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Selective Presence of Ubiquitin in Intracellular Inclusions

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The authors have shown previously that ubiquitin, a protein involved in the degradation of short-lived and abnormal proteins, is present in several cytoplasmic inclusions of neurons. This study used a library of antibodies to ubiquitin and immunohistochemically examined for the presence of ubiquitin in nonviral intracytoplasmic inclusions that form in different cell types under various pathologic conditions. Membrane-bound lysosomal and nonlysosomal inclusions such as those of storage diseases, Russell bodies, alpha-1-antitrypsin and alpha-fetoprotein as well as nonmembrane-bound inclusions were examined. Ubiquitin epitopes were detected in some of the nonmembrane-bound inclusions only. The ubiquitin-containing inclusions were the Rosenthal fibers, Mallory bodies, Crooke bodies, Lafora bodies, amyloid bodies, and the giant axons of giant axonal neuropathy. Nemaline bodies and the inclusions of juvenile digital fibromatosis, both of which contain actin and actin-binding proteins, did not show immunoreaction. These findings, as well as those of the previous study, show that the presence of ubiquitin in cellular inclusions is selective. The ubiquitin-containing inclusions are not membrane bound; they are fibrillary and most contain also intermediate filament-related proteins. The role of ubiquitin in the formation of these inclusions remains to be elucidated. (Am J Pathol 1989, 134:505–513)

Cytoplasmic inclusions can form in most cell types in response to a variety of conditions. Morphologically, nonviral cytoplasmic inclusions can be divided into two main types according to whether or not they are bound by a membrane. The most common, and best known, membrane-bound cytoplasmic inclusions are lysosomal in origin. The formation of these inclusions is commonly due to a defect in a lysosomal enzyme, which leads to the intralysosomal accumulation of undigested substrate.[1] Other membrane-bound inclusions result from the abnormal accumulation of proteins inside cisterns of rough endoplasmic reticulum (RER).

The mode of formation of nonmembrane-bound inclusions is less well known. Until recently, it was not known whether cytoplasmic proteolytic systems were involved in the pathogenesis of these inclusions, many of which have a fibrillary structure and contain cytoskeletal proteins.

The recent identification of ubiquitin (Ub) in neurofibrillary tangles (NFT), the intraneuronal cytoplasmic nonmembrane-bound inclusions found in Alzheimer disease (AD),[2,3] has provided a new approach to the study of the mechanisms that lead to the formation of intracytoplasmic inclusions. Ub is a 76-amino acid, highly conserved protein that is thought to play an essential role in cellular function.[4–6] It is present in cells as a free molecule and conjugated to selected proteins.[6] Whether the free form of Ub has functions that differ from those of the conjugated form remains to be established. Two types of proteins conjugated to Ub may be distinguished: those that are targeted by Ub for rapid degradation through an ATP-dependent, nonlysosomal proteolytic system and those that are relatively stable.[7,8] Abnormal and short-lived normal proteins are more likely to be candidates for Ub-mediated rapid proteolysis.[9] The more stable conjugates include nuclear proteins such as histones H2A and H2B, plasma membrane proteins such as the lymphocyte homing receptor, and possibly cytoskeletal proteins such as microtubule-associated proteins.[10–14] The role of Ub in these stable conjugates has not been established. It has been shown, however, that Ub also has an intrinsic proteolytic activity under appropriate conditions.[15] In addition, it has been suggested that, as a protease conjugated to proteins, Ub may modulate the function of the conjugates.[15]

The finding that Ub is present in the NFT of AD raised questions concerning the selectivity and the role of Ub

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Table 1. *Inclusions Examined*

Inclusion type	Cell type	Ultrastructure	Composition	UB
Membrane bound				
GM1-gangliosidosis lysosomal inclusions	Neurons	Membranous bodies	GM1-gangliosides	−
Hurler disease lysosomal inclusions	Neurons	Membrane-bound amorphous vacuoles	Dermatan and heparan sulphate	−
Pompe disease lysosomal inclusions	Myocytes	Membrane-bound glycogen containing vacuoles	Glycogen	−
Ceroid lipofuscinosis lysosomal inclusions	Neurons	Membranous bodies	Undetermined	−
Alpha-1-antitrypsin deficiency inclusions	Hepatocytes	Amorphus material in distended RER	Alpha-1-antitrypsin variant	−
Russell bodies	Plasma cells	Amorphous material in distended RER	Immunoglobulins	−
Alpha-fetoprotein inclusions	Ovarian cells	Amorphous material in distended RER	Alpha-fetoprotein	−
Nonmembrane-bound rosenthal fibers	Astrocytes	Amorphous material surrounded by 10–12 nm filaments	GFAP; 19 kd polypeptide	+
Mallory bodies	Hepatocytes	Randomly oriented 10–20 nm filaments and amorphous material	Cytokeratin	+
Crooke bodies	Pituitocytes	Bundles of 13 nm filaments	Cytokeratin	+
Lafora and amyloid bodies	Neurons and astrocytes	Randomly arranged 15–20 nm filaments and amorphous material	Glucose polymer and proteins	+
Giant axons	Neurons	10 nm filaments and amorphous material	Neurofilaments	+
Infantile digital fibromatosis inclusions	Fibroblasts	Bundles of 6–8 nm filaments	Actin	−
Nemaline bodies	Myocytes	Z-disklike fibrillary structure	Actin and alpha-actinin	−

in intracellular inclusions. We recently reported that Ub epitopes are selectively present in several other neuronal inclusions characteristic of degenerative conditions of the central nervous system, such as Lewy bodies of Parkinson disease, Pick bodies of Pick disease, and NFT of progressive supranuclear palsy (PSP).[16] They could not, however, be demonstrated in other neuronal inclusions associated with aging and AD such as in Hirano bodies and granulovacuolar degeneration.[16]

In this study, we examined intracytoplasmic inclusions that form in different cell types under various pathologic conditions. Using antibodies that recognize epitopes located in different regions of the Ub molecule, we obtained evidence that Ub is selectively present in nonmembrane-bound filamentous inclusions, most of which also contain intermediate filament proteins.

Material and Methods

Tissue Source

Nonviral cytoplasmic inclusions, membrane-, and nonmembrane-bound, were examined from a variety of tissues obtained at surgical biopsy and autopsy (Table 1). Biopsy tissues were fixed for 1 day in formalin or in Perfix (Fisher Scientific, Orangeburg, NY) and embedded in paraffin. The autopsy tissue was fixed for 10–20 days in 10% buffered or unbuffered formalin and embedded in paraffin. Appropriate control tissues from autopsy and surgical specimens also were examined. These inclusions displayed their respective histopathologic features and could readily be identified with common histologic stains.

Membrane-Bound Inclusions

1) Lysosomal inclusions were examined in cerebral tissue obtained at autopsy, from subjects with GM1-gangliosidosis, ceroid lipofuscinosis, and Hurler disease as well as skeletal muscle from a subject with Pompe disease. All these cases with storage diseases had clinical histories and pathologic changes typical of their condition. The clinical diagnosis was established by demonstrating the lack of activity of the appropriate lysosomal enzyme, except for the ceroid lipofuscinosis, which was diagnosed by electron microscopic examination of a brain biopsy.

2) Nonlysosomal inclusions: A needle core biopsy of the liver that contained a large number of cytoplasmic bodies in periportal hepatocytes was obtained from a 7-

month-old girl with alpha-1-antitrypsin deficiency. Russell bodies were examined in the plasma cell infiltrates present in biopsies from two patients, one with acute and the other with chronic osteomyelitis of the femur and the other with *in situ* squamous cell carcinoma of the vulva. Alpha-fetoprotein inclusions were studied in an endodermal sinus tumor of the ovary.

Nonmembrane-Bound Inclusions

1) Rosenthal fibers: Brain tissue rich in Rosenthal fibers was obtained by biopsy from two patients with juvenile cerebellar astrocytoma, and at autopsy from a case of Alexander disease.[17]

2) Mallory (alcoholic hyaline) bodies were examined in liver tissue obtained at autopsy from three subjects with alcoholic hepatitis and micronodular cirrhosis.

3) Crooke (hyaline) bodies: Pituitary tissue that contained numerous Crooke bodies was obtained at biopsy from two subjects with ACTH-secreting pituitary adenomas.

4) Tissue was obtained at biopsy and autopsy from the cerebral cortex of three cases of Lafora body disease.[18]

5) Tissue from the brain stem, spinal cord and peripheral nerves that contained numerous enlarged axons was obtained at autopsy from two cases of familial giant axonal neuropathy (GAN).[19,20]

6) Corpora amylacea were examined in cerebral and spinal cord tissue obtained at autopsy from cases with various diseases including amyotrophic lateral sclerosis and tuberous sclerosis.

7) Fibromatosis: Soft tissue was obtained at excisional biopsies from the toes of two children with infantile digital fibromatosis. The fibroblasts contained the eosinophilic inclusions that are characteristic of this condition.

8) Nemaline myopathy: Skeletal muscle was obtained at biopsy from an 8-month-old boy. The clinical and pathologic findings were typical of nemaline myopathy.

Control Tissues

Control tissues were used for all the positive inclusions. Normal liver, skin, pituitary and cerebral tissue and peripheral nerve were obtained from subjects of similar age as that of those whose tissues contained Mallory bodies, Crooke bodies, Rosenthal fibers, and giant axons.

Antibodies

Three monoclonal antibodies (MAbs), 4-2D8, 4-3H8,[12] and 5-25[20,21], and two immunoaffinity purified antisera to Ub were used. The first two MAbs recognize epitopes located between residues 34 and 54 of Ub and the third recognizes an epitope located between residues 64 and 76.[21] The two antisera were raised in rabbits with SDS-denatured Ub cross-linked to keyhole limpet hemocyanin as immunogen and immunoaffinity purified.[16] Both antisera recognize epitopes along the entire Ub molecule.[22] All MAbs and antisera to Ub employed in this study immunoreacted with NFT in AD and PSP as well as with Lewy and Pick bodies.[16] As controls, we used preimmune sera from the rabbits immunized with Ub and an irrelevant mAb to Sindai virus (a gift from Dr. J. Nedrud, CWRU). In addition, we used MABs to a phosphorylated epitope of the high molecular weight subunit of neurofilaments (NF) (1.1.1),[23] to actin (C4)[24] and to cytokeratin (34BE12)[25] as well as rabbit antisera to the low molecular weight subunit of NF[26] and to GFAP.[27]

Immunocytochemistry

Tissue sections were immunostained using peroxidase-antiperoxidase (PAP) complexes (Sternberger-Meyer Inc., Baltimore, MD) with 3,3-diaminobenzidine as cosubstrate. The reaction was enhanced by treatment with 1% OsO_4 for 30 seconds.

Results

Immunocytochemistry

Inclusions

Among the nonmembrane-bound inclusions examined, only the Rosenthal fibers, Mallory bodies, Crooke bodies, enlarged axons of giant axonal neuropathy, Lafora bodies, and amyloid bodies were recognized by all the antibodies to Ub used (Table 1). These antibodies, however, did not immunostain the nonmembrane-bound inclusions of infantile digital fibromatosis nor the nemaline bodies of nemaline myopathy. The inclusions in these two entities immunostained with an antibody to actin. None of the membrane-bound inclusions that comprised the lysosomal inclusions of GM1 gangliosidosis, Hurler disease, ceroid lipofuscinosis, and Pompe diseases as well as nonlysosomal inclusions such as alpha-1-antitrypsin, Russell bodies, and alpha-fetoprotein were stained with antibodies to Ub.

The pattern of immunostaining was not the same in the various inclusions that reacted with the Ub antibodies. The Rosenthal fibers were immunostained preferentially or exclusively at their periphery. Occasionally, however, only a thin rim around the fibers was positive. This staining

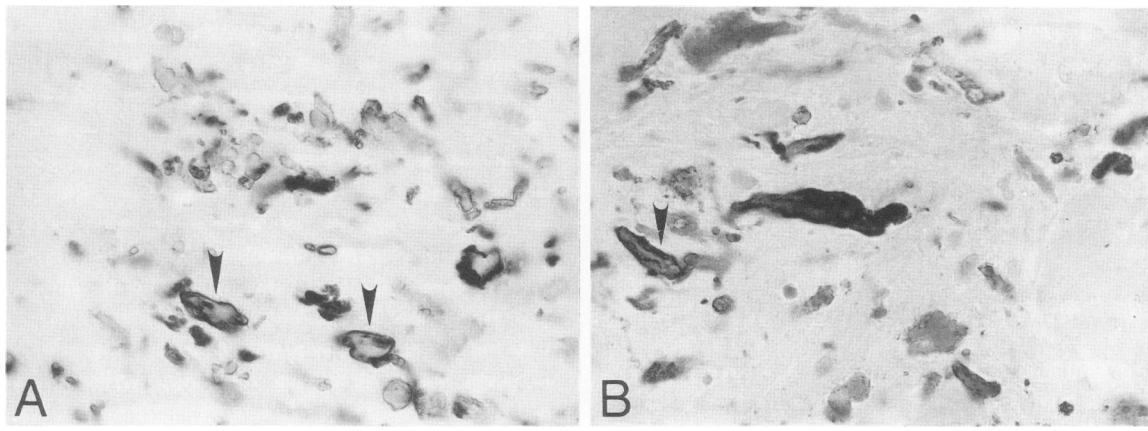

Figure 1. *Rosenthal fibers. Monoclonal antibodies (MAb) to Ub (4-2D8) (A), glial fibrillary acidic protein (GFAP) (B). Note the variable immunostaining patterns with preferential peripheral staining of the fibers (arrowheads, ×450).*

pattern was indistinguishable from that obtained with an antibody to glial fibrillary acidic protein (Figure 1). In contrast, the immunostaining was homogeneous in the Mallory bodies and Crooke bodies and in a manner similar to that obtained with an antibody to cytokeratin (Figure 2). The staining pattern and intensity also varied in the enlarged axons of GAN (Figure 3). The staining pattern of the enlarged axons was similar to that produced with antibodies to the 68 kd and to the 200 kd subunits of NF, although fewer enlarged axons were immunostained by the NF antibodies than by the Ub antibodies (Figure 3). Lafora and amyloid bodies also demonstrated variety in staining patterns, including peripheral, central, and target-like staining (Figure 4). None of the inclusions were stained with either preimmune sera or the irrelevant mAb used as controls.

Other Components of Abnormal Tissues

The nuclei were the cell structure most often immunostained in all the tissues examined. They were stained more consistently in hepatocytes, fibroblasts and pituitary cells. Glial cells and Schwann cell nuclei were immunostained more often than neuronal cell nuclei. Occasional cell bodies of hepatocytes located in areas of intense cirrhosis and pituitary adenoma tumor cells were immunostained diffusely.

Normal Tissues

Tissues from the central nervous system, liver, and pituitary gland were unstained except for scattered nuclei. Nuclei of fibroblasts and Schwann cells were stained more often. Cell perikarya and nuclei were consistently stained in the squamous cell layer of the skin.

Discussion

This study demonstrates that antibodies to Ub immunoreact with intracellular inclusions located in various cell types and formed under different pathologic conditions. Because the antibodies that we used are directed to epitopes located in different regions of Ub, it is likely that these inclusions contain the entire Ub molecule, or a large portion of it.

In a previous study, we reported also that not all the inclusions examined were recognized by the Ub antibodies.[16] Of the 15 inclusion types examined in the current study (Table 1) and of the 5 examined previously, Ub was found only in 9.[16] This selective presence of Ub in cellular inclusions raises questions concerning the characteristics of the ubiquinated inclusions and the role that Ub plays in these inclusions. Although we examined only a relatively small number of inclusions and cannot exclude that Ub was undetected in some of the negative inclusions due to the inaccessibility of epitopes recognized by our antibodies, tentative conclusions concerning the general features of the ubiquitinated inclusions can be drawn. Ub could not be demonstrated in the membrane-bound inclusions, whether they were lysosomal or nonlysosomal. The lack of Ub in abnormal inclusions of lysosomal origin is not surprising because the Ub-dependent proteolytic pathway is distinct from the lysosomal pathway of protein degradation, and it is unlikely that Ub becomes part of the lysosomal system in abnormal conditions such as in lysosomal storage diseases.[9] Russell bodies, the eosinophilic inclusions of alpha-1-antitrypsin deficiency, and the intracellular hyaline bodies of alpha-fetoprotein are located inside cisterns of the RER of plasma cells, hepatocytes, and ovarian endodermal sinus tumor tissue, respectively.[28-30] Russell bodies result from an accumulation of immunoglobulin and perhaps of mucoproteins as

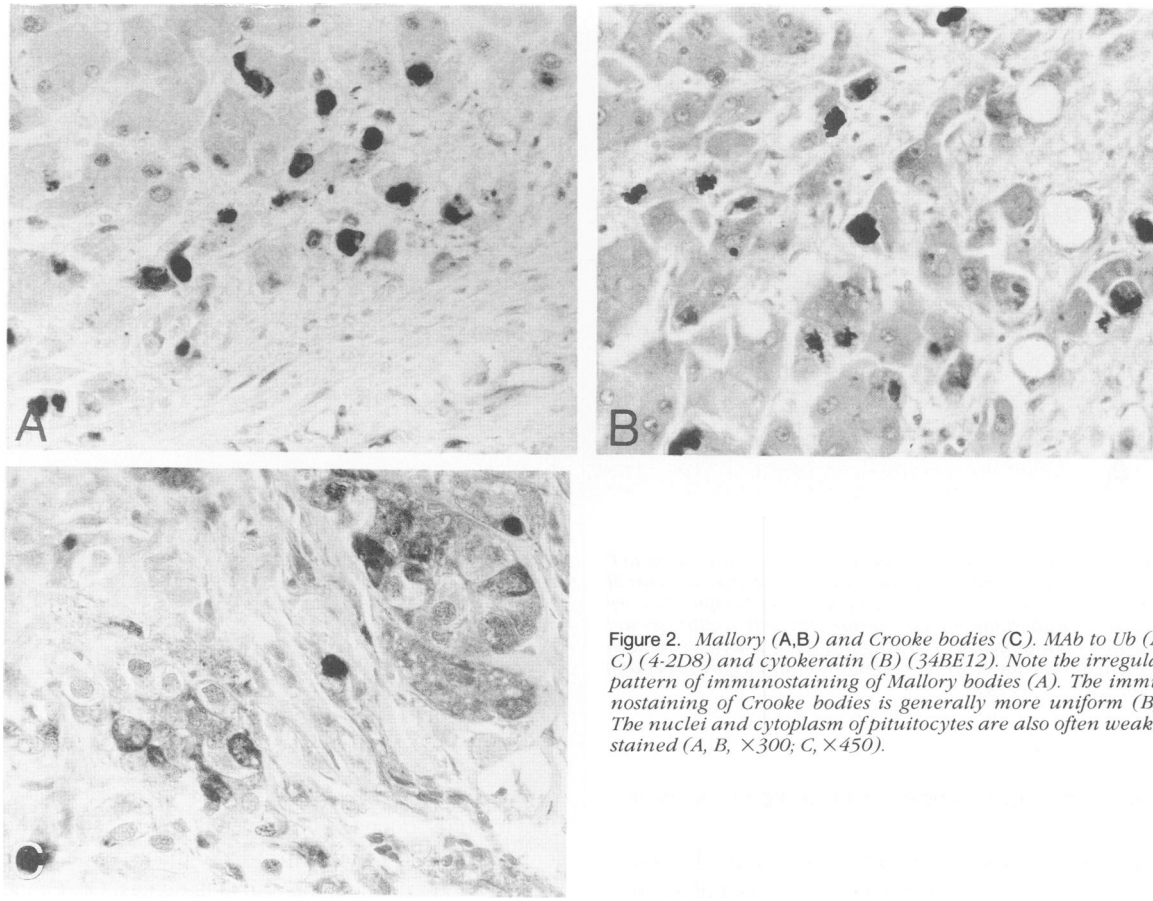

Figure 2. *Mallory (A,B) and Crooke bodies (C). MAb to Ub (A, C) (4-2D8) and cytokeratin (B) (34BE12). Note the irregular pattern of immunostaining of Mallory bodies (A). The immunostaining of Crooke bodies is generally more uniform (B). The nuclei and cytoplasm of pituitocytes are also often weakly stained (A, B, ×300; C, ×450).*

a result of impaired immunoglobulin secretion.[31,32] In alpha-1-antitrypsin deficiency the inclusions are made of a variant of the alpha-1-antitrypsin glycoprotein in which lysine substitutes for glutamic acid.[33,34] The mutant protein is not efficiently transported beyond the RER.[35] Because these inclusions are made of an abnormal protein, it might be expected that the Ub-dependent proteolytic system plays a role in the formation of these inclusions. Because the variant alpha-1-antitrypsin protein remains segregated from the cytosol by the membrane of the RER, however, it may be inaccessible to the Ub system. The intracellular hyaline bodies of alpha-fetoprotein in endodermal sinus tumors consist of electron-dense basement membranes material. This material is similar to the basement membrane that is present between the endoderm and the mesenchyme in the yolk sac of a 7-week pregnancy.[36]

Although all the Ub-containing inclusions were not membrane bound, not all the nonmembrane-bound inclusions are ubiquinated. Ubiquitin presence, therefore, is also selective among nonmembrane-bound inclusions. Of the nine types of nonmembrane-bound inclusions examined, two—the inclusions of the infantile digital fibromatosis and the nemaline bodies of nemaline myopathy—were not immunostained. Both these inclusions are fibrillary and both have been shown to have a similar composition.[37-39] The eosinophilic inclusions of infantile digital fibromatosis are made largely of actin.[37] Nemaline bodies contain both F-actin and alpha-actinin.[38,39] Moreover, previous studies failed to demonstrate an association of Ub with Hirano bodies, an intraneuronal inclusion thought to contain actin, alpha-actinin, and other actin-associated proteins.[16,24] Because none of the three cellular inclusions containing actin and actin-associated proteins that we examined are recognized by Ub antibodies, it seems unlikely that Ub plays a role in the formation of actin-containing inclusions. This finding is unexpected because insect muscle cells have been found to contain a significant amount of ubiquitinated actin.[40]

The Rosenthal fibers of astrocytes, the Mallory bodies of hepatocytes, the Crooke bodies of pituitary cells, the Lafora bodies of neurons, the corpora amylacea of astrocytes, and the giant axons were all immunostained by all the MAbs and antisera to Ub that we tested. These inclusions have common features with respect to their morphology and composition. They are all fibrillary, and the fibrillary structures may be present in the entire inclusion as in the Crooke bodies[41,42] or associated with an amorphous component as in Rosenthal fibers, Mallory,

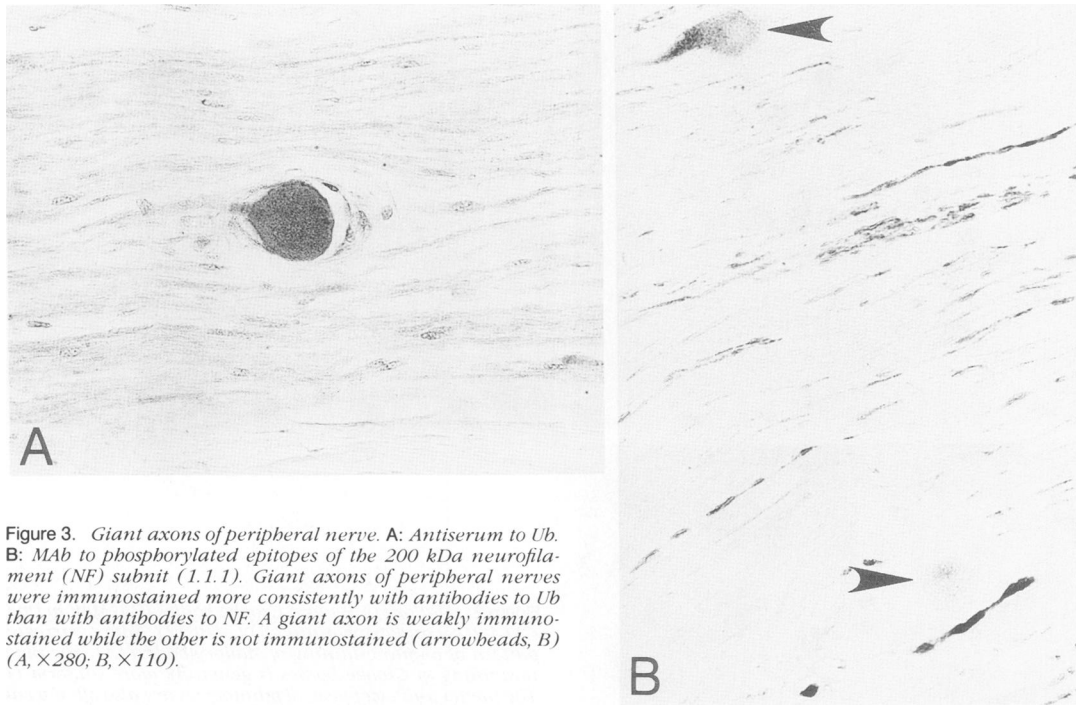

Figure 3. *Giant axons of peripheral nerve.* A: *Antiserum to Ub.* B: *MAb to phosphorylated epitopes of the 200 kDa neurofilament (NF) subnit (1.1.1). Giant axons of peripheral nerves were immunostained more consistently with antibodies to Ub than with antibodies to NF. A giant axon is weakly immunostained while the other is not immunostained (arrowheads, B) (A, ×280; B, ×110).*

Lafora, and amyloid bodies, and enlarged axons of GAN.[43-46]

The filaments that compose Mallory and Crooke bodies contain cytokeratin, the protein component of the intermediate filaments (IF) of epithelial cells. Detailed studies of Mallory bodies have shown that the filaments that make up these bodies are related to, but morphologically and antigenically distinct from, the normal IF and are likely to result from changes in cytokeratin assembly and conformation.[43,47] Moreover, Mallory bodies are only partially soluble in SDS.[47]

In the Rosenthal fibers the IF that are immediately adjacent to and surround the amorphous component are often morphologically abnormal.[44,45] Immunocytochemical evidence indicates that the filaments contain GFAP, the protein component of the astrocytic IF, whereas a 19 kd protein has been isolated from the amorphous mass.[48] Preliminary sequencing has revealed that Ub is present in this protein and it has been suggested that Ub is conjugated to another protein, possibly a fragment of GFAP.[45] Neurofilaments are closely associated with an amorphous or granular component in the enlarged axons of GAN.[46]

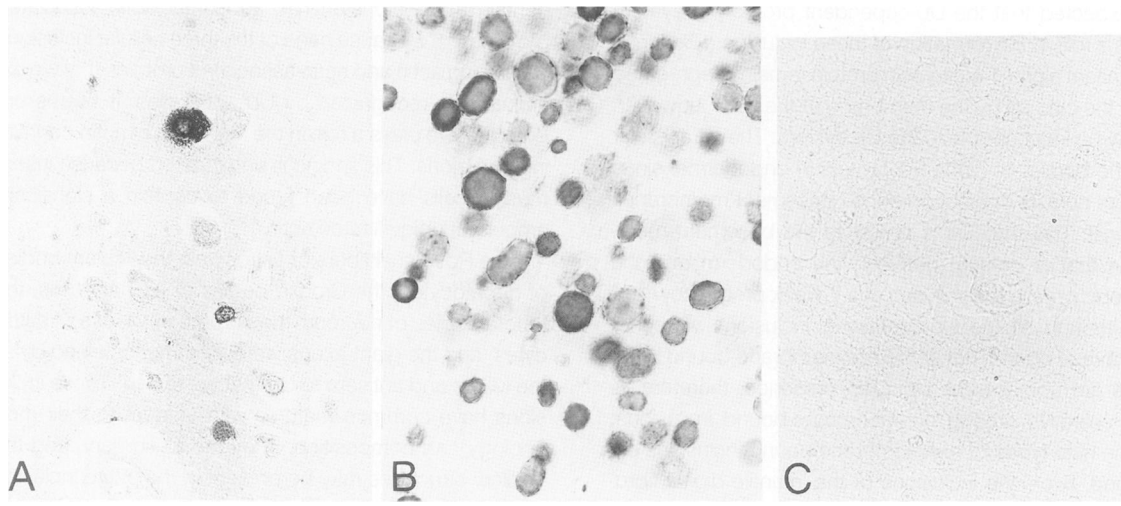

Figure 4. *Lafora bodies (A) and amyloid bodies (B, C).* A,B: *Antiserum to Ub.* C: *Preimmune serum. Both inclusions are consistently immunostained but with variable intensity. Often a targetlike pattern of immunostaining is present in both bodies (A, C, ×450; B, ×670).*

The amorphous component has not been characterized.[18,46] Similarly, with the possible exception of the NFT of PSP, all the neuronal inclusions that reacted with Ub antibodies, ie, NFT of AD, Lewy bodies of Parkinson disease, Pick bodies of Pick disease, were composed of morphologically abnormal filaments which share epitopes with NF.[2,3,16,49–54]

Studies of the ultrastructural localization of Ub epitopes carried out in NFT, Pick, Lewy, and Mallory bodies have unequivocally shown that they are located in the abnormal filament component in all these inclusions.[16,54,55]

At variance with other Ub-containing inclusions, the Lafora and amyloid bodies have not been shown to contain IF protein. These two inclusions are morphologically similar and are generally referred to as polyglucosan bodies.[56] They are composed of irregular branching filaments and amorphous material distributed in aggregates of different sizes.[56] Definitive data on the composition of these inclusions are unavailable, but both have been reported to contain principally glucose polymers and less than 5% proteins.[56–59] Four main polypeptides have been demonstrated in the amyloid bodies.[59] Preliminary studies indicate that Ub is present in at least two of these polypeptides, whereas no definite evidence for the presence of GFAP or other IF proteins has been obtained (Gauvreau D, Perry G, personal communication). The presence of Ub in intracellular inclusions, therefore, may not be invariably related to the presence of IF proteins.

The Ub-containing inclusions examined in this study share the following features: 1) they are not membrane bound; 2) they are filamentous; 3) most, but not all, contain IF-related proteins.

The selective association of Ub with cellular inclusions allows for a whole range of hypotheses. One may postulate that the presence of Ub is irrelevant to the formation of inclusions because Ub may be normally bound to proteins that subsequently become major components of inclusions. The presence of Ub epitopes in IF of normal hepatocytes is consistent with this hypothesis. At the other extreme, however, one may hypothesize that Ub-containing inclusions result from a failure of the Ub system to clear the cells of cytoskeletal and possibly other abnormal proteins that accumulate as a result of chronic cellular stress. The known role of Ub in the rapid breakdown of proteins altered during various forms of cellular stress is consistent with this hypothesis.

After completion of this study, Lowe et al reported that Rosenthal fibers, Mallory bodies, and cytoplasmic bodies of muscle contain ubiquitin epitopes as demonstrated by the immunostaining of these inclusions with an antiserum to ubiquitin.[60]

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