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Ubiquitin is associated with abnormal cytoplasmic filaments characteristic of neurodegenerative diseases

(Alzheimer disease/Parkinson disease/paired helical filaments/cytoskeleton/proteolysis)

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ABSTRACT **Several degenerative diseases of the nervous system are characterized by the presence of neuronal inclusions. Most of these inclusions are made of abnormal filaments and share epitopes with cytoskeletal proteins. One of these inclusions, the neurofibrillary tangle of Alzheimer disease, has recently been shown to contain ubiquitin, a regulatory protein thought to play a role in the degradation of abnormal proteins. We carried out light and electron microscopic immunocytochemistry with several polyclonal and monoclonal antibodies to investigate the presence of ubiquitin in neuronal inclusions of neurodegenerative diseases. Ubiquitin was present not only in paired helical filaments that form the neurofibrillary tangle of Alzheimer disease, but also in the filamentous components of the inclusion characteristic of Parkinson disease, Pick disease, and progressive supranuclear palsy. In contrast, ubiquitin was not detected in other neuronal inclusions often found in aging and in Alzheimer disease, such as Hirano bodies and granulovacuolar degeneration. Reactivity with monoclonal antibodies suggests differences in the ubiquitin-acceptor proteins present in the inclusions studied. It is concluded that ubiquitin is selectively present in neuronal inclusions of degenerative diseases.**

The neurofibrillary tangle (NFT), a prominent neuronal inclusion in Alzheimer disease (AD), is composed mainly of paired helical filaments (PHFs) (1). PHFs have been shown to contain epitopes of cytoskeletal proteins, such as the microtubule-associated proteins τ and MAP2 and neurofilament proteins (2, 3). Recently, ubiquitin has also been found to be present in PHFs (4, 5). Ubiquitin, a highly conserved protein of 76 amino acids, is thought to play a regulatory role in eukaryotic cells by forming conjugates with acceptor proteins (6–8). Some of these conjugates are believed to be obligatory intermediates in an ATP-dependent nonlysosomal proteolytic system that is particularly active in the degradation of damaged or abnormal proteins (9, 10). Thus, one of the roles of ubiquitin may be to prevent or limit cell damage caused by the presence of altered proteins. In other conjugates, ubiquitin is thought to play a role in functions other than degradation, since histones H2A and H2B (11, 12) and some membrane receptors have been shown to be ubiquinated (13–15). A proteolytic activity intrinsic to ubiquitin has also been reported (16). The presence of such a multifunctional protein in PHFs raises several questions.

In the present study we investigated whether ubiquitin is specifically associated with PHFs or is also present in inclusions characteristic of other neurodegenerative diseases. Pick bodies of Pick disease, Lewy bodies of Parkinson disease, and NFTs of progressive supranuclear palsy (PSP) are all made of 15-nm straight filaments (17–19) and they share epitopes with normal components of the neuronal cytoskeleton (2–4, 20). We found that ubiquitin is present in straight filaments of all these inclusions. In contrast, ubiquitin is not present in two other inclusions often found in hippocampal neurons during aging and in AD: the Hirano bodies, paracrystalline structures made of sheets of 6- to 10-nm filaments (21) containing actin and actin-binding proteins (22–23), and the granulovacuolar degeneration, a nonfilamentous inclusion reported to contain tubulin epitopes (24). Thus, ubiquitin is not uniquely present in NFTs of AD, nor is it indiscriminately present in all neuronal inclusions.

MATERIALS AND METHODS

Tissue Source. Tissue was obtained at autopsy from two cases each of histologically confirmed Alzheimer, Pick, and Parkinson diseases and PSP. Tissue was sampled from the hippocampus (AD), temporal cortex (Pick disease), locus ceruleus (Parkinson disease), and pontine tegmentum (PSP). Autopsy tissue from the same regions was also obtained from age-matched, neurologically normal brains. Tissue was fixed in buffered 10% (vol/vol) formalin or in Bouin's fixative. For immunocytochemistry, fixed tissue was embedded in paraffin for light microscopy or cut in 40-μm-thick sections with a Vibratome (Oxford) for electron microscopy.

Antibodies. Eight mouse monoclonal antibodies (mAbs) (15, 16) to epitopes located between residues 34 and 53 of ubiquitin (H.T.S. and V.A.F., unpublished data) were used: 5-2E6, 2-3D7, 3-3G6, 4-3H8, 5-2F3, 7-2E7, 1-2H11, and 4-2D8. These mAbs recognize different conformational determinants of ubiquitin, depending on the nature of the conjugate. Antisera were raised in rabbits by using $NaDodSO_4$-denatured ubiquitin crosslinked to keyhole limpet hemocyanin as immunogen (25). The rabbit antibodies were immunopurified on a column of ubiquitin coupled to Sepharose CL-4B. These antibodies reacted with both free and conjugated ubiquitin on heat-treated nitrocellulose blots (26) (Fig. 5).

Light-Microscope Immunocytochemistry. Sections of paraffin-embedded tissue were immunostained by the peroxidase-antiperoxidase procedure (27) with 3,3'-diaminobenzidine as cosubstrate. Reaction product was enhanced by treatment with 1% (wt/vol) OsO_4 for 30 sec.

Immunoelectron Microscopy. Vibratome sections were autoclaved at 120°C for 30 min (26), a treatment that was required for immune reaction of these sections with all the mAbs to ubiquitin. Sections were then incubated for 10 min with 0.01% Triton X-100 in 50 mM Tris·HCl, pH 7.6/150 mM

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Abbreviations: AD, Alzheimer disease; mAb, monoclonal antibody; NFT, neurofibrillary tangle; PHFs, paired helical filaments; PSP, progressive supranuclear palsy.

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NaCl (TBS) and for 1 hr with 10% normal goat serum in TBS. Sections were incubated overnight at 4°C with the primary antibodies and then for 48 hr with the secondary antibody complexed to 17-nm colloidal gold (28). The sections were rinsed in TBS, fixed for 2 hr in 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), postfixed for 2 hr in 2% OsO_4, dehydrated in graded solutions of acetone, and flat-embedded in Spurr's medium. Thin sections (silver interference color) were contrasted with uranyl acetate and lead citrate, carbon-coated, and viewed at 60 kV in a Jeol 100 CX electron microscope.

Immunoblots. Ubiquitin (Sigma) and hippocampus samples from AD and age-matched controls were fractionated by PAGE and transferred to nitrocellulose (29). The membranes were autoclaved for 30 min at 120°C (26), blocked in 10% nonfat dry milk, and incubated overnight at 4°C with affinity-purified antibodies. Reaction was visualized by the indirect peroxidase method with 3,3'-diaminobenzidine as cosubstrate.

RESULTS

All the affinity-purified antibodies immunostained NFTs of AD and PSP as well as Lewy and Pick bodies (Fig. 1). However, mAbs showed a selective reactivity with the ubiquitin epitopes present in these inclusions. Although five of the eight mAbs (4-2D8, 4-3H8, 3-3G6, 5-2E6, and 5-2F3) immunostained NFT of AD and Lewy bodies, only mAb 4-2D8 reacted with Pick bodies and NFTs of PSP. The pattern of immunostaining was similar for all the antibodies. The staining was evenly distributed over all the inclusions except for the Lewy bodies, in which only the rim was stained. Neurites were immunostained in all cases of AD, Parkinson disease, and PSP but not in those of Pick disease. None of the antibodies reacted with Hirano bodies or granulovacuolar degeneration. In sections of hippocampus, cerebral cortex, and pons from age-matched controls, only occasional NFT and neurites were immunostained. Electron microscopic immunocytochemistry showed that ubiquitin epitopes were present in the filamentous components of each of the reacting inclusions; the PHFs in NFTs of AD (Fig. 2) and the straight filaments in NFTs of PSP (Fig. 3*a*), Pick bodies (Fig. 3*b*), and Lewy bodies (Fig. 4) were invariably decorated with gold particles. In addition, immunoreaction also occurred in the amorphous material present in NFTs of AD and PSP and in Pick bodies. However, in Lewy bodies the fibrillar component at the edge of the inclusion was decorated, whereas the compact amorphous core was not. The patterns of immunodecoration obtained with affinity-purified rabbit antibodies were indistinguishable from those obtained with mAbs.

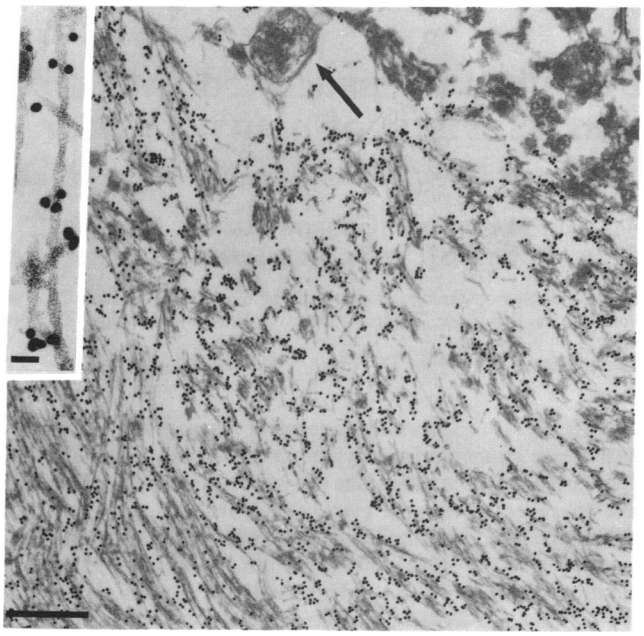

FIG. 2. Electron micrograph of a NFT of AD immunostained with mAb 4-2D8. Gold particles are seen only over the filamentous material, whereas organelles such as mitochondria (arrow) are not immunostained. (Bar = 0.3 μm.) (*Inset*) At higher magnification, the filamentous components show the helical conformation of PHFs. (Bar = 0.05 μm.)

On immunoblots, high molecular weight ubiquitin conjugates were present in hippocampus of both AD and age-matched controls. Reaction with insoluble material not entering the stacking gel and known to contain PHFs (30) was markedly increased in AD (Fig. 5).

DISCUSSION

In this study we used several antibodies to demonstrate that ubiquitin is present not only in PHFs of AD but also in straight filaments of Lewy and Pick bodies and of NFTs of PSP. The presence of ubiquitin is thus not a feature unique to PHFs but is shared by inclusions of other degenerative diseases. However, ubiquitin is not a component of all neuronal inclusions associated with aging and AD, as we did

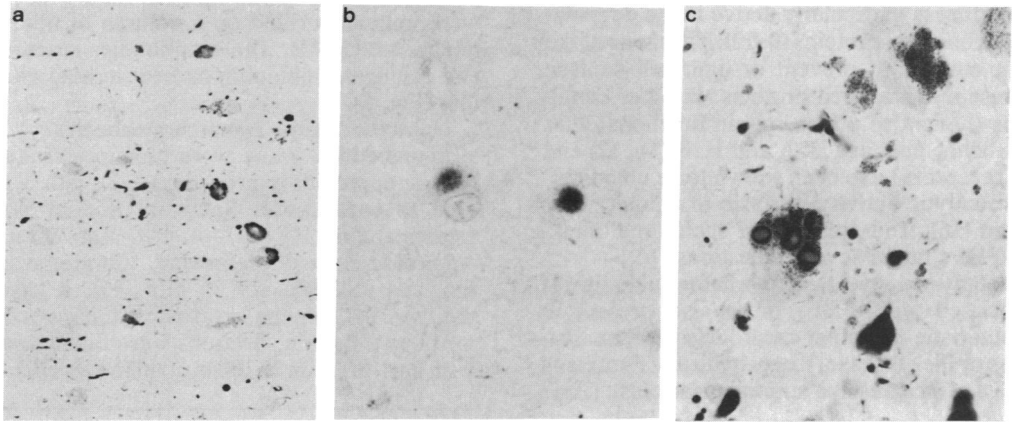

FIG. 1. Immunostaining with mAb 4-2D8, seen by light microscopy. (*a*) Pons from a case of PSP; several NFTs are immunostained. (×65.) (*b*) Temporal cortex from a case of Pick disease; Pick bodies are immunostained. (×260.) (*c*) Locus ceruleus from a case of Parkinson disease; the rims of Lewy bodies are intensely immunostained. (×260.)

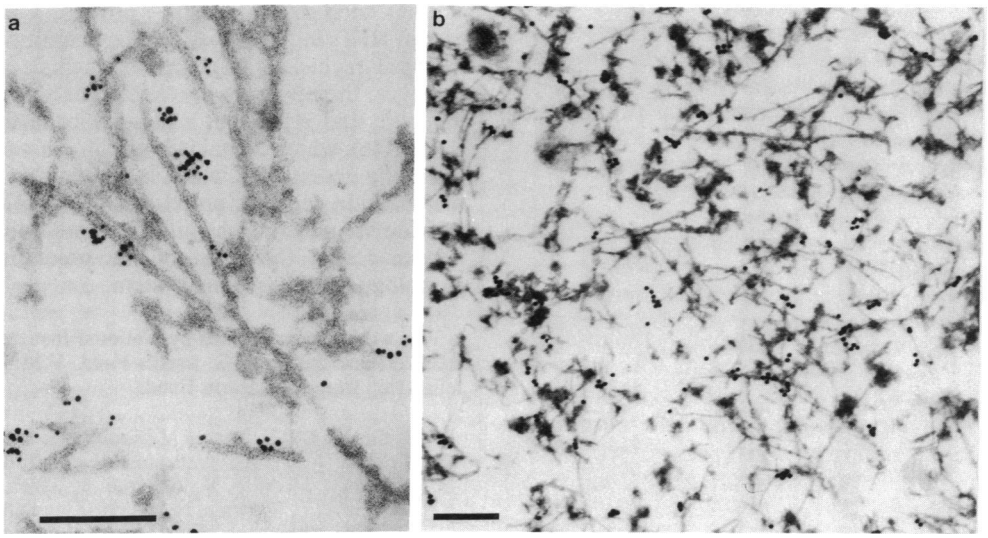

FIG. 3. Immunodecoration of straight filaments in NFTs of PSP (*a*) and straight filaments and amorphous material of a Pick body (*b*) with mAb 4-2D8. (Bars = 0.2 μm.)

not detect it in Hirano bodies and granulovacuolar degeneration.

The significance of the selective presence of ubiquitin in intraneuronal inclusions can be speculated on the basis of available knowledge of the function of ubiquitin in cells under normal and pathological conditions. There is evidence that the ubiquitin-dependent proteolytic system is involved in the selective disposal of short-lived and, perhaps as part of the heat shock system, of altered proteins that are formed as a result of a variety of injuries (8). The ubiquitin system is likely to be operative in the nervous system, since, as we showed in this study, substantial amounts of ubiquitin conjugates are present in immunoblots of both control and AD cortex. In tissue sections, however, only the abnormal inclusions were immunostained. A possible explanation of this finding is that ubiquitin conjugates are detected by immunocytochemical methods only when associated with a particular structure, whereas the conjugates present in normal cells may be diffusely distributed and therefore not visualized. Other cell constituents, such as microtubule components, are detected by immunohistochemistry only when they are assembled (31).

Ubiquitin conjugates that are intermediates in an ATP-dependent proteolytic system are rapidly deubiquitinated under normal conditions, and some of the acceptor proteins undergo proteolysis (32, 33). After heat shock, however, there is an increase in the amount and stability of Triton-insoluble high molecular weight ubiquitin conjugates, suggesting an association with the cytoskeleton (34). Moreover, the rate of protein degradation decreases after heat shock (34, 35) and during cellular aging (36, 37). The increase in stable ubiquitin conjugates has been tentatively attributed to a saturation of the ubiquitin-mediated proteolytic system by an increased amount of altered proteins, as the ubiquitinating and ubiquitin-dependent proteolytic activities have been found to be maintained (34, 38). Similar mechanisms may be postulated to explain the presence of ubiquitin in intracellular inclusions that form during normal aging and in neurodegenerative diseases. In these conditions, the ubiquitin system might become saturated by an excess of altered cytoskeletal proteins, which would aggregate in highly ubiquitinated, morphologically abnormal filaments. The presence of abnormal cytoskeletal proteins may be due to either errors in protein synthesis or a decrease in editing capabilities, both of

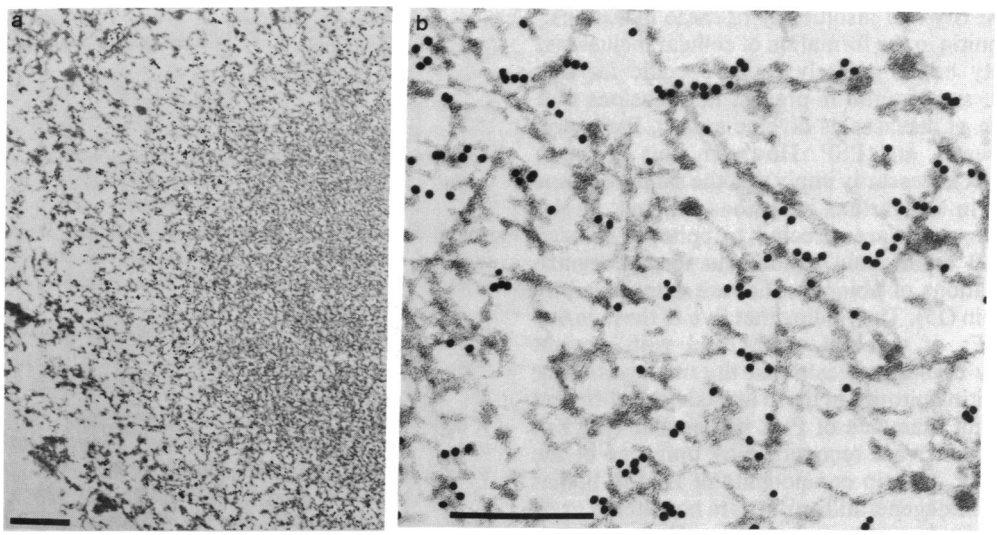

FIG. 4. Electron micrograph of a Lewy body immunostained with mAb 4-2D8. (*a*) Immunoreaction is seen at the rim but not at the core. (*b*) At higher magnification, only the filaments of the peripheral rim are decorated with gold particles. (Bars = 0.5 μm.)

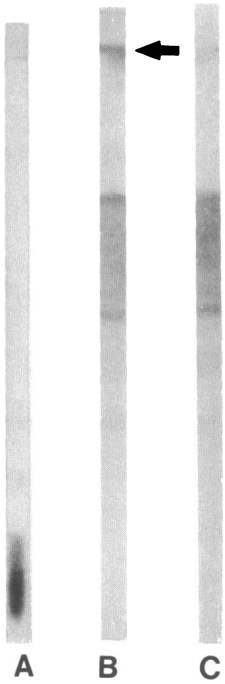

FIG. 5. Immunoblots with affinity-purified rabbit antibodies to ubiquitin. Lane A: ubiquitin (1 μg of protein; 14% polyacrylamide gel). Lanes B and C: hippocampus (10% polyacrylamide gel) from an AD (40 μg) and a control (40 μg) brain, respectively. Reaction is seen in the high molecular weight region in both AD and control samples, whereas reaction with material on top of the stacking gel (arrow) is very prominent in the AD sample.

which are known to occur with increased frequency during aging (37); it could also be due to sustained cellular stress related to the disease process, or to a combination of these events.

Although the saturation of the ubiquitin-dependent proteolytic system is an attractive hypothesis consistent with available data, other explanations for the presence of ubiquitin in intracellular inclusions cannot be ruled out. For example, stable high molecular weight ubiquitin conjugates not targeted for proteolysis (32, 39, 40) or ubiquitinated cytoskeletal components (41, 42) may become constituents of the filaments that form the inclusions. Morever, the possibility, suggested by Rechsteiner (6), that ubiquitinated substrates may become crosslinked to other proteins, could explain the complexity and insolubility of these inclusions.

The role of ubiquitin in the formation of cellular inclusions, whatever this may be, is unlikely to be specific for any disease condition, as ubiquitin is present in inclusions that form in the course of diseases as diverse as AD, Parkinson disease, Pick disease, and PSP. However, this common occurrence does not necessarily imply that the same acceptor protein is present in the various inclusions. Indeed, all the mAbs used in this study are directed to epitopes located within the same 21 amino acid region, but they recognize different conformations of ubiquitin that are determined by the acceptor protein (15). The finding that five of these mAbs reacted with PHFs of cerebral cortex and with straight filaments of Lewy bodies suggests that the organization or exposure of ubiquitin epitopes in these inclusions is different from that in straight filaments of Pick bodies and NFTs of PSP that, in contrast, were recognized by only one of the mAbs. Interestingly, protein components of Hirano bodies and granulovacuolar degeneration either are not altered or do not seem to be ubiquitin-acceptor proteins. However, it is still possible that ubiquitin is present but not accessible for reaction with the tested antibodies.

It has been suggested that τ protein, a prominent constituent of NFTs in AD (43–45), is the ubiquitin-acceptor protein in these inclusions (4). In the inclusions we examined, however, there was no correlation between the presence of ubiquitin and τ protein. τ has not been detected in Lewy bodies (46), which contain ubiquitin; conversely, τ is present in Hirano bodies (47), which lack ubiquitin. Identification of the ubiquitin-acceptor proteins in these inclusions is likely to provide valuable insight into their mechanism of formation and to reveal the roles of the ubiquitin system in the pathology of the nervous system.

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