Purification and Characterization of Lewy Bodies from the Brains of Patients with Diffuse Lewy Body Disease

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Lewy bodies (LBs) are the pathological hallmarks of degenerating neurons in the brains of patients with Parkinson’s disease and diffuse Lewy body disease. We developed a novel purification procedure for LBs using sucrose density separation followed by fluorescence-activated particle sorting, and we raised >15 monoclonal antibodies to LBs purified from diffuse Lewy body disease brains. The monoclonal antibody that stained the largest number of LBs most intensely did not recognize ubiquitin in free or monoubiquitinated forms nor the ubiquitin conjugating enzymes, but it did react with polyubiquitin chains as well as with high molecular weight polyubiquitinated LB-derived proteins. Thus, these results suggest that LBs contain polyubiquitin chains. Although polyubiquitination of LB proteins may trigger ubiquitin-proteasome proteolytic pathways, the incomplete activation of these pathways could play a mechanistic role in the formation of LBs in neurodegenerative diseases. (Am J Pathol 1996, 148:1517–1529)

Lewy bodies (LBs) are the most characteristic intraneuronal inclusions found in the cytoplasm of degenerating neurons in the brainstem (ie, substantia nigra and locus ceruleus) of Parkinson’s disease (PD) patients,1–4 and PD is the most common human neurodegenerative disease affecting the extrapyramidal system. LBs are spherical intraneuronal inclusions composed of filaments approximately 10–25 nm in diameter that frequently radiate out from a central core into the periphery or “halo” region. The “core” is composed of a dense meshwork of filaments intermixed with abundant amorphous material and membranous profiles.5 The presence of LBs in neurons of substantia nigra and other brainstem nuclei is diagnostic of PD, and distinguishes PD from other clinically similar extrapyramidal disorders.2

However, telencephalic LBs are pathological signatures of diffuse Lewy body disease (DLBD), the second most common dementing neurodegenerative disease after Alzheimer’s disease (AD).3,4,6 The LBs in DLBD show a less stereotyped core and peripheral organization, and the LBs in DLBD are found in neurons of selected cerebrocortical regions (ie, cingulate, temporal, and insular cortices) in addition to brainstem nuclei. Although DLDB may occur independently, it frequently coexists with AD lesions including abundant amyloid β protein deposits and neurofibrillary pathology.7 Moreover, it has been shown that some familial AD patients with βAPP717 (Val to Ile) mutations develop cortical LBs and classical AD pathology.8 Furthermore, degenerating spinal cord neurons in patients with amyotrophic lateral sclerosis, a fatal progressive motor neuron disease, sometimes exhibit intraneuronal inclusions (termed Lewy body-like inclusions) that are morphologically and immunocytochemically similar to LBs.9,10 Thus, the elucidation of the molecular nature of LBs is crucial to understanding the pathogenesis of neuro-

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Table 1. Summary of the Clinicopathological Information of the Patients Included in This Study

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Clinical course*</th>
<th>Cortical pathology†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86</td>
<td>F</td>
<td>Parkinsonism → Dementia</td>
<td>+++ +   + +   +</td>
</tr>
<tr>
<td>2</td>
<td>82</td>
<td>F</td>
<td>Dementia</td>
<td>+++ +   + +   +</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
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<td>Dementia</td>
<td>++ +    + +   + +</td>
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<td>Dementia</td>
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<td>F</td>
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<td>M</td>
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<td>7</td>
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<td>Dementia</td>
<td>+   +    + +   +</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>F</td>
<td>Dementia and Parkinsonism</td>
<td>+ + +    + +   +</td>
</tr>
<tr>
<td>9</td>
<td>57</td>
<td>M</td>
<td>Dementia → Parkinsonism</td>
<td>+   -    -   -</td>
</tr>
<tr>
<td>10</td>
<td>58</td>
<td>M</td>
<td>Mental change → Parkinsonism</td>
<td>+   -    -   -</td>
</tr>
</tbody>
</table>

*The major presenting clinical features of the cases are summarized. An arrow signifies the emergence of second major clinical feature which followed the initial presenting clinical feature.
†Cortical pathology was evaluated in the cingulate cortex. LB: + 0–5, ++ 5–10, +++ >10/mm². SP and NFT: − none, + 0–15, ++ >15/mm².

Degenerative conditions characterized by parkinsonism, dementia, and motor weakness.

The biochemical composition of LBs is still incompletely characterized largely because of the paucity of LBs in a given volume of brain tissue. For these reasons, immunohistochemical studies have contributed the most information on the composition of LBs. The first proteins that were shown to be components of LBs by immunocytochemistry were the neurofilament (NF) subunits. NFs are neuron-specific intermediate filaments that constitute the major cytoskeletal elements in neuronal processes. Although the abnormal filaments in LBs resemble NFs ultrastructurally, LB filaments are thinner, more regular, and lack the side arms typical of normal NFs. Further immunocytochemical studies have shown that a limited number of LBs are NF-positive, suggesting that the NFs in LBs may be highly modified.

Another component consistently detected in LBs is ubiquitin (Ub). Ub is a 76-amino-acid highly conserved polypeptide that is conjugated to a target protein through an isopeptide bond between its carboxyl terminal Gly and the ε-amino group of Lys in a target protein. Further, the polyubiquitin chains formed by the Lys-48-linked Ub-Ub conjugation appear to serve to bind the targeted protein to the 26S proteasome complex. However, the mode of conjugation of Ub to proteins in LBs has not yet been elucidated.

Because much of the most dramatic recent progress in AD research was made possible by the successful purification and biochemical analysis of β-amyloid fibrils in plaques and tau-rich paired helical filaments (PHFs) in the tangles of AD brains, the development of a consistent method for the purification of LBs could accelerate the pace of research on DLBD or PD. However, there are as yet only a few attempts that have sought to isolate and biochemically characterize LBs, among these, Pollanen et al. have recently described a method to isolate LBs from DLBD brains and have suggested the presence of NF epitopes in LB-derived proteins. Here we describe an effective procedure for the purification of LBs from DLBD cortices using sucrose density fractionation, immunofluorescence labeling, and fluorescence-activated cell sorting (FACS) followed by detergent extraction to purify LBs to near homogeneity. Monoclonal antibodies (MAbs) raised to these purified LBs demonstrated that LBs contained polyubiquitin chains, which is in sharp contrast to the monoubiquitin species in AD PHFs. Thus, these findings suggest that the mode of activation of the Ub pathway is different in PD and DLBD from that in AD. Further, we speculate that incomplete activation of this proteolytic pathway could play a mechanistic role in the formation of LBs in PD and DLBD.

Materials and Methods

Cases

We used ten human brains obtained from patients diagnosed with DLBD as described (Table 1). At autopsy, one hemisphere was fixed in 10% formalin, or small blocks from various regions of a hemisphere were taken and fixed in 70% EtOH, 150 mmol/L NaCl. They were embedded in paraffin and cut at 6 µm for histological examination. These tissues were also examined by immunocytochemistry using antibodies to Ub and RMO32, (a MAb that exclusively labels LBs), to evaluate the amount of LBs. Five cases showed multiple LBs throughout neocortex and few or no neurofibrillary tangles (NFTs), except in hippocampus, whereas the other
cases contained a greater admixture of NFTs and LBs in neocortical samples (Table 1 and see below).

Antibodies

The antibodies used in this study (see Table 2) were characterized in a number of previously published reports; these antibodies included the following MAbs: MAbs 1510,24 5–25,25 and DF226 to Ub; MAbs RMO32,13 TA51, and RMO21727 to NF subunits; MAb C528 to phosphorylated tau; and MAb BAN50 to amyloid β protein (Aβ).29 In addition, we used a rabbit antisera to MAP2 and Ub30 (Table 2).

Preparation of Crude LB Fractions by Sucrose Density Gradient Centrifugation

Crude LB fractions were prepared as outlined in Figure 1. Briefly, 2 gm of cortical gray matter from DLBD brains free of meninges and white matter were minced and then homogenized with 4 volumes of TSI buffer (50 mmol/L Tris HCl, pH 7.6, 150 mmol/L NaCl, 3 mmol/L CaCl₂, 0.1 mmol/L diisopropylfluorophosphate, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 µg/ml Na-p-tosyl-L-lysine chloromethyl ketone, 1 µg/ml antipain, 0.1 µg/ml leupeptin, 0.1% NaN₃) by weight of the sample (8 ml) in a Dounce homogenizer (Wheaton, Millville, NJ, pestle B) for 20 strokes, which was adjusted to a final concentration of 1.0 mol/L sucrose by the addition of 2.2 mol/L sucrose solution and homogenized again by 10 additional strokes. The homogenate was centrifuged at 500,000 x g for 30 minutes on a TL100.4 rotor in a TLX microcentrifuge (Beckman, Palo Alto, CA), and the floating myelin-rich layer and supernatants were discarded. The pellet was resuspended in TSI buffer containing 1.0 mol/L sucrose to a final volume of 12 ml in a Dounce homogenizer (pestle B, 10 strokes). Samples (2 ml/tube x 6) were layered over a sucrose gradient composed of 2.2 mol/L (1 ml), 1.4 or 1.5 mol/L (1 ml), and 1.2 mol/L (1 ml) sucrose layers in distilled water. For those brains with more abundant SP amyloid and NFT contaminants, the use of a 1.5 mol/L sucrose layer instead of a 1.4 mol/L layer reduced the amount of contaminants in the 1.5 mol/L/2.2 mol/L interface. On the other hand, more LBs were effectively recovered in the 1.4 mol/L/2.2 mol/L than 1.5 mol/L/2.2 mol/L interface when the brains contained little or no SP amyloid and NFTs. The gradients were spun at 243,000 x g (45,000 rpm) for 2 hours at 2°C on a SW50.1 rotor in a XL-100 ultracentrifuge (Beckman, Palo Alto, CA). The 1.4 or 1.5/2.2 mol/L gradient interface and the 2.2 mol/L fraction were collected, diluted with TSI buffer (final sucrose concentration: 0.3 mol/L), and spun at 30,000 x g for 30 minutes. The resulting pellet was designated the postsucrose LB fraction, and this pellet was used for immunocytochemical analysis and further purification by FACS. Two DLBD brains that were richest in LBs but lacked cortical NFTs (case 1 and 2) were mainly used to prepare immunogens for MAb production and for the immunoblot and amino acid analyses of LBs.

Immunocytochemistry and Immunoelectron Microscopy of Isolated LBs

The postsucrose LB fraction was resuspended in a small volume of TSI buffer and a 0.5-µl aliquot was smeared onto poly-L-lysine coated glass slides. Smears were immunostained by a standard avidin-biotin-complex method as previously described.29 Ethanol-fixed floating sections of DLBD cortices were cut on a freezing microtome and similarly im-
munostained. Confocal immunofluorescence microscopic observations were performed as previously described.\textsuperscript{30} Immunoelectron microscopic observations were performed as follows. Briefly, a postsucrose LB fraction was resuspended in 10% calf serum in 10 mmol/L phosphate-buffered saline (PBS), kept at 4°C for 30 minutes to block nonspecific binding, and then a prediluted primary antibody was added and incubated with the LBs for 24 hours at 4°C. The suspension was spun in a 1.5-ml tube at 11,000 rpm for 5 minutes, and the antibody solution was discarded while the pellet was washed three times with 1 ml of PBS. The pellet was resuspended and incubated with a secondary antibody tagged with 5- or 10-nm colloidal gold particles (Amersham, Bucks, UK) for 24 hours at 4°C and similarly washed 3 times in PBS. The resulting pellet was fixed in 4% glutaraldehyde in 0.1 mol/L sodium cacodylate (pH 7.4), postfixed by 2% OsO\textsubscript{4} and processed for electron microscopy (EM) as previously described.\textsuperscript{30,31} In double immunogold labeling experiments, the pellet was initially incubated in a mixture of primary antibodies of two different species (ie, mouse and rabbit), and then secondary antibodies tagged with 5- or 10-nm colloidal gold were used to label the bound primary antibodies. EM observations on the detergent-treated LBs were similarly performed without prior immunostaining.

**FACS Purification of LBs**

The postsucrose LB fraction from 2 gm of cortex was resuspended in 10% calf serum in PBS to block nonspecific binding for 30 minutes, and then probed with either of the primary antibodies (ie, MAb 1510 (concentration: 1:400) or RMO32) by incubating the suspension on a rotary shaker overnight at 4°C. After three washes with 1.5 ml of TSI buffer by vigorous agitation with a Vortex mixer (Scientific Industries, Bohemia, NY) for 1 minute followed by spinning in a minicentrifuge, the fraction was incubated with FITC-conjugated anti-mouse IgG (Jackson, West Grove, PA) diluted 1:50 for 6 hours at 4°C. After washing three times, the FITC-labeled fraction was resuspended in 1.0 ml of TSI buffer, sieved through 45-μm nylon mesh, and applied to a cell sorter (Epics Elite, Coulter, Miami, FL). Particles were sorted at a flow rate of 4000–5000 particles/sec using a 76- or 100-μm flow-cell tip nozzle and an argon laser with an excitation maximum set at 488 nm, and emission signals were picked up by a 525- and 575-nm band pass filter followed by a 675-nm long pass filter. Samples were analyzed by FACS to assess fluorescence intensity and forward angle light-scattering.
Acid Analysis as a and FGF (Takeda content, (Hitachi, hours. hydrolyzed in formic and cine (7000 D differential solubilization of LBs. FACS-purified LBs were incubated in 0.1 mol/L glycine HCl (pH 2.4) for 2 hours to remove the antibodies and then incubated in 1% Triton-X, 1% Sarkosyl, 2% SDS, or 70% formic acid at room temperature for 2 hours, with or without homogenization (20 strokes) in a small Dounce homogenizer. After centrifugation (7000 × g, 20 minutes) and a wash with PBS, the pellets were examined by EM or smeared on glass slides and examined by immunocytochemistry with anti-Ub MAbs or RMO32.

Determination of Protein Content by Amino Acid Analysis.

FACS and Sarkosyl purified LBs were denatured in 70% formic acid overnight, lyophilized, and then hydrolyzed in 1% phenol/6 N HCl at 110°C for 22 hours. The resulting materials were derivatized by orthophtalaldehyde and analyzed by amino acid analyzer (Hitachi, L-8500) and the protein content of a single LB was calculated from the total amino acid content, using purified recombinant basic FGF (Takeda Chemical Industries, Osaka, Japan) as a standard protein.

Production of Monoclonal Antibodies

LB derived proteins extracted with 70% formic acid from 2 million twice sorted LBs (25 µg proteins) were used as immunogens for MAb production. Immunogens were resuspended in 0.1 ml of 50 mmol/L Tris HCl (pH 7.6) with 1% SDS. They were injected subcutaneously into the foot pads of BALB/c mice twice a week for 3 weeks. After the seventh immunization, the popliteal and inguinal lymph nodes were harvested, and two separate fusions were performed as described. Screening of hybridoma supernatants was performed by immunostaining with smears of the post sucrose fractions to detect clones producing MAbs that labeled LBs in these smears.

Characterization of LB-Specific Monoclonal Antibodies

Bovine Ub was purchased from Sigma (St. Louis, MO), and 500,000 × g pellets of normal or DLBD brain tissue homogenates in TSI buffer were used as a source of histone H2A. Lysozyme-polyubiquitin conjugates were prepared as previously described. Briefly, bovine lysozyme (20 µg/ml) and Ub (6 mg/ml) were coincubated with Ub conjugating enzymes (0.15 mg/ml of E1/E2 mixture and 0.45 mg/ml of E3) prepared from bovine reticulocytes together with 100 mmol/L Tris HCl (pH 9.0), 5 mmol/L MgCl₂, 1 mmol/L DTT, 2 mmol/L ATP, 2.4 U/ml of pyrophosphatase, 10 µg/ml creatine kinase and 10 mmol/L phosphocreatine. Pure polyubiquitin chain fractions (without substrate proteins, eg, lysozyme) were prepared following the method of Chen et al., except that a mixture of multiple E2 isoforms (including the 25 kd isoform) were coincubated with bovine Ub in the presence of E1. Ub conjugating enzyme fraction (E1, E2, E3, pyrophosphatase, creatine kinase, phosphocreatine at amounts equal to those used above; ref.32) was also probed with LB112 to rule out the possibility of the crossreaction of the MAb with these components. To probe LB-derived proteins by immunoblot, twice-sorted, Sarkosyl treated LBs (1.5 µg) were dissolved in 70% formic acid, desiccated in a SpeedVac (Savant Instruments, Holbrook, NY) in the presence of SDS, and adjusted with Laemmli’s sample buffer. Dot blotting, SDS-PAGE, and immunoblotting were done as previously described.
Results

Purification of Lewy Bodies from DLBD Cortices

Purification of LBs from DLBD brains was accomplished by a procedure that combined sucrose gradient fractionation, FACS, and detergent extraction (Figure 1). LBs were recovered by discontinuous sucrose-density gradient centrifugation (1.0 mol/L/1.2 mol/L/1.4 or 1.5 mol/L/2.2 mol/L) of the homogenized DLBD cortices and were found primarily at the 1.4 or 1.5/2.2 mol/L interface as demonstrated by immunostaining of unfixed smears of different gradient fractions (Figure 2A, see below). The major contaminants, as identified by phase-contrast microscopy or by immunostaining with anti-tau (C5) or anti-Aβ (BAN50) antibodies, were nuclei of neurons and glia admixed with fragments of thick neurites, blood vessels, amyloid debris, and NFTs.

interfaces of the gradients (1.0 mol/L/1.2 mol/L and 1.2 mol/L/1.4 or 1.5 mol/L) contained abundant cellular processes, membranous debris, and small fragments of amyloid. The 1.4 or 1.5 mol/L/2.2 mol/L fraction was then incubated with anti-Ub antibodies or an anti-NF MAb (RM032) followed by a secondary FITC-conjugated antibody. Because RM032 recognizes LBs but not NFTs, this antibody was extremely useful for sorting when the post-sucrose LB fraction contained some NFTs, although RM032 occasionally labeled some normal neurites. Fluorescence-labeled post-sucrose fractions were loaded on a cell sorter (Epics Elite, Coulter, Miami, FL) and sorted by particle size and FITC-fluorescence intensity. Approximately 0.1–0.5% of the total number of particles in the post-sucrose fraction were identified by FACS as possible LBs (Figure 2A). Lipofuscin granules were the major fluorescent contaminant, but they
were eliminated by FACS because they emitted yellow to orange autofluorescence (Figure 3B). The recovery rate of LBs after the first FACS sort (in 20 sorts of samples from cingulate or temporal cortex of six different cases) ranged from 25–80% (mean: 45.9%), and after the first sort, the fraction was 10–15% pure by computer reanalysis of the sorted LB particles. The recovery rate on the second FACS sort was similar to that in the first sorting (range: 35–70%, mean: 46.9%, in 10 sorts of once sorted samples from 5 different cases), and the purity of the twice-sorted LBs was approximately 60–70% by computer reanalysis of these particles. Twice sorted LBs were washed with 1% Sarkosyl. This step dramatically reduced the amount of soluble contaminants in the LB fraction. Although these purified, detergent extracted LBs were nearly homogenous by EM while retaining the spherical morphology and filamentous ultrastructure of authentic LBs in situ, they also contained a few electron-dense, membranous contaminants (Figure 4A). When DLBD cortices from LB-rich areas (ie, cingulate and temporal cortices) were used as starting materials, the LB yield was 20,000–240,000 LBs/2 gm of wet brain (mean: 72,000 in the 20 sorts described above) after the 1st FACS sort, whereas 20,000–40,000 LBs/2 gm (mean: 28,000, in the 10 sorts described above) were obtained after the second FACS sort.
ments of dendrites and neuronal cell bodies were MAP2 positive. The anti-NF MAbs TA51 and RM0217 recognized the multiphosphorylation repeat domain of NF-H in a phosphorylated state, and both antibodies stained fragmented neurites; but they stained <5% of the Ub or RMO32 positive LBs, and most of these LBs contained a labeled core region. These heterogeneities with respect to the positivity for anti-NF antibodies were constantly observed in samples derived from various neocortical areas of different DLBD cases. Anti-tau antibodies stained only NFTs and neuropil threads (NTs) but no LBs.

Immunoelectron microscopy performed on isolated LBs using anti-Ub, anti-MAP2, and RMO32 antibodies showed immunoreactivity chiefly on small amorphous aggregates between filaments, predominantly at the periphery of LBs as well as in some filaments. Double immunolabeling experiments using the anti-Ub MAb/anti-MAP2 polyclonal and RMO32/anti-Ub polyclonal antibodies showed that neither of the immunoreactivities colocalized on the amorphous deposits in LBs (Figure 2B). FACS-purified LBs were treated with one of the following detergents or denaturants: 1% Triton-X, 1% Sarkosyl, 2% SDS or 70% formic acid; and the ultrastructure and Ub-immunoreactivity of these LBs were examined. Filamentous structures were preserved even after homogenization in Triton-X, Sarkosyl, and SDS (Figure 4C), but all filaments disappeared after being incubated in formic acid for 2 hours. LBs retained immunoreactivity for Ub after incubation in Triton-X, Sarkosyl, and SDS, but they still contained some amorphous materials associated with the filaments when viewed by EM (Figure 4B, arrows). However, LBs lost Ub-immunoreactivity when homogenization was followed by incubation with the same agents, while leaving many well-preserved filaments (Figure 4C).

**Protein Content Determination of LBs**

FACS-purified LBs were denatured by formic acid and then hydrolyzed by HCl and subjected to amino acid analysis (Table 2). The total amounts of amino acids recovered in two independent quantitations were 2.9 and 2.1 µg/200,000 LBs, respectively.
Thus, the protein content of a single LB was estimated to be approximately 12.5 pg.

Establishment of Anti-LB Monoclonal Antibodies

LB-derived proteins extracted from 2 million twice-sorted LBs (approximately 25 μg protein) were used as immunogens for MAb production as described. Two separate fusions were performed and of the >15 positive hybridomas, only one MAb (LB112) strongly labeled LBs in unfixed LB smears and in EtOH-fixed frozen sections of DLBD cortices (Figure 5A). Significantly, LB112 only weakly stained NFTs and NTs, and it did not label normal structures (Figure 5A). Immunelectron microscopy of LBs probed with LB112 showed positive immunoreactivity chiefly on the amorphous materials as well as on LB filaments (Figure 5B).

Identification of LB-Associated Polyubiquitin Chains

Because the immunostaining pattern produced by LB112 resembled that produced by antibodies to Ub, we examined the reactivity of LB112 with Ub. LB112 did not recognize free Ub on dot- or immunoblots (Figure 6A, C and E), but LB112 did show positive reaction to the lysozyme-Ub conjugates that were generated by the coincubation of lysozyme, Ub and Ub-conjugating enzymes (E1, E2, and E3) (Figure 6, B and C). Notably, the anti-Ub MAb 1510 reacted equally well with multiple high molecular weight bands corresponding to polyubiquitinated lysozyme as well as with free Ub with a molecular weight of 8.5 (Figure 6C, arrow); whereas LB112 reacted only with polyubiquitinated proteins, and especially with the high molecular weight species (Figure 6C). To determine whether LB112 recognized any conjugated forms of Ub (ie, monoubiquitin as well as polyubiquitin chains), or if it was specific only for polyubiquitin chains composed of multiple Ub molecules, we studied the reactivity of LB112 with two types of Ub conjugates, ie, histone H2A and pure polyubiquitin chains. Histone H2A is found in cellular nuclei and it is a well known example of a monoubiquitinated protein. We found that MAb 1510 and 5–25, whereas LB112 preferentially reacted with high molecular weight components in addition to Ub dimers and trimers (Figure 6E). The Ub conjugating enzyme fractions were not reactive with LB112 on immunoblots (data not shown). These results strongly suggest that LB112 specifically recognizes polyubiquitin chains but not monoubiquitin conjugates nor the components of Ub conjugating enzyme fraction.

To confirm that polyubiquitinated proteins are incorporated into LBs, twice-sorted, formic acid denatured LBs were probed with MAb LB112 and 1510 on immunoblots. LB112 reacted with middle to high molecular weight smears, whereas 1510 showed similar smearing reaction extending to the middle to low molecular weight ranges (Figure 6F). These results suggest that high molecular weight polyubiquitinated proteins are constituents of LBs.

Discussion

Here we describe an effective and reproducible procedure for the purification of LBs from DLBD cortices. Differential centrifugation and solubilization are popular methods for the purification of insoluble AD lesions, such as β amyloid plaque cores and PHFs from human AD brains. However, these strategies are not as suitable for the purification of LBs, because LBs co-fractionate with numerous contaminants (eg, lipofuscin, amyloid, etc.) with densities similar to LBs. To overcome these difficulties, we utilized immunolabeling methods and FACS to discriminate LBs from other contaminating particles, and to purify LBs to near homogeneity as monitored by electron microscopy (Figure 4A). Although previous investigators used FACS to purify other pathological structures including β amyloid cores from AD brains and Mallory bodies from gilsofuvin-fed rats, the yields in these experiments often were low, even though the particles of interest in the starting materials were more numerous than the LBs in our post-sucrose fraction. Anti-Ub antibodies and an anti-NF protein MAb (RM032) served as powerful immunochemoic probes for the FACS purification of LBs. Current FACS facilities equipped with video-assisted droplet monitor systems enabled us to minimize the loss of positive particles, and this was instrumental for effective recovery of fluorescence-labeled LBs because of their very low abundance. We performed particle sorting on the LB fraction before the solubilization of contaminants because the labeling intensity of LBs tended to decrease after detergent treatment, and because nucleic acids
were released after solubilization, leading to large aggregates that hampered accurate particle sorting. However, effective solubilization of most contaminating particles was possible after particle sorting. Thus, our purification procedure enabled us to directly investigate the biochemical composition of LBs purified to near homogeneity for the first time, and this strategy may be applicable to the purification of other low abundant cellular inclusions.

Using highly purified LBs as immunogens, we sought to identify structural components of LBs by raising MAbs to isolated LBs. One of the MAbs (LB112) stained LBs very intensely, but it crossreacted weakly with NFTs and NTs although it did not label normal structures. Biochemically, LB112 did not react with free-Ub, Ub-conjugating enzymes nor with monoubiquitin conjugates. However, it did react strongly with lysozyme-Ub conjugates containing abundant polyubiquitin chains, as well as with pure polyubiquitin chain fractions and especially with the high molecular weight forms of these Ub polymers. Also, LB112 recognized a middle to high molecular weight smear in immunoblots of purified LB proteins. Recently, we have raised MAbs to lysozyme-Ub conjugates, and these MAbs preferentially recognize polyubiquitinated proteins. Notably, these antibodies exhibit properties that are very similar to those of LB112 (unpublished data). Taken together, these data strongly suggest that Ub is present in purified LBs in the form of abundant polyubiquitin chains that are conjugated to as yet unidentified proteins.

Although little is known about the conjugation state of Ub in the abnormal intracellular inclusions in AD and PD, ubiquitinated PHF-tau in NFTs has been most extensively studied, and 70–80% of the Ub in PHF is present as monoubiquitin. However, monoubiquitin cannot serve as a degradation signal, while polyubiquitin (multoubiquitin) chains trigger the Ub-proteasome proteolytic pathway leading to the ATP-dependent degradation of the target proteins. Thus, the present results complement and extend previous immunocytochemical findings showing that LBs contain proteasome immunoreactivity. Taken together, these data suggest that Ub-proteasome proteolytic pathways are activated in LBs, but that some proteins are resistant to degradation. This could be because of the fact that some proteins are not polyubiquitinated and therefore accumulate in LBs. Alternatively, faulty activation of the Ub-proteasome pathway may lead to the failed elimination of LBs. In either case, our data suggest that ubiquitination is more active in LBs compared to PHFs, although the degradation of LB-associated proteins seems to be incomplete, resulting in the deposition of polyubiquitinated proteins in LBs. The novel MAb LB112 that specifically reacts with polyubiquitin chains should serve as an effective probe in pathological as well as biological studies of Ub and LBs including studies of the biochemical nature of LB filaments. Although LB filaments and NFs are similar, it seems unlikely that unmodified NFs are incorporated into LBs as only a small percentage of LBs are recognized by these antibodies except for RMO32, which labels nearly all LBs. Second, unlike NFs, LB filaments are resistant to solubilization by harsh detergents such as SDS. Thus, the precise subunit proteins of LB filaments remain to be characterized, and our LB purification procedure should facilitate the direct analysis of the molecular composition of LB filaments, and this will facilitate the elucidation of the role of LBs in DLBD and PD.

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