Microtubule-associated protein tau (τ) is a major antigenic component of paired helical filaments in Alzheimer disease

(cytoskeleton/neurofibrillary tangles/neuronal degeneration)

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ABSTRACT  The detailed protein composition of the paired helical filaments (PHF) that accumulate in human neurons in aging and Alzheimer disease is unknown. However, the identity of certain components has been surmised by using immunocytochemical techniques. Whereas PHF share epitopes with neurofilament proteins and microtubule-associated protein (MAP) 2, we report evidence that the MAP tau (τ) appears to be their major antigenic component. Immunization of rabbits with NaDdSO4-extracted, partially purified PHF (free of normal cytoskeletal elements, including τ) consistently produces antibodies to τ but not, for example, to neurofilaments. Such PHF antibodies label all of the heterogeneous fetal and mature forms of τ from rat and human brain. Absorption of PHF antisera with heat-stable MAPs (rich in τ) results in almost complete loss of staining of neurofilibrillary tangles (NFT) in human brain sections. An affinity-purified antibody to τ specifically labels NFT and the neurites of senile plaques in human brain sections as well as NaDdSO4-extracted NFT. τ-Immunoreactive NFT frequently extend into the apical dendrites of pyramidal neurons, suggesting an aberrant intracellular locus for this axonal protein. τ and PHF antibodies label τ proteins identically on electrophoretic transfer blots and stain the gel-excluded protein representing NaDdSO4-insoluble PHF in homogenates of human brain. The progressive accumulation of altered τ protein in neurons in Alzheimer disease may result in instability of microtubules, consequent loss of effective transport of molecules and organelles, and, ultimately, neuronal death.

The progressive formation of paired helical filaments (PHF) and other abnormal cytoplasmic fibers in degenerating human neurons is a major manifestation of Alzheimer disease (AD). Qualitatively identical fibrous changes affect certain neurons of the limbic system during normal aging of the human brain. PHF accumulate in large, nonmembrane-bound aggregates in the perinuclear cytoplasm of neurons. Such masses are referred to as neurofibrillary tangles (NFT) and represent one of the two classical cytopathological features of AD when brain sections are examined by light microscopy. The other characteristic lesion is the neuritic (senile) plaque; it consists of a focal collection of dystrophic neurites, both axonal terminals and dendrites, many of which contain PHF. Senile plaques often contain a central core of extracellular 5- to 10-nm filaments that are morphologically distinct from PHF and have the structural and tinctorial properties of amyloid fibrils.

Available information about the identity of the proteins comprising PHF is conflicting and incomplete. The principal obstacles preventing further biochemical analysis of PHF are their marked insolubility (1-3) and the inability to purify them to homogeneity. As a result, most data regarding the composition of the fibers in NFT have derived from studies of antigenic crossreactivities. The proteins with which NFT have already been shown to crossreact at the light and electron microscopic levels are normal cytoskeletal elements, particularly neurofilaments and microtubule-associated proteins (MAPs). The most compelling identifications have used specific monoclonal antibodies. Epitopes shared with the fibers of NFT are contained in the high and middle molecular weight polypeptides of the neurofilament (4, 5), in vimentin (6), and in MAP 2 (7, 8). Recently, evidence that the MAP tau (τ) is also a constituent of the fibers of NFT has been presented (9, 33). τ protein consists of four to seven highly related phosphoproteins that range in size from 50–70 kDa and share with other MAPs the characteristics of cosassembling with tubulin and promoting its polymerization when microtubules are purified by repetitive cycles of temperature-dependent assembly and disassembly (10).

In this report, we present data implicating τ as a major antigenic component of NFT. For this purpose, we have utilized antibodies previously reported to react specifically with PHF but not with any cellular structures or proteins in age-matched normal brain (11). These antibodies react with NaDdSO4-extracted NFT and specifically decorate individual PHF using colloidal gold at the electron microscopic level (12). We have compared the reactivities of NFT and τ protein using PHF antibodies as well as affinity-purified antibodies specific for τ (10).

METHODS

Polyclonal antibodies to PHF were raised by injecting partially purified PHF fractions into New Zealand White rabbits as described (11). Absorptions were performed by incubating the PHF antibodies with an estimated 100-fold excess (weight for weight) of the absorbing protein for 16 hr at 4°C in Tris-buffered saline (pH 7.6) containing 2 mM EDTA. Affinity-purified polyclonal antibodies that are monospecific for the τ protein (10) were obtained through the generosity of David Drubin. All antibodies were analyzed with regard to their reactivity on neurons containing NFT (i.e., aggregates of PHF) in 10% formalin-fixed, paraffin-embedded sections of AD brain tissue and on isolated AD NFT prepared by NaDdSO4 extraction as described (11, 13). Formalin-fixed, human post-mortem brain sections were not adequate for the detection of the normal distribution of the τ antigen. Therefore, PHF and τ antisera were studied on rat cerebellum that was immersion-fixed in 2% paraformaldehyde/lysine/periodate (14) and sectioned on a cryostat. Sections of fresh frozen rat cerebellum fixed on the slides in acetone were also allowed to react. Antibodies were analyzed with regard to their reactivity with τ proteins on electrophoretic transfer blots (immunoblots) by the method of Towbin et al. (15).

In all cases, reaction product was detected by using peroxidase-

Abbreviations: NFT, neurofibrillary tangles; PHF, paired helical filament(s); MAP, microtubule-associated protein; AD, Alzheimer disease.
conjugated secondary antibodies and visualized with diaminobenzidine according to the method of Sternberger (16).

\( \tau \) protein was prepared from fetal and mature rat and fetal (20 weeks) human brain tissue. Microtubule-enriched fractions from these species were prepared by using the taxol method (17). Heat-stable MAP2A and MAP2B, free of tubulin were obtained by making the microtubule fraction 0.75 M in NaCl, boiling for 5 min, and centrifuging at 20,000 \( \times \) g for 20 min (18). The supernatants were analyzed by \( {\text{NaD}}_{2}\text{SO}_4 \)/polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (19) using 28-cm slab gels with a 5–15% acrylamide gradient. For some electrophoretic transfer blotting experiments, PHF-enriched fractions were prepared from affected regions of AD cerebral cortex by using extraction in 1% sarkosyl and differential centrifugation.

**RESULTS**

**Characterization of PHF Antibodies.** Three rabbits were injected with partially purified fractions of PHF prepared from human cerebral cortex affected with AD (11). The resultant antisera were characterized on paraffin-embedded, formalin-fixed sections of AD brain tissue. All three antisera labeled NFT and the neurites of senile plaques, both of which contain PHF. Randomly distributed PHF-bearing neurites frequently observed in some areas of AD cerebral cortex were also labeled (11). One of the antisera has been shown to label the PHF specifically with colloidal gold particles at the electron microscopic level (12). In addition to labeling NFT and abnormal neurites, two of the antisera produced variable staining of the extracellular amyloid fibrils that form the cores of senile plaques and the deposits in some blood vessel walls in AD brain. Such amyloid staining is probably due to the presence of contaminating amyloid filaments in some of the PHF-enriched fractions used as immunogens (20). None of the three antisera labeled any normal neuronal, glial, or vascular elements in formalin-fixed, paraffin-embedded sections of normal aged and AD brain tissue. A fourth rabbit was immunized with a low molecular weight amyloid protein extracted from a PHF-enriched fraction by treatment with 88% formic acid/1% \( {\text{Na}}_2\text{SO}_4 \) (20). The resultant antiserum did not react with NFT, either in situ or after their isolation, but instead selectively stained senile plaque cores and vascular amyloid deposits in AD brain.

In previous attempts to identify the antigens recognized by the PHF antiserum, we utilized electrophoretic transfer blotting (11). Homogenates of AD brain contained immunoreactive insoluble protein excluded at the top of the stacking gel as well as a diffuse smear of immunoreactive material seen throughout the running gel. Homogenates of normal human brain showed neither the insoluble material nor the smear but, like the AD homogenates, contained several poorly resolved protein bands between Mr 40,000 and Mr 60,000 (11). These bands were faintly stained by all three PHF antiserum at 1:250 dilution but were not visible at \( \geq 1:500 \), at which dilution NFT and neurites in AD brain still stained intensely. These bands were also not reactive following absorption of the PHF antiserum with normal human brain homogenates, whereas staining of the gel-excluded band and the diffuse smear in AD brain homogenates as well as of NFT and neurites in tissue sections persisted.

**PHF Antibodies Specifically Label \( \tau \) Proteins.** Because of previous reports (9, 33) suggesting that \( \tau \) was a component of NFT, we investigated the immunoreactivity of the PHF antiserum with fractions highly enriched in the heat-stable proteins from taxol-stabilized microtubules. Heat-stable MAPs were prepared from rat and human forebrain. Rats were obtained at the following times during development: embryonic days 15–16 and 18–19, postnatal days 0 and 10, and maturity. Human brain was obtained at 18–20 weeks of gestation. In rat preparations from all time points, MAP 2 and \( \tau \) were the major Coomassie-stained proteins of the heat-stable fraction (Fig. 1). With equal protein loading, there was less \( \tau \) protein relative to MAP 2 in the mature rat than in the fetal animals in the heat-stable MAP fraction (Fig. 1). Mature rat MAP 2 migrated as a doublet and mature rat \( \tau \) was seen in only trace amounts at Mr 50,000 by Coomassie blue staining (Fig. 1, lane a). Heat-stable MAPs from earlier developmental time points in the rat demonstrated the shifts in electrophoretic mobility of \( \tau \) and MAP 2 that have been well-described (10, 21–24). Fetal rat \( \tau \) consisted of the Mr 50,000 moiety and a doublet at Mr 46,000 and Mr 48,000 (Fig. 1, lane b). The fetal human heat-stable MAPs were highly enriched in \( \tau \) with only trace amounts of MAP 2; two prominent \( \tau \) bands at Mr 48,000 and Mr 50,000 and a fainter band at Mr 46,000 were detected (Fig. 1, lane c). Due to protein degradation, heat-stable MAP preparations from adult human brain were not interpretable. \( \tau \) proteins were identified in the various preparations described above by four specific criteria: their fractionation with microtubules, their heat stability, their characteristic migration pattern, and their crossreactivity with a well-characterized antibody to \( \tau \) (10).

Such heat-stable MAP preparations were then used to characterize the PHF antibodies by electrophoretic transfer blotting (Fig. 2). All of the \( \tau \) proteins from rat (Fig. 2, lane a) and human (lane b) fetal brains and mature rat brain (lane g) were stained by all three PHF antisera. Included in the immunoblot analysis were samples of the heat-stable MAPs stained with the affinity-purified \( \tau \) antibodies. Anti-PHF (lanes a, b, and g) and anti-\( \tau \) (lanes c, d, and h) stained identical bands in adjacent lanes. Absorption of one of the PHF antisera with mature rat and fetal human heat-stable MAPs virtually abolished the staining of \( \tau \) on electrophoretic transfer blots (lanes e and f). The antiserum to the low molecular weight amyloid protein from AD brain (20) did not stain any proteins in human or rat heat-stable MAP fractions.

**\( \tau \) Antibodies Selectively Label PHF-Bearing Neurons in AD Brain.** These findings led us to examine whether \( \tau \) antibodies would immunolabel NFT (i.e., aggregates of PHF) and PHF-containing senile plaque neurites in AD brain. In formalin-fixed brain sections, NFT in the hippocampus and the neocortex were intensely stained with anti-\( \tau \) (1:200 dilution) (Fig. 3). Characteristic ring- and flame-shaped NFT were readily detected. \( \tau \)-Immunoreactive NFT appeared to extend into the apical dendirites of pyramidal cells, despite the reported axonal localization of \( \tau \) in normal mammalian neurons (25). Light microscopic counts of adjacent sections stained with anti-\( \tau \) or PHF antibodies showed that the antibodies recognized approximately the same number of intraneuronal NFT. We previously found that the PHF antibodies label essentially all NFT in AD brain sections (11).

**FIG. 1.** Coomassie brilliant blue-stained \( {\text{NaD}}_{2}\text{SO}_4 \)/PAGE of the heat-stable fraction from taxol-stabilized microtubules. The upper arrowhead points to the MAP 2 bands and the lower arrowhead indicates the \( \tau \) bands. Lane a, mature rat forebrain; lane b, embryonic day 18 rat forebrain; lane c, 20-week-gestation monkey forebrain. Molecular weight (lane MW) standards in ascending order are lysozyme (Mr 14,000), soybean trypsin inhibitor (Mr 22,000), carbonic anhydrase (Mr 30,000), ovalbumin (Mr 43,000), bovine serum albumin (Mr 68,000), phosphorylase b (Mr 92,000).
NFT that appeared to be extracellular—i.e., lying free in the neuropil without an associated nucleus or other definable cell structure (so-called "ghost tangles" or "tombstones")—did not react or reacted very slightly with anti-\(\tau\). In contrast, PHF antisera consistently labeled such ghost tangles, albeit more lightly than adjacent intraneuronal NFT, as reported (13). The dystrophic PHF-containing neurites of senile plaques were prominently labeled by the \(\tau\) antibody in a manner very similar but not identical to PHF antisera (Fig. 3B). The \(\tau\) antibodies appeared to stain more slender neuritic processes in the senile plaques than did anti-PHF; both reagents labeled the larger dystrophic neurites characteristically observed in the plaques. The number of neuritic plaques recognized by each reagent was the same. Both antibodies identified innumerable isolated neurites in the cortical neuropil not organized into discrete plaques (Fig. 3C); such staining has been reported for the PHF antibodies (11, 13) and suggests a more extensive abnormality of the cortical neuropil than the plaques and tangles alone would indicate. The amyloid cores of senile plaques and vascular amyloid deposits showed no reaction with anti-\(\tau\). One of the PHF antisera was absorbed with heat-stable MAP fractions from mature rat and fetal human brain. The absorption resulted in almost complete abolition of NFT and neurite staining.

NFT were isolated from AD cerebral cortex by extraction with a buffer containing 2% NaDodSO\(_4\) and 0.1 M 2-mercaptoethanol (11, 13). Many of the NFT in this preparation were immunolabeled by the \(\tau\) antibodies; however, the intensity of the reaction was less than that observed on the same preparation stained with a PHF antiserum. Amyloid cores also present in such preparations were unlabelled by anti-\(\tau\). When NaDodSO\(_4\)-extracted PHF-enriched fractions were examined by electrophoretic transfer blotting (Fig. 4), the gel-excluded, insoluble material at the top of the stacking gel [which contains PHF (1, 11)] was labeled by anti-\(\tau\) as well as by the three PHF antisera (Fig. 4). No such insoluble material was detected by the antibodies in identically treated normal human brain fractions.

For comparison, we also studied the distribution of \(\tau\) immunoreactivity in normal brain. Formalin-fixed human brain sections, when allowed to react with anti-\(\tau\), showed only staining of NFT and abnormal neurites in AD cases and no staining whatsoever in normal cases. Because \(\tau\) is thought to be localized to axons in mammalian brain (25), the lack of axonal staining in the human sections was thought to be due to either post-mortem conditions or fixation. Rat cerebellum, fixed in 2% paraformaldehyde/lysine/periodate and cryostat-sectioned, demonstrated an axonal pattern when allowed to react with anti-\(\tau\) [as reported (25)] or with anti-PHF (data not shown). Most prominently stained were the parallel fibers in the molecular layer. Staining of deep white matter tracts was present but less robust.
DISCUSSION

Several distinct cytoskeletal proteins or their fragments have been identified in AD PHF based on immunocrossreactivities. The most extensively studied of these proteins are elements of the microtubule and neurofilament systems. The presence of unique or altered epitopes in PHF has been suggested by the inability to find crossreacting antigens in normal brain tissue using polyclonal or monoclonal antibodies raised specifically against PHF-enriched fractions (11, 26-28). The findings of this study indicate that the τ proteins (or portions thereof) are the principal antigenic determinants of three polyclonal PHF antibodies and represent a major antigenic component of the PHF. Several lines of evidence support this conclusion: (i) immunization of rabbits with NaDodSO₄-extracted, partially purified NFT (free of normal cytoskeletal elements, including τ) consistently produces antibodies to τ (but not, for example, to neurofilaments); (ii) absorption of antisera to PHF with heat-stable MAPs results in a marked reduction of NFT immunoreactivity; (iii) PHF antisera, which react with τ, have been shown to immunolabel PHF directly at the electron microscopic level (12); (iv) all τ antibodies described to date specifically label NFT (refs. 9, 29, and 33; this report); (v) the affinity-purified τ antibodies used here react with NFT even after extraction in NaDodSO₄, a step that removes normal τ proteins; (vi) a newly produced monoclonal antibody to τ also selectively labels NFT in situ and after isolation (K.S.K. and D.J.S., unpublished data). The fact that the PHF used as immunogens to raise antisera were extensively extracted in NaDodSO₄ and the retention of τ immunoreactivity on NaDodSO₄-extracted NFT suggest that τ proteins are integrally incorporated into the PHF.

The reactivity of PHF antisera with τ is not apparent when homogenates of total adult human brain proteins are immunoblotted (11, 28). However, PHF antisera from three different rabbits consistently labeled the τ proteins found in high amounts in heat-stable MAP fractions of fetal human, fetal rat, and mature rat brain. Fetal material was most immunoreactive because heat-stable taxol preparations from fetal brain show a greater relative concentration of τ protein than those from adult brain. The anti-PHF labeling pattern of individual τ bands in adult and fetal brain preparations was identical to the labeling obtained with the τ antibodies. Anti-τ also labeled NFT and the neurites of senile plaques in a highly specific fashion in AD brain tissue sections. There was no reaction product in the region of vascular or senile plaque amyloid deposits and no labeling of normal brain structures. NFT extracted in the presence of NaDodSO₄ retained τ immunoreactivity. Under these conditions, however, the intensity of the staining was reduced.

Under routine fixation conditions of human post-mortem brain tissue, the normal τ antigen is not well-preserved for immunocytochemical analysis. Staining of structures other than NFT and abnormal neurites in formalin-fixed AD brain sections was not detected by either anti-τ or anti-PHF (11), despite the presence of τ in axons (25) and its reaction on immunoblots of unfixed human brain tissue. The preservation of τ immunoreactivity in NFT and PHF-bearing neurites under conditions in which it is lost in normal structures is presently unexplained. Normal τ protein could be immobilized in the highly inert PHF (i.e., tightly bound) and therefore visualized in tangle-bearing neurons even after fixation or detergent extraction. The τ protein in the fibers could be present in a chemically modified form, since it resists NaDodSO₄ solubilization and does not lose antigenicity after formalin fixation. The possibility that the τ protein found in PHF is modified from normal τ could explain, in part, the fact that absorption of PHF antisera with large amounts of normal τ does not completely abolish NFT and neurite staining in AD brain. Indeed, τ-absorbed PHF antisera continue to stain NFT isolated from brain in physiologic buffers (9). Therefore, some antibodies in the polyclonal PHF antisera are either not reactive with τ in the form found in the heat-stable MAP fractions used for absorption or are directed against PHF epitopes entirely distinct from τ. The latter possibility plus the fact that the extracellular residua of NFT—i.e., ghost tangles or tombstones—are not stained by anti-τ raises the prospect that other, as yet unidentified, proteins occur in PHF and that the strong antigenicity of the τ component reflects exposed τ epitopes on the surface of the fibers.

PHF antibodies and τ antibodies produce intense staining of the abnormal neurites found in senile plaques and in the cortical neuropil. The majority of these dystrophic neurites are thought to be axons (30–32), a conclusion that is consistent with the finding of an axonal cytoskeletal element—i.e., τ—in these abnormal processes. It is unclear to what extent the dendritic MAP 2, which is also antigenically represented in NFT, is present in dystrophic neurites in AD brain tissue. A polyclonal antibody to MAP 2 reacted with the neurites of senile plaques (34), whereas a monoclonal antibody did not (7). The detailed protein composition of individual PHF could, in part, be a function of their intracellular locus.

Several protein components of the neurofilament and microtubular systems appear to be present in NFT based on immunocytochemical analyses. To what extent each of these proteins is an integral part of the PHF versus a closely associated protein is presently unclear. One criterion for considering a protein integral to the PHF is retention of immunoreactivity after extraction and heating in the ionic detergent NaDodSO₄. Neurofilament immunoreactivity has been reported after NaDodSO₄ extraction of PHF (8, 35). In the case of τ protein, NFT immunoreactivity to anti-τ and the antigenicity of τ in PHF are retained after NaDodSO₄ extraction.

τ is a heterogeneous protein that undergoes major electrophoretic shifts during development. The fetal forms of τ are highly reactive with PHF antisera. The particular molecular form(s) of τ present in PHF are unknown, but it is possible
that fetal τ antigens contribute to the composition of PHF. An immature (non-neuronal) form of enolase has been detected in hippocampal pyramidal neurons in AD (36). Axotomized neurons undergoing regeneration may also reexpress some fetal proteins (37). Axonal sprouting has been observed within senile plaques (38) and a more generalized sprouting response has been shown in AD hippocampus (39). Whether the neurites and cell bodies of these sprouting fibers contain PHF is not known. The possible presence of fetal cytoskeletal elements—e.g., τ proteins—within an otherwise mature neural milieu could result in abnormal protein interactions that lead to formation of highly stable pathological fibers. The presence of τ in PHF also provides one possible explanation for accumulation of aluminum in NFT (40–42), since calmodulin binds strongly to τ (43) and aluminum (44).

The locus of τ immunoreactivity in NFT-bearing neurons suggests an aberrant topography of the τ protein in these neurons. Most cortical NFT are found in pyramidal cells, and within these cells they typically fill the soma and extend into the proximal portion of the apical dendrite. This intracellular distribution gives NFT their classical flame-shaped appearance. The human apical dendrite is very rich in MAP 2 (7, 34); τ, in contrast, is largely localized to the axon (25). Altered segregation of these axonal and dendritic populations of τ and MAP 2, respectively, may play a role in the genesis of NFT. The presence of τ in the proximal dendrite might also lead to its abnormal phosphorylation by those kinases associated with MAP 2, particularly type II-cAMP-dependent protein kinase. Some evidence exists that phosphorylated forms of τ are present in the NFT (9). The presence of phosphorylated forms of the neurofilament proteins within the NFT of the neuronal soma may represent a similar phenomenon since such epitopes are normally distributed to the distal axons (45, 46). An impairment in the expression, transport, and/or processing of τ and other cytoskeletal proteins in selected neurons in AD could result in a reduction in neurotransmitter production and, ultimately, neuronal death.

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