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Ultrastructural localization of β-amyloid, τ, and ubiquitin epitopes in extracellular neurofibrillary tangles

(Alzheimer disease/antigenic characteristics/paired helical filaments/straight filaments)

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ABSTRACT **Neurofibrillary tangles (NFTs), a hallmark of Alzheimer disease, are commonly located in perikarya of neurons. In advanced cases of Alzheimer disease, however, NFTs are observed also in the extracellular space. As extracellular NFTs (E-NFTs), and occasionally intracellular NFTs (I-NFTs), are recognized by antibodies to β-amyloid protein (βAP), βAP may be present not only in amyloid deposits but also in paired helical filaments (PHFs), the primary components of NFTs. We compared the antigenic characteristics of I-NFTs and E-NFTs with light- and electron-microscopic immunocytochemistry by using several antibodies to noncontiguous epitopes of the microtubule-associated protein τ and of ubiquitin (Ub) as well as an antiserum to βAP. At variance with I-NFTs, E-NFTs were made predominantly of straight filaments (SFs), rather than PHFs, that were often separated by astroglial processes and in close association with small βAP deposits. Occasionally, E-NFTs were made of bundles of amorphous material, which showed no resemblance to SFs, PHFs, or amyloid fibrils. The antigenic changes in E-NFTs suggest that when NFTs become extracellular they lose the N and, possibly, the C termini of τ while maintaining the intermediate region of the molecule; they also lose the N-terminal two-thirds of Ub while the C-terminal conjugation site of Ub is preserved. A small subset of E-NFTs reacted with antibodies to both βAP and τ. Although in most E-NFTs, the epitopes recognized by τ and Ub antibodies were located in typical PHFs and SFs, the epitopes recognized in this subset of anti-βAP and anti-τ-positive E-NFTs were located exclusively in the bundles of amorphous material. It is suggested that either βAP epitopes are present but inaccessible in PHFs and SFs and become exposed after conformational changes occurring in the extracellular space or PHFs and SFs become closely associated with βAP in the extracellular space.**

Neurofibrillary tangles (NFTs) and amyloid deposits are the two most conspicuous structural lesions of Alzheimer disease (AD) (1). NFTs form in neurons and consist of bundles of abnormal, twisted filaments called paired helical filaments (PHFs) (2). In severe and advanced cases, numerous NFTs lie in the extracellular space. Extracellular NFTs (E-NFTs) are believed to result from the death of the neurons that contained them (ref. 3; quoted in ref. 4). E-NFTs are morphologically and antigenically distinct from intracellular NFTs (I-NFT). They have been reported to be made mostly of straight filaments (SFs) (5), which, in contrast to the filaments of I-NFTs, are more spaced, stain more lightly with silver stains, lack several τ epitopes, and become reactive with antibodies to glial fibrillary acidic protein (6–8).

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Amyloid deposits, another lesion in AD, are aggregates of fibrillar material that form in the extracellular spaces of the cerebral parenchyma and in the walls of cerebral and meningeal vessels, leading to the formation of senile plaques and to amyloid angiopathy (1). The main component of amyloid fibers is a 39- to 42-amino acid polypeptide, the β-amyloid protein (βAP) (9–14).

An important and necessary step in understanding the pathogenesis of AD is to establish whether and how these two lesions are related. Protein chemical analyses of fractions enriched in PHFs (15, 16) have detected βAP. Immunocytochemical observation that an antibody to full-length βAP reacted with E-NFTs but not I-NFTs suggested that βAP epitopes are masked in I-NFTs but become exposed in the E-NFT by degradation of external constituents by proteases (17, 18). Moreover, it was recently reported that an antibody to the N terminus of βAP reacts also with I-NFTs (19, 20). These findings raise the intriguing possibility that NFTs and amyloid deposits derive from the same protein. However, these immunocytochemical studies were carried out by light microscopy and did not provide information on the ultrastructure of the filament components of the NFT labeled by the antibodies to βAP or the fine localization of the βAP epitopes within the NFT.

Using both light- and electron-microscopy immunocytochemistry, we compared the antigenic characteristics of I-NFTs and E-NFTs by using antibodies to τ proteins (τ), ubiquitin (Ub), and βAP. We observed that the antigenic characteristics of E-NFTs differ from those of I-NFTs and may be related to the morphological changes of E-NFTs. Our findings that E-NFTs cease to react with some, but not all, antibodies to τ and to Ub indicates that E-NFTs selectively lose τ and Ub epitopes.

A small number of E-NFTs were found to react with both τ and βAP antibodies. However, ultrastructural examination showed these E-NFTs to be made of atypical filamentous material, while those made of characteristic PHFs or SFs did not react with anti-βAP. We conclude that either βAP epitopes are present but inaccessible in PHFs and SFs and become exposed when these structures undergo profound structural rearrangement, or PHFs and SFs of some E-NFTs become intimately associated with amyloid or preamyloid deposits and this association leads to marked morphological changes.

MATERIALS AND METHODS

Tissue. Hippocampus and entorhinal cortex from 10 patients with pathologically confirmed AD, 5 with predominant

Abbreviations: AD, Alzheimer disease; βAP, β-amyloid protein; NFT, neurofibrillary tangle; E-NFT, extracellular NFT; I-NFT, intracellular NFT; mAb, monoclonal antibody; PAP, peroxidase-antiperoxidase; PHF, paired helical filament; SF, straight filament; Ub, ubiquitin.

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E-NFTs and 5 with predominant I-NFTs, were fixed with buffered formalin.

Antibodies. Antibodies known to recognize I-NFTs or βAP were used (Table 1). Antibodies to τ: (*i*) Anti-PHF, an antiserum to PHFs (21) that recognizes epitopes in the C-terminal one-third of τ (22) partially overlapping with the Alz-50 epitope (23); (*ii*) anti-τ, an antiserum to τ proteins (24, 25); (*iii*) τ-1, a monoclonal antibody (mAb) to a nonphosphorylated epitope within τ residues 131–149 (26, 27); (*iv*) Alz-50, a mAb thought to recognize an epitope within the last 47 C-terminal amino acids of τ (23, 28). Antibodies to Ub: (*i*) Anti-Ub, an antiserum to Ub (29) that presumably recognizes epitopes along the entire Ub molecule; (*ii*) 3-39, a mAb to an epitope between Ub residues 50 and 65 (30, 31); (*iii*) 5-25, a mAb to an epitope between Ub residues 64 and 76 (30, 31); these antibodies were generated to PHF fractions (30); (*iv*) 4-2D8, a mAb to an epitope between Ub residues 34 and 54 (31–33). Antibodies to βAP: Anti-βAP, a rabbit antiserum to a synthetic peptide corresponding to the full length (residues 1–42) of βAP protein (10). Similar immunostaining was obtained also with an antiserum to βAP-(1–28) (34) and with a mAb to βAP-(1–10) but they were not used for quantitative analyses.

Antisera Absorption. The anti-PHF and the anti-τ antisera, diluted 1:500 and 1:200, respectively, in Tris-buffered saline, were incubated overnight at 4°C with 5–100 μg of bovine brain τ per ml (35), or with 100 μg of bovine serum albumin per ml.

Light-Microscopy Immunostaining. Serial paraffin sections were immunostained by the peroxidase-antiperoxidase (PAP) method (36) with 3,3′-diaminobenzidine. Antibody dilutions were as follows: Alz-50, 1:20; anti-βAP, 1:200; anti-τ, anti-PHF, and τ-1, 1:500; anti-Ub, 1:1000; 3-39 and 3-25, 1:10,000; 4-2D8, 1:50. Sections were treated with 90% formic acid for 5 min prior to anti-βAP incubation and with alkaline phosphatase (37) prior to τ-1 immunostaining. Formic acid-treated sections were double immunostained with an anti-βAP and Alz-50 mixture and then successively incubated with mouse PAP and developed with 3,3′-diaminobenzidine, and then with rabbit PAP and developed with 4-chloro-1-naphthol.

Quantitative Analysis. The number of immunostained I-NFTs and E-NFTs was determined in three adjacent subiculum fields at ×250. We classified NFT as intra- or extracellular on the basis of (*i*) the presence of adjacent nuclei and cytoplasmic profiles under phase-contrast illumination, (*ii*) their compactness, and (*iii*) the intensity of immunostaining with anti-PHF antibodies. Only the definitely identified E-NFTs and I-NFTs were used for quantitative analysis. The number of I-NFTs and E-NFTs recognized by each antibody was expressed as a percentage of those immunostained with anti-PHF, since this antiserum immunostained the highest number of I- and E-NFTs.

Table 1. Antigenic characteristics of I- and E-NFTs

Antibody	Epitopes	I-NFTs, %	E-NFTs, %
τ			
Anti-PHF	C-terminal one-third	100	100
Anti-τ	Entire τ?	97 ± 4	99 ± 4
τ-1	Residues 131–149	85 ± 1	0
Alz-50	Residues 402–448?	100 ± 3	0–5*
Ub			
Anti-Ub	Entire Ub?	85 ± 3	10 ± 1
3-39	Residues 50–65	81 ± 2	67 ± 4
5-25	Residues 64–76	82 ± 2	69 ± 2
4-2D8	Residues 34–54	78 ± 3	0
βAP			
Anti-βAP	Residues 1–42	0	2 ± 2

*Reactive only after formic acid treatment.

Immunoelectron Microscopy. Hippocampal paraffin sections (50 μm thick) of patients with predominant E-NFTs were processed for the preembedding colloidal gold method using anti-τ, anti-Ub, or anti-βAP (38).

RESULTS

Immunocytochemistry. I-NFTs and E-NFTs were easily identified after immunostaining and examination under phase-contrast illumination (Fig. 1). The immunostaining of I-NFTs was strong and dense, whereas that of E-NFTs was weaker because of dispersed fibrils. The immunoreaction of E-NFTs and I-NFTs with both anti-PHF and anti-τ was blocked by absorption with 50 or 100 μg/ml, respectively. The E-NFTs that reacted with anti-βAP were stained less intensely than with other reactive antibodies (Fig. 1*B*). Occasionally, βAP-positive E-NFTs also reacted with Alz-50 (Fig. 1*B*).

Quantitative Analysis. Virtually all detectable I-NFTs immunoreacted with anti-PHF, anti-τ, and Alz-50 (Table 1); 75–85% were also recognized by the other antibodies to τ and by those to Ub, but none was immunostained with anti-βAP, which, however, reacted with amyloid of senile plaques and vessel walls (data not shown). Some neurons not bearing

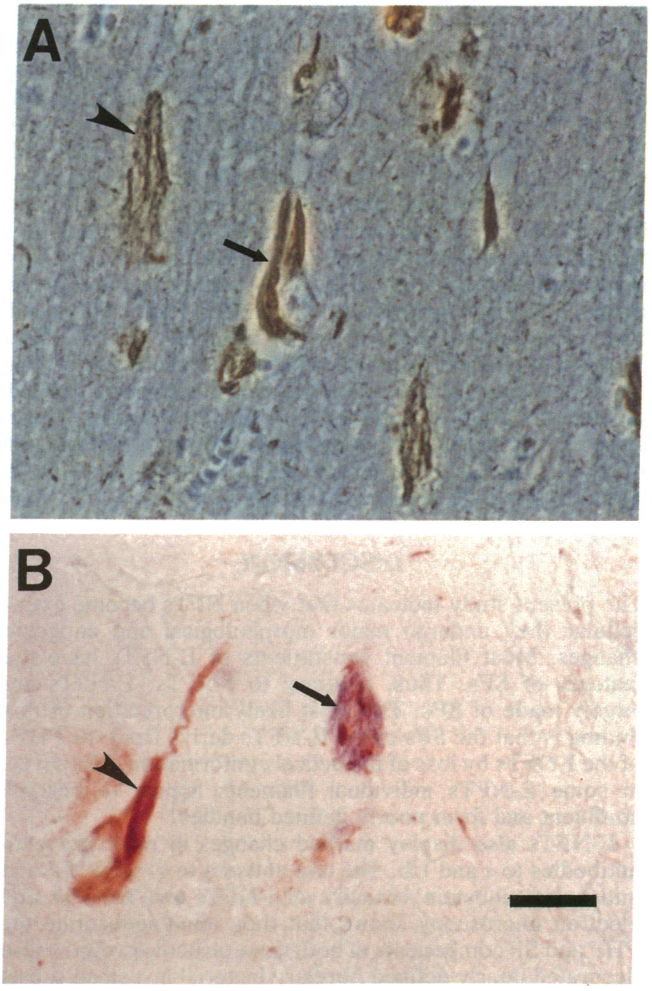

FIG. 1. Immunocytochemistry of I-NFTs and E-NFTs. (*A*) Immunostaining with anti-PHF. E-NFTs (arrowhead) have a looser appearance and immunostain less intensely than the I-NFT (arrow). (*B*) Double immunostaining with Alz-50 (brown) and anti-βAP (magenta). Some E-NFTs (arrow) reacted with both antibodies. The adjacent I-NFT (arrowhead) reacted only with Alz-50. Phase-contrast microscopy. (Bar = 25 μm.)

NFTs were immunostained by anti-τ, Alz-50, and, to a lesser extent, by anti-PHF (7, 39).

Six of the nine antibodies recognized E-NFTs (Table 1). The two antisera to τ, anti-PHF and anti-τ, recognized all identifiable E-NFTs. τ-1, which is directed to an epitope in the N-terminal half of human τ, showed no reaction with E-NFTs. Alz-50, whose epitope is controversial (23, 40) but by sequence analysis appears to be located at the C terminus of τ (23), was also negative; however, in formic acid-pretreated tissues, it reacted with $\approx$5% of the E-NFTs. mAbs 3-39 and 5-25, which recognize epitopes within the C-terminal one-third of Ub, reacted with 68–70% of the E-NFTs. In contrast, E-NFTs were not immunostained by mAb 4-2D8, which recognizes an epitope in the middle region of Ub. Approximately 10% of the E-NFTs reacted with anti-Ub, an antiserum that presumably recognizes epitopes defined by sequences located along the entire Ub molecule. Only 2% of E-NFTs were labeled by anti-βAP. Some of the βAP-positive E-NFTs reacted also with Alz-50 after formic acid treatment (Fig. 1*B*).

Ultrastructure and Immunoelectron Microscopy. E-NFTs showed great ultrastructural variability. Some were composed mostly of typical SFs (20 $\pm$ 3 nm), which only occasionally had the appearance of PHFs (24 $\pm$ 2 nm) (Fig. 2). In some of the E-NFTs, the filaments were packed as in I-NFTs (data not shown). More often, they formed small bundles that were widely separated either by empty spaces or by packed bundles of astroglia-like fibers (Fig. 2*B*). These fairly typical PHFs and SFs were invariably immunodecorated with anti-τ (Fig. 2 *A* and *B*). Only E-NFTs composed predominantly of PHFs were immunodecorated with anti-Ub (Fig. 2*C*). On the contrary, the E-NFTs immunodecorated with anti-βAP were made of parallel-oriented irregular bundles of poorly defined filamentous profiles, quite different from PHFs, SFs, and amyloid fibrils (Fig. 3 *A* and *B*). Occasionally, similar filamentous structures were also immunodecorated by anti-τ (Fig. 3*C*). These structures were occasionally seen in close association with typical PHFs and SFs, apparently as components of the same E-NFTs (Fig. 3*D*). Small clusters of amyloid fibrils were sometimes seen adjacent to both SFs and PHFs as well as the poorly defined filamentous profiles of the E-NFTs (Fig. 3 *A* and *E*). These amyloid deposits were immunodecorated by anti-βAP but not by anti-τ (Fig. 2*A*). Conversely, anti-βAP was never seen to decorate typical PHFs and SFs (Fig. 3*E*).

DISCUSSION

The present study indicates that when NFTs become extracellular they undergo major morphological and antigenic changes. Most filament constituents of E-NFTs have the features of SFs. Thus, contrary to I-NFTs, E-NFTs are largely made of SFs. The most likely interpretation of this finding is that the SFs of the E-NFTs derive from the PHFs of the I-NFTs by loss of the helical conformation. Moreover, in some E-NFTs individual filaments appear to become confluent and form poorly defined bundles.

E-NFTs also display marked changes in reactivity with antibodies to τ and Ub. The two antisera to τ, anti-PHF and anti-τ, immunostain virtually all I-NFTs and E-NFTs and electron microscopy shows that they immunodecorate the PHF and SF components of both these structures. Anti-τ also decorated poorly defined fibrillary material in a small subset of E-NFTs (see below). On the contrary, few or none of the E-NFTs are immunostained by mAbs that recognize epitopes located in the N-terminal half of the τ molecule (τ-1) (27) or possibly in the C-terminal region (Alz-50) (23). However, formic acid treatment, which probably produces a conformational change, reestablished Alz-50 immunoreactivity in a small subpopulation of E-NFTs, as was reported for τ-1 (41).

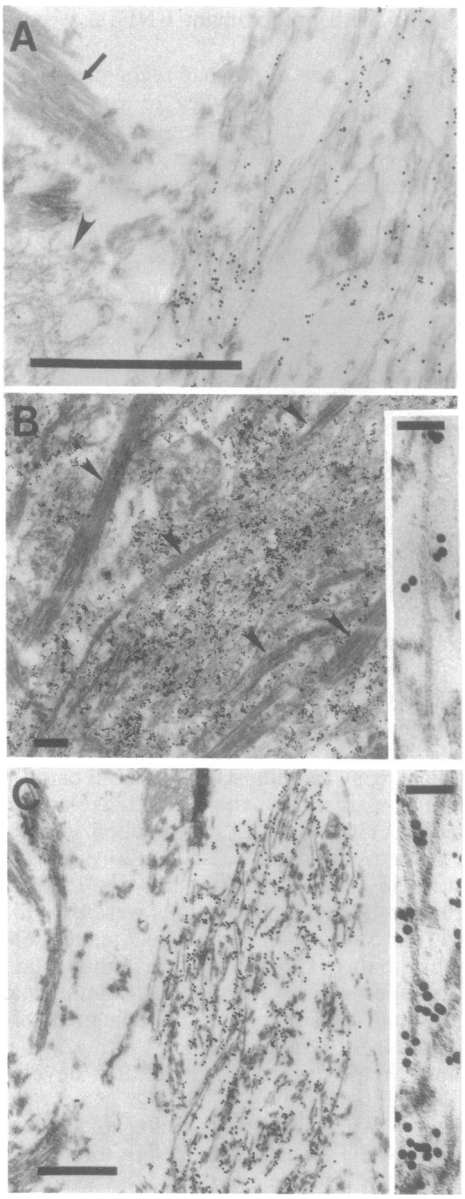

FIG. 2. Immunoelectron microscopy of E-NFTs with anti-τ (*A* and *B*) and anti-Ub (*C*). (*A*) Immunodecoration of an E-NFT made of a loose bundle of predominantly SFs. Typical amyloid fibrils (arrowhead) and glial filaments (arrow) are not decorated. (*B*) In many E-NFTs small bundles of immunodecorated SFs (*Inset*) were separated by astroglia-like processes (arrowheads). (*C*) E-NFTs immunoreacting with anti-Ub contained predominantly PHFs (*Inset*). (Bars = 1.0 μm; *Insets*, bars = 0.1 μm.)

Together these data indicate that when NFTs become extracellular, epitopes located near the C terminus and the N-terminal half of τ are either lost or become inaccessible to the antibodies. The latter could explain why, in some E-NFTs, Alz-50 epitopes were revealed after formic acid treatment. The staining of E-NFTs with anti-τ, which is likely to recognize epitopes along the entire τ molecule, and with anti-PHF, which recognizes the C-terminal one-third (22), indicates that other τ epitopes are maintained.

The extracellular location of NFTs is also associated with changes in reactivity with antibodies to Ub. The three mAbs and the antiserum to Ub recognized 78–85% of I-NFTs. Of the mAbs, 3-39 and 5-25 also immunoreacted with a high number of E-NFTs, while 4-2D8 recognized none. Anti-Ub recognized only 10% of the E-NFTs. Interestingly, the reactive E-NFTs were made predominantly of PHFs rather

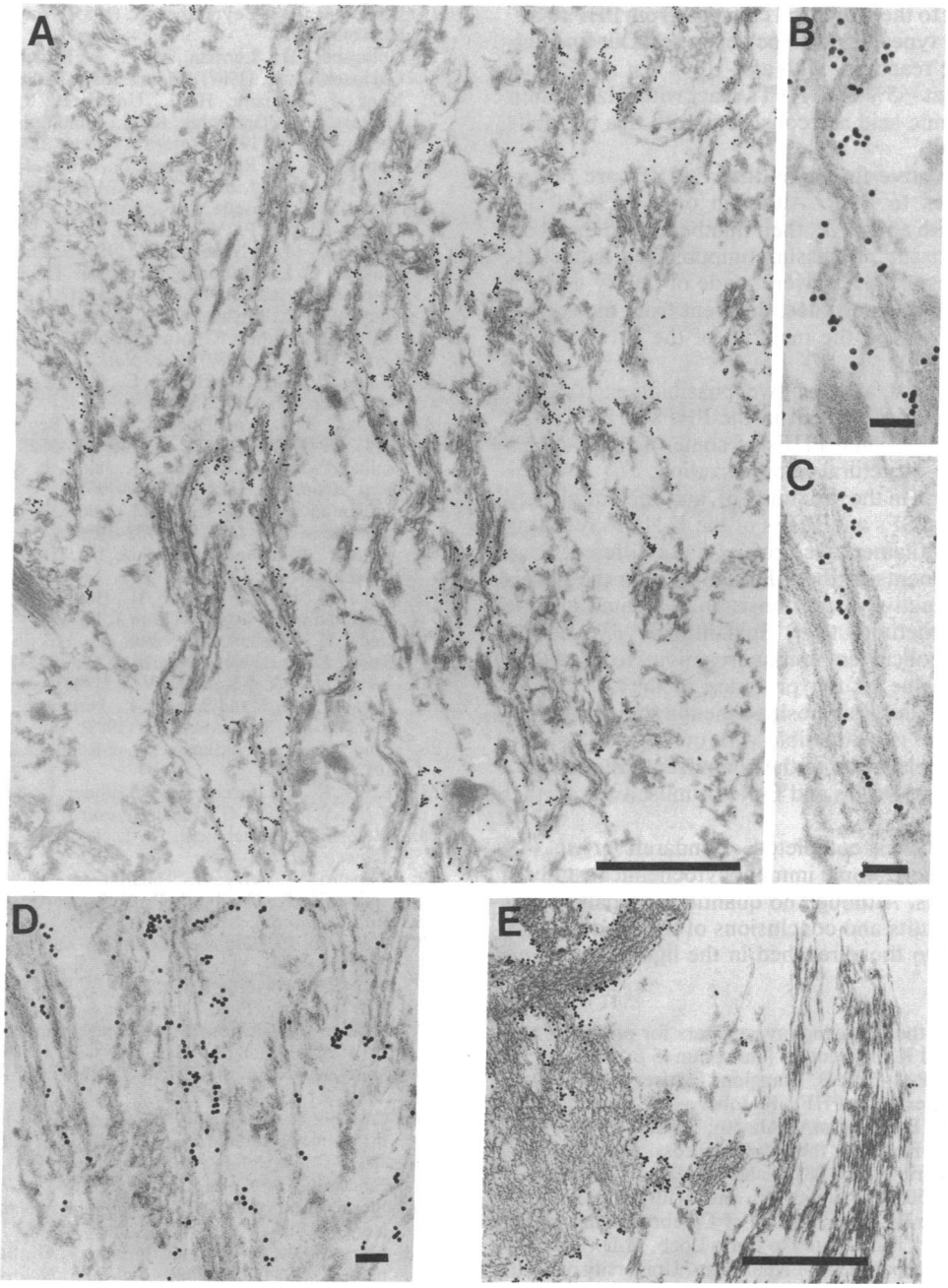

FIG. 3. E-NFTs immunodecorated with anti-βAP (*A*, *B*, and *E*) and with anti-τ (*C* and *D*). (*A*) Anti-βAP-decorated E-NFTs contained widely separated bundles of poorly defined fibrillary material that lacked identifiable SFs, PHFs, or amyloid fibrils. (*C*) Bundles similar to those reacting with βAP were immunodecorated with anti-τ. (*D*) These bundles were occasionally seen adjacent to distinct SFs or PHFs, all components of apparently the same E-NFT and all immunodecorated by anti-τ. (*E*) A small amyloid deposit with the characteristic random arrangement of fibrils is labeled by anti-βAP while an adjacent NFT, apparently extracellular and made predominantly of PHFs, is unlabeled. (*A* and *E*, bars = 1.0 μm; *B–D*, bars = 0.1 μm.)

than SFs. The three mAbs to Ub used in this study recognize epitopes located in different regions of the Ub molecule (31). While 3-39 and 5-25 recognize epitopes within the 26 C-terminal residues, the epitope recognized by 4-2D8 is within residues 34–54, closer to the N terminus of Ub (30–33). Therefore, it is likely that when NFTs become extracellular they lose the N-terminal two-thirds of Ub while maintaining epitopes near the C terminus at which conjugation to acceptor proteins takes place (42). Alternatively, the changes in reactivity with antibodies to Ub may be due to conformational differences of the Ub epitopes that may take place when NFTs become extracellular.

The finding that after Pronase treatment PHFs lose reactivity with antibodies raised to normal τ but the protease-resistant "core" still contains sequences of the τ repeat region (43, 44) suggests that the decreased τ and Ub reactivity of E-NFTs is due to extracellular proteases stripping off the superficial components of PHFs. If E-NFTs represent a "natural version" of *in vitro* Pronase treatment, then the intermediate region of τ and the C-terminal region of Ub are definitely components of the PHF "core" (43). Moreover, since the C-terminal region of Ub is the site of Ub conjugation, it is likely that the filaments of E-NFTs contain the unidentified Ub acceptor protein(s). An important difference between the Pronase-treated PHFs and the E-NFT filaments is that the former maintain the original helical structure, while the latter are straight or even more altered (44). Therefore, the decrease in immunoreactivity may be related not to the

loss of epitopes but to the structural changes, from PHF to SF or to other filament types, which block epitopes. Our findings that in E-NFTs the reactivity with anti-Ub is lost in SFs but not in PHFs and that ≈5% of E-NFTs react with Alz-50 after treatment with formic acid are consistent with this possibility.

The most provocative finding is that E-NFTs are recognized by antibodies to βAP. Although only ≈2% of the E-NFTs reacted with anti-βAP, their number may depend on the conditions of tissue processing (unpublished data). Ultrastructurally, these E-NFTs were made of poorly defined and irregular filamentous profiles, different from the typical PHFs and SFs present in the majority of the E-NFTs, yet recognized by anti-τ.

Our findings suggest at least two possibilities: (*i*) Concealed βAP epitopes are present in the PHFs of NFTs and become exposed only when PHFs of some of the E-NFTs undergo profound structural reorganization; (*ii*) βAP or preamyloid may form in the proximity of, and become closely associated with, the SFs and PHFs of the E-NFTs resulting in the formation of filamentous profiles quite different from the original components of the NFTs. Based on the observation that βAP reactivity was consistently located outside and around the reactivity to τ, Spillantini *et al.* (19, 20) suggested that the concurrent immunoreactivity to τ and βAP in the E-NFTs is due to the presence in these NFTs of τ-positive filaments and βAP-positive membranous material. Although this model is compatible with our second hypothesis, a definitive explanation of the concurrent presence of τ and βAP epitopes in I-NFTs and E-NFTs must await further studies.

After this work was completed, Bondareff *et al.* (45) published a light-microscopic immunocytochemical study of extracellular tangles. Although no quantitative analysis was carried out, the results and conclusions of these authors are virtually identical to those reached in the light-microscopic part of our study.

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