Neurofibrillary tangles of Alzheimer disease share antigenic determinants with the axonal microtubule-associated protein tau (τ)

(paired helical filaments/cytoskeleton/phosphorylation/immunohistochemistry)

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ABSTRACT   The relationship of the neurofibrillary tangle, found in Alzheimer disease and aged brains, to normal or abnormal cytoskeletal proteins remains elusive. Although immunohistochemical studies have yielded disparate results, most antigenic determinants localized to neurofibrillary tangles are cytoskeletal constituents normally present in neuronal perikarya or dendrites. We report light and electron microscopic immunolabeling of neurofibrillary tangles by a monoclonal antibody to the microtubule-associated protein tau (τ). Dephosphorylation of tissue slices not only increased the number of τ-positive tangles but also produced marked positive immunoreactivity of neuritic plaques. The localization of τ, an axonal protein, to neurofibrillary tangles in the perikaryon in particular suggests that abnormal synthesis, modification, or aggregation of τ may induce aberrant cytoskeletal–cell organelle interactions, subsequent interference with axonal flow, and resultant tangle formation.

The neurofibrillary tangle (NFT) is a neuropathologic hallmark of Alzheimer disease (AD) found to a lesser extent and in a more restricted distribution in nondemented elderly patients. In AD, this cytoplasmic inclusion is found within neurons within the hippocampus, amygdala, neocortex, and deep gray matter. Structurally, the NFT consists of unique paired helical filaments (PHF) (25, 26) that exhibit distinctive chemistry in that they are insoluble under a variety of denaturing conditions (1). It is not yet clear whether the NFT is composed of normal cytoskeletal polypeptides, modified cytoskeletal polypeptides, or polypeptides unique to the NFT (cf. refs. 2–14). We postulate that abnormal cytoskeletal polypeptides may predispose the neuron to pathologic cytoskeletal–organelle interactions with resultant NFT formation. Although NFTs have been probed for cytoskeletal elements normally present in cell bodies and dendrites, axonally distributed polypeptides have not been extensively studied. Using a monoclonal antibody to the microtubule-associated protein (MAP) tau (τ), found in axons of normal brains, we have assessed the distribution of this family of cytoskeletal polypeptides in AD brain tissue.

METHODS

Antibody Production. The τ-1 monoclonal antibody used in these experiments has been recently described (15). τ-1 is of the IgG2a subclass and, although the original immunogen was bovine τ, it is known to crossreact with rat and chicken τ (ref. 15 and L.I.B., unpublished observations). The immunoglobulin used in these experiments was derived from spent serum-free medium taken from the τ-1 hybridoma line that had been subcloned previously three times by limiting dilution.

Gel Electrophoresis Procedures. Frozen hand-dissected blocks of hippocampus and frontal cortex from an AD case and an age-matched nondemented control case were solubilized in sodium dodecyl sulfate sample buffer and subjected to polyacrylamide gel electrophoresis exactly as described (15). Equal aliquots of sample were loaded based upon the wet weight of the original tissue samples. The separated polypeptides were transferred to nitrocellulose, and immunoblot analysis using the τ-1 antibody was performed as described (15). The blots were photographed using Kodak contrast-process ortho black and white sheet film.

Tissue Preparation and Immunocytochemical Procedures. Hippocampus and frontal cortex used in this procedure were derived from autopsy brains of three cases of neuropathologically confirmed AD in patients 61, 66, and 70 years of age as well as from a 66-year-old man without dementing illness. The tissue had been fixed in 10% buffered formalin within 2–6 hr of death. For immunolabeling procedures, the tissue was dissected into pieces 7 × 7 mm, mounted in 4% agar on a balsa wood block, and sliced in 50-μm sections on a Lancer vibratome. The best sections were selected under a dissecting microscope for immunocytochemical experiments.

All steps were performed with constant gentle agitation. The sections were incubated in τ-1 antibody (1:10–1:25 dilution) in 50 mM Tris-buffered saline (TBS) (pH 7.2) for 16–18 hr at 0–4°C. The sections were then processed according to the instructions in the Vectastain biotin-avidin-peroxidase-based kit (Vector Laboratories, Burlingame, CA), except that 2- to 3-hr washes were used. The last wash step was at 0–4°C, and the peroxidase was developed for 4–8 min with 3,3′-diaminobenzidine (22 mg) and 30% hydrogen peroxide (10 μl) in 50 ml of ice-cold TBS. The sections were then washed for 1–15 hr in TBS and processed for light microscopy as described (16). For electron microscopy, the areas of intense label were selected for plastic embedding using a dissecting microscope. This tissue was postfixed in 2% osmium tetroxide, dehydrated in ascending concentrations of alcohol, treated with propylene oxide, and embedded in Epon. For light microscopy, the sections were photographed with and without hematoxylin counterstaining; ultrathin sections were examined on an RCA EMU-4 electron microscope with and without staining with 4% uranyl acetate and 0.1% lead citrate. In some experiments, the tissue slices were digested for 16 hr with alkaline phosphatase (Sigma, type VII S; 150 μg/ml) in 0.1 M Tris·HCl (pH 8.0) (11) containing 2 mM phenylmethylsulfonyl fluoride and 10 μg of leupeptin per ml or in buffer without enzyme prior to performing τ immunocytochemistry.

Immunocytochemical controls included the substitution of normal mouse ascites fluid or IgG for the τ-1 antisera in the first step of the staining.

Abbreviations: AD, Alzheimer disease; MAP, microtubule-associated protein; NFT, neurofibrillary tangle; NP, neuritic plaque; PHF, paired helical filaments.

RESULTS AND DISCUSSION

The immunochemical and immunocytochemical properties of the τ-1 antibody have been described for bovine and rat tissue (15). This antibody recognizes all of the known electrophoretic forms of τ on immunoblots of mammalian brain and on immunoblots of microtubules from different brain regions (15). Immunocytochemical analysis showed that τ-1 immunoreactivity was exclusively localized in axons of neurons from several brain regions and was not detectable in somata and dendrites (15).

The results of immunoblot analysis of τ polypeptides in hippocampus from an AD patient and an age-matched nondemented individual are shown in Fig. 1 at two concentrations of sample. All images are from the same blot, which was immunostained as a single piece. Lanes 1 and 2 and lanes 3 and 4 are duplicates of two different loadings of sample from the control (nondemented individual) tissue. Lanes 6 and 7 are derived from samples of AD tissue that were loaded in an identical concentration to lanes 1 and 2 or lanes 3 and 4 based on the original wet weight of tissue. In all cases, five prominent immunostained bands are present that are equivalent to those observed in bovine tissue (15). In addition, three or four lower molecular weight bands are recognized by the antibody, which we believe are proteolytic fragments from the major τ bands generated postmortem in this autopsy tissue. The results indicate that the monoclonal antibody we are using recognizes the major τ antigens in human control and AD tissue. The greater intensity of labeling of bands from the AD tissue, together with the observation that we can label pathological structures in AD tissue using dilutions of antibody that do not normally detect τ, suggest that τ may be expressed to a greater degree in AD tissue. This intriguing possibility requires further investigation. For purposes of this
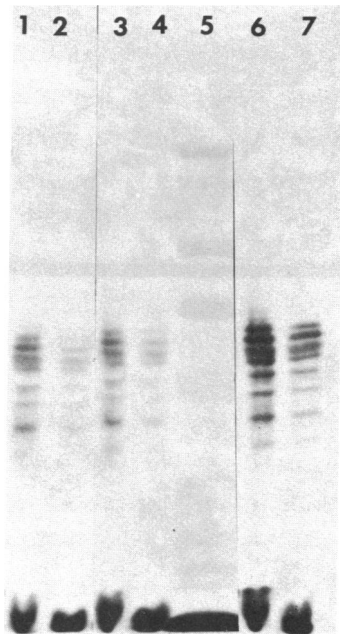

FIG. 1. Immunoreactivity of τ in brain homogenates. Immunoblot analysis of brain homogenates from age-matched nondemented human brain control tissue (lanes 1–4) and AD tissue (lanes 6 and 7). Lanes 1 and 2 and lanes 3 and 4 are duplicates of two different concentrations of sample. Lanes 6 and 7 are samples that were loaded at the same concentrations as the control based on wet weight of tissue. Lane 5 contains molecular weight standards from top to bottom: 200,000, 97,400, 68,000, 43,000, 25,700, 18,400, and 14,300. The τ-1 antibody recognizes five prominent bands and three minor lower molecular weight bands that probably represent proteolytic fragments of the primary τ family of polypeptides.

communication, the immunoblot analysis validates the use of our antibody to study τ distribution in human tissue.

Examination of temporal lobe white matter from AD tissue as well as hippocampus from the brain of an age-matched nondemented patient showed that the τ-1 antibody labeled axons but not cell somata in human tissue at 1:10 dilutions of the primary antibody, although not as intensely as in rat tissue. Disruption of axonal architecture in this autopsy tissue precludes adequate immunoelectron microscopic assessment of the distribution of τ in the axon. At all dilutions of τ-1 antibody, a population of NFTs was intensely labeled at the light microscopic level, particularly in the pyramidal cells of Ammon's horn of the hippocampus. For these studies, we concentrated on tissue stained with higher dilutions (lower concentrations) of primary antibody, which yielded intense NFT staining (Fig. 2). At higher magnifications, the label had a linear pattern (Fig. 2 C and D) similar to that of the NFT on silver stains. Control tissue for immunocytochemical analysis never revealed specific reaction product for peroxidase (Fig. 2B). By electron microscopy, striking label by antibody was localized almost exclusively to the NFT-bearing neurons (Fig. 2E). Conventional electron microscopy showed typical PHF within NFTs (Fig. 2F). Immunocytochemistry confirmed the presence of reaction product decorating the PHF (Fig. 2G). In addition, immunoreactivity was observed along some apparent straight tubules within the NFT. Determination of the relationship of these straight tubules and PHF requires further investigation.

The AD tissue also exhibited senile or neuritic plaques (NPs) along with the NFTs on silver staining. We did not, however, observe prominent labeling of the NPs with our monoclonal antibody to τ. Only focal positivity was noted in the abnormal neurites comprising the periphery of the NP. In contrast, Kosik *et al.* found intense labeling of NPs using a polyclonal antibody to τ (17). To examine the possibility that our monoclonal antibody is recognizing an epitope that is masked in the NP, we performed phosphatase digestion of AD tissue prior to immunocytochemical analysis. Slices of hippocampus treated with buffer containing protease inhibitors revealed the same number of labeled NFTs and absence of labeling of NPs as the untreated tissue (Fig. 2H). In contrast, phosphatase digestion produced a striking increase in the number of NFTs observed, and, in addition, many NPs were now labeled (Fig. 2 I and J).

Our results show that antigenic determinants recognized by the monoclonal antibody τ-1 are highly concentrated or favorably exposed for antibody binding in certain NFT in AD tissue. Other NFTs and many NPs also contain τ but the epitope is masked by phosphate groups. This suggests that abnormal phosphorylation of τ may be a seminal event in the formation of tangles and plaques.

τ is an electrophoretically heterogeneous complex of polypeptides that can copurify with brain microtubules through repetitive cycles of assembly and disassembly (cf. ref. 18). Other MAPs identified are high molecular weight polypeptides termed MAP1 and MAP2 (cf. ref. 15). τ and MAP2 are particularly interesting since they stoichiometrically stimulate microtubule assembly *in vitro* (cf. refs. 15 and 27). τ and MAP2 undergo changes in electrophoretic mobility as the brain develops, suggesting either developmentally regulated gene activation or posttranslational modification (cf. ref. 15). Furthermore, immature forms of τ and MAP2 less efficiently promote microtubule assembly *in vitro* than do adult forms (19), indicating age-related functional changes in these proteins.

MAP2 is highly concentrated in dendrites (cf. refs. 20 and 21) where presumably its role may include regulation of the assembly of dendritic microtubules. By contrast, τ immunoreactivity appears complementary to MAP2 in brain in that it is highly concentrated in axons but virtually undetectable in
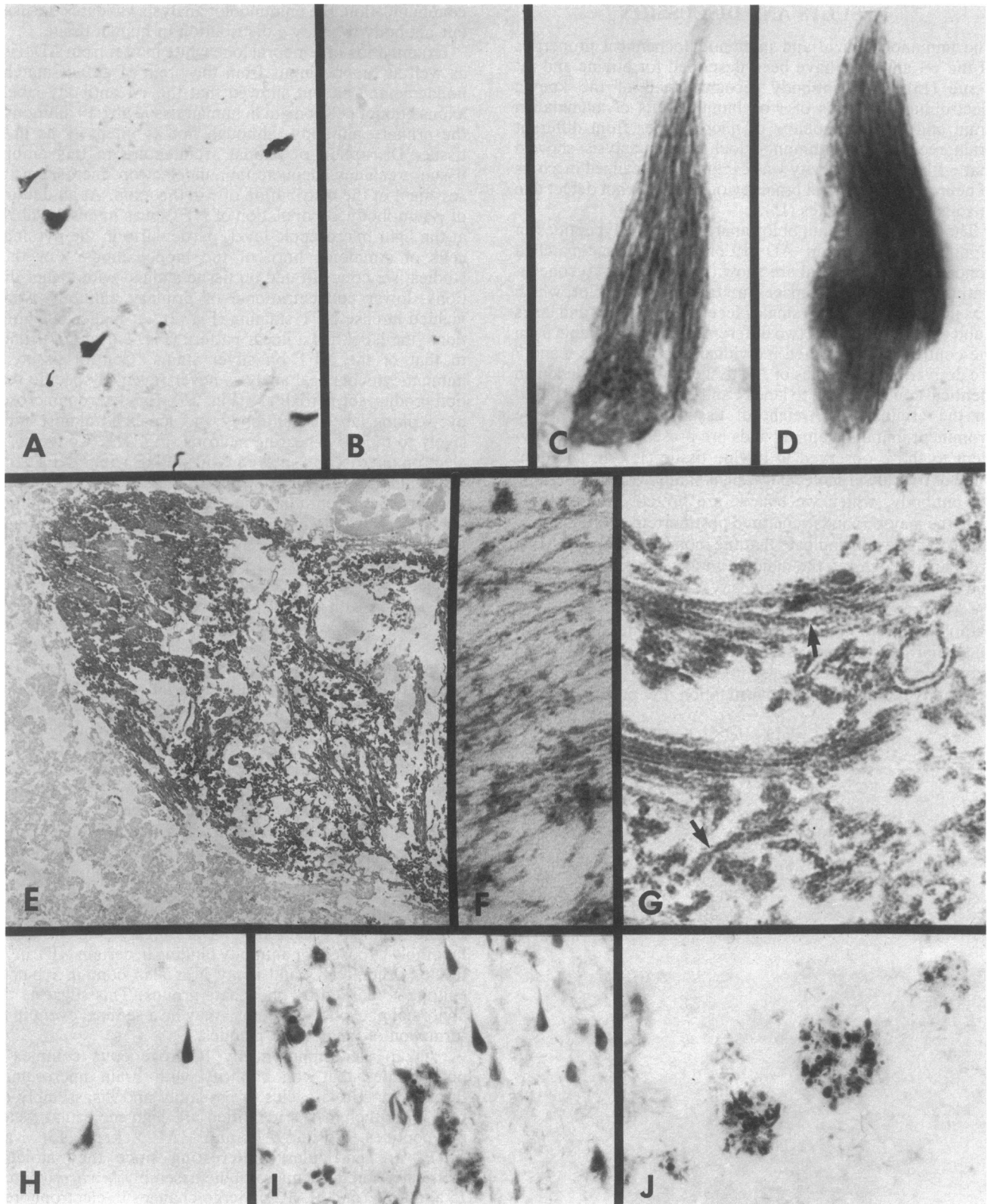
FIG. 2.   Immunoreactivity of τ with NFTs and NPs. (*A*) Light micrograph of peroxidase label using anti-τ antibody (1:25 dilution) shows several NFTs in the hippocampus from a case of AD. (×100.) (*B*) Immunocytochemical control using ascites fluid shows no peroxidase reactivity. (×100.) (*C* and *D*) NFTs from a case of AD show a linear distribution of peroxidase reaction product. (×1000.) (*E*) Peroxidase label of NFT-bearing neuron using τ antisera is noted. (Unstained by lead citrate or uranyl acetate. ×5350.) (*F*) NFT from nonimmunolabeled tissue shows typical PHF. (Lead citrate and uranyl acetate stain. ×58,800.) (*G*) Decoration of PHF (arrows) by reaction product is seen. Adjacent apparent straight tubules are also labeled. (Unstained by lead citrate or uranyl acetate. ×48,900.) (*H*) Light micrograph of hippocampus using buffer control for phosphatase digestion. Label of several neurofibrillary tangles is observed. (×100.) (*I* and *J*) τ-1 immunoreactivity following phosphatase treatment of hippocampal slices shows label of many additional NFTs (*I*) and intense label of NPs (*I* and *J*).

neuronal somata and dendrites (15). Since τ and MAP2 can compete for the same binding sites on the microtubule wall

(22), their compartmentalization in the neuron may be significant with regard to their function. If both τ and MAP2 are

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Proc. Natl. Acad. Sci. USA 83 (1986) 4043

involved in the assembly of microtubules, their physical separation would suggest that one function of τ *in vivo* might be the regulation of assembly and stability of axonal microtubules required for ongoing neuronal functions such as axonal transport.

This presence or redistribution of τ immunoreactivity within the NFT, a cell body structure, is therefore particularly intriguing. Recent immunohistochemical studies of AD tissue have focused on the presence or absence of various cytoskeletal elements or novel polypeptides within the NFT. The results are conflicting, but positive immunolabeling has been achieved with antisera to neurofilament, tubulin, MAP2, and "microtubule proteins" (2–14). These varied observations may be partially explained by the use of antibodies that recognize phosphorylated vs. nonphosphorylated epitopes on the same translational product of protein synthesis (11). Interestingly, with one exception, cytoskeletal proteins associated with NFTs have been those normally found in the perikarya or dendrites. One monoclonal antibody recognizing phosphorylated epitopes on the 200-kDa neurofilament polypeptide normally restricted to axons labels the NFT, suggesting that altered specific kinases (or phosphatases) modify neurofilament polypeptides and contribute to the formation of NFT (11). Alternatively, nonspecific crossreactivity must be considered. Some monoclonal antibodies to the heavily phosphorylated 200-kDa neurofilament polypeptide, for example, recognize epitopes on other heavily phosphorylated but unrelated proteins such as rhodopsin (23). Notwithstanding this caveat, the finding of "axonal" polypeptides in NFTs warrants further investigation.

Our work demonstrates that τ antigen is a major component of the NFT and NP. This conclusion is supported by the observation that purified PHF proteins share molecular weight (24) with τ. τ immunoreactivity is not normally detected in the perikaryon or dendrite, although τ must be synthesized in the cell body. It seems unlikely that the absence of uninvolved neuronal cell body staining is due to the presence of τ in modified form. Instead, it is more likely that newly synthesized τ is preferentially shuttled to the axon and is present in nondetectable amounts in the cell body. The τ immunoreactivity seen within the NFT of the neuronal perikaryon has several possible interpretations. τ may become modified in AD so that it cannot be preferentially shuttled to the axon where it presumably stabilizes microtubules and mediates interactions with the surrounding cytoskeleton. Alternatively, as a result of a generalized defect in transport of synthesized proteins to axons, τ may accumulate in the somata and perhaps form aberrant complexes with somal cytoskeletal elements. The observation that phosphorylation masks the epitope recognized by τ-1 in NFTs and NPs suggests that populations of τ are abnormally phosphorylated in AD. This aberrant phosphorylation might then lead to abnormal interactions and functions of cytoskeletal polypeptides. This does not preclude the possibility that the accumulation of axonal and dendritic proteins in the cell body leads to formation of NFTs in AD tissue simply because these usually separated proteins are now in apposition.

Finally, the NFT may act as a sink, trapping τ and other proteins. All of these scenarios might result in less stable, "τ-poor" axonal microtubules leading to impaired axonal transport and function.

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