Lewy Bodies Contain Epitopes Both Shared and Distinct from Alzheimer Neurofibrillary Tangles

P. G. GALLOWAY, M.D., I. GRUNDE-IQBAL, PH.D., K. IQBAL, PH.D., AND G. PERRY, PH.D.

Abstract. Most of the identified constituents of the filamentous inclusions characteristic of the neurodegenerative diseases of aging are derived from the cytoskeleton. This study was undertaken to define immunocytochemically the cytoskeletal constituents of the filamentous cytopathologic marker of idiopathic Parkinson disease, the Lewy body (LB). An array of antibodies specific to neurofilaments, tubulin, microtubule associated proteins (tau, MAP1 and MAP2) and Alzheimer neurofibrillary tangles (NFT) were used to immunostain sections containing LB. All the antibodies to tubulin, MAP1 and MAP2 and the majority of the antibodies to neurofilaments and NFT recognized LB. The two monoclonal antibodies to NFT that recognize LB also react with ubiquitin, which has been identified in NFT. The prominent NFT component, tau, is apparently not incorporated into LB. These findings suggest that the presence of tau might not be a prerequisite to the formation of abnormal filaments. Therefore, although LB contain elements of neurofilaments, microtubules and ubiquitin, as do other abnormal neuronal filaments, they are distinct in composition. These distinctive and shared features may provide useful insights regarding the mechanisms underlying the formation of filaments in LB as well as those of other neuronal inclusions.

Key Words: Cytoskeleton; Lewy body; Microtubule associated protein; Neurofilament; Parkinson’s disease; Tubulin; Ubiquitin.

INTRODUCTION

Lewy bodies (LB) are the characteristic intracytoplasmic inclusion of neurons in idiopathic Parkinson’s disease, a degenerative condition of the central nervous system (CNS). They are composed of a dense core, often containing granules, circular profiles and vesicles, surrounded by radiating filaments varying in diameter from 7.5 to 20 nm (1, 2). Primarily located in pigmented neurons of the locus ceruleus and substantia nigra (3), they have been reported in other sites in the CNS and peripheral nervous system, including the non-pigmented brainstem nuclei, the nucleus basalis of Meynert, hypothalamus, and less commonly in the fifth and sixth layers of the cerebral cortex and the intermediolateral cell columns of the spinal cord (4).

The components and cellular precursors of LB have been difficult to define. This is because (as for other neuronal inclusions in degenerative disease of the CNS) their direct biochemical examination has been impeded by the lack of a pure fraction. Consequently, there has been a reliance on structural, histochemical and immunocytochemical approaches to identify the elements of LB. Consistent with the non-
filamentous structure of the LB core, the central region reacts positively with histochemical stains to sphingomyelin (5). Immunocytochemical approaches have followed two methods to understand the composition of LB: 1) the search for antibodies related to normal proteins that react with LB, and 2) the identification of normal proteins recognized by antibodies raised to fractions enriched in LB. The latter method was applied to monoclonal antibodies raised to substantia nigra and locus ceruleus fractions, and resulted in the identification of two proteins, of approximately 40 and 70 kDa, whose cellular identity is unknown (6). Studies using the complementary approach have been used to demonstrate neurofilament subunits (7–9) in the peripheral halo of the LB suggesting a cytoskeletal derivation for the filaments in this region.

In light of numerous studies which have been reviewed (10) that have demonstrated immunocytochemical cross reactivity of microtubules and neurofilaments with the NFT of Alzheimer disease (AD) (8, 11–17), Pick bodies of Pick disease (8, 18, 19) and NFT of progressive supranuclear palsy (20, 21), we undertook this immunocytochemical study to further define the identity of LB components and to gain a better understanding of the mechanisms underlying the formation of LB and other neuronal inclusions, with particular reference to NFT. An array of antibodies raised to neurofilaments and their subunits, several proteins present in microtubules and enriched fractions of paired helical filaments (PHF) (the primary component of NFT) were used to immunostain sections containing LB.

We found that LB contain epitopes in common with many of the proteins comprising microtubules and neurofilaments in addition to ubiquitin and some as yet undefined epitopes found in NFT. However, crossreactivity with the microtubule-associated protein, tau, which is a component of NFT (8–10), was not detected.

Preliminary findings from this study have been reported (22–24).

MATERIALS AND METHODS

Immunostaining and Antibodies

Sections of locus ceruleus and substantia nigra obtained at autopsy from seven patients with idiopathic Parkinson disease were fixed in 10% formalin, embedded in paraffin and sectioned 8 micrometers (μm) thick. Ten μm thick frozen sections of unfixed substantia nigra were cut for immunostaining with tau antibodies, and fixed in 95% ethanol. The age at death of the patients ranged from 54 to 74 years. Immunostaining was performed using the peroxidase–antiperoxidase procedure (PAP) (25). The following antibodies were used (Table 1): rabbit antisera (K68, K145 and K200) raised to each of the three neurofilament subunits purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (26); monoclonal antibodies reacting with epitopes of the high molecular weight neurofilament subunit which were either phosphorylated (SMI 31 and 34) or non-phosphorylated (SMI 33) (Sternberger-Meyer, Inc.); monoclonal antibodies 1.2, 2.1, 1.1, and 5.14A raised to human neurofilaments (27); a sheep affinity purified antisera to bovine tubulin (28), antiserum to a synthetic peptide containing tyrosinated tubulin sequences (29); monoclonal antibodies (30, 31) and an antisera (32) to tau protein; antisera (1B2, 1A1 and 2-2) to high molecular weight microtubule associated proteins, MAP1 and MAP2 (33); three antisera (34–36) and three monoclonal antibodies 5-25, 3-39 and 3-13, raised to PHF fractions isolated from Alzheimer NFT (37). Sections were incubated with the primary antibody for 18 hours (h) at 4°C. Development time was less than three minutes (min), using 3,3′-diaminobenzidine (0.75 mg/ml) (Sigma Chemicals, St. Louis, MO), with hydrogen peroxide (0.015%) in 0.05 M Tris-HCl, pH 7.6. Immunofluorescence was done on frozen sections using the antibodies to tau protein by incubating with primary antibody, then with fluorescein-conjugated goat anti-mouse or rabbit immunoglobulin (DAKO) both for twenty min at room temperature. Sections were rinsed three

### TABLE 1
Specificity of Antibodies Used to Stain Lewy Bodies*

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Lewy body</th>
<th>PHF</th>
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<th>145NF</th>
<th>68NF</th>
<th>Tub</th>
<th>Tau</th>
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* The antibody reactivity of all but the results for Lewy bodies refers to previously published characterization cited in text.
† Sheep affinity purified antibody.
‡ Tyrrosinated tubulin antibody.
§ Non-tyrosinated tubulin antibody
‖ Grundke-Iqbal (35).
‖ Ibara et al (36).

PHF = paired helical filament. NF = neurofilament subunit. Tub = tubulin. ND = not determined. ICN Pharmaceutical. HMW MAP = high molecular weight microtubule associated protein.

Times between incubations with phosphate buffered saline, pH 7.6, then mounted in aquamount (Lerner Laboratories, New Haven, CT). Mouse pre- and non-immune, and rabbit and sheep non-immune sera instead of primary antibody served as negative controls. Positive controls for neurofilament and microtubule antibodies consisted of the appropriate structure (dendrites or axons) on the same tissue section as the LB. Immunostaining for a given tissue section was considered positive when the appropriate structure on the same section was immunostained. Positive controls for the PHF antibodies consisted of hippocampal sections from age matched patients with AD. These sections were processed in the same way as sections of locus ceruleus and substantia nigra. Sections in which immunostained LB were not seen were counterstained with eosin to confirm their presence. The sections were viewed at ×20 to ×1,000 under bright field with an Olympus microscope or under epi-illumination with a Zeiss fluorescence microscope with 50 watt tungsten illumination.
Fig. 1. Lewy body in the locus ceruleus immunostained with monoclonal antibody SMI 34. This is an example of the common pattern of immunostaining, with a peripheral immunostained ring surrounding the Lewy body core (arrowhead). PAP. ×1,248.

Phosphatase Treatment

Dephosphorylation of sections from the locus ceruleus, substantia nigra and AD patient hippocampus (as control) was carried out before application of the two monoclonal antibodies to tau, by treating the specimen with calf intestinal alkaline phosphatase (Type VII, Sigma), 43 μg/ml in 0.1 M Tris-HCl, pH 8.0, containing 0.01 M phenylmethyl-sulfonyl fluoride at 32°C for 2.5 h. Controls (AD hippocampal sections) were incubated under similar conditions using a buffer alone. Treatment of AD hippocampal sections with alkaline phosphatase, as described above, was effective in blocking the reaction of an antibody to phosphorylated neurofilaments (SMI 34) (38) with axons.

RESULTS

The results of our immunostaining of LB, and immunoreaction characteristics of the antibodies used to other epitopes based on previous characterization (26–37), are summarized in Table 1. Lewy bodies were immunostained by 1) the antisera (K68, K145 and K200) which specifically react with each of the three neurofilament subunits, 2) the monoclonal antibodies to neurofilaments (1.2) whether non-phosphorylated (SMI 33) or phosphorylated (SMI 31 and 34), 3) all the antisera recognizing tubulin epitopes, whether tyrosinated or non-tyrosinated, 4) the antibodies to high molecular weight MAPs, MAP1 and MAP2, 5) two of the antisera to PHF (34, 35), and 6) two of the monoclonal antibodies to paired helical filaments (5-25 and 3-39).

With the exception of two antibodies, the pattern of LB immunostaining was the same with all reactive antibodies. The common pattern consisted of a peripheral
Fig. 2. Lewy body in the substantia nigra immunostained with the affinity purified tubulin antibody. PAP. ×1,248.

Fig. 3. Lewy body in locus ceruleus immunostained with the microtubule associated protein antibody 1B2. PAP. ×1,248.
ring surrounding the core of the LB. The immunostaining was located inside the ill-defined border of the inclusion, so that an unstained external ring of the inclusion could still be discerned (Figs. 1–3). This pattern differed from that seen with SMI 33 and the ICN antiserum to PHF (34), both of which immunostained the innermost and outermost borders of the halo region. These two positively stained areas were separated by an unstained ring (Fig. 4). This exceptional staining pattern was consistent in all of the Parkinson cases. Not all LB within one neuron stained with equal intensity (Fig. 5), although this may be the result of differing planes of section. In no cases were the cores of the Lewy bodies immunostained with these antibodies. There was no difference in immunostaining intensity or pattern between Lewy bodies located in the substantia nigra and those in the locus ceruleus.

No LB were immunostained with one of the antisera (36) and one monoclonal antibody (3-13) to PHF, the antibodies 1.1, 2.1 and 5.14A to neurofilaments, and the antibodies to tau protein. Previous studies (11, 12, 14) have demonstrated that one of the monoclonal antibodies to tau (tau-1) recognizes a phosphatase dependent epitope of tau. Therefore, we also tested tau-1 and the other antibodies to tau on sections treated with phosphatase. After phosphatase treatment, the tau antibodies did not immunostain Lewy bodies, even though immunostaining of neurofibrillary tangles by tau-1 was enhanced in the same sections and in similarly treated sections of hippocampus from patients with AD. Because paraffin sections typically show little or no tau reactivity, frozen sections were also used for immunofluorescence staining. Reactivity was not seen in LB.
Fig. 5. Lewy bodies in substantia nigra immunostained with 5-25. Note multiple Lewy bodies with variable immunostaining intensity. PAP. ×1,248.

DISCUSSION

This study demonstrated the presence of a broader array of cytoskeletal components in LB than previous reports. We have confirmed the presence of all three neurofilament subunits, including both phosphorylated and non-phosphorylated epitopes, but we have also detected the microtubule protein tubulin, MAP1 and MAP2. The presence of low molecular weight neurofilament subunit, MAP1 and tubulin is novel, for none of these proteins has been detected in other neuronal inclusions, including NFT. Further, NFL, the low molecular weight neurofilament subunit (also called NF68), and tubulin which are both present in LB constitute the core of a cytoskeletal structure and thus differ from identical NFT components which are external elements of microtubules and neurofilaments.

One of the most prominent antigenic components of neurofibrillary tangles, the microtubule associated protein, tau, was not demonstrated in LB. Although it is possible that our findings are the result of factors intrinsic to tissue preparation, tissue containing NFT of Alzheimer's disease (AD), Pick bodies and NFT of progressive supranuclear palsy were recognized by all the antibodies to tau which were used in this study when prepared similarly (10–14, 19–21). Immunoreactivity was also not seen in alcohol-fixed frozen sections suggesting that the results are not an artifact of paraffin embedding. Phosphatase treatment had no effect on staining of LB with antibodies to tau. Although the tau epitopes may be masked, it is more likely that LB are the first cytoskeleton derived neuronal inclusions to be shown to lack tau. This suggests that the presence of tau may not be a prerequisite to the formation of abnormal neuronal filaments, even though this protein is incorporated into filaments of NFT of AD (11–14), the NFT of progressive supranuclear palsy (20, 21), Pick bodies (19, 20) and Hirano bodies (30).
The results we obtained with the antibodies to PHF are equally interesting and perplexing. The epitopes recognized by these antibodies have only been partially identified. Of the three antisera to PHF, two (34, 35) immunostained LB. The third (36), which does not immunostain LB, does recognize tau (13). Although the protein(s) in LB recognized by the two antisera to PHF (34, 35) have not been identified, they are probably distinct epitopes, since different LB regions are stained by each. Also, two of the three monoclonal antibodies to PHF (5-25 and 3-39) recognize epitopes present in ubiquitin (39), a highly conserved 76 amino acid residue protein important in regulating many cellular functions (40) and recently found in NFT (41, 42). The present findings indicate that at least these two ubiquitin epitopes are present in both LB and NFT. Further support for the presence of ubiquitin in LB comes from the recent observation that an affinity purified antiserum to ubiquitin (43) and several monoclonal and polyclonal antibodies raised to ubiquitin also recognize LB (44). Although the role ubiquitin plays in LB and NFT remains to be clarified, our findings may be important in this regard. Although there is no definitive evidence that ubiquitin in LB is present as a covalent conjugate to an undefined component, it seems likely since many of the antisera that stain LB have a low affinity for free ubiquitin (Perry and Fried, unpublished data). Further, although antibodies to tau or ubiquitin recognize similar structures in brain sections from cases of AD (41), their difference in immunostaining of LB suggests that conjugates other than ubiquitinated tau are present in AD and Parkinson disease neurons.

None of the antibodies employed in this or previously reported studies has recognized the LB core, which along with its distinct morphology and prominent lipid components, is clearly delineated from the radiating filaments surrounding it. We are unable to define the relationship between these two components. In the absence of ultrastructural localization of the cytoskeletal epitopes to the halo, we can only suggest an association of the epitopes with the halo filaments. Of interest in this regard are the two distinct staining patterns observed. The common pattern, that is recognition of the halo, has been previously reported (22–24). Clearly distinct from this pattern were the inner and outer regions of the halo recognized by SMI 33 and the ICN antiserum (ICN Pharmaceutical) to paired helical filaments. This finding suggests regional heterogeneity of at least some LB components. The significance of the latter pattern is unclear since proteins recognized by the ICN antiserum to PHF have not been identified, and the role non-phosphorylated neurofilaments play in LB formation is not known.

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REFERENCES

21. Tabaton M, Manetto V, Fried V, Perry G. Epitopes of neurofibrillary tangles (NFT) are influenced by the neuronal type in Alzheimer disease (AD) and progressive supranuclear palsy (PSP). (Abstract) J Cell Biol 1987;105 (4 pt 2):316a
42. Mori H, Kondo J, Ihara Y. Ubiquitin is a component of paired helical filaments in Alzheimer's disease. Science 1987;235:1641–4

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