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Structural characterization of the core of the paired helical filament of Alzheimer disease

(molecular pathology/neurodegenerative disease/neurofibrillary tangles/scanning transmission electron microscopy)

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ABSTRACT **The paired helical filament, the principal constituent of the neurofibrillary tangles characteristic of Alzheimer disease, is shown to consist of two structurally distinct parts. An external fuzzy region can be removed by Pronase treatment to leave a Pronase-resistant morphologically recognizable core. Scanning transmission electron microscopy gives an estimate for the mass per unit length as 79 kDa·nm^{-1} before Pronase treatment and 65 kDa·nm^{-1} after treatment. The fuzzy region carries all the epitopes recognized by two different antisera against microtubule-associated protein tau. By contrast, a monoclonal antibody (mAb) we have raised to paired helical filament cores (mAb 423) decorates Pronase-treated filaments much more strongly than it does untreated ones. We have shown in previous papers that the epitope recognized by mAb 423 is carried by a central 9.5-kDa fragment of tau protein, which therefore forms part of the Pronase-resistant core structure. The remainder of the tau protein incorporated into the filaments must contribute part, if not all, of the fuzzy region. The mass per unit length measurements imply that the three-domain structural subunit of the core that we visualized previously by image reconstruction has a molecular mass of ≈100 kDa.**

Neurofibrillary tangles and neuritic plaques of Alzheimer disease are both sites of accumulation of a distinctive type of abnormal filament, the so-called paired helical filament (PHF) (1). We have previously shown (2, 3) that the PHF consists of a double-helical stack of transversely oriented subunits, such as might be generated by polymerization of an aberrant protein. Attempted identification of the subunit protein(s) of the PHF by immunocytochemical methods has led to confusing and conflicting claims (reviewed in refs. 4 and 5). Part of the difficulty arises from the inability of light microscopy to distinguish between different components of a tangle and the likelihood that parts of the normal neuronal cytoskeleton become associated with or trapped within the tangle. This can be overcome by using immunoelectron microscopy to study the labeling of individual identifiable PHFs. Even here, one must be careful to distinguish between components decorating the surface of the PHF, either specifically or nonspecifically, and components intrinsic to the core of the PHF.

Here we establish the existence of a Protease-resistant core within the PHF. We have measured by scanning transmission electron microscopy (STEM) the mass per unit length of untreated and Pronase-treated PHFs and show that the Pronase treatment removes ≈17% of the material, leaving a core that retains the characteristic morphology. The results indicate that the three-domain core subunit we have described (3) has a molecular mass of ≈100 kDa. Electron microscopy of negatively stained specimens shows that untreated filaments have a fuzzy outer covering, which is removed by the protease. Antisera raised against microtubule-associated tau protein decorate untreated filaments but not Pronase-treated ones, showing that the fuzzy material carries tau-like epitopes. Previous studies have pointed to the presence of tau epitopes in tangles (6–10) and, more specifically, in isolated PHFs (11), but without distinguishing between the fuzzy region and the core.

As described in the second paper of this series (12), the Pronase-stripped filaments were used as immunogen to raise a monoclonal antibody (mAb) against the PHF core (mAb 423), which reacts with Pronase-stripped filaments much more strongly than it does with unstripped ones. As described (12), mAb 423 does not react with intact isolated mammalian tau protein. A 9.5-kDa protein fragment carrying the mAb 423 epitope was isolated from Pronase-treated PHF cores and, with protein sequence derived from this fragment, the corresponding cDNA was cloned (13). Despite the apparent counterindications from the immunological tests on the Pronase-stripped filaments, it turned out that the cDNA clones encode tau protein, of which the 9.5-kDa fragment represents the internal repeated region. Thus, a portion of the core of the PHF, as defined here, is formed by part of tau or a tau-like protein. If the 9.5-kDa fragment we have isolated represents the stub of tau, which anchors it to the core, the molecular identity of the remaining 90 kDa of PHF core protein is still to be discovered.

MATERIALS AND METHODS

Preparation of PHFs. Pronase-treated PHFs were isolated according to the ifII protocol reported (12). For electron microscopy, however, the SW28 centrifugation step was omitted. Instead, ifII fractions were extensively dialyzed against distilled water in 50-kDa cut-off dialysis tubing. Non-Pronase-treated PHFs were prepared in the same way but the Pronase digestion step was omitted.

Scanning Transmission Electron Microscopy (STEM). PHF preparations were suspended in 25 mM ammonium bicarbonate and applied to copper grids coated with a thin layer of carbon. Subsequently, a drop of tobacco mosaic virus (TMV) (used as internal calibration) in 25 mM ammonium bicarbonate was also applied to the grid, which was then blotted and allowed to dry in air. Pictures were taken with the VG HB5 STEM at the European Molecular Biology Laboratory in Heidelberg. They were recorded at an operating voltage of 100 kV in the dark field mode at ×50,000 magnification

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Abbreviations: PHF, paired helical filament; STEM, scanning transmission electron microscope; TMV, tobacco mosaic virus; mAb, monoclonal antibody.

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(giving a frame size of 1.9 μm), using a 512 × 512 raster, with a dwell time of 16 μs/pixel and with a beam current of 2×10^{-11} A. To minimize mass loss, the specimen was kept at a temperature of 120 K and areas for recording images were preselected at low magnification (×5000).

Digital processing of STEM images was carried out on a Tektronix 4012 graphics terminal by using standard programs (14). Fields were selected that contained clearly identifiable PHFs and TMV particles well away from any contaminating material. Histograms were produced that show the mass per unit length of the filaments, expressed as a fraction of the known mass per unit length of TMV (131 kDa·nm^{-1}). Radial density profiles were obtained from STEM images of unstained PHFs by integrating parallel to the filament axis. The data from many particles were averaged after referring each particle to its best midline.

Antibodies. The preparation of mAb NOAL 6/66.423.2 (referred to as mAb 423) against PHF core will be described elsewhere (M.N., P.C.E., R. Pannell, C.M.W., and C. Milstein, unpublished data) (see also ref. 12). The polyclonal antibodies with anti-tau reactivity come from two sources. One is the ICN 65-095 anti-tubulin preparation, which contains substantial anti-tau reactivity, having been raised against a twice-cycled tubulin preparation. The second, kindly supplied by G. Perry (Case Western Reserve University, Cleveland), is a polyclonal antibody raised against porcine tau and affinity purified for tau by G. Perry, who has shown it to decorate isolated PHFs (11).

Immunoelectron Microscopy. A small droplet of PHF suspension was placed on a carbon-coated copper grid (400 mesh) and allowed to evaporate partially. The grid was then placed on a drop of 0.1% gelatin in phosphate-buffered saline and blocked for 5–10 min. The grid was lifted, excess solution was blotted, and the grid was placed on a solution of the first antibody for 1 hr. The grid was then washed with a few drops of a 0.1% solution of gelatin in phosphate-buffered saline, blotted, and placed on a solution of the appropriate second antibody conjugated to gold (Janssen Auroprobe EM goat anti-rabbit or goat anti-mouse IgGG5) for 1 hr. The grid was washed with a few drops of the gelatin solution, blotted, stained with a few drops of 1% lithium phosphotungstate, and allowed to dry in air. Micrographs were recorded on a Philips EM301 or EM420, at an operating voltage of 80 kV, and at nominal magnifications between ×25,000 and ×45,000. Unlabeled filaments were prepared by negative staining with 1% lithium phosphotungstate.

RESULTS

Negatively Stained PHFs. PHFs that have not been treated with Pronase (Fig. 1*a*) appear fuzzy when viewed in the electron microscope and are ≈19 nm wide at the widest part. Pronase-treated PHFs (Fig. 1*b*) have a sharper outline, are ≈16 nm wide, and show more clearly the staggered dark patches ("barber's pole" appearance), arising from the characteristic alternating deposition of stain beneath the edges of the twisted ribbon. They also show more clearly the ultrastructural features we have described (2). These comprise alternating regions of 3 and 4 longitudinal striations, arising from the changing aspect of the subunit in the helical twist, and transverse striping at 3-nm intervals, which represents the axial periodicity of the subunit (3). Thus, the essential morphological features of the core remain intact after Pronase treatment. All that is lost is fuzzy outer material, which lacks sufficient order to generate a consistent signal in diffraction patterns from PHF images.

STEM Measurements. We used STEM to measure the mass per unit length in treated and untreated PHFs, so we could determine the likely subunit molecular mass and also discover how much material was removed by the Pronase treatment. The electron scattering from unstained PHFs was compared to that from TMV deposited on the same grid (Fig. 2*a*). Pronase-treated PHFs have a mass per unit length that is 0.5 (SD = 0.06) that of TMV, whereas for untreated ones the fraction is 0.6 (SD = 0.12) (Fig. 2*b*), giving values of the mass per unit length as 65 kDa·nm^{-1} and 79 kDa·nm^{-1}, respectively. The difference between treated and untreated PHFs is statistically highly significant ($t' = 9.7$; $P < 0.001$) (15) and demonstrates that 17% of the mass of the PHF is lost on Pronase treatment. The mass loss can also be seen as a reduction in the radial extent of the PHF (Fig. 2*c*), confirming that the material comes from the periphery of the filament, as indicated by the negatively stained specimens (Fig. 1).

On the basis of these results, we draw a distinction between the core of the PHF, which corresponds to the three-domain structural subunit defined in our earlier work (3), and the Pronase-sensitive peripheral region. The measured mass per unit length implies that the core subunit has a molecular mass of ≈100 kDa, assuming it has an axial extent of 3 nm. The three-domain structural subunit may of course consist of more than one polypeptide chain.

Immunoelectron Microscopy. To identify the material removed in the course of Pronase digestion, we compared filaments isolated with and without Pronase treatment for

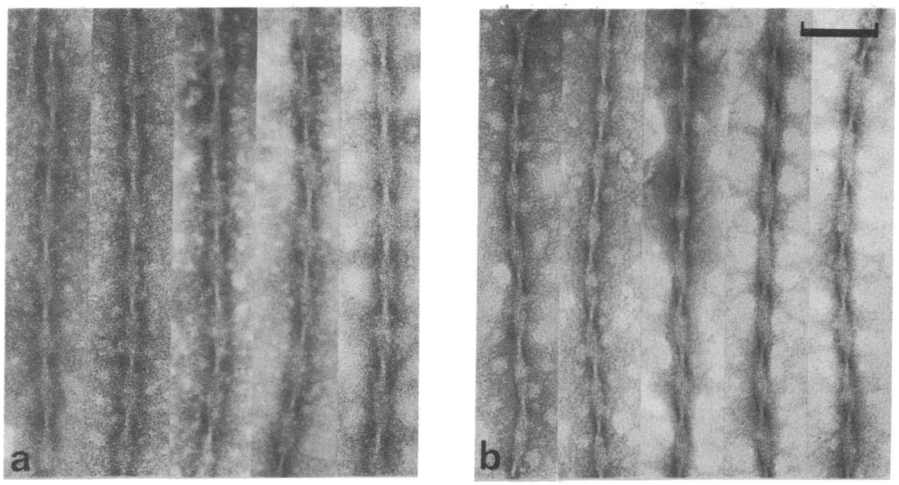

FIG. 1. Electron micrographs of PHFs negatively stained with lithium phosphotungstic acid without (*a*) and with (*b*) Pronase treatment. In *a*, the filaments have a fuzzy outline; in *b*, the edges are sharply defined and the structural details are more clearly visible. (Bar = 100 nm.)

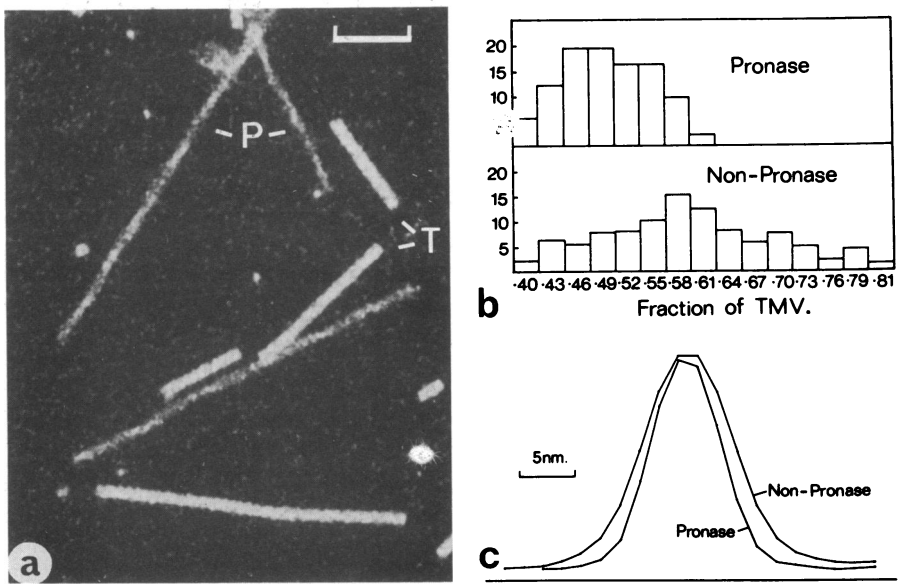

FIG. 2. (*a*) STEM picture of an unstained field of Pronase-treated PHFs (P) and reference TMV particles (T). The characteristic twist of the PHFs is clearly visible. (Bar = 100 nm.) (*b*) Histograms showing the mass per unit length of Pronase-treated and nontreated PHFs, expressed as a fraction of the mass per unit length of TMV. (*c*) Average radial density profiles of Pronase-treated and nontreated PHFs, showing that the nontreated filaments are wider than the treated ones.

reactivity with antibodies against tau protein. Untreated filaments, in which the fuzzy outer domain remains intact, show strong ImmunoGold labeling with anti-tau antisera from two different sources (Fig. 3 *a* and *c*). Reactivity to both antisera was lost in Pronase-treated PHFs (Fig. 3 *b* and *d*). We conclude from this that the epitopes recognized by both antisera are present only in the fuzzy outer region of the PHF and are not present in the stripped PHF core filament.

mAb 423, which was selected for its specific binding to PHF core (12), labels Pronase-stripped PHFs strongly (Fig. 4*b*) and remains positive down to antibody dilutions of 1:5000. It will label untreated PHFs (Fig. 4*a*), but only down to antibody dilutions of ≈1:200. Thus, the epitope recognized by mAb 423 is not present in the amorphous material at the periphery of the PHF structure. Nevertheless, the fact that some reactivity can be demonstrated in the absence of Pronase digestion implies that the mAb 423 recognition site does not contain a Pronase cleavage site. Rather, the epitope is more favorably exposed by the removal by Pronase of the occluding fuzzy outer material.

Although amyloid fibrils contaminate ifII preparations, mAb 423 shows no ImmunoGold labeling of these structures (Fig. 4*c*) and no histological labeling of amyloid deposits.

DISCUSSION

We have shown here that the PHF has a Pronase-resistant core, distinct from fuzzy material, which coats it and is removed by Pronase treatment. The STEM measurements indicate that the molecular mass of the three-domain core subunit identified previously (3) is ≈100 kDa after Pronase treatment, which removes ≈20 kDa per subunit.

Antibody labeling experiments with two anti-tau polyclonal antisera produced by others show that the tau epitopes recognized by these sera reside entirely in the fuzzy region and that Pronase-treated PHFs do not react with these antisera. Thus, previous reports implying the presence of tau protein in neurofibrillary tangles (6–10), or even in individual filaments (11), do not establish its presence in the core of the PHF. However, mAb 423 raised to PHF cores decorates Pronase-stripped PHF cores much more strongly than it does untreated filaments. Thus, the epitope recognized by mAb 423 does not reside in the fuzzy region. It is important to note that this mAb was selected on the basis that it strongly decorated morphologically recognizable PHF cores and is therefore clearly against an epitope in the core, as defined here.

The previous paper (12) described the extraction from the PHF core of a 9.5-kDa protein fragment and a related 12-kDa fragment, both of which are strongly positive on immunoblots with mAb 423 antibody. The protein sequence derived from the 9.5-kDa fragment was used to isolate a human cDNA clone, which proved to encode tau protein (13). The 9.5-kDa fragment represents ≈87 amino acids of the 352 in this tau sequence and thus corresponds to about one-quarter of the 38-kDa tau molecule (starting at about residue 211). Our immunological and biochemical results taken in conjunction establish unequivocally the presence of part of tau protein in the core of the PHF.

A possible explanation for the apparently paradoxical observations that tau is present chemically but is not observable by anti-tau antibodies other than mAb 423 could be as follows. Suppose that there is one tau molecule or tau-like molecule associated with each PHF core subunit. Pronase treatment could then remove the larger N- and smaller C-terminal region of the tau polypeptide, representing roughly the 20-kDa difference per subunit found by the STEM measurements and corresponding to the fuzzy material, which carries the epitopes recognized by two standard anti-tau antisera used here. What remains behind would be the protected 9.5-kDa fragment of tau, closely associated with the core and carrying the epitope recognized by mAb 423. The 9.5-kDa fragment is itself extremely Pronase sensitive once it has been freed from the core, suggesting that its association with the core either explicitly shields it from the action of Pronase or puts it into some conformation that renders it Pronase resistant.

What we have proved in this and the companion papers (12, 13), by using structural, biochemical, immunological, and molecular biological techniques, is that part of the tau protein is intimately associated with the PHF core: the precise form of the tau molecule incorporated into PHFs requires more investigation. For example, only a subset of the various different forms of tau protein might be incorporated and the protein might well have been subjected to some form of

(giving a frame size of 1.9 μm), using a 512 × 512 raster, with a dwell time of 16 μs/pixel and with a beam current of 2×10^{-11} A. To minimize mass loss, the specimen was kept at a temperature of 120 K and areas for recording images were preselected at low magnification (×5000).

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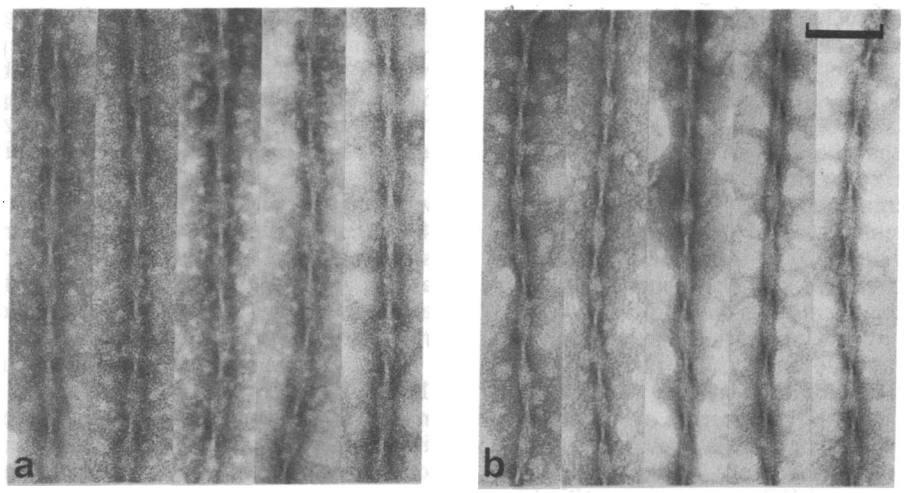

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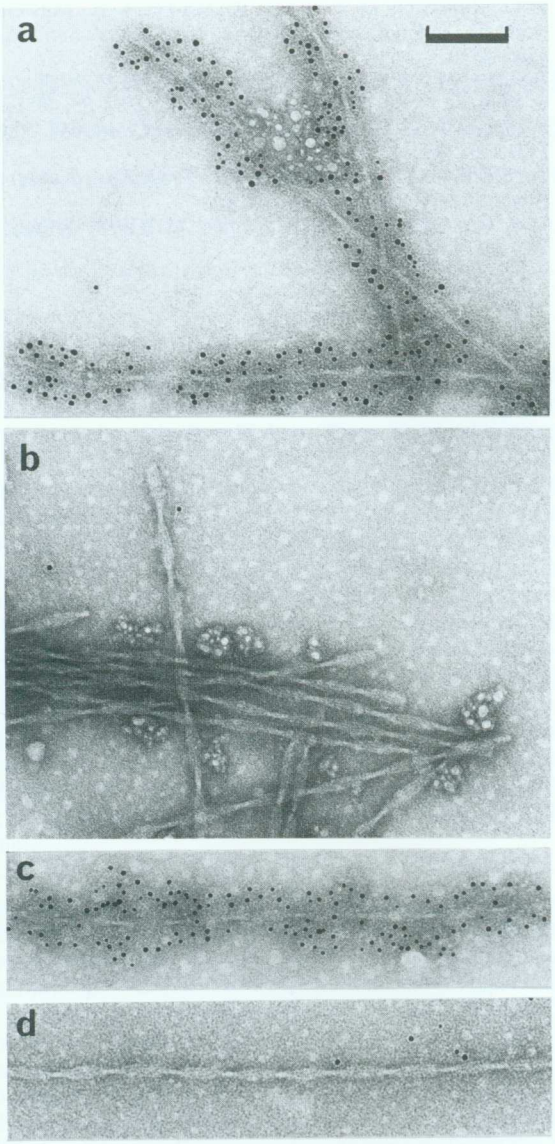

FIG. 3. Antibody labeling experiments on PHFs with anti-tau antibodies. Filaments that have not been treated with Pronase show strong labeling (*a* and *c*), which is abolished in filaments that have been treated with Pronase (*b* and *d*). In *a* and *b*, the first antibody is the ICN 65-095 anti-tubulin antiserum with strong tau reactivity. In *c* and *d*, the first antibody is an affinity-purified antiserum to tau provided by G. Perry. (Bar = 100 nm.)

posttranslational modification. It may be significant that the protected part of the tau molecule corresponds to the conserved triple repeat in human (13) and mouse (16) tau protein, which could well represent the microtubule binding site. The identified tau protein fragment contributes only 9.5 kDa of the 100-kDa structural subunit of the PHF core. What remains to be discovered is the identity of the much larger remainder of the core protein. The image reconstruction (3) shows three domains within the structural subunit. It is conceivable, though unlikely in our view, that the structural unit could consist of three compactly folded tau-like molecules. More likely, it is formed by some quite different molecule. Whichever is the case, the nature of the tight association between the demonstrated tau-like part and the remainder, whether covalent or noncovalent, peptide or nonpeptide linkage, must be elucidated.

In conclusion, our proof that at least part of tau protein constitutes a component of the core of the PHF in Alzheimer

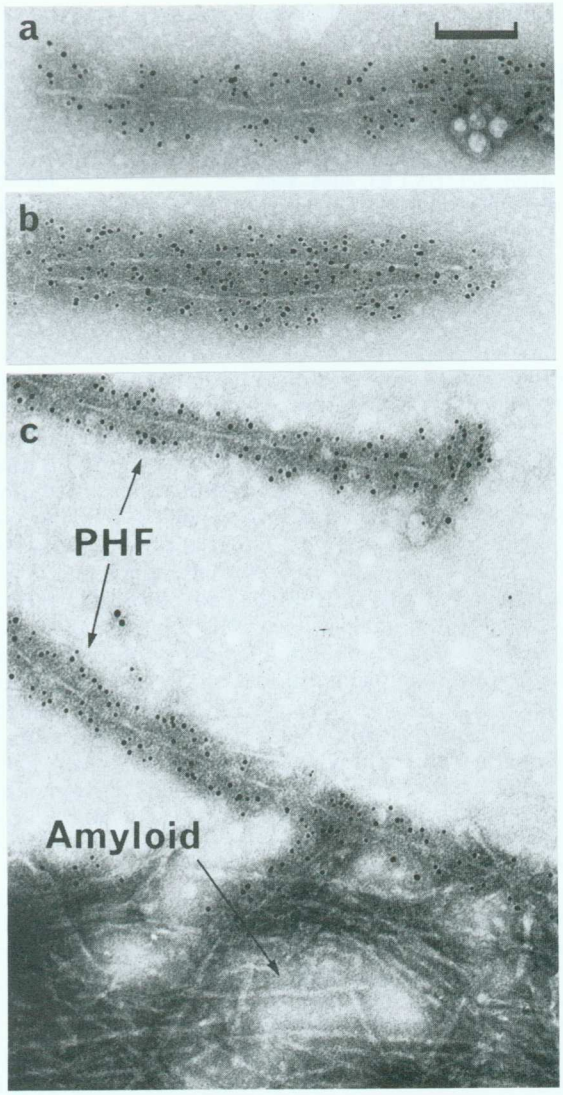

FIG. 4. Labeling of PHFs with anti-PHF core mAb 423. (*a*) Filament not treated with Pronase, showing labeling at 1:200 dilution of antibody. (*b*) Pronase-treated filament showing labeling at 1:1000 dilution of antibody. (*c*) Field showing Pronase-treated PHFs strongly labeled with mAb 423 but amyloid fibrils in the same field completely unlabeled. (Bar = 100 nm.)

disease now makes it possible to start to unravel the mechanisms that lead to PHF formation.

We are grateful to Dr. G. Perry for providing affinity-purified anti-tau antibodies. We thank Dr. P. Brook (Fulbourn Hospital, Cambridge) for clinical assessment of patients with Alzheimer disease. We thank Mr. R. Pannell for technical assistance and Dr. C. Milstein for helpful discussions. The tissue collection of the Medical Research Council, Cambridge Brain Bank Laboratory was indispensable for this work. We thank Imperial Chemical Industries PLC and ICI Americas, Inc., for financial support during the later stages of this work. C.M.W. is a Meres Senior Student at St. John's College, Cambridge, and a Lister Institute Research Fellow.

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