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# Ubiquitin and microtubule-associated protein tau immunoreactivity each define distinct structures with differing distributions and solubility properties in Alzheimer brain

(Alzheimer disease/neurofibrillary tangle/paired helical filament/neurofilament/tau ubiquitin)

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ABSTRACT **Several cytoskeletal polypeptides as well as the protein ubiquitin have been implicated as components of the neurofibrillary tangles of Alzheimer disease. We have examined the relationship of ubiquitin staining with immunoreactivity for some of these proteins, both in frozen sections and in cytoskeletal fractions of Alzheimer brain material. We noted (*i*) antibodies specific solely for neurofilament and glial filament proteins failed to stain the fibrils stainable with ubiquitin. Tau-1 antibody stained some but not all of the ubiquitin-stained profiles; fibers staining only for tau or only for ubiquitin were also seen. (*ii*) The Tau-1-stained material was rather diffuse and granular, in contrast to the very sharply defined ubiquitin-positive profiles. (*iii*) When Tau-1 and ubiquitin stain the same fiber, Tau-1 immunoreactivity is often visualized as a diffuse cortical layer of material surrounding a core of ubiquitin immunoreactivity. (*iv*) The tau immunoreactivity can be almost totally removed by boiling Alzheimer brain cytoskeletal material in 2% $NaDodSO_4$ containing a sulfhydryl reducing agent, this procedure apparently having no effect on the ubiquitin immunoreactivity. If similar material is boiled in 2% $NaDodSO_4$ in the absence of a sulfhydryl reducing agent, the tau immunoreactivity is removed less efficiently, suggesting that tau epitopes are bound to the ubiquitin reactive material in a manner partially dependent on covalent disulfide bridges. These results show that the tau and ubiquitin distributions, both characteristic of Alzheimer disease, are qualitatively different, and that the two markers define immunologically and biochemically distinct structures.**

The neurofibrillary tangles (NFT) of Alzheimer brain can be stained with antibodies specific for ubiquitin (1, 2). Other studies have indicated that various neuronal intermediate filament epitopes are incorporated into NFTs (see, for example, refs. 3–5). Recently, several groups have shown that microtubule-associated protein tau is aberrantly expressed in Alzheimer brain and can be detected immunologically in NFTs (6–10). The tau staining pattern appears to closely resemble that seen with ubiquitin antibodies. Two obvious questions are how is the intermediate filament immunoreactivity related to that of ubiquitin, and is the ubiquitin pattern identical to that of tau? Here we have used double-labeling methodologies to compare the distribution of ubiquitin immunoreactivity with the distribution of immunoreactivity for tau, neurofilaments, and glial filaments.

## MATERIALS AND METHODS

**Tissues.** Human brain material was obtained from brain banks at the Universities of Florida at Gainesville and California at San Diego. Material was derived from patients with a long clinical history of dementia who on autopsy revealed the histopathological lesions characteristic of Alzheimer disease. We made use of material from six separate Alzheimer brains. Material from two brains of nondemented individuals were used as controls.

**Antibodies.** The majority of the neurofilament antibodies have been characterized in previous reports (see Table 1 and ref. 11). BD4, DA2, and DA3 are newly produced mouse monoclonal antibodies that have not been previously described and were raised against enzymatically dephosphorylated pig neurofilament proteins. Antibodies NE14, NN18, and NR4 are identical to BM200, BM160, and BM68, respectively, obtainable commercially from Boehringer Mannheim. SM1-31, -32, -33, and -34 were purchased from Sternberger-Meyer Immunochemicals (Jarrettsville, MD). 8D8, RT97, and BF10 were obtained from Brian Anderton (Saint George's Hospital Medical School, London). The antibodies against components of glial filaments, V9 (anti-vimentin) and GA-5 (anti-glial fibrillary acidic protein), were provided by K. Weber and M. Osborn (Max-Planck-Institut for Biophysical Chemistry Goettingen, F.R.G.) (14, 15). Tau-1 was the kind gift of L. Binder (University of Alabama, Tuscaloosa, AL) and has been described (16). Ubiquitin antibodies were raised against ubiquitin conjugated to keyhole limpet hemocyanin (KLH) as described by Haas and Bright (17). The two rabbit polyclonal antisera have been described (1, 18). Several BALB/c mice were injected repeatedly with ubiquitin–KLH conjugates. Peritoneal fluid from these mice contained strong anti-ubiquitin immunoreactivity and stained tangle material on histological sections. Fluid from two of these mice was affinity-purified on a ubiquitin affinity column to produce, in each case, several milliliters of polyclonal mouse anti-ubiquitin. These eluates behaved identically to the rabbit polyclonal antisera in immunocytochemical experiments, as determined by appropriate double-label immunofluorescence on Alzheimer brain material. They were used in double-label experiments with the various rabbit polyclonal antibodies.

Monoclonal antibodies were used as undiluted hybridoma supernatant or, in the case of Tau-1, BD4, DA2, DA3, SMI-31, -32, -33, and -34, as dilutions from affinity-purified ascites fluids to give an IgG concentration of ≈10 μg/ml. Polyclonal antisera were affinity-purified on the appropriate antigen and diluted to a final concentration of 10 μg/ml.

Second antibodies were fluorescein, rhodamine, and Texas red-conjugated anti-mouse and anti-rabbit antibodies and were obtained from Sigma or Jackson Immunochemicals (West Grove, PA). Micrographs were made on a Zeiss Axiophot microscope fitted with a ×100 planapo lens.

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Abbreviation: NFT, neurofibrillary tangle(s).

Table 1. Reactivity of pelleted ubiquitin-positive fibrils from Alzheimer brain with antibodies to cytoskeletal proteins

| Name | Specificity | Reactivity on ubiquitin-positive fibrils | Ref(s). for antibody |
|---|---|---|---|
| H297 | NFL | − | 11 |
| NR4 | NFL | − | 11 |
| DA2 | NFL | − | ND |
| BD4 | NFL | − | ND |
| H298 | NFM | − | 11 |
| NN18 | NFM | − | 11 |
| DA3 | NFM | − | ND |
| BF10 | NFM | − | 12, 13 |
| H301 | NFH + P | − | 11 |
| NE14 | NFH + P | − | 11 |
| RT97 | NFH + P, NFM + P, tau + P | + (weak) | 12, 13 |
| SMI31 | NFH + P, NFM + P, tau + P | − | 12, 13 |
| NA34 | NFM, NFH | − | 11 |
| NC43 | NFM + P, NFH + P | − | 11 |
| SMI34 | NFM + P, NFH + P | − | 12, 13 |
| NC52 | NFH | − | 11 |
| SMI32 | NFH | − | 12, 13 |
| SMI33 | NFH − P | − | 12, 13 |
| 8D8 | NFM + P, NFH + P, tau + P | + (strong) | 12, 13 |
| V9 | Vimentin | − | 14 |
| GA5 | GFA | − | 15 |
| Tau-1 | Tau | + (strong) | 16 |

NFH, NFM, and NFL refer to the heavy, medium, and light neurofilament polypeptides, respectively. +P indicates that an antibody reacts only with the phosphorylated form of a molecule, and −P indicates reactivity only with the dephosphorylated form. ND, Antibody in question has not been described in detail in a previous publication. GFA, glial fibrillary acid protein.

**Frozen Sections.** Alzheimer brain specimens were removed at autopsy and frozen by placing in a −70°C freezer. These tissues showed ice crystal damage but were still usable for these experiments. Frozen sections (8 μm) were cut and immunostained by standard procedures.

**Enrichment and Visualization of Ubiquitin-Positive Fibers from Alzheimer Brain.** Alzheimer brain gray matter (0.5–1 g) was gently homogenized in 10 vol of Tris-buffered saline (TBS; 10 mM Tris·HCl/9 g of NaCl per liter, pH 7.5) plus 1 mM EDTA in a 7-ml Wheaton manual tissue grinder with an "A" pestle. This material was centrifuged at 300× *g* for 10 min. The supernatant was centrifuged at 100,000× *g* for 30 min. The resulting pellet was taken up in TBS and applied to a discontinuous gradient of 1.0 M, 1.5 M, and 2.0 M sucrose in TBS and was spun at 35,000 rpm in an SW.50.1 for 4 hr at room temperature. Typically, large bundles of neurofilaments and glial filaments could be detected by immunostaining at both the 1.0–1.5 M and the 1.5–2.0 M interfaces. Ubiquitin-positive profiles were seen predominantly at the 1.5–2.0 M interface, where they appeared as very sharply defined linear structures. The material from 1.0–1.5 M interface also contained shorter but equally well defined ubiquitin-positive fibrils. Material from the 1.5–2.0 M interface was taken and diluted between 1:3 and 1:9 in TBS. Sixty-five-microliter volumes were centrifuged onto square glass coverslips (4 × 4 mm) in a Beckman Airfuge fitted with the EM 90 rotor, run at 88,000 rpm for 30 min at room temperature. Pelleted material was stained for double-label immunofluorescence by standard methodologies.

**Detergent Extraction of Native Material.** Material from the 1.5–2.0 M interface was boiled for 30 min in TBS containing 2% $NaDodSO_4$ with either 0.1 M 2-mercaptoethanol, 2 mM dithiothreitol, or without additive. Undissolved material was visualized by centrifugation and immunostaining as described above.

## RESULTS

**Double-Label Immunocytochemistry of Frozen Sections of Alzheimer Brain.** Frozen cerebral cortex from six Alzheimer patients and hippocampus from one of these patients were examined. Prominent ubiquitin-immunoreactive fibers were seen throughout cortical regions of these specimens. In contrast, sections from control brains showed few or no ubiquitin-immunoreactive fibrils. Many tau-positive fibers were seen in the specimens of Alzheimer brain examined here, although very little tau staining was seen in the cerebral cortex or hippocampus of control brains.

We performed a series of double-label immunofluorescence studies to compare the distribution of ubiquitin immunoreactivity to that for neurofilament, glial filament, and tau proteins in frozen sections. All of the neurofilament antibodies tested showed strong staining for large numbers of neurofilamentous profiles. However, only 2 of 19 antibodies showed any overlapping staining with ubiquitin. A typical result is shown in Fig. 1 *a* and *b*. Only 8D8 strongly and clearly stained ubiquitin-immunoreactive structures. RT97 also showed some staining of the ubiquitin-associated material, but with a much lesser intensity than that of 8D8. Antibodies to glial intermediate filament proteins, vimentin, and glial fibrillary acidic protein showed strong staining of astrocytic processes, but no overlap was seen between the staining pattern for either of these proteins and ubiquitin.

The distribution of tau, as revealed by Tau-1 staining, proved to be distinct from that for ubiquitin. We observed some tau-immunoreactive fibers that were negative for ubiquitin, some that apparently showed colocalization of the two markers, and some that were only immunoreactive for ubiquitin. The tau-stained material usually looked somewhat diffuse, in contrast to the very sharply defined ubiquitin profiles. Interestingly, where tau and ubiquitin immunoreactivity partially overlapped, we frequently observed that the tau profile was more extensive than the ubiquitin pattern. Typically we saw a larger tau-positive fiber with a thinner and shorter ubiquitin profile apparently inside (Fig. 1 *c* and *d*).

**Immunocytochemistry of Extracted Undenatured NFT Material.** To increase the resolution beyond that obtainable with sectioned material, we extracted cytoskeletal material under nondenaturing conditions, as described in *Materials and Methods*. Immunofluorescence with appropriate antibodies revealed many long neurofilament-positive profiles and, in separate experiments, we were also able to see fiber bundles positive for glial fibrillary acidic protein and for vimentin. Ubiquitin immunoreactivity was localized to very strongly stained fibrils, which were generally rather shorter than the neurofilament and glial filament profiles. These ubiquitin-positive fibers must correspond to the ubiquitin-immunoreactive material seen in the frozen sections.

Table 1 summarizes the results of a series of double-label immunofluorescence experiments using this material with antibodies to neurofilament, glial filament, tau, and ubiquitin. In general, ubiquitin-immunoreactive filaments could not be stained with antibodies to neurofilament or glial filament proteins, as we had seen in the sectioned material. Tau-1 immunoreactivity did not exactly correlate with that for ubiquitin. Some short Tau-1-positive profiles were not stainable for ubiquitin. Profiles stained with ubiquitin but not Tau-1, apparently not morphologically different from those stainable with both markers, were also seen, although the majority of ubiquitin-stained fibers also stained with Tau-1. When we were able to resolve a difference in staining pattern

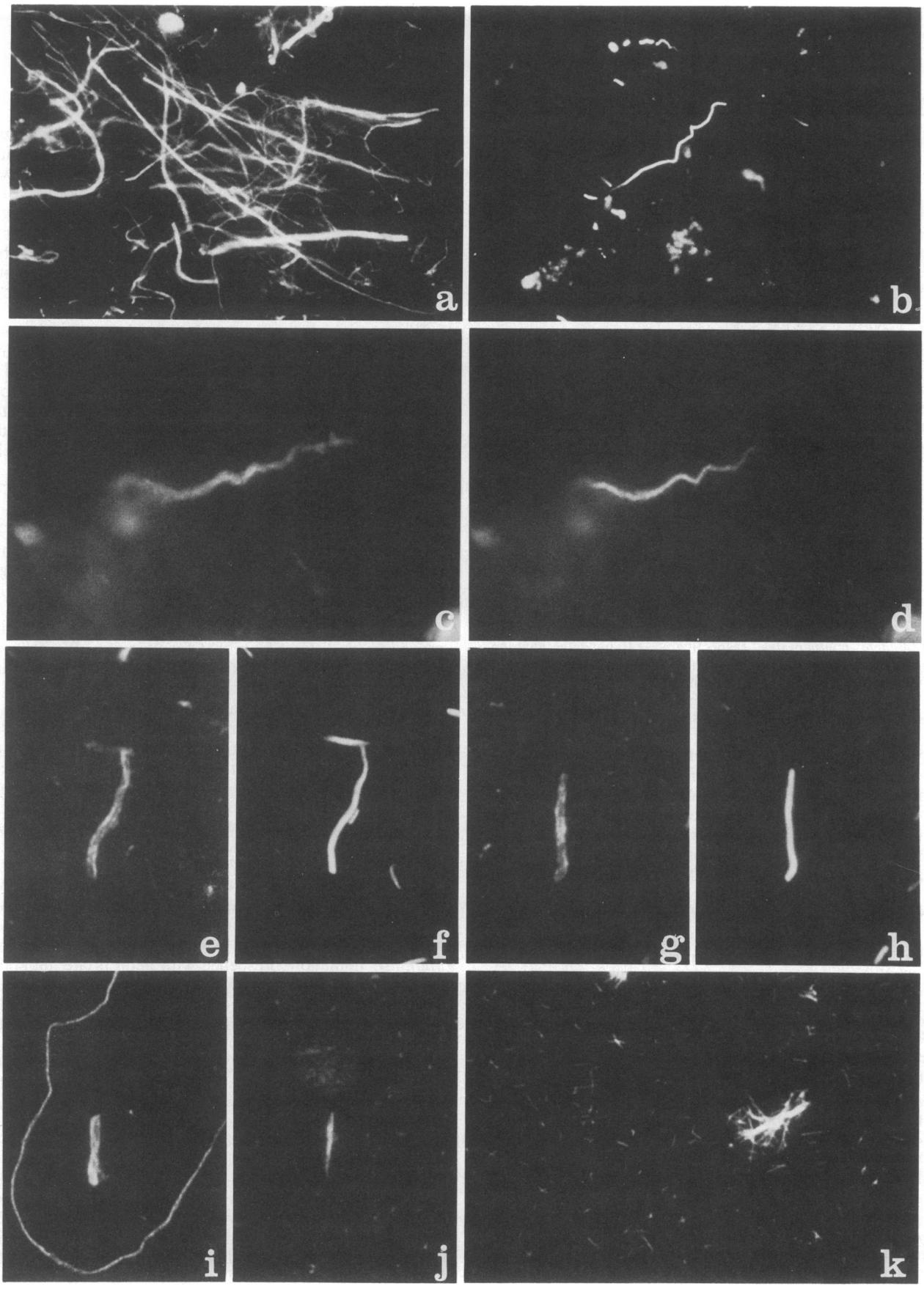


FIG. 1. Double-label immunofluorescence microscopy of Alzheimer brain material. Double-label immunofluorescence of a frozen section stained with the neurofilament antibody NE14 (*a*) and with ubiquitin polyclonal antibody (*b*). Circular objects in the ubiquitin channel are lipofuscin granules, which are also visible in non-antibody-stained controls. (*c*) Staining of a frozen section with antibody Tau-1; (*d*) corresponding view stained with ubiquitin antibody. (*e–h*) Double-label immunofluorescence of extracted undenatured cytoskeletal material

for these two markers, the difference was always that Tau-1 immunoreactivity formed a coating around ubiquitin-immunoreactive material (see Fig. 1 *e–h*). There was also a qualitative difference in the two staining patterns; while ubiquitin was extremely sharp and well-defined, tau staining was usually much more diffuse, an impression we had already obtained from our study of frozen sectioned material.

Single-label experiments with either Tau-1 or the ubiquitin antibodies alone gave results similar to those obtained with the same antibodies when used in double-label experiments. Thus, Tau-1 frequently revealed the sheath-like staining pattern when applied without a ubiquitin antibody. This renders it unlikely that the results reported here are due to competition between antibodies, with ubiquitin being more successful at binding in the tangle material than Tau-1.

When we stained pelleted material with neurofilament monoclonal antibody 8D8, we saw long well-defined neurofilamentous fibers, as we had seen with the other neurofilament antibodies, one of which is clearly shown in Fig. 1*i*. However, additional staining was a coating of immunoreactivity for 8D8 surrounding some of the ubiquitin-immunoreactive profiles, comparable to that seen with Tau-1 antibody (compare Fig. 1 *i* and *j*). The staining pattern for neurofilament monoclonal antibody RT97 was similar to 8D8, although in this case the ubiquitin-associated staining was much weaker.

**Immunochemistry of $NaDodSO_4$ Extracted Material.** Material treated identically to that described above was boiled for 30 min in 2% $NaDodSO_4$ prior to pelleting onto coverslips. This procedure was expected to remove loosely adherent material from insoluble tangle components as well as to dissolve the neurofilaments and glial filaments. We stained this material with a selection of antibodies and noted that the diffuse tau immunoreactivity associated with the ubiquitin-positive fibers was not completely removed. Inclusion of 0.1 M 2-mercaptoethanol or 2 mM dithiothreitol followed by boiling under the same conditions as before also resulted in almost total removal of immunoreactivity for Tau-1, although ubiquitin staining remained following either treatment. Under these conditions, the larger well-defined ubiquitin-positive profiles were converted to a mixture of frayed bundles of filaments and small, very fine filaments (Fig. 1*k*). We were able to see a small amount of very weak and possibly nonspecific Tau-1 immunoreactivity associated with some of these larger ubiquitin-staining profiles. We never saw Tau-1 staining of the very fine ubiquitin-positive filaments.

## DISCUSSION

Our results show that the fibrillar material obtained from Alzheimer brain, which is stainable with ubiquitin, is not recognized by antibodies solely specific for neurofilaments or glial filaments. Tau-1 staining showed a limited codistribution with that of ubiquitin-positive fibrils, although it was clear that not all ubiquitin-positive fibrils stained for tau, and not all tau-positive profiles stained for ubiquitin. However, when a particular profile did stain for both tau and ubiquitin, the staining patterns were usually obviously different. This was already apparent in frozen sectioned material, but it was particularly clearly seen in material pelleted onto coverslips. Tau-1 staining was peripheral to a core of ubiquitin immunoreactivity. The tau staining was often more extensive than the ubiquitin staining, so a long tau-positive profile could frequently be seen with a shorter and thinner ubiquitin profile apparently inside. The tau-stained material usually appeared granular or diffuse, in contrast to the very sharply defined ubiquitin staining. There can be little doubt that, while the tau and ubiquitin-stained profiles may be related to one another, they must represent very distinct structures.

This conclusion was further strengthened by studies of the sensitivity of the tangle material to chaotropic agents. Boiling the material for 30 min in 2% $NaDodSO_4$ with 0.1 M 2-mercaptoethanol or 2 mM dithiothreitol caused the tau immunoreactivity to be almost totally removed, but it did not appear to affect ubiquitin staining. This finding further underlines the difference between the staining pattern of the tau- and ubiquitin-reactive regions of the fibrils and indicates the existence of two distinct structures with different distributions and solubility properties. These experiments also show a correlation between the presence of ubiquitin and the extreme insolubility of the $NaDodSO_4$/sulfhydryl reducing agent-resistant material. Ubiquitin immunoreactivity, and not that for tau, neurofilaments, or glial filaments, is the essential marker for the totally insoluble material in Alzheimer brain. It seems that the presence of ubiquitin defines this insoluble material.

Many previous studies have suggested that various intermediate filament proteins may be constituents of NFT (3–5). Our immunological evidence shows that no antibody directed solely against any neuronal or glial intermediate filament protein showed any staining for the ubiquitin- or tau-positive material. The antibodies we have used here were both monoclonal and polyclonal and include antibodies made in four different laboratories. Furthermore, the majority of them are well-defined in their specificity for the different intermediate filament subunit proteins. Our results show that a variety of different neurofilament and glial filament epitopes are not detected on the tau- and ubiquitin-positive material found in Alzheimer brain. Two neurofilament antibodies, 8D8 and RT97, which did not stain the tau- and ubiquitin-reactive material, proved to stain in the same diffuse peripheral way as Tau-1 antibody. We concluded that 8D8 and RT97 stain the ubiquitin-associated material because of tau and not neurofilament immunoreactivity, and we were pleased when recent studies showed that these antibodies do in fact recognize tau (12, 19). Presumably, other immunological results that suggest intermediate filaments are constituents of NFT may reflect similar cross-reactivities or may be due to normal constituents of the cytoskeleton adjacent to the insoluble NFT material.

Boiling the Alzheimer brain preparations in the presence of 2% $NaDodSO_4$ and a sulfhydryl reducing agent resulted not only in removal of almost all Tau-1 immunoreactivity but also in fragmentation of most of the larger ubiquitin-stained fibrils down to very fine filaments. These results suggest that Tau-1 immunoreactivity is bound to the ubiquitin-immunoreactive material in a manner partially dependent on disulfide bonds, and that disulfide bonds may also play a part in holding the ubiquitin-positive material in bundles. Electron microscopic examination of this $NaDodSO_4$/2-mercaptoethanol-treated material shows the presence of numerous single PHF, which can be continuously immunolabeled with ubiquitin antibodies (data not shown). We conclude that the finer profiles we saw in the light microscope represent single PHF, and indeed the standard PHF preparation includes a similar $NaDodSO_4$/2-mercaptoethanol extraction step (13).

A recent report has shown that tau protein, in the absence of tubulin, can assemble into filaments with a paired helical

immunostained on coverslips, with Tau-1 staining to the left and ubiquitin to the right of each pair of figures. (*g* and *h*) Double-labeling of extracted material performed with neurofilament antibody 8D8 (*i*) and ubiquitin (*j*). (*k*) Ubiquitin-stained $NaDodSO_4$/2-mercaptoethanol-extracted cytoskeletal material. (*a* and *b*, ×925; *c* and *d*, ×1800; *e–k*, ×2080.)

morphology (20). This polymerization is induced by the conversion of glutamine residues in tau to glutamic acid. Perhaps the diffuse tau-stained material we have seen here represents a polymer of modified tau similar to that reported by these workers. We would be surprised if microtubules would stay polymerized in frozen sectioned tissues or in the gradient centrifugation as performed in this study, especially as our starting material was frozen tissues from autopsy brain. Our preliminary experiments have indicated that several tubulin antibodies do not stain any fibrillar material either in frozen sections of these brain tissues or in undenatured extracted cytoskeletal material, suggesting that the tau epitopes detected here are not associated with tubulin. Presumably, the tau staining represents tau protein organized into a stable linear structure. Future work should define the composition of this unusual tau-reactive material.

A major unsolved problem is the constitution of the highly insoluble material in Alzheimer brain. Ubiquitin antibodies strongly stain NFT and PHF material, and there is firm biochemical evidence that certain amino acid sequences derived from ubiquitin are incorporated into PHFs (2). What is the ubiquitin bound to? An obvious suggestion is that it becomes attached to aberrant tau-containing structures. The results presented here show that tau antibody binds to material associated with the highly insoluble fibrillar material but does not effectively decorate the insoluble material itself. It would not be surprising if tau immunoreactivity were lost following ubiquitinylation due to epitope masking. Ubiquitinylated tau could therefore be the essential PHF constituent. However, the possibility that PHFs are made from ubiquitin in combination partially or wholly with non-tau proteins cannot be excluded. Future experiments aimed at identifying the nature of the ubiquitin acceptor in NFT should resolve these questions.

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