American Journal of Pathology, Vol. 145, No. 6, December 1994

Ultrastructure and Biochemical Composition of Paired Helical Filaments in Corticobasal Degeneration

Hanna Ksiezak-Reding, Karen Morgan, Linda A. Mattiace, Peter Davies, Wan-Kyng Liu, Shu-Hui Yen, Karen Weidenheim, and Dennis W. Dickson

From the Department of Pathology, Albert Einstein College of Medicine, Bronx, New York

Corticobasal degeneration (CBD) is a neurodegenerative disorder associated with extensive cytoskeletal abnormalities. These include tau-positive neuropil threads and grains, ballooned or swollen neurons, neurofibrillary tangles, and glial inclusions. Given the presence of tau-positive structures in CBD, we investigated whether abnormalities in tau proteins associated with CBD were similar to those in Alzheimer's disease (AD). Fractions of abnormal tau proteins were isolated as Sarkosyl-insoluble pellets. By electron microscopic examination, the fraction from CBD contained twisted filaments that differed from paired helical filaments of AD. In CBD, filaments were shorter in length, rarely longer than 400 nm, 10 to 20% wider in the maximum and minimum widths (26 to 28 nm and 13 to 14 nm, respectively), and the periodic twist (169 to 202 nm) was twice as long as that in AD. Immunogold labeling with a panel of tau-reactive antibodies (Alz 50, Tau 14, AH-1, E-11, PHF-1, and Tau 46) showed no apparent differences in the pattern of tau immunoreactivity between filaments of CBD and AD. Western blots revealed that polypeptides of abnormal tau were present in both fractions; however, only two polypeptides (68 and 64 kd) were present in CBD as compared with three (68, 64, and 60 kd) in AD. Both of these polypeptides were reactive with additional antibodies (E-9, Tau-1 after dephosphorylation, AT8, and NP8). Only one polypeptide (68 kd) bound an antibody to adult-specific tau sequence encoded by exon 2, but neither was reactive with antibodies to adult-specific sequences encoded by exons 3 and 10. The results suggest that abnormalities in the number and heterogeneity of isoforms of tau may be one of the factors contributing to ultrastructural differences in pathological filaments of CBD and AD. (Am J Pathol 1994, 145:1496–1508)

Corticobasal degeneration (CBD) is an uncommon, late-onset neurodegenerative disorder. Clinical symptoms are associated with cognitive (apraxia and aphasia) and extrapyramidal motor dysfunction (rigidity and dystonia), and moderate dementia.[1–3] Although original neuropathological studies emphasized achromatic or neurofilament-positive ballooned neurons[4] in the cortex and nuclei of the extrapyramidal system, recent immunocytochemical studies of CBD have demonstrated more extensive cytoskeletal alterations, including tau-positive neuropil threads in the white matter, tau-positive grains in the gray matter, tau-positive glial inclusions (so called coiled bodies[5]), and occasional neurofibrillary tangles.[3,6,7] The accumulation of tau-reactive lesions suggests a similarity to Alzheimer's disease (AD) or progressive supranuclear palsy (PSP)[8,9] even though these disorders are histopathologically and clinically distinct. One major difference between AD and CBD is the absence of senile (amyloid) plaques in CBD.[2,5]

It is well established that in AD neurofibrillary tangles, neuropil threads, and some of the abnormal neurites in senile plaques are composed primarily of paired helical filaments (PHF).[10,11] A main component of PHF has been characterized as hyperphosphorylated tau proteins and designated PHF-tau or A68.[12–16] Although ultrastructural studies of lesions in CBD and related conditions suggest that the neuronal

Supported by NIH Grants AG06803 and NS30027.

Accepted for publication August 15, 1994.

Address reprint requests to Dr. H. Ksiezak-Reding, Department of Pathology, Room F-538, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.

and glial inclusions contain filaments distinct from PHF, their biochemical composition and characterization is unknown. In particular, it is unknown whether filaments that accumulate in CBD are composed of hyperphosphorylated tau proteins similar to PHF-tau from AD.

Tau proteins belong to the family of microtubule-associated proteins, and they play an important role in microtubule assembly/disassembly and stability.[17,18] There are multiple isoforms of tau; in adult human brain five to six different polypeptides of 65 to 50 kd can be distinguished by polyacrylamide SDS-gel electrophoresis.[19–21] The heterogeneity of tau is partially due to alternative splicing of the tau gene[22] located on chromosome 17.[23] Furthermore, expression of sequences encoded by exons 2, 3, and 10 is known to be developmentally regulated.[24,25] Additional microheterogeneity of tau is due to post-translational modifications such as phosphorylation.[26,27] There are at least two major differences between tau and PHF-tau of AD. By polyacrylamide SDS-gel electrophoresis, PHF-tau contain three major polypeptides of 68, 64, and 60 kd and the phosphate content of PHF-tau (6 to 8 mol phosphate/mol protein) is three to four times more than normal tau.[28,29] The role of these differences in formation of abnormal filaments is unclear inasmuch as PHF-like structures can be polymerized *in vitro* from bacterially expressed nonphosphorylated isovariant of normal human tau.[30]

Abnormal, tau-immunoreactive filaments have been demonstrated in other neurodegenerative disorders including Pick's disease and PSP. Ultrastructurally, they differ from classic PHF, which are described as helically twisted ribbons of 20-nm maximum and 10-nm minimum widths, with a periodic twist of 80 nm.[10,11,31] In Pick's disease, two kinds of filaments have been described: 10- to 15-nm straight filaments and 24-nm-wide long period PHF-like filaments, twisted every 130 to 160 nm.[32,33] In PSP, filaments are predominantly 15- to 18-nm straight filaments.[8,34,35] As in AD, abnormal tau proteins have been detected in PSP by Western blotting. Unlike PHF-tau in AD, however, the abnormal tau proteins in PSP were composed of two polypeptides of 69 and 64 kd, and the PHF-tau polypeptide with the lowest molecular weight was absent.[36]

It is unclear whether the structural diversity of filaments in neurodegenerative diseases is related to the composition of PHF-tau proteins. In the present paper, using ultrastructural, morphological, and biochemical methodologies, we investigated whether abnormalities of tau proteins associated with CBD are similar to those observed in AD.

Materials and Methods

Tissues

Brain tissue from two patients with CBD (case 1: 71-year-old woman, 6-hour postmortem delay; and case 2: 74-year-old woman, 24-hour postmortem delay) and a patient with AD (88-year-old woman, 8-hour postmortem delay) were examined histopathologically at the time of autopsy and kept frozen at −70 C until used. The diagnosis of CBD was based on pathological findings of neuronal loss in cortex and substantia nigra and ballooned neurons in cortex, basal ganglia, and brainstem as well as presence of characteristic tau-positive neuronal and glial lesions[6,7] (see below). The diagnosis of AD was based on the modified, age-adjusted quantitative criteria of Khachaturian[37] in which neocortical neurofibrillary tangles were required in all ages.

Isolation of PHF

PHF-enriched fractions were obtained from frontal lobe white and gray matter as Sarcosyl-insoluble 100,000 × *g* pellets.[28] The Sarcosyl-insoluble pellets from 10 grams of brain tissue were suspended in 0.5 to 1 ml of 50 mmol/L 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.8, and these fractions were used in most experiments. In other experiments, PHF-enriched fractions were further purified on sucrose density gradients according to previously described procedures[38] and fraction A2, which sedimented in the 1-mol/L sucrose layer, was collected.

Electron Microscopy (EM) and Immunogold EM

Samples (25 to 50 μl) were deposited on copper grids (200 mesh), precoated with Formvar and carbon (E. Fullam, Latham, NY), and stained with 2% uranyl acetate before or after labeling with 10-nm immunogold.[39]

SDS-Gel Electrophoresis and Western Blots

Samples were subjected to electrophoresis on 10% polyacrylamide SDS-gels according to Laemmli[40] and transferred to nitrocellulose paper.[41] Electroblots were incubated with 5% fat-free milk in 10 mmol/L Tris/HCl, pH 7.4, containing 150 mmol/L NaCl before incubation with primary antibodies. Secondary antibodies were conjugated to biotin (ABC VectaStain;

Vector Laboratories, Burlingame, CA). Diaminobenzidine and hydrogen peroxide were used as peroxidase substrates. Some electroblots were preincubated with alkaline phosphatase (*Escherichia coli*, 10 IU/ml; Sigma Chemical Co., St. Louis, MO) before immunostaining, as previously described.[28]

Antibodies

Monoclonal antibodies and antisera that recognize distinct regions of the tau molecule (Figure 1) included Alz 50,[42,43] NP8 and NP18,[44,45] PHF-1,[21,46] Tau-1[47] (provided by Lester Binder), Tau 14 and Tau 46[47] (provided by Virginia Lee University of Pennsylvania, Philadelphia, PA), antiserum AH-1[22] from Genentech (San Francisco, CA), and AT8[48] from Innogenetics (Ghent, Belgium). A monoclonal antibody Ab39, raised to Alzheimer neurofibrillary tangles, recognizes a conformational epitope unique to PHF and straight filaments in PSP[49] and does not bind to PHF-tau on Western blots.[50] Antisera E-2, E-3, E-9,[51,52] and E-11[38] were raised to synthetic peptides corresponding to tau sequences encoded by exons 2, 3, 9, and 11, respectively. Antiserum E-10 was raised in rabbits (Immunodynamics, La Jolla, CA) to a 15-mer synthetic peptide of tau according to the procedure described earlier.[38] For Western blotting, antibodies were diluted 1:1 (Alz 50), 1:5 (Tau 14), 1:10 (Tau-1, E-10, and E-11), 1:50 (E-3), 1:100 (NP-8 and E-2), 1:200 (PHF-1, AT8, AH-1, and E-9), and 1:2000 (Tau 46). For immunogold EM, antibodies were diluted 5- to 10-fold more than for Western blotting. Amyloid was detected with a rabbit polyclonal antiserum to [beta]-amyloid synthetic peptide which has been characterized previously.[53] Oligodendroglia were detected with Leu7, a monoclonal antibody commercially available from Becton Dickinson (Mountain View, CA).

Histochemistry and Immunocytochemistry

Brain tissue obtained at the time of autopsy was immersion fixed for 12 to 16 hours in 4% paraformaldehyde, stored in 30% sucrose, and subsequently sectioned with a vibratome at a thickness of 50 μ. For immunostaining, sections were preincubated with 0.3% peroxide to block endogenous peroxidase and 5% nonfat milk to block nonspecific antibody binding and then incubated with primary and secondary antibodies as described before.[50] Primary monoclonal antibodies (NP18, Ab39, and Alz 50) were diluted 1:5 or 1:10 (Leu7), the antibody to [beta]-amyloid was diluted 1:250 to 1:400. Antibody binding was detected with peroxidase-conjugated, isotype-specific secondary antibodies. Paraffin sections (7 μ thick) were stained with hematoxylin and eosin (H&E), Luxol fast blue stain for myelin, and Bodian's silver stain for neurofilaments.

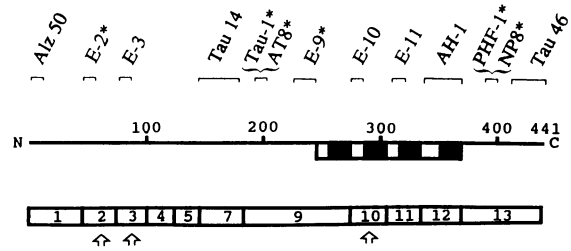


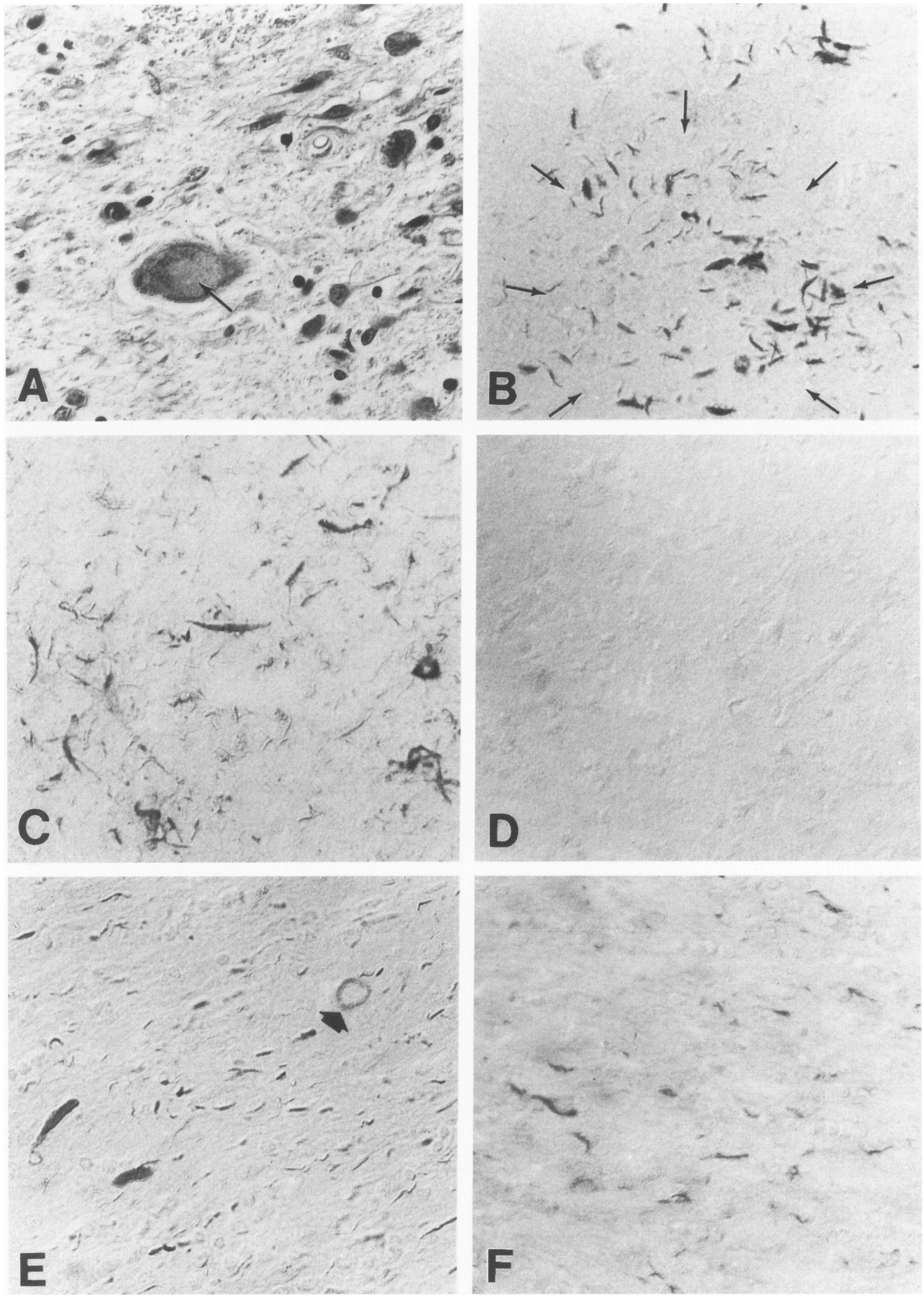
Figure 1. *Schematic diagram of the longest isoform of tau with 441 amino acid residues[56] and location of epitopes for antibodies used in the present study. Asterisks denote antibodies to epitopes that are phosphorylated in AD. The microtubule-binding domain of tau has four repeats, each containing a variable (white rectangle) and a constant (black rectangle) region. Exons encoding different regions of tau are numbered 1 to 13 and regions that are adult specific are marked (arrows).*

Results

Neuropathology

H&E-stained sections of cortex, including the parietal cortex, of both CBD cases revealed ballooned achromatic neurons that are one of the hallmarks of the disease (not shown). Other characteristic features such as neuronal loss, gliosis, and neurons with basal bodies were also detected in the substantia nigra (Figure 2A). The ballooned neurons were positive with antibody NP18 for phosphorylated neurofilament.[4,6] A characteristic type of neuritic plaque, composed of clusters of grain-like neurites,[6] was present in the cortex of CBD. The neurites in CBD could be immunostained with a PHF-specific antibody, Ab39 (Figure 2B). Neuritic changes in the cortex were also stained with Alz 50 (Figure 2C), suggesting their relationship to abnormal tau. In contrast to AD, however, immunostaining with an antibody to [beta]-amyloid was completely negative in the cortex (Figure 2D). In the CBD cases, white matter changes were more pronounced than in most cases of AD. In the white matter, Luxol fast blue staining revealed depletion of myelinated fibers that was also detected with Bodian's stain (not

Figure 2. *Neuropathological changes in neurons and the white matter from CBD.* **A:** *Neuronal loss, gliosis, and swollen neurons (basal bodies, arrow), one of the hallmarks of CBD, in the substantia nigra (H&E stain).* **B:** *Cluster of neurites in the cortex immunostained with Ab39.* **C:** *Neuritic changes in the cortex immunostained with Alz 50.* **D:** *In the cortex, negative immunostaining with an antibody to [beta]-amyloid. In the white matter, abnormal profiles in processes and glia (arrow) detected with antibody Ab39 (E) and Alz 50 (F).*


A
B
C
D
E
F

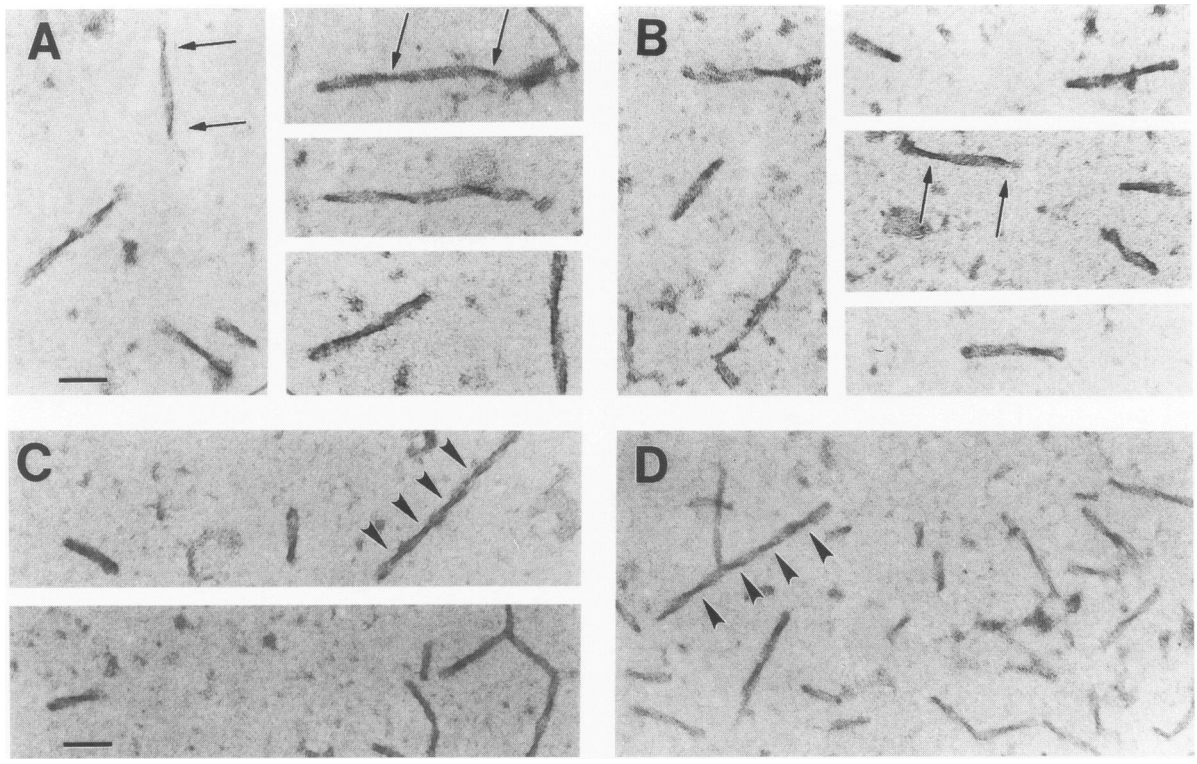


Figure 3. *Electron micrographs of filaments isolated from brain from CBD* (**A** *and* **B**) *or AD* (**C** *and* **D**) *and stained with uranyl acetate.* **A** *and* **C**: *white matter;* **B** *and* **D**: *gray matter. In CBD, filaments were dispersed and short in length, rarely longer than two twists, with a periodic twist every 169 to 202 nm* (*arrows*). *Filaments were more numerous in white than gray matter and aggregates were not observed. In AD, PHF were longer, four twists or more, with a periodic twist every 85 to 93 nm* (*arrowheads*), *and they were more numerous in gray than white matter. A variable number of bundles and aggregates of filaments was detected* (*not shown*[35]). *Scale bars correspond to 100 nm.*

shown). In addition, abnormal argyrophilic inclusions in glia that were demonstrated with Bodian's stain were also positive with Ab39 and Alz 50 (Figure 2E, F, respectively). The white matter inclusions stained with tau and PHF markers were double stained with Leu7, a marker for oligodendroglia (unpublished observation).

Ultrastructure of PHF

In both cases of CBD studied, Sarcosyl-insoluble pellets of frontal lobe white and gray matter contained abnormal, twisted filaments with some similarities to PHF of AD (Figure 3). Although some of the appearance could be due to the effects of isolation procedure, the filaments were usually short, rarely longer than two twists, and less aggregated than filaments from AD.[38] Ultrastructural examination of unfixed preparations revealed differences from PHF of AD. Whereas PHF in AD had maximum and minimum widths of 22 to 24 nm and 11 to 12 nm, respectively, and periodic twists every 85 to 93 nm, filaments in CBD had the maximum and minimum widths of 26 to 28 nm and 13 to 14 nm, respectively, and their periodic twist was 169 to 202 nm apart (Table 1). Thus, filaments in CBD were wider by 10–20% and their periodic twists were twice as long. In both disorders, filaments tended to be wider by 5–10% in white matter than in gray matter, although the difference in width was not statistically significant (Student's *t*-test).

Immunogold Labeling

In CBD, filaments from both white and gray matter were immunolabeled with anti-tau antibodies that recognize N-terminal epitopes of tau (Alz 50 and Tau 14), microtubule binding domain (E-11 and AH-1), and either phosphorylated (PHF-1) or nonphosphorylated (Tau 46) C-terminal epitopes of tau (Figures 4 and 5). The intensity and pattern of immunogold decoration were similar in filaments from both diseases regardless of whether they were obtained from gray or white matter. The similarity was evident in both the marked labeling with antibodies to N- or C-terminal epitopes and in less intense and polar labeling with antibodies to the microtubule-binding domain. The polar pattern of labeling has been observed previously in PHF of

Table 1. *Comparison of Physical Dimensions of PHF from CBD and AD*

Source of PHF	Dimensions (*nm*)*		
	Maximum Width	Minimum Width	Periodic Twist
CBD			
White matter	28 ± 4† (37)	14 ± 3† (49)	202 ± 42‡ (17)
Gray matter	26 ± 3‡ (11)	13 ± 2† (16)	169 ± 8‡ (3)
AD			
White matter	24 ± 4 (13)	12 ± 2 (14)	85 ± 7 (6)
Gray matter	22 ± 3 (30)	11 ± 2 (35)	93 ± 12 (25)

* Values are means ± SD for the number of measurements in the parentheses. Note that in CBD, periodic twist of filaments is twice as long as that in AD.
† $P < 0.01$.
‡ $P < 0.001$ as compared with respective values in AD (Student's *t*-test).

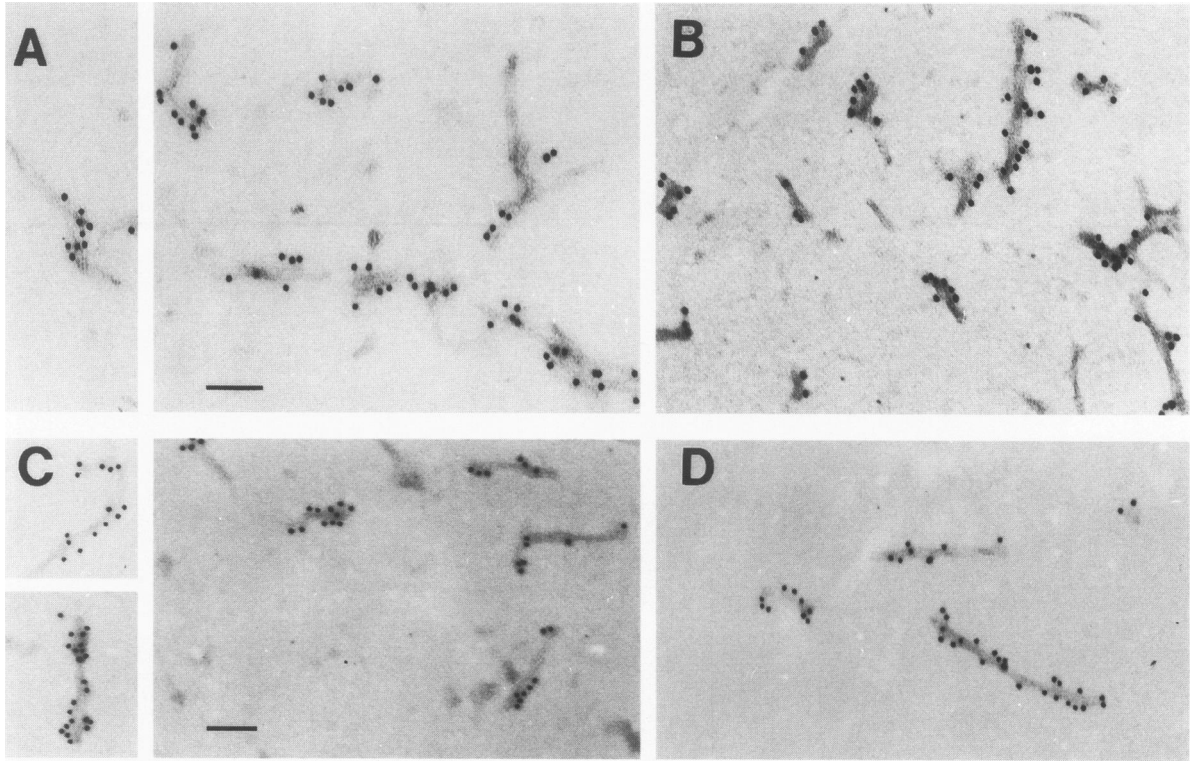


Figure 4. *Immunogold EM of filaments from CBD* (**A** *and* **C**) *and AD* (**B** *and* **D**) *with antibodies to N-terminal tau epitopes, Alz 50* (*in* **A** *and* **B**) *and Tau 14* (*in* **C** *and* **D**). *In both disorders, a heavy immunodecoration was detected along the entire length of filaments. Scale bars correspond to 100 nm.*

AD.[38] This pattern has been attributed to the limited access of the microtubule-binding domain to antibody binding in nonaggregated and nondigested filaments. As tested with various dilutions, the distinct pattern and low intensity of labeling of filaments were not due to the antibody titer.

Western Blotting: CBD

Western blotting of filament-enriched fractions from CBD white and gray matter revealed the presence of abnormal tau-immunoreactive proteins. In contrast to PHF-tau of AD, however, only two polypeptides of 68 and 64 kd were consistently detected (Figure 6A, B). The third polypeptide of 60 kd, usually present in AD samples, could not be demonstrated with most of the antibodies. With antibody Tau 46, however, a weakly reactive band of 60 kd was noticed in some preparations, suggesting that the third polypeptide of abnormal tau may be present in trace quantities. Alternatively, the Tau 46-reactive 60-kd polypeptide could represent a degradation product of 68- or 64-kd polypeptides. As detected with a panel of well characterized, tau-reactive antibodies, both 68- and

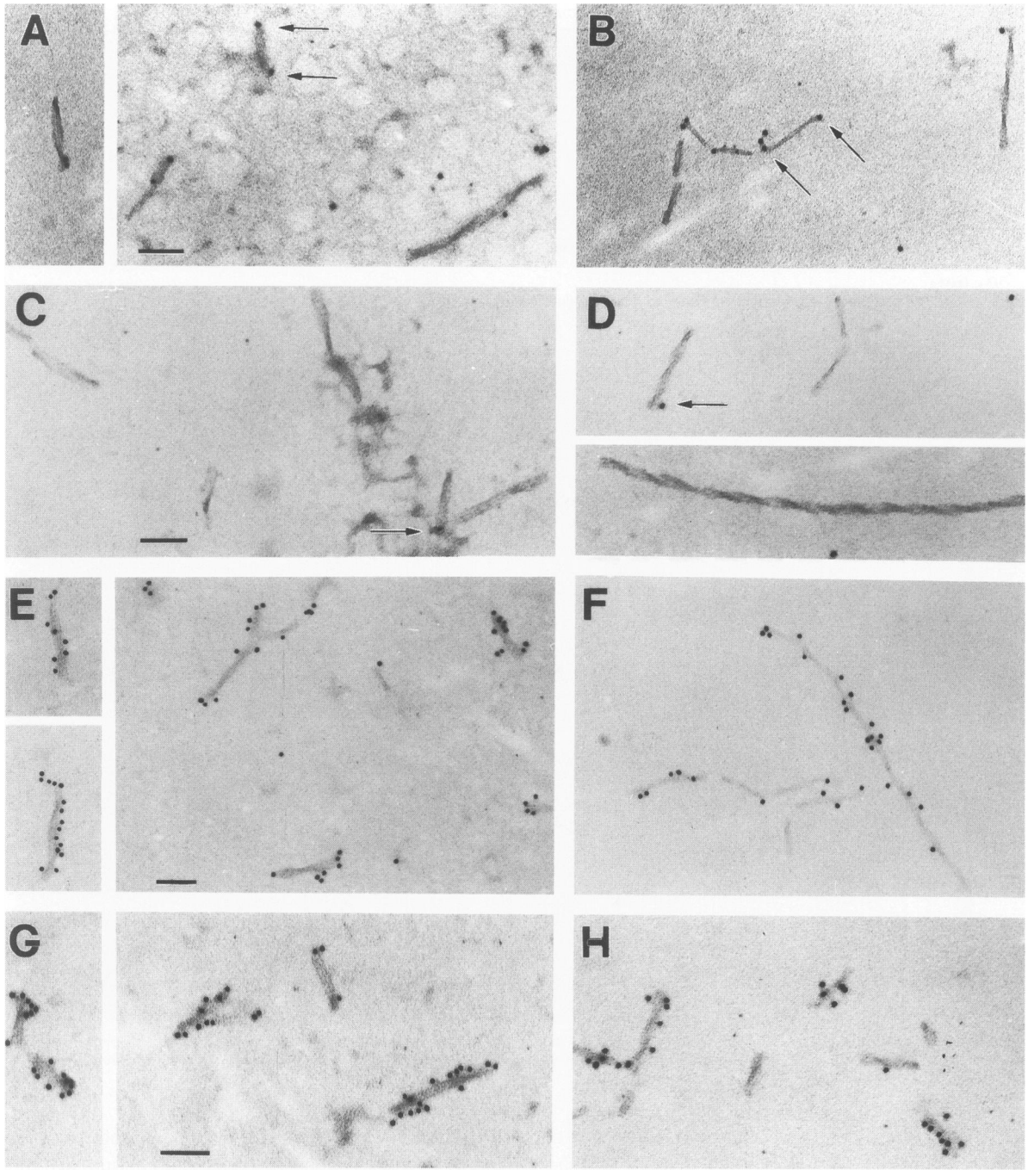


Figure 5. *Immunogold EM of filaments from CBD* (A, C, E, *and* G) *and AD* (B, D, F, *and* H) *with antibodies to microtubule-binding domain, AH-1* (A *and* B) *and E-11* (C *and* D), *and C-terminal tau epitopes, PHF-1* (E *and* F) *and Tau 46* (G *and* H). *Immunodecoration with AH-1 and E-11 was sparce, mostly at the ends of filaments (arrows in* A *to* D). *Heavy immunodecoration with PHF-1 and Tau 46 was found along the entire length of filaments. Scale bars correspond to 100 nm.*

64-kd polypeptides contained multiple epitopes from N- and C-termini of tau and the microtubule-binding domain. The common epitopes included nonphosphorylated (Alz 50 and Tau 46) as well as phosphorylated (AT8, PHF-1, and NP8) epitopes and epitopes blocked by phosphorylation (Tau-1). Both polypeptides also contained the E-9 epitope, which has recently been reported to be phosphorylated in some PHF-tau from AD.[52] Two epitopes, E-3 and E-10, could not be detected in either polypeptide. Another epitope, E-2, was demonstrated only in the 68-kd polypeptide. The binding of the E-2 antibody to the 68-kd polypeptide was marginally improved after phosphatase treatment suggesting a partial phos-

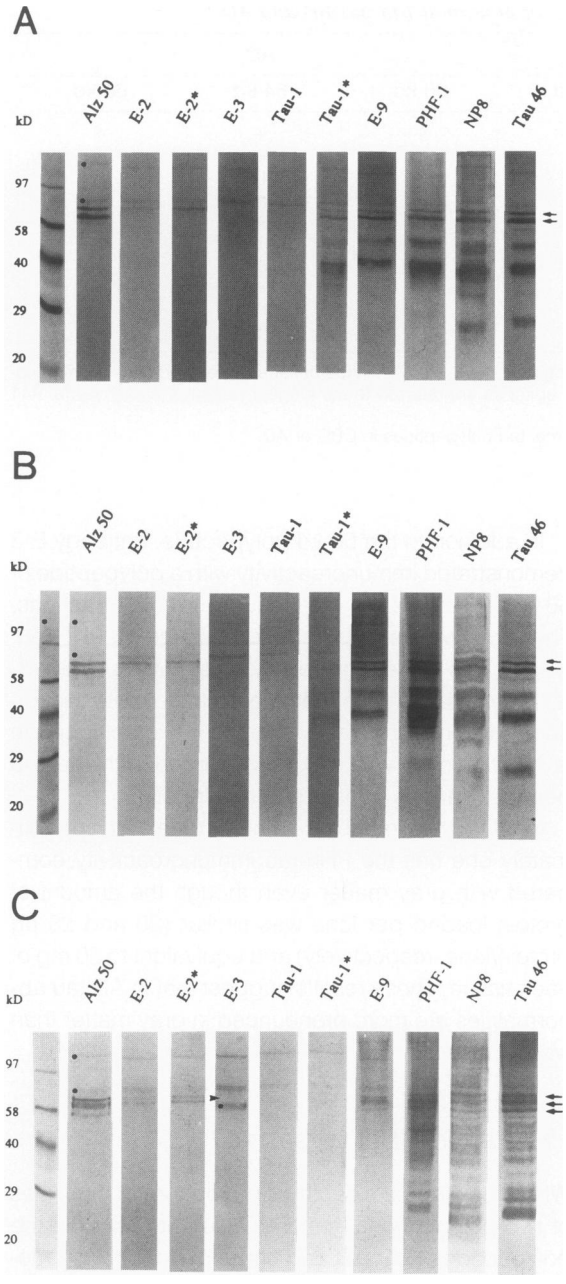


Figure 6. *Immunoblots of filament-enriched fraction from white matter* (**A**) *or gray matter* (**B**) *of CBD and white matter of AD* (**C**) *with a panel of tau antibodies. Asterisk* (*) *denotes blots treated with alkaline phosphatase. In both fractions from CBD* (**A** *and* **B**), *two polypeptides of 68 and 64 kd* (*arrows*) *were detected with most antibodies, one polypeptide* (*68 kd*) *was immunoreactive with E-2, but none with E-3. By comparison, in white matter from AD* (**C**) *three polypeptides of PHF-tau* (*68, 64, and 60 kd, arrows*) *were detected with most antibodies, two polypeptides* (*68 and 64 kd*) *were recognized by E-2 and only one polypeptide* (*68 kd, arrowhead*) *was recognized by E-3. The staining of polypeptides migrating at 120 kd and 70 to 75 kd in all blots* (*marked with dots in lane Alz 50*) *and the 58-kd polypeptide in a blot with E-3* (*marked with a dot in* **C**) *are considered nonspecific. Amount of protein used for immunoblotting:* (**A**), *23 μg,* (**B**), *50 μg, and* (**C**) *20 μg per lane.*

phorylation of this polypeptide in CBD as in AD.[51] The overall results of immunoblotting suggested that the abnormal polypeptides closely resembled PHF-tau from AD.

The CBD fractions contained a polypeptide of 120 kd and a doublet at 70 to 75 kd, which were detected with all antibodies with the avidin-biotin system (see Materials and Methods). The staining of these polypeptides was considered nonspecific because it was observed after the omission of the primary antibodies. Most likely, the staining was due to the binding of the secondary antibody (avidin-peroxidase) to those proteins that contain the covalently bound biotin, eg, carboxylases.[54]

In CBD, more immunoreactive PHF-tau polypeptides per gram tissue was detected in the white matter than in the gray matter fractions. This was demonstrated by the fact that the PHF-tau immunoreactivity, observed in samples of white matter equivalent to 20 mg of tissue (23 μg protein/lane), was similar to that seen in samples of gray matter equivalent to 40 mg of tissue (50 μg protein/lane Figure 6A, B). The results of immunoblotting together with neuropathological findings indicate that abnormalities in tau proteins in CBD are present in both white and gray matter. The results suggest also that tau abnormalities are possibly more pronounced in white matter than gray matter.

Western Blotting: AD

For comparisons with CBD, PHF-enriched fractions were prepared from white and gray matter of AD brain. In both fractions, three PHF-tau polypeptides of 68, 64, and 60 kd were detected as illustrated for white matter (Figure 6C). The immunoreactivity of PHF-tau polypeptides with tau-reactive antibodies was similar to that reported elsewhere.[16,19,42,51,55] In summary (Table 2), all three polypeptides were immunoreactive with antibodies to nonphosphorylated (Alz 50 and Tau 46) and phosphorylated (AT8, PHF-1, and NP8) epitopes. They were also immunoreactive with antibody E-9, which has recently been reported to recognize an epitope phosphorylated in less than 50% of the PHF-tau.[52] As previously demonstrated, the Tau-1 epitope was blocked by phosphorylation but could be detected after phosphatase treatment (Figure 6C, Tau-1*). From the triplet PHF-tau, only two polypeptides of 68 and 64 kd were immunoreactive with E-2 and E-10 (Figures 6C and 7, respectively). The immunoreactivity of these polypeptides with E-2 improved after phosphatase treatment indicating that the epitope was phosphorylated in some of the PHF-

Table 2. *Summary of Immunoreactivities of Individual Polypeptides of Abnormal Tau in CBD and AD*

Antibody	Epitope	CBD		AD		
		68 kd	64 kd	68 kd	64 kd	60 kd
Alz 50	2–10	+*	+	+	+	+
E-2†	44–55	+	0	+	+	0
E-3	75–86	0	0	+	0	0
Tau-1†	192–199	+	+	+	+	+
AT8‡	202	+	+	+	+	+
E-9†	226–240	+	+	+	+	+
E-10	274–283	0	0	+	+	0
PHF-1‡	389–402	+	+	+	+	+
NP8‡	235/396?	+	+	+	+	+
Tau 46	404–441	+	+	+	+	+

* Presence (+) or absence (0) of epitopes was based on Western blotting (Figures 6 and 7). Antibodies that were used only for immunocytochemistry or in EM immunogold studies are not listed. Positions of the epitopes are referred to the longest variant of human tau of 441 amino acid residues.[56]

† Antibodies to epitopes that are phosphorylated in all or some of abnormal tau polypeptides in CBD or AD.

‡ Antibodies to phosphorylated epitopes.

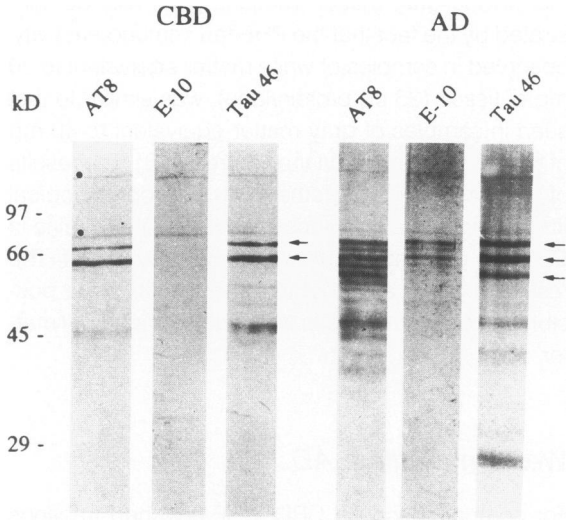


Figure 7. *Immunoblots of filament-enriched fractions from white matter (CBD) or gray matter (AD) with tau antibodies AT8, E-10, and Tau 46. Two polypeptides (68 and 64 kd) in CBD and three polypeptides (68, 64, and 60 kd) in AD were detected with both AT8 and Tau 46 (arrows). Note that E-10 recognized PHF-tau of 68 and 64 kd in AD, but not these in CBD. In addition to PHF-tau triplet, AD samples also contained N-terminal degradation products of PHF-tau (66, 62, and 58 kd) that were positive with AT8 or E-10 but not Tau 46. Their presence was apparently due to the C-terminal proteolysis of samples. Nonspecific staining of bands at 70 to 75 kd and 120 kd (marked with dots in lane AT8) was observed in most blots.*

tau as reported previously.[51] Consistent with the previous studies,[51] antibody E-3 was immunoreactive with only one of the triplet PHF-tau, the 68-kd polypeptide (Figure 6C, arrowhead). It is interesting to note that the immunoreactivity of the 68-kd polypeptide with E-3 was much less intense in white matter than in gray matter with similar amounts of protein loaded per lane (20 and 28 μg/lane, respectively). Also, in comparison with other antibodies (eg, E-2), the immunoreactivity of the 68-kd polypeptide with E-3 appeared to be much lower in white matter than gray matter.

In addition to the 68-kd polypeptide, antibody E-3 demonstrated immunoreactivity with a polypeptide of 58 kd present in both white and gray matter fractions. This polypeptide is probably unrelated to PHF-tau, since it was not recognized by other anti-tau antibodies. As demonstrated in previous studies with this antibody, the immunoreactivity of the 58-kd polypeptide is not detected with E-3 preadsorbed with the E-3 peptide[51] and is considered nonspecific.

In AD, white matter fractions contained approximately one half the PHF-tau immunoreactivity compared with gray matter even though the amount of protein loaded per lane was similar (20 and 28 μg protein/lane, respectively) and equivalent to 20 mg of each tissue. These results suggest that in AD tau abnormalities are more pronounced in gray matter than white matter.

PHF-tau in CBD versus AD

When run side by side, the electrophoretic mobilities of two polypeptides from CBD were identical to the polypeptides from AD and corresponded to the molecular weight of 68 and 64 kd (Figure 7). We were interested to determine whether tau immunoreactivities of these co-migrating polypeptides were also identical. In Table 2, we compared the pattern of immunoreactivity of all polypeptides from CBD and AD with a panel of tau antibodies. We focused on adult-specific epitopes E-2, E-3, and E-10 because their presence or absence could distinguish individual tau isoforms. From this comparison, it is apparent that CBD polypeptides are not identical to similarly sized polypeptides from AD. Instead, they have closer similarity to the 64- and 60-kd polypeptides, respectively. In particular, the CBD polypeptide of 68 kd resembled the AD polypeptide of 64 kd by the presence of one

adult epitope (E-2) but differed by the absence of another adult-specific epitope (E-10). The polypeptide of 64 kd from CBD was identical to the polypeptide of 60 kd from AD by the absence of all three adult-specific tau epitopes.

Discussion

These results have demonstrated that brain tissue of patients with CBD display cytoskeletal changes similar to, but not identical to, those in AD. These abnormalities were present in both white and gray matter and consisted of changes in neurons and glia. Neuronal changes included neurofilament protein-positive swollen perikarya and tau-positive neuritic processes (grains and neuropil threads). Glial changes consisted of tau-positive filamentous inclusions. Only a small number of neurofibrillary tangles were detected in cortex and hippocampus. Although most of the neuronal and glial changes in CBD were immunoreactive with antibodies to tau, they were morphologically distinct from neuronal alterations that characterize AD. Moreover, they were observed in the absence of immunoreactivity with a [beta]-amyloid antibody. Therefore, the neurofibrillary degeneration in CBD supports the hypothesis that widespread cytoskeletal changes are not necessarily directly related to amyloid formation as was postulated for AD.[57]

Fractions isolated from brain tissue of CBD patients contained twisted PHF-like filaments, which were less abundant than in most cases with AD. The abnormal, ultrastructurally similar filaments were present in fractions from both white or gray matter. Although effects of isolation procedure could not be discerned, the obtained filaments were short, rarely longer than two twists in length (approximately 400 nm) and dispersed. At their maximum width, the filaments were 26 to 28 nm wide. They narrowed to 13 to 14 nm at their minimum width every 169 to 202 nm. These dimensions differed from those of a population of nonaggregated PHF from AD by a 10 to 20% increase in width and longer periodicity. Moreover, the filaments from CBD shared only limited morphological resemblance to those found in other neurodegenerative disorders. For instance, filaments in PSP and postencephalitic Parkinson's disease are mostly straight 15- to 18-nm filaments.[8,34,35] Occasional 15- to 22-nm twisted filaments found in both disorders have variable periodicity[58] and the similarity with filaments in CBD is limited. There is strong possibility, however, that some of the reported differences may be due to examination of fixed tissues compared with isolated filament fractions. Nevertheless, ultrastructurally, CBD filaments showed the most similarity with the twisted 24-nm filaments found in Pick's disease.[32] The twisted 24-nm filaments, which coexist with straight 10- to 15-nm filaments in Pick's bodies, have overall dimensions nearly identical to that in CBD. They are 12 nm wide at the minimum width and have a periodic narrowing every 130 to 160 nm.

Although the ultrastructure of filaments in CBD differed from those in AD, the pattern of immunogold labeling with a panel of tau-reactive antibodies was indistinguishable. Intense labeling was observed with antibodies directed to either N- or C-termini of tau, including antibodies to phosphorylated epitopes. Moreover, a distinct polar pattern of immunodecoration was observed with antibodies to the microtubule-binding domain (eg, AH-1). This pattern was consistent with a domain buried in the core of filament and inaccessible to the antibody binding except for the ends. It was not due to low antibody titer. Polar binding of antibodies to filaments has previously been described for nonaggregated and nondigested PHF in AD,[38] suggesting that the arrangement of tau molecules is similar in both kinds of filaments.

The present data, as well as recent studies from several laboratories,[14–16,31,36,59] indicate that filaments from various neurodegenerative disorders, having different ultrastructural appearances, have a common structural component, hyperphosphorylated tau. Variability in tau may be one of the factors that lead to the ultrastructural heterogeneity of filaments. At present, the heterogeneity of abnormal tau has been recognized by differences in the number of isoforms with different electrophoretic mobility on SDS-gels. Among the diseases, this number varies from two to three polypeptides of 60 to 70 kd. In CBD, with a relatively homogeneous population of Sarkosyl-insoluble filaments, we have demonstrated the presence of two polypeptides of abnormal tau. Although these polypeptides migrated at 68 and 64 kd, their immunoreactivity more closely resembled the 64- and 60-kd PHF-tau polypeptides of AD when probed with antibodies recognizing adult-specific sequences of tau. These results suggest that electrophoretic mobility alone is not sufficient to distinguish among tau isoforms and that isoform-specific antibodies are needed to define more accurately the nature of tau abnormalities in these disorders.

Judging from the retardation on SDS-gels, the abnormal polypeptides from CBD may be phosphorylated to a higher degree than those in AD.[28,29] The degree to which tau proteins are phosphorylated is likely to be an additional important factor contributing to the diversity of tau and ultrastructural features of

filaments in neurodegenerative disorders. Further studies that include direct phosphate analysis are needed to confirm such a hypothesis. Alternatively, other post-translational modifications may also be involved in the alterations of tau in CBD. Ubiquitination, which has been demonstrated in neurofibrillary degeneration in AD[60] and other disorders, could be another such modification. The glial and neuronal lesions in CBD are indeed positive with ubiquitin immunocytochemistry.[61]

In the present paper, abnormal tau proteins from CBD have been found to be phosphorylated at several sites. These phosphorylation sites have been detected with the following antibodies: Tau-1, AT8, NP8/NP18, and PHF-1. Phosphorylation of tau in CBD is similar to that described previously for PHF-tau from AD.[38,44,46,48] Moreover, similar phosphorylation sites have been reported in abnormal tau proteins in other disorders.[62] Interestingly, some of these sites have been demonstrated to be phosphorylated in fetal but not adult tau,[48,63] suggesting that developmentally specific kinases responsible for phosphorylation of fetal tau may be expressed in neurodegenerative diseases.

The content of tau proteins in gray matter of normal brains has been reported to be similar,[19] higher,[59] or lower[64] than in white matter; however, variability in the number of tau isoforms has not been described with respect to gray and white matter.[19] There are indications, however, that in gray matter, more tau proteins are phosphorylated at the Tau-1 site than in white matter.[65,66] In the present study of CBD and AD, we found no significant differences between white and gray matter in terms of heterogeneity of tau on Western blots, as well as pattern of immunogold labeling and ultrastructure of filaments. White matter in CBD, however, contained approximately twice as much abnormal tau immunoreactivity as did the gray matter. Moreover, this ratio was reversed in AD, in agreement with other studies.[59] The comparison on the protein level supports the conclusion on the basis of immunocytochemical observations of intense involvement of white matter in CBD. It is possible that distinct patterns of tau abnormalities in CBD, AD, and other neurodegenerative disorders may be related to the degenerative process affecting different populations of cells.

Note Added in Proof

Since submission of this paper other published reports have emphasized the presence of widespread cytoskeletal changes in CBD, *eg*, Wakabayashi K, Oyanagi K, Makifuchi T, Ikuta F, Honna A, Honna Y, Horikawa Y, Tokiguchi S: Corticobasal degeneration: etiopathological significance of the cytoskeletal alterations. Acta Neuropathol (Berl) 1994, 87:545–553.

Acknowledgments

We thank Drs. L. Binder and V. Lee for their generous gift of Tau-1 and Tau 46, respectively. We also thank Genentech (San Francisco, CA), and Innogenetics (Ghent, Belgium) for providing us with antibodies AH-1 and AT8, respectively.

References

1. Rebeiz JJ, Kolodny EH, Richardson EP: Corticodentatonigral degeneration with neuronal achromasia. Arch Neurol 1968, 18:20–33
2. Gibb RG, Luthert PJ, Marsden CD: Corticobasal degeneration. Brain 1989, 112:1171–1192
3. Paulus W, Selim M: Corticonigral degeneration with neuronal achromasia and basal neurofibrillary tangles. Acta Neuropathol (Berl) 1990, 81:89–94
4. Dickson DW, Yen S-H, Suzuki KI, Davies P, Garcia JH, Hirano A: Ballooned neurons in select neurodegenerative diseases contain phosphorylated neurofilament epitopes. Acta Neuropathol (Berl) 1986, 71:216–223
5. Braak H, Braak E: Argyrophilic grains: characteristic pathology of cerebral cortex in cases of adult onset dementia without Alzheimer changes. Neurosci Lett 1987, 76:124–127
6. Mattiace LA, Wu E, Aronson M, Dickson DW: A new type of neuritic plaque without amyloid in corticonigral degeneration with neuronal achromasia (abstract). J Neuropathol Exp Neurol 1991, 50:310
7. Dickson DW, Mattiace LA: Immunocytochemical studies distinguish corticobasal degeneration from progressive supranuclear palsy (abstract). J Neuropathol Exp Neurol 1992, 51:321
8. Tabaton M, Whitehouse PJ, Perry G, Davies P, Autillo-Gambetti L, Gambetti P: Alz 50 recognizes abnormal filaments in Alzheimer's disease and progressive supranuclear palsy. Ann Neurol 1988, 24:407–413
9. Yamada T, McGeer PL, McGeer EG: Appearance of paired nucleated, tau-positive glia in patients with progressive supranuclear palsy brain tissue. Neurosci Lett 1992, 135:99–102
10. Kidd M: Alzheimer's disease - an electron microscopical study. Brain 1964, 87:307–320
11. Braak H, Braak E, Grundke-Iqbal I, Iqbal K: Occurence of neuropil threads in the senile human brain and in Alzheimer's disease: a third location of paired helical filaments outside of neurofibrillary tangles and neuritic plaques. Neurosci Lett 1986, 65:351–355
12. Brion JP, Passareiro H, Nunez J, Flament-Durand J: Mise en evidence immunologique de la proteine tau

au niveau des lesions de degenerescence neurofibrillaire de la maladie d'Alzheimer. Arch Biol (Bruxelles) 1985, 95:229–235
13. Grundke-Iqbal I, Iqbal K, Tung YC, Ouinlan M, Wisniewski HM, Binder LI: Abnormal phosphorylation of the microtubule-associated protein tau in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci USA 1986, 83:4913–4917
14. Greenberg SG, Davies P: A preparation of Alzheimer paired helical filaments that displays distinct tau proteins by polyacrylamide gel electrophoresis. Proc Natl Acad Sci USA 1990, 87:5827–5831
15. Lee VM-Y, Balin BJ, Otvos L, Trojanowski JQ: A68: a major subunit of paired helical filaments and derivatized forms of normal tau. Science 1991, 251:675–678
16. Ksiezak-Reding H, Yen S-H: Structural stability of paired helical filaments requires microtubule-binding domains of tau: a model for self-association. Neuron 1991, 6:717–728
17. Cleveland DW, Hwo S-Y, Kirschner MW: Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. J Mol Biol 1977, 116: 227–247
18. Wiche G, Oberkanins C, Himmler A: Molecular structure and function of microtubule-associated proteins. Int Rev Cytol 1991, 124:217–273
19. Ksiezak-Reding H, Binder LI, Yen S-H: Immunochemical and biochemical characterization of tau proteins in normal and Alzheimer's disease brains with Alz 50 and Tau-1. J Biol Chem 1988, 263:7948–7953
20. Goedert M, Jakes R: Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. EMBO J 1990, 9:4225–4230
21. Greenberg SG, Davies P, Schein JD, Binder LI: Hydrofluoric acid-treated tau-PHF proteins display the same biochemical properties as normal tau. J Biol Chem 1992, 267:564–569
22. Himmler A: Structure of bovine tau gene: alternatively spliced transcripts generate a protein family. Mol Cell Biol 1989, 9:1389–1396
23. Neve RL, Harris P, Kosik KS, Kurnit DM, Donlon TA: Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. Mol Brain Res 1986, 1:271–280
24. Kosik KS, Orecchio LD, Bakalis S, Neve RL: Developmentally regulated expression of specific tau sequences. Neuron 1989, 2:1389–1397
25. Goedert M, Spillantini MG, Potier MC, Ulrich J, Crowther RA: Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. EMBO J 1989, 8:393–399
26. Lindwall G, Cole RD: The purification of tau protein and the occurrence of two phosphorylation states of tau in brain. J Biol Chem 1984, 259:12241–12245
27. Butler M, Shelanski ML: Microheterogeneity of microtubule-associated tau proteins is due to differences in phosphorylation. J Neurochem 1986, 47: 1517–1522
28. Ksiezak-Reding H, Liu W-K, Yen S-H: Phosphate analysis and dephosphorylation of modified tau associated with paired helical filaments. Brain Res 1992, 597:209–219
29. Kopke E, Tung YC, Shaikh S, Alonso AC, Iqbal K, Grundke-Iqbal I: Microtubule-associated protein tau; abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. J Biol Chem 1993, 268:24374–24384
30. Crowther RA, Olesen OF, Smith MJ, Jakes R, Goedert M: Assembly of Alzheimer-like filaments from full-length tau protein. FEBS Lett 1994, 337:135–138
31. Crowther RA: Structural aspects of pathoiogy in Alzheimer's disease. Biochim Biophys Acta 1991, 1096: 1–9
32. Takauchi S, Hosomi M, Marasigan S, Sato M, Hayashi S, Miyoshi K: An ultrastructural study of Pick bodies. Acta Neuropathol 1984, 64:344–348
33. Yen S-H, Dickson DW, Peterson C, Goldman JE: Cytoskeletal abnormalities in neuropathology. Progress in Neuropathology, vol. 6. Edited by HM Zimmerman. New York, Raven Press, 1986, pp 63–90
34. Powell HC, London GW, Lampert PW: Neurofibrillary tangles in progressive supranuclear palsy. J Neuropathol Exp Neurol 1974, 33:98–106
35. Takauchi S, Mizuhara T, Miyoshi K: Unusual paired helical filaments in progressive supranuclear palsy. Acta Neuropathol 1983, 59:225–228
36. Flament S, Delacourte A, Verny M, Hauw J-J, Javoy-Agid F: Abnormal tau proteins in progressive supranuclear palsy: similarities and differences with the neurofibrillary degeneration of the Alzheimer type. Acta Neuropathol 1991, 81:591–596
37. Khachaturian ZS: Diagnosis of Alzheimer's disease. Arch Neurol 1985, 42:1097–1105
38. Ksiezak-Reding H, Morgan K, Dickson DW: Tau immunoreactivity and SDS solubility of two populations of paired helical filaments that differ in morphology. Brain Res 1994, 649:185–196
39. Wischik CM, Novak M, Edwards PC, Klug A, Tichelaar W, Crowther RA: Structural characterization of the core of the paired helical filament of Alzheimer disease. Proc Natl Acad Sci USA 1988, 85:4884–4888
40. Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 1970, 227:680–685
41. Towbin H, Staehelin R, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979, 76:4350–4354
42. Ksiezak-Reding H, Chien C-H, Lee VMY, Yen S-H: Mapping of the Alz 50 epitope in microtubule-associated protein tau. J Neurosci Res 1988, 25:412–419

43. Goedert M, Spillantini MG, Jakes R: Localization of the Alz 50 epitope in recombinant human microtubule-associated protein tau. Neurosci Lett 1991, 126:149–154
44. Ksiezak-Reding H, Dickson DW, Davies P, Yen S-H: Recognition of tau epitopes by anti-neurofilament antibodies that bind to Alzheimer neurofibrillary tangles. Proc Natl Acad Sci USA 1987, 84:3410–3414
45. Lee VMY, Otvos L, Carden MJ, Hollosi M, Dietzschold B, Lazzarini RA: Identification of the major multiphosphorylation site in mammalian neurofilaments. Proc Natl Acad Sci USA 1988, 85:1998–2002
46. Lang E, Szendrei GI, Lee VM-Y, Otvos L: Immunological and conformational characterization of a phosphorylated immunodominant epitope on the paired helical filaments found in Alzheimer's disease. Biochem Biophys Res Commun 1992, 187:783–790
47. Kosik KS, Orecchio LD, Binder LI, Trojanowski J, Lee VM-Y, Lee G: Epitopes that span the tau molecule are shared with paired helical filaments. Neuron 1988, 1:817–825
48. Goedert M, Jakes R, Crowther RA, Six J, Lubke U, Vandermeeren M, Cras P, Trojanowski JQ, Lee VM-Y: The abnormal phosphorylation of tau proteins at Ser-202 in Alzheimer disease recapitulates phosphorylation during development. Proc Natl Acad Sci USA 1993, 90:5066–5070
49. Dickson DW, Kress Y, Crowe A, Yen S-H: Monoclonal antibodies to Alzheimer neurofibrillary tangles. II. Demonstration of common antigenic determinant between ANT and neurofibrillary degeneration in progressive supranuclear palsy. Am J Pathol 1985, 120: 292–302
50. Dickson D, Ksiezak-Reding H, Liu WK, Davies P, Crowe A, Yen S-H: Immunocytochemistry of neurofibrillary tangles with antibodies to subregions of tau protein: identification of hidden and cleaved tau epitopes and a new phosphorylation site. Acta Neuropathol 1992, 84:596–605
51. Liu W-K, Dickson DW, Yen S-H: Heterogeneity of tau proteins in Alzheimer's disease: evidence for increased expression of an isoform and preferential distribution of a phosphorylated isoform in neurites. Am J Pathol 1993, 142:387–394
52. Liu W-K, Dickson DW, Yen S-H: Amino acid residues 226–240 of tau, which encompass the first Lys-Ser-Pro site of tau, are partially phosphorylated in Alzheimer paired helical filament-tau. J Neurochem 1994, 62: 1055–1061
53. Dickson DW, Crystal H, Mattiace L, Kress Y, Schwagerl A, Ksiezak-Reding H, Davies P, Yen S-H: Diffuse Lewy body disease: light and electron microscopic immunocytochemistry of senile plaques. Acta Neuropathol (Berl) 1989, 78:572–584
54. Chandler CS, Ballard FJ: Distribution and degradation of biotin-containing carboxylases in human cell lines. Biochem J 1985, 232:385–393
55. Ksiezak-Reding H, Binder L, Yen S-H: Alzheimer disease proteins (A68) share epitopes with tau but show distinct biochemical properties. J Neurosci Res 1990, 25:420–430
56. Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA: Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 1989, 3:519–526
57. Tabaton M, Mandybur TI, Perry G, Onorato M, Autillo-Gambetti L, Gambetti P: The widespread alteration of neurites in Alzheimer's disease may be unrelated to amyloid deposition. Ann Neurol 1989, 26:771–778
58. Ishii T, Nakamura Y: Distribution and ultrastructure of Alzheimer's neurofibrillary tangles in postencephalitic Parkinsonism of Economo type. Acta Neuropathol (Berl) 1981, 55:59–62
59. Delacourte A, Flament S, Dibe EM, Hublau P, Sablonniere B, Sherrer V, Defossez A: Pathological proteins Tau 64 and 69 are specifically expressed in the somatodendritic domain of the degenerating cortical neurons during Alzheimer's disease. Acta Neuropathol (Berl) 1990, 80:111–117
60. Morishima-Kawashima M, Hasegawa M, Takio K, Suzuki M, Titani K, Ihara Y: Ubiquitin is conjugated with amino-terminally processed tau in paired helical filaments. Neuron 1993, 10:1151–1160
61. Dickson DW, Yen SH: Cytoskeleton and neurodegenerative diseases. Heat Shock Proteins in the Nervous System. Edited by J Mayer and I Brown. London, Academic Press, 1994, pp 226–235
62. Greenberg SG: Filament-associated tau proteins in neurodegenerative diseases (abstract). Soc Neurosci Abst 1993, 19:196
63. Kenessey A, Yen S-HC: The extent of phosphorylation of fetal tau is comparable to that of PHF-tau from Alzheimer paired helical filaments. Brain Res 1993, 629: 40–46
64. Binder LI, Frankfurter A, Rebhun LI: Distribution of tau in the mammalian central nervous system. J Biol Chem 1985, 101:1371–1378
65. Papasozomenos SC, Binder LI: Phosphorylation determines two distinct species of tau in the central nervous system. Cell Motil Cytoskeleton 1987, 8:210–226
66. Garcia de Ancos J, Ledesma MD, Avila J: Tau isoforms from grey and white brain matter. Mol Biol Cell 1993, 4:391a