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Measurement of distinct immunochemical presentations of tau protein in Alzheimer disease

(neurofibrillary tangles/paired helical filaments/neurodegeneration)

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ABSTRACT **The tau protein is a microtubule-associated protein that is normally located in nerve axons. In Alzheimer disease, it is a constituent of paired helical filaments (PHFs), which are the principal fibrous component of the characteristic neurofibrillary tangles. The tau protein, therefore, is abnormally sequestered in an insoluble form in PHFs in the cell body and dendrites in Alzheimer disease. We have used two monoclonal antibodies (mAbs) to selectively measure the levels of normal, soluble tau protein and of PHF-associated tau protein in the brain. mAb 423 binds to PHFs and recognizes a 12-kDa fragment of tau protein released by formic acid treatment of PHFs, but it does not recognize normal tau protein. In contrast, mAb 7.51 recognizes normal tau protein as well as the PHF core-derived tau fragment, but its epitope is concealed in the PHF-bound form. The differential binding properties for these two mAbs have enabled us in this study to quantify insoluble PHF-associated tau protein in the somatodendritic compartment as well as normal soluble tau protein in its predominantly axonal location. Our findings demonstrate that a distinct immunochemical presentation of tau protein recognized by mAb 423, a PHF-specific marker, can be used to quantify neurofibrillary pathology in Alzheimer disease independently of the presence of normal tau proteins.**

The neurofibrillary tangles (NFTs) that form within neurons of the brain in Alzheimer disease have as their principal fibrous component the paired helical filament (PHF) (1). The tau protein is bound to microtubules predominantly in nerve axons (2), where it probably serves to promote the stability of such microtubules, though its precise function is unclear. In Alzheimer disease tau protein, abnormally sequestered in an insoluble form in PHFs, is found in the cell body and dendrites, the sites of NFT formation (3–7).

We have previously shown that tau protein is incorporated into the PHF in such a way that the N-terminal 200-amino acid domain contributes largely to a protease-sensitive fuzzy coat that surrounds the PHF (8, 9). A central region consisting of three tandem repeats, corresponding to the microtubule-binding domain, is firmly attached within the protease-resistant core structure of the PHF in some form of tight association that differs from the normal tau/microtubule association. In a further study (10), we have mapped the repeat region of PHF tau with two monoclonal antibodies (mAbs) raised against PHF core preparations, both of which recognize a major 12-kDa band released from the Pronase-resistant core. mAb 423 binds to PHFs and recognizes a conformation of the repeat region that is specific to PHF tau, but does not recognize any form of normal adult tau protein that we have been able to prepare. mAb 423 is therefore a reagent specific for PHF core-derived tau. The second antibody, mAb 7.51, recognizes a segment in the C-terminal half of the repeat region that is common to all known forms of tau, and hence can be used as a generic tau marker.

Until now there has been no way to differentiate immunochemically between normal tau proteins and those involved in neurofibrillary pathology. Attempts to measure tau proteins in brain homogenates have been based on antibodies that crossreact with normal tau proteins, making interpretation of the findings difficult. mAbs 423 and 7.51 provide the means to discriminate between normal forms of soluble or microtubule-associated tau protein and tau protein that is incorporated into the PHF core in Alzheimer disease. We have used immunoassays based on these antibodies (11) to show that normal and PHF-derived tau protein can be quantified independently in the same brain homogenates. These immunoassays provide a direct immunochemical measure of the neurofibrillary pathology in Alzheimer disease.

MATERIALS AND METHODS

Human Brain Tissue. Postmortem brain specimens were obtained from 26 patients (13 male, 13 female) with pathologically confirmed Alzheimer disease (mean age 79.3 years, range 57–97) and six controls (5 male, 1 female; 19–88 years) presenting no neurological disorders. Blocks from frontal and temporal cortex and hippocampus were fixed in methanol/acetic acid, 95:5 (vol/vol), for immunohistochemistry to reveal NFTs and senile plaques with mAbs as described (12). NFTs and plaques were also visualized in tissue fixed in 10% formalin, using a methenamine silver stain (13). Remaining brain tissue was frozen at −70°C for subsequent isolation of tau protein or PHFs. The interval between death and tissue processing was in the range of 1–21 hr.

Preparation of PHFs and Their Subfractions. Bulk PHF preparations treated with Pronase were isolated according to the ifII protocol (9, 14). These were treated in one of two ways: (*i*) formic acid was used to extract two major peptide fragments, corresponding to the repeat region of tau protein (9), in a fraction termed F5.5; (*ii*) fragmented PHFs were prepared by sonication of PHFs in 100 mM NH_4HCO_3 (pH 8.5); insoluble material was removed by centrifugation and the supernatant fraction obtained was termed ABCsup. These PHF fragments retained their characteristic morphology and periodicity when examined by electron microscopy.

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Abbreviations: NFT, neurofibrillary tangles; PHF, paired helical filament; mAb, monoclonal antibody; HRP, horseradish peroxidase.

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For analyses of specific regions, smaller quantities (0.5 g, wet weight) of brain tissue were dissected and a scaled-down version of the ifII protocol was used to prepare "insoluble" fractions that were treated as in procedures *i* and *ii* above. The initial supernatant from the brain homogenate (S1) was dialyzed against distilled water and heat-treated (100°C for 5 min with 1 M NaCl), and thermostable microtubule-associated proteins were concentrated by precipitation using 60% saturated $(NH_4)_2SO_4$. Pellets were dissolved in 50 mM NH_4HCO_3 (pH 8.0), dialyzed against the same buffer overnight, and concentrated to dryness with a SpeedVac concentrator (Savant). This fraction was termed the "soluble fraction."

Preparation of tau Protein. tau protein was isolated from the brain of a 23-year-old man (case 531): microtubule proteins were obtained by three cycles of temperature-dependent assembly–disassembly (15) and tau protein was purified from thermostable microtubule-associated proteins by gel filtration (16). tau protein was also purified by immunoaffinity chromatography of the initial supernatant from the brain homogenate (S1), using mAb 7.51 linked to Affi-Gel Hz (Bio-Rad) according to the manufacturer's recommendations.

mAbs and Immunoassays. mAbs 423 and 7.51 were raised against differently treated PHF preparations (8–10). The competitive immunoassay, using purified mAbs conjugated with horseradish peroxidase (HRP), has been described (11).

Other Analytical Procedures. SDS/PAGE and immunoblotting with mAb–HRP conjugates were performed as described (11). Protein was determined using a modified Lowry method in which SDS is included to permit solubilization of proteins (17). Bovine serum albumin was used as standard protein.

RESULTS

Immunoreactivity of Purified and Microtubule-Associated tau Proteins. Intact human tau protein isolated by three cycles of temperature-dependent assembly–disassembly of microtubules contained the normal set of six tau proteins having apparent molecular mass of 52–65 kDa (Fig. 1 *B* and *D*). All forms were recognized on immunoblots (lanes 3 and 4) by mAb 7.51, whereas none was detected with mAb 423. tau proteins were also isolated from the first soluble brain homogenate (S1) prepared from both control and Alzheimer brain tissue by immunoaffinity chromotography using mAb 7.51. In this case, proteins having lower apparent molecular mass than the normal tau bands were also present that reacted with mAb 7.51 but not with mAb 423. Thus mAb 423 did not recognize any of the forms of tau that bind to microtubules, and furthermore, it did not react with any other soluble tau species that might lack the ability to interact with microtubules. Similarly, mAb 423 did not label microtubules by immunoelectron microscopy (data not shown).

Competitive immunoassays based on mAbs 423 and 7.51 were developed to measure tau proteins (11) and these have been used to quantify the differences observed on immunoblots (Fig. 1 *A* and *C*). In these assays, the F5.5 PHF core-derived tau fraction is used as the solid-phase competitive antigen since it contains species immunoreactive with both mAbs (Fig. 1 *B* and *D*; lanes 5). The tau protein purified from the S1 fraction by immunoaffinity purification was shown to compete for the binding of mAb 7.51 to F5.5. Soluble tau protein, in contrast, did not compete for the binding of mAb 423 to F5.5. In addition, tau isoforms containing either three or four repeats and expressed in bacteria were found to react with mAb 7.51 but not with mAb 423 in these immunoassays (see below). It is important to have demonstrated that mAb 423 does not recognize any normal soluble tau protein by either immunoblot or competitive immunoassay.

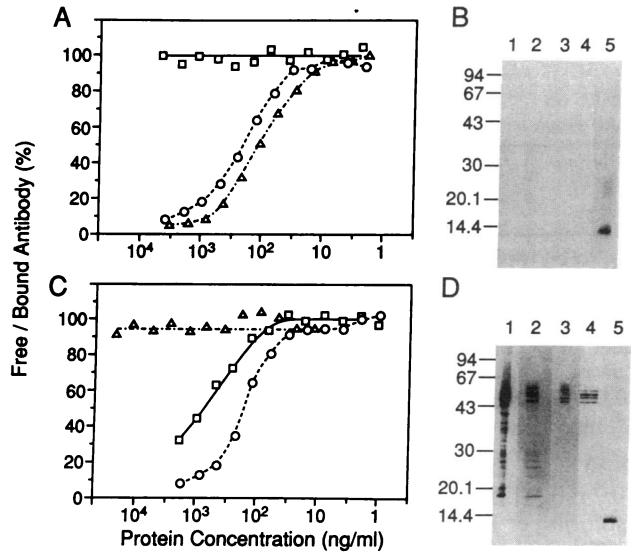


FIG. 1. Immunoreactivity of normal and PHF-associated tau protein by competitive immunoassay and by immunoblot. Immunoaffinity purified tau protein (□) from the Sl supernatant fraction of an Alzheimer brain and F5.5 (○) and ABCsup (△) fractions prepared from PHFs of an Alzheimer brain were tested by competitive ELISA (11) with mAb 423 (*A*) and mAb 7.51 (*C*). Individual points are the mean values from three separate determinations. Competition curves demonstrate that mAb 423 reacted with F5.5 and ABCsup but not with tau protein, whereas mAb 7.51 reacted with tau protein and F5.5 but not with ABCsup. The difference in mAb 7.51 reactivity for tau protein (molecular mass, 37–46 kDa; ref. 18) compared with F5.5 (apparent molecular mass, 12 kDa), on a weight basis, reflects an equimolar ratio of the mAb 7.51 epitope for the two preparations. Lack of mAb 423 reactivity with normal tau protein was confirmed by immunoblot (*B* and *D*): immunoaffinity-purified tau protein from the S1 of a control (lane 1) and an Alzheimer case (lane 2), microtubules plus their associated tau protein (lane 3), and purified tau protein (lane 4) did not react with mAb 423 (*B*) whereas the 12-kDa F5.5 band from an Alzheimer brain (lane 5) did. In contrast, all the tau proteins (lanes 1–4) and the 12-kDa F5.5 band (lane 5) reacted with mAb 7.51 (*D*). Positions of molecular mass markers (kDa) are indicated at left of the immunoblots.

Immunoreactivity of PHF Core-Derived tau Protein. Having established the distinctive patterns of immunoreactivity for mAbs 423 and 7.51 with normal tau protein both on immunoblots and by immunoassay, we examined immunoreactivity with PHF core-derived tau present in the F5.5 fraction and in a highly enriched preparation of morphologically intact PHF fragments, termed ABCsup. PHF-core tau fragments remain in the bound state in the ABCsup preparation but can be extracted from PHFs into the F5.5 fraction. By competitive immunoassay, both the F5.5 and the ABCsup fraction reacted with mAb 423 (Fig. 1*A*). In contrast, mAb 7.51 reacted only with tau fragments that had been released from the PHF core, found in the F5.5 fraction (Fig. 1*B*).

Sensitivity of immunoassay. Since mAb 423 does not react with normal tau protein, it is not possible to express the results directly in terms of tau content. Relative immunoreactivities, therefore, have been expressed with respect to a reference preparation of F5.5, which reacts with both mAb 423 and mAb 7.51. Nevertheless, the detection limits for the immunoassays can be determined for mAb 7.51 by using purified, bacterially expressed tau proteins. The concentrations of tau protein with three repeats (htau23) or four repeats (htau40) (18) required for 50% competition in an immunoassay with mAb 7.51 were 1.55 nM and 0.91 nM, respectively. The effective range for measuring tau protein in the assay was

Table 1. Two-site immunometric assay

mAb on solid phase	Rate of change of absorbance, milliunit/min			
	mAb 7.51–HRP		mAb 423–HRP	
	+ F5.5	− F5.5	+ F5.5	− F5.5
None	0.8	0.6	1.6	1.7
7.51	4.7	1.8	112.0	7.2
423	170.0	6.1	26.0	9.0

HRP-conjugated mAbs were diluted 1:1000 in phosphate-buffered saline (pH 7.4) containing 1% dried milk and 0.05% Tween 20, with or without F5.5 (0.325 μg of protein per ml), and incubated at 4°C for 16 hr. Aliquots were tested (0.05 ml; 10 replicates) on two independent plates coated with antibody. Purified mAbs 7.51 and 423 were coated on microtitration plates in carbonate buffer at concentrations of 1.0 and 10 μg/ml, respectively. The mean values for rate of change of absorbance were calculated for each condition.

2–50 ng of three-repeat tau protein in the adsorption mixture (0.2 ml). This gives a lower limit for detection of 1 ng of this isoform per individual replicate in the assay.

Two-site immunometric assay using F5.5. To demonstrate that the same tau fragments released from the PHF core are reactive with both mAbs, we examined the immunochemical properties of the F5.5 tau species in a two-site immunometric sandwich assay. In this experiment, F5.5 was trapped between two mAbs: one adsorbed on the solid phase and the second (HRP-conjugated) preincubated with F5.5. The experiment was carried out under saturating conditions such that when the same antibodies were present on both sides in the assay, there was no trapping of the F5.5 peptides (Table 1). Only when a combination of different mAbs was used could the reactive molecular species, already labeled with mAb–HRP, be trapped by the second antibody attached to the solid phase.

These results demonstrate that the repeat region of PHF core-derived tau can present both epitopes for the simultaneous binding of the two mAbs. The experiment further confirms that the epitopes for these mAbs must exist on the same molecular species, since the species labeled by the first mAb–HRP conjugate must be the only one that is detected when trapped by the second mAb. The possibility of mAbs reacting with different species of tau protein in F5.5 was circumvented in this experiment by using HRP-conjugated mAbs.

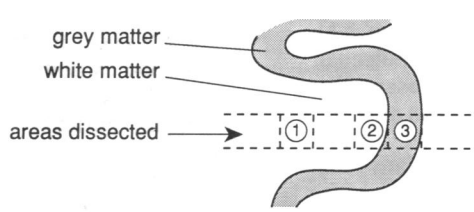
Evidence that the epitopes for the two mAbs are on a shared molecule is also supported by the close linear relationship between immunoreactivity for the two mAbs that was obtained for F5.5 preparations from 18 different brains (data not shown).

Exposure and release of PHF core-derived tau fragments. Although the mAb 423 and 7.51 epitopes are located on the same tau species, mAb 7.51 can discriminate between PHF-bound tau and tau released from the core. mAb 7.51 does not decorate isolated PHFs by immunoelectron microscopy nor does it react in competitive immunoassay with morphologically intact PHF fragments (ABCsup; Fig. 1). mAb 423, on the other hand, both labels intact PHFs (8) and reacts with ABCsup (Fig. 1). From the results of further immunoassays, it has been found that the mAb 7.51 epitope is exposed only under acid conditions sufficient to release the repeat region of tau from the core. Under these conditions an acid-stable core conformation of tau protein still exists, which has a diminished affinity (approximately halved) for mAb 423 (C.R.H., unpublished data).

Distribution of tau Protein in Alzheimer Disease. The results presented so far demonstrate the feasibility of measuring distinct immunochemical presentations of tau protein in Alzheimer disease. In particular, these results impose clear criteria for distinguishing between normal tau protein and tau proteins bound within the core of the PHF. Normal tau proteins ought to be detected in the "soluble" phase, by mAb 7.51, whereas PHF-associated tau protein, in the "insoluble" phase, should be detected by mAb 423. If the tau detected in the latter is indeed bound within the core of the PHF, the mAb 7.51 epitope ought to remain in the hidden configuration, unless revealed under acidic conditions.

Using a scaled-down version of the ifII protocol, we have analyzed the distribution of tau protein in different layers of the temporal cortex from a 91-year-old Alzheimer patient (case 64), dividing the protein into "soluble" and "insoluble" fractions.

There exists a gradient for soluble mAb 7.51 immunoreactivity: it is greatest in deep white matter, less in the marginal zone adjacent to cortical layer VI, and least in grey matter (Fig. 2). Such a gradient would be consistent with the expected distribution of normal axonal tau protein. Approximately 40% of the axonal volume can be found in grey matter (19), accounting for tau observed in this region, and tau protein is also found in astrocytes in the grey matter (20). By



mAb	Formic acid	Soluble			Insoluble		
		1	2	3	1	2	3
7.51	−	100*	30	15	0	0	0
	+				0	43	108
423	−	11	13	5	1	50	100*
	+				0	13	37

FIG. 2. Distribution of tau protein immunoreactivity in Alzheimer temporal cortex. Grey and white matter (≈0.5 g of tissue, wet weight) was dissected from three regions, as indicated, and fractionated into a thermostable "soluble" fraction and an "insoluble" fraction (in equal volumes). The insoluble fraction was treated with or without formic acid as indicated. Concentrations of immunoreactivity were normalized for a starting value of 0.50 g (wet weight) of tissue. Tabulated results are related to immunoreactivity found in a particular region (denoted by a star), for each separate mAb, which has been given a value of 100.

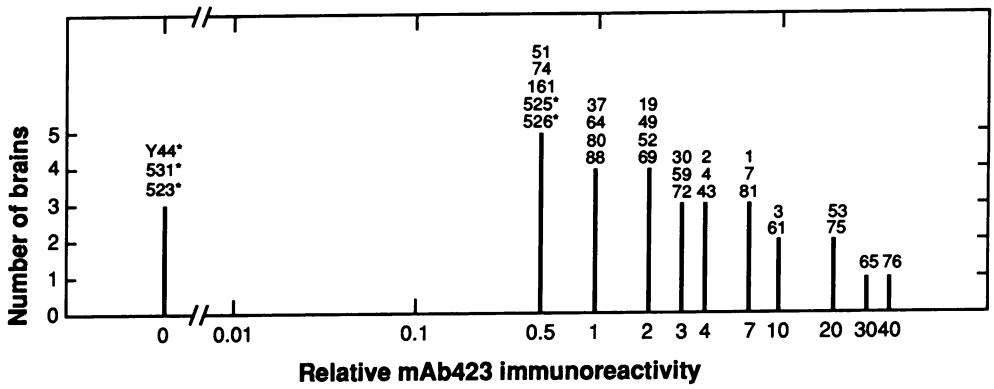


FIG. 3. Relative mAb 423 immunoreactivity for PHF preparations from 31 brains. Grey matter was mixed from frontal, temporal, and parietal cortex and PHFs were prepared from 80 g (wet weight) of tissue (9, 14). Immunoreactivity in F5.5 and ABCsup was measured by competitive immunoassay (11). Numbers refer to different cases having a given relative immunoreactivity; all were neuropathologically confirmed as Alzheimer brains except for control cases, which are marked with stars (cases Y44, 531, 523, 525, and 526). A relative immunoreactivity value of 0.005 is the lower limit of detection.

contrast, mAb 423 immunoreactivity predominates in the insoluble fraction, the distribution being greatest in grey matter, less in the marginal zone, and absent from the white matter. These results are consistent with sequestration of tau in PHFs, which occurs predominantly in the somatodendritic compartment of cortical pyramidal cells. Reactivity with mAb 7.51 was observed in the insoluble fraction only following acidic exposure. Since we have demonstrated that the mAb 7.51 and mAb 423 epitopes exist on the same molecular species, these results confirm the selective detection of insoluble PHF-specific tau by mAb 423.

The same experiment was done using the brain tissue of an 88-year-old control individual (case 533). In this case no immunoreactivity was observed in the insoluble fractions with mAb 423 or with mAb 7.51 after acid treatment.

Quantitative Immunoassays for PHFs in Alzheimer Brain Tissues. The results presented above validate the use of mAb 423 immunoassays to measure bulk PHF yield from Alzheimer brain tissue homogenates. PHF-associated mAb 423 immunoreactivity in 26 Alzheimer cases was spread over a broad range, reflecting variations in the severity of the disease for these cases (Fig. 3). The intrabrain variation (bulk preparative runs from different brain quadrants) was small when compared with the interbrain variation shown in Fig. 3. For example, the immunoreactivity in case 49 varied over the range 1.6–3.1 (2.1 ± 0.2, mean ± SEM, n = 7). PHF-associated mAb 423 immunoreactivity was absent from brains of controls aged 23 (case 531), 19 (case Y44), and 57 (case 523) years despite the fact that the ABCsup preparation from these brains contained up to 5–10% of the protein normally obtained in the corresponding fraction from an Alzheimer brain. Two of the aged control cases (case 525, aged 68, and case 526, aged 73) had PHF-associated mAb 423 immunoreactivity at the lower end of the clinical Alzheimer range. Occasional NFTs were observed both immunohistochemically and by silver stain in the cortex of case 526, whereas all the clinically demented cases had at least 10 NFTs per mm^2 (10-μm sections) in one or more of the cortical regions examined. In a study to be described elsewhere, a statistically significant 25-fold increase in mAb 423 immunoreactivity in ABCsup preparations has been found in 7 Alzheimer disease cases, when compared with 3 age-matched controls (E.B.M.-L., unpublished data).

DISCUSSION

The results of this and our other study (10) demonstrate that mAb 423 fails to recognize any form of normal tau protein that we have been able to prepare, whether isolated from adult human brain or expressed in bacteria, other than that which is released from the Pronase-resistant core of the PHF. In contrast, mAb 7.51 recognizes all forms of isolated or expressed tau protein. mAb 423 recognizes a PHF-specific conformation of the repeat region of tau protein, whereas the mAb 7.51 epitope has been mapped to a segment in the C-terminal half of the repeat region that is common to all isoforms of tau protein (10). Nevertheless, the mAb 7.51 epitope is inaccessible when the 12-kDa tau fragment is bound within the PHF core and is exposed only in acidic conditions that release the repeat region from the core of the PHF. In contrast, the PHF-specific conformation of the repeat region of tau protein that is detected by mAb 423 survives release from the PHF core, but the binding affinity for the antibody is decreased by approximately half. These results show that the repeat region of tau protein can exist in three immunochemically distinct forms in Alzheimer disease: (*i*) bound within the PHF core (full mAb 423 reactivity, mAb 7.51 nonreactive); (*ii*) released from the PHF core (half mAb 423 reactivity, mAb 7.51 reactive); (*iii*) non-PHF-core tau species; i.e., normal microtubule-associated or free tau protein (mAb 423 nonreactive, mAb 7.51 reactive).

These immunochemical differences have provided the basis for distinguishing between normal tau proteins and those which have become integrated into the PHF core. These distinctions have been demonstrated in this study by measuring different presentations of tau protein in layers of the temporal cortex. Normal tau protein immunoreactivity is soluble and predominant in the axonal compartment, as shown previously by immunohistochemical methods (2, 21). PHF-specific tau immunoreactivity predominates in the grey matter and is largely insoluble. That tau immunoreactivity in the insoluble fraction, measured using mAb 423, must exist in a PHF-like configuration was confirmed by the observation that the mAb 7.51 epitope remained hidden in this fraction unless revealed with formic acid treatment. These data also confirm that the mAb 423 immunoassays specifically measure PHF-associated tau immunoreactivity.

PHFs are the main structural constituents both of NFTs and of the dystrophic neurites that form in neuritic plaques and also appear diffusely in the neuropil as so-called "curly fibers." NFTs have long been known to correlate with cognitive deficit (22, 23) and it has been shown that tau-immunoreactive neuritic plaques correlate better with dementia than senile plaques detected with anti-β-amyloid antibodies (24). The ability to measure PHF-specific tau immunoreactivity in Alzheimer brains is important for clinicopathological correlation studies in Alzheimer disease. The findings of the present study demonstrate that it is now

possible both to distinguish and to measure PHF tau in the presence of normal tau protein. This depends on the absence of mAb 423 reactivity with normal tau protein.

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