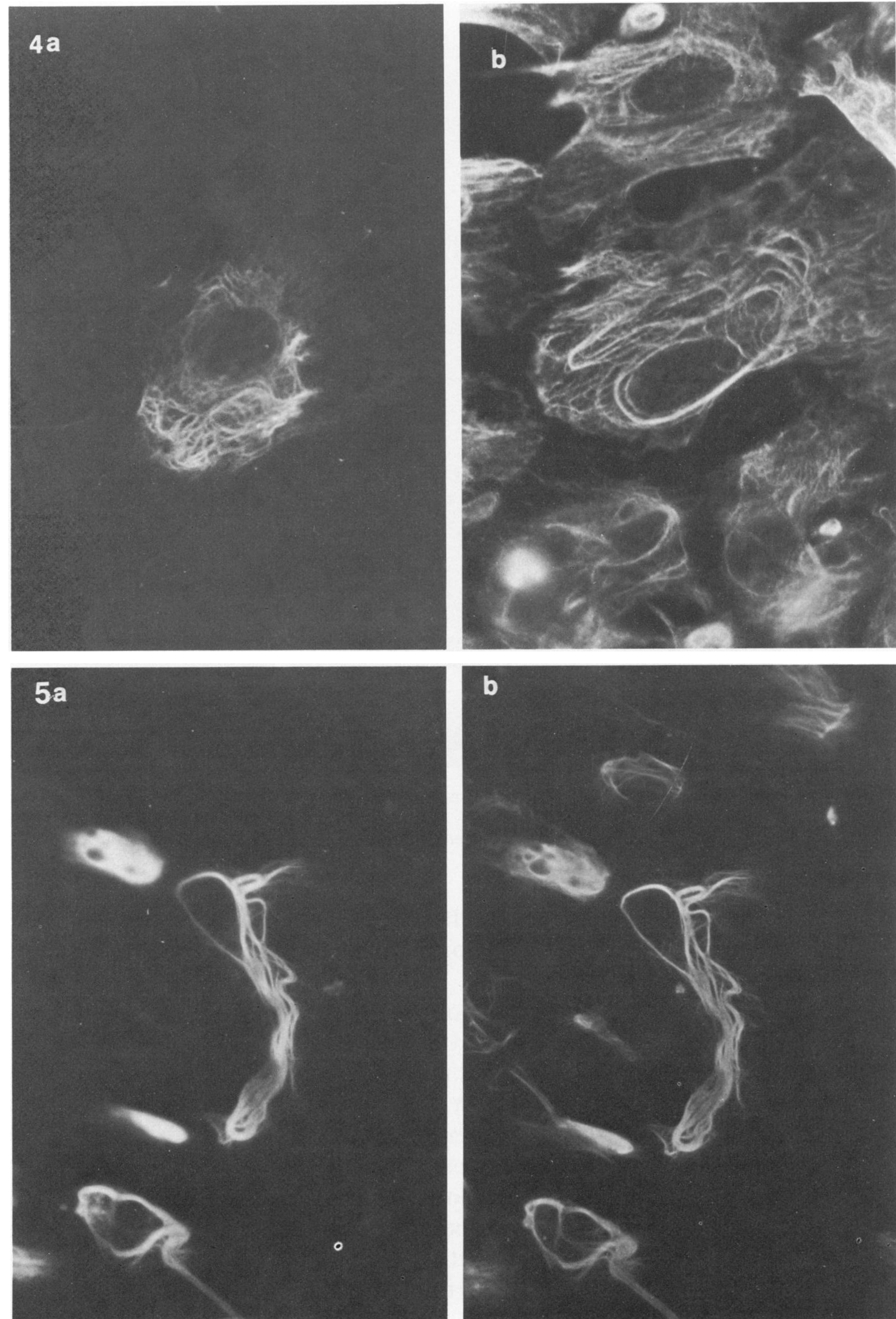

Figure 4—Immunofluorescent staining of colchicine-treated (**a**) HeLa cells and fibroblasts (**b**) with the monoclonal antibody. Coiled fibers were visible in all cells. (×700) **Figure 5**—Double labeling of cerebral cells which had been pretreated with colchicine with (**a**) rabbit anti-GF antibodies and (**b**) monoclonal antibody. Both GF-positive and GF-negative cells were stained by the monoclonal antibody. (×420)

tion. This reaction did not alter the binding sites by a polyclonal antiserum to a human microtubule fraction.[6] These sites could be detected with a rhodamine-labeled antiserum. We used this double fluorochrome immunofluorescence method to determine whether tangle-containing neurons were also stained by the monoclonal antibody. Over fifty percent of tangle-containing neurons in isolated preparations or cryostat sections of SDAT brain were stained by the monoclonal antibody (see Figures 1 and 2 for double-labeling experiments). For simplicity the antigen detected by the monclonal antibody will be referred to as tangle-related antigen.

The antibody also bound fibroblasts and newborn rat cerebrum dissociated cells (Figure 3b and 3c). The staining patterns of these cultured cells as well as HeLa cells (Figure 3a) appeared to be filamentous. Treatment of the cells with colchicine or colcemid prior to the incubation with monoclonal antibody resulted in collapsing or coiling of the filamentous

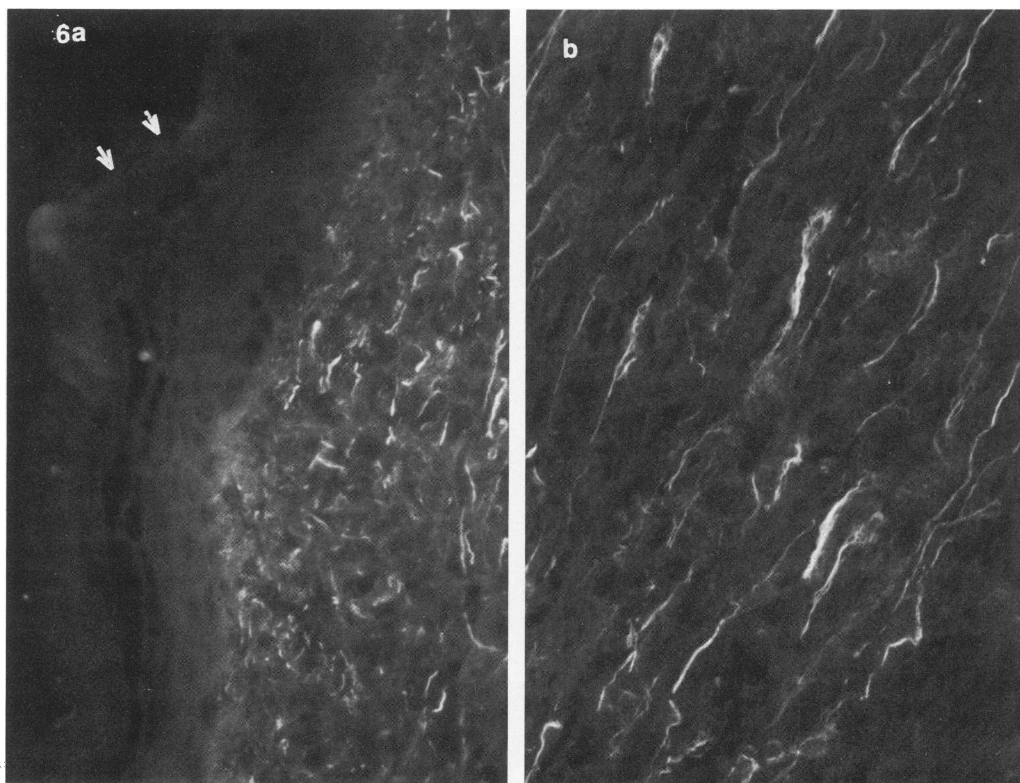

Figure 6—Immunofluorescent staining of newborn rat skin with the monoclonal antibody. **a**—Epidermis (indicated by *arrows*) was not stained, whereas the dermis was labeled. (×420) **b**—In a deeper layer of dermis the immunofluorescence-positive elements often were running parallel to each other. The staining pattern resembles that of anti-vimentin antibodies (not shown).

network (Figure 4). Also, it appears that all of the cultured cells reacted with the monoclonal antibody.

Newborn rat cerebrum dissociated cell cultures contain astrocytes and fibroblasts.[11] In order to study the relationship between tangle-related antigen and glial filaments (GF), we double-labeled the cerebrum cells with rabbit antibodies to human GF proteins and the monoclonal antibody that stains neurofibrillary tangles. The results showed that cells containing GF antigens (presumably astrocytes) and cells negative for anti-GF were both stained by the monoclonal antibody (Figure 5). The staining pattern with GF antibody in astrocytes was similar to that with the monoclonal antibody.

Incubation of the monoclonal antibody with fibroblasts, cytoskeleton, or purified vimentin removed the anti-tangle activity as well as the ability to stain filamentous networks in cultured cells. Repetitive adsorptions of the antibody with human brain microtubule fractions diminished its staining intensity. Complete removal of the anti-tangle activity was not achieved. The incomplete adsorption might be due to an insufficient amount of tangle-related antigen in the microtubule fraction.

Immunostaining of cryostat sections of newborn rat skin showed that epidermis, enriched with tonofilaments, was not stained, whereas the dermis that contains vimentin filaments was labeled (Figure 6). The fluorescence-positive elements were long, thin fibers alone or in bundles. In some areas of the section they were associated with cell bodies. The staining pattern was similar to that of rabbit anti-vimentin antibody. In rat skeletal muscle, the antigens were distributed in a pattern similar to that of Z bands (Figure 7) which have been reported to have intermediate filament proteins of vimentin and desmin.[15] Axons in sciatic nerve or spinal cord sections, known to contain neurofilaments, did not bind the antibodies, but Schwann cells surrounding the myelinated axon in sciatic nerves were stained.

The monoclonal antibody was further analyzed by the immunoblotting technique. The antibody reacted with proteins of molecular weight of 58,000 daltons in HeLa cell or cerebrum cell preparations (Figure 8) and stained weakly a 56,000-dalton protein band of the HeLa cell samples. It did not recognize proteins in both human microtubule fractions and human brain filament fractions which contain GF protein

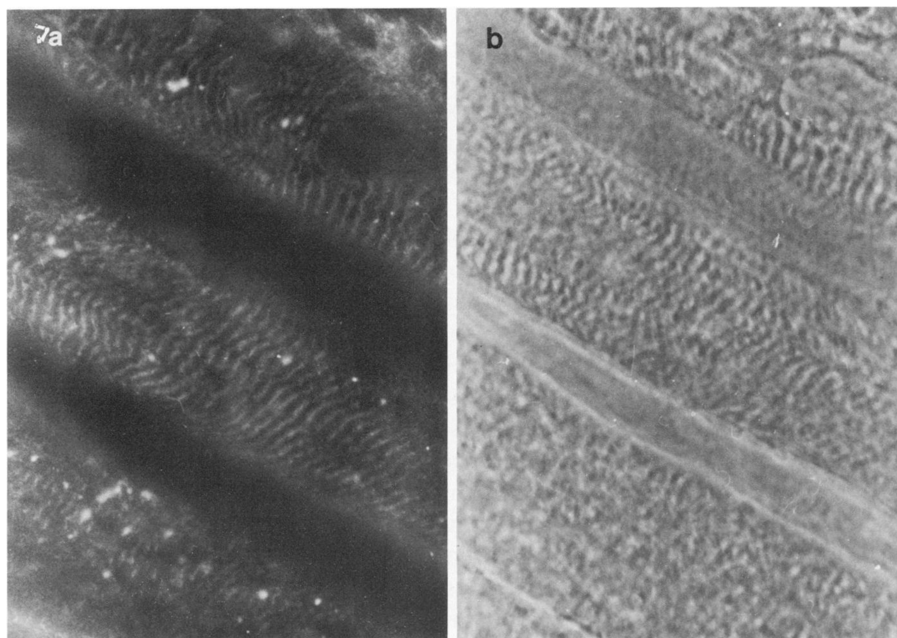

Figure 7a – Immunofluorescent staining of rat skeletal muscle. The antigens were located in Z bands, as shown in (b) the phase-contrast image of the same field. (×700)

and neurofilament triplet proteins. GF proteins in the latter preparation were easily recognized by anti-GF antibodies with the biotin-avidin-peroxidase method.

Discussion

The present study demonstrates that neurofibrillary tangles of SDAT react with a monoclonal antibody raised against an antigen from the brain of an apparently normal elderly person. The antibody, in addition, recognizes filamentous networks in a variety of cultured cells. These filaments after incubation with colchicine or colcemid form coils. This response is characteristic of 10-nm intermediate-sized filaments of the vimentin class. In tissue sections, the antigens are located in cell types which are enriched with vimentin filaments but not in cells enriched with neurofilaments or tonofilaments. Immunoblotting studies show that the monoclonal antibody stained a 58,000-dalton protein band, which is similar to vimentin in

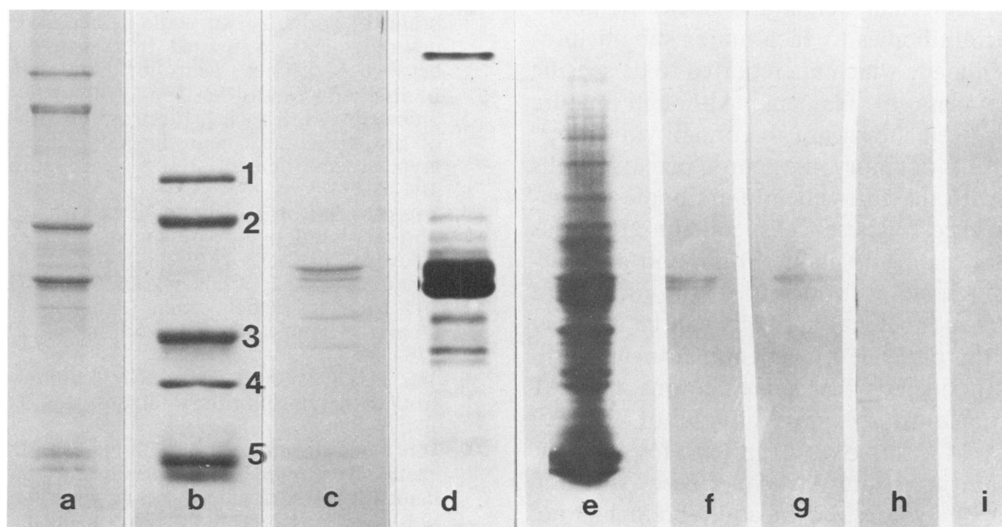

Figure 8 – The gel electrophoretic pattern of rat brain filament fraction (a), molecular weight standards (b), cerebral cells (c), two-cycle purified human brain microtubule fraction preparation (d), and Hela cells (e). Immunoblotts showing the antigenic specificities of monoclonal antibodies on triton insoluble cytoskeletal proteins of HeLa cells (f), cerebral cells (g), rat brain filaments (h), and two-cycles purified human brain microtubule fraction (i). Molecular weight standards: 1) phosphorylase (94 kd), 2) bovine serum albumin (68 kd), 3) ovalbumin (43 kd), 4) carbonic anhydrase (30 kd), 5) trypsin inhibitor (20 kd).

molecular weight and peptide maps.[11] These results suggest that neurofibrillary tangles contain a protein sharing an antigenic determinant with vimentin. The antibody gave a weak staining of a 56,000-dalton protein of HeLa cells as well. We do not know the relationship between vimentin and the 56,000-dalton protein. The smaller protein may be a variant or a degraded vimentin. We have tested a polyclonal antibody against vimentin[16] and a monoclonal antibody against intermediate filament[17] for their binding to tangles. Although these antibodies recognize vimentin of rat origin,[10,17] they do not bind well to vimentin of normal human cells, nor do they react with tangles.

Only slightly over 50% of the tangles stained with the monoclonal antibody. This may be due to the lability of the binding sites secondary to the isolation and fixation procedures. Tangles found in routine autopsy tissue sections fixed in formalin and embedded in paraffin did not stain with our monoclonal antibody. Whether this can account for the inability of a number of antibodies to vimentin and neurofilaments to stain the tangles remains to be determined. The affinity of the antibody may also play a role. Alternatively, not all of the neurofibrillary tangles contain filaments with the same antigenic determinants.

Currently, five major classes of intermediate filaments are known, including the cytokeratin of the true desmosome-expressing epithelia, the desmin fibers of certain muscle types, the glial filaments of astrocytes, the neurofilaments of neurons and the vimentin filaments in cells of mesenchymal origin.[15,18] The main protein subunit of each class of filaments has a distinct molecular weight and isoelectric point. Monospecific antibodies to each major subunit protein have been made which are reported to be specific to only one class of filament. Although mature neurons have been shown not to contain vimentin,[10] neuroblastic cells or embryonic neuron precursor cells are positive with the anti-vimentin antibodies by immunofluorescence.[19] These cells contain numerous filaments with diameters about 10 nm and of molecular weight, resembling vimentin. If the staining of tangles by the monoclonal antibody can be taken as evidence of the presence of vimentin in neurofibrillary tangles, it suggests that some neurons in SDAT are differentiated to reexpress a type of filament characteristic of embryonic neurons. However, the possibility that in certain Alzheimer cases vimentin continues to be synthesized in adult brain has not been completely ruled out. This possibility can be examined with suitable clinical material. At any rate, the role of vimentin in the pathogenesis of tangles remains to be elucidated.

Although different classes of intermediate filaments contain different proteins, recent studies on the amino acid sequences of intermediate filament proteins show that some filament proteins are structuraly related.[20,21] Moreover, several monoclonal antibodies have been shown to recognize antigenic determinants common to several classes of intermediate filament proteins.[17,22] Thus, caution should be taken in applying the immunologic data to identify proteins in the neurofibrillary tangle. It remains a possibility that the tangle-related antigen is not due to the presence of vimentin but rather due to the modification of neurofilaments. Because of the difficulty in solubilizing the tangles,[23,24] this possibility cannot be ruled out at the present time.

Recently a small number of monoclonal antibodies against neurofilament proteins have been reported to bind to tangles.[25] The interpretation of this data is subject to constraints similar to those in our studies. At any rate, our monoclonal antibody did not react with neurofilaments. These results indicate the possibility that both vimentin and neurofilaments are present in the tangle. The exact chemical composition and the role of intermediate filaments in the pathogenesis of tangles will not be resolved until they can be solubilized.

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