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RESEARCH ARTICLE

Lewy Bodies in Alzheimer's Disease: A Neuropathological Review of 145 Cases Using α-Synuclein Immunohistochemistry

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Antibodies to α-synuclein (AS) now provide a sensitive and specific method for the detection of Lewy bodies (LBs) and their use will allow a more accurate determination of the prevalence of LBs in Alzheimer's Disease (AD). Studies using AS immunohistochemistry (IHC) have found LBs in the amygdala of over 60% early onset familial AD and in 50% of Down's syndrome patients with AD, however, no studies have reported the use of AS IHC to detect LBs in a large cohort of sporadic AD. This study examined 145 sporadic AD cases diagnosed using CERAD criteria from 1995-1999 for the presence of LBs using AS IHC. AS IHC detected LBs in 88/145 (60.7%) of sporadic AD cases. Similarly, LBs were found in 56.8% of the 95 cases fulfilling the more stringent NIA-RI criteria for the diagnosis of AD (Braak stage 5-6). In all cases with LBs, the amygdala was involved and LBs were always most numerous in this area, however, in some cases LBs in the substantia nigra were rare or not present. In conclusion, this study found that AS IHC detects LBs in the majority of sporadic AD cases and that the amygdala is the most commonly affected region.

Introduction

It has long been recognized that a significant number of Alzheimer's Disease (AD) cases have concomitant Lewy bodies (LBs). Estimates of the number of AD cases with LBs have generally ranged from 7-30% (8, 9, 11, 12, 14, 17, 35), however, one study reported a remarkable 71% of 48 AD cases with cortical LBs (20). Although part of the variability in these studies may stem from the use of different criteria for the diagnosis of AD (13), the major difficulty in an accurate assessment of LBs in AD has been the lack of a specific marker for the LB. Many previous studies have used ubiquitin immunohistochemistry (IHC) to identify LBs in AD, however, ubiquitin is a non-specific marker for LBs and ubiquitin-positive globose neurofibrillary tangles (NFTs) can be mistaken for LBs unless tau and ubiquitin double immunostaining are used (23). Current criteria for the assessment of LBs in dementia allow for evaluation to be performed either on standard hematoxylin and eosin (H&E) stained sections or with ubiquitin IHC, although it is suggested that in the presence of NFTs, tau immunostaining should be used to confirm the identity of the LB (28). In practice, the identification of LBs in H&E stained sections of substantia nigra is relatively easy even in AD cases with only scattered nigral LBs. In many of these cases cortical LBs are common and can be readily identified on H&E stained sections, where the eosinophilic LB is easily distinguishable from the slightly basophilic globose NFT. In some AD cases, however, cortical LBs are rare and can be missed even with careful scrutiny of H&E stained sections. Thus, while ubiquitin IHC is sensitive, it lacks specificity and in AD cases with rare LBs and numerous NFTs, accurate assessment of the number of LBs may be difficult. On the other hand, H&E-based LB assessment suffers from a lack of sensitivity and probably results in an underestimation of the number of cortical LBs (30).

Recently, it was demonstrated that LBs are composed primarily of aggregated α-synuclein (AS) and well-characterized monoclonal antibodies specific for AS for use on paraffin-embedded sections have become available (2). Studies using AS IHC have demonstrated that LBs can be detected with AS IHC in the amygdala of over 60% of familial AD cases (24). Similar studies have also shown that 50% of Down's syndrome cases with AD pathology have LBs as well (25). It is clear

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from these initial studies that the sensitivity and specificity of AS antibodies for LBs will allow for a more precise determination of the extent of LB pathology in AD, however, no studies using AS IHC to detect LBs in a large cohort of sporadic AD have been reported.

Materials and Methods

Patients and neuropathological evaluation for diagnosis of AD. The study population consisted of 145 neuropathologically diagnosed AD cases from 1995-99 that had been evaluated by the Pittsburgh ADRC and clinically diagnosed as "Probable AD" using National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (29). All 145 patients included in this study met the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropathological criteria for the diagnosis of "definite AD" (31). Brains were fixed in 10% formalin for 7-10 days and sections taken following CERAD guidelines (31). Sections in all cases included middle frontal gyrus, inferior parietal lobule, superior temporal gyrus, hippocampus with entorhinal cortex and transentorhinal cortex (at the level of the lateral geniculate nucleus [LGN]), amygdala with periamygdaloid cortex, head of caudate, putamen, globus pallidus, insular cortex, substantia nigra, and rostral pons as well as other areas (thalamus, cerebellum, medulla, nucleus basalis). H&E stained sections were examined on all patients. Semiquantitative evaluation of the frequency of neuritic plaques and NFT were performed on Bielschowsky stained sections and converted to an age-related plaque score for the diagnosis of "definite AD" as defined by CERAD (31). The extent of NFT formation was staged following the guidelines established by Braak (3). Stages 1 and 2 indicate NFT confined to the mesial temporal lobe, stages 3-4 indicate some neocortical (limbic) NFT, while stages 5-6 indicate frequent neocortical NFT.

LB pathology and scoring. For some cases in this study, the extent of LB pathology was estimated using a scoring system based on that proposed in the LB consensus criteria (28) with three modifications. In this system, specified portions of five areas of the brain transentorhinal cortex (at the level of the red nucleus), cingulate gyrus, mid-frontal gyrus, inferior parietal lobule and superior temporal gyrus) are examined and the number of LBs in each area determined. If there are greater than 5 LBs, the area is given a score of "2," if there are 1-5 LB, the score is "1." The scores for the five areas are then summed to yield a LB score for the case. Cases are further categorized into three groups: cortical (score 7-10), limbic/transitional (score 3-6), and brainstem predominant (score 0-2). The LB scores reported in this study are based on a similar protocol with the following modifications: 1) AS IHC was used instead of ubiquitin or H&E to identify LBs, 2) the inferior lip of the superior temporal gyrus was used instead of the superior lip of the middle temporal gyrus, as the latter was not present in many sections prior to 1998, and 3) the transentorhinal LB score was taken at the level of the LGN, which is slightly posterior to the section at the level of the red nucleus.

AS Immunostaining. Immunostaining for AS was performed with protease pre-treatment for antigen enhancement according to the following protocol: 4-to-6-micron thick paraffin sections are placed on Superfrost Plus slides (Fischer Scientific, Pittsburgh), dried in an oven for one hour at 50-58°C (do not exceed 60°C) and then allowed to dry overnight at room temperature (rt). The slides are then heated to 58°C for 45 minutes and immediately dewaxed in xylene (2×10 minutes), followed by absolute ethanol (3×5 minutes) and 95% ethanol (3×5 minutes). Endogenous peroxidase activity is quenched by a 30 minute incubation in methanol-hydrogen peroxide (0.3%) solution, followed by rinses in distilled water. The slides are then incubated for one minute at 37°C in protease solution which consists of 350 ml distilled water with 100 mg of type XXIV Protease (Sigma). Following rinses in distilled water and PBS, apply Protein Blocking Agent (Lipshaw Immunos, Pittsburgh) and incubate 30 minutes. Tap off excess reagent and incubate for 60 minutes at rt with primary antibody diluted in Common Antibody Diluent (Biogenex, San Ramon), then rinse in PBS and incubate with secondary (link) antibody from Dako LSAB-2 kit (Dako, Carpenteria). Following PBS washes, incubate 30 minutes rt with streptavidin (Dako LSAB-2 kit), rinse, develop with freshly prepared and filtered diaminobenzadine (DAB) and counter stain with Mayer's hematoxylin. For this study, we used the well-characterized monoclonal antibody to AS, LB509 (2, 19) at 1:1200 dilution. LB509 was a generous gift from Drs. Trojanowski and Lee. In preliminary studies we also used a polyclonal rabbit anti-AS obtained from Chemicon (Temecula, CA), which (at a dilution of 1:1600) showed results essentially identical to those obtained with LB509.

LB Score	Number of cases
0	5
1	1
2	2
3	1
4	2
5	1
6	2
7	0
8	0
9	2
10	7
total LB	23
neg	15

Table 1. 1999 LB Scores using AS IHC.

LB Score	Number of cases
0	11
1	2
2	5
3	6
4	2
5	2
6	1
7	1
8	2
9	0
10	0
total LB	32
neg	42

Table 2. LB Scores using AS IHC on cases in which LBs had not been previously detected.

Results

AS IHC staining detects LBs in most AD cases: From 1995-99 a total of 145 cases of AD were diagnosed. AS IHC was performed on the section containing the amygdala in all 145 cases and LBs were detected in 88/145 (60.7%) while 57/145 (39.3%) had no LBs. To determine if LBs might be detected in areas other than the amygdala, 39 of the 57 AS-negative cases were selected for additional AS IHC on sections of substantia nigra, pons with locus ceruleus, mid-frontal cortex, cingulate gyrus, and hippocampus with entorhinal cortex and transentorhinal cortex (at the level of the LGN); 25 of the AS-negative cases also had sections of insular cortex, inferior parietal cortex, and superior temporal cortex stained. No LBs were found in the substantia nigra, locus ceruleus, or in any of the other sections in the 39 cases that had no LBs in the amygdala by AS IHC.

In 1999, 38 cases of sporadic AD were diagnosed and 15/38 (39.5%) cases had no LBs, while 23/38 (60.5%) had LBs in one or more sections. Using modified consensus guidelines a LB score was determined for these cases (Table 1). Approximately one-third (9/23) had widespread, numerous neocortical LBs (LB score 7-10), while one-third had minimal LB pathology (LB score 0-2). Two of the cases with a score of "0" had LBs only in the amygdala. There were no LBs in the substantia nigra, locus ceruleus, transentorhinal cortex, cingulate gyrus, or other cortical areas. Cases with scores of 0-3 typically had numerous LBs in the amygdala, and periamygdaloid cortex and often had LBs in the entorhinal cortex at the level of the LGN, while in the substantia nigra, only one or two AS-positive foci of cytoplasmic staining in pigmented neurons were found, usually accompanied by widely scattered LN. Even upon painstaking scrutiny of H&E stained sections, no classic LBs could be identified in the substantia nigra in such cases. In the nine cases with LB scores of 9 or 10, LBs were frequent in neocortical areas, but these areas also contained many delicate thread-like LN, which were more numerous than LBs. In these cases, LBs were easily detected by H&E stained sections in the substantia nigra and AS IHC revealed numerous LN.

Prior to the availability of AS antibodies, ubiquitin IHC and H&E stained sections had been used to detect LBs. From 1995-98, LBs were identified in 33/107 (30.8%) AD cases, comparable to results in other studies using similar methodology (14). The use of AS IHC, however, identified LBs in additional AD cases that had not been detected using ubiquitin and H&E stains. Out of 74 AD cases from 1995-98 in which LBs were not previously identified, 32/74 were found to have LBs in the amygdala by AS IHC. These 32 cases had additional AS IHC performed on sections of substantia nigra, pons with locus ceruleus, mid-frontal cortex, cingulate gyrus, and hippocampus with entorhinal cortex and transentorhinal cortex (at the level of the LGN), insular cortex, inferior parietal cortex, and superior temporal cortex stained and a LB score was determined (Table 2). As expected, more than half of these cases (18/32) had very low LB scores (LB score 0-2). In these cases, LBs were found primarily in the amygdala and transentorhinal cortex, and in a few cases, there were <5 LBs in the cingulate gyrus. Most of these cases had numerous ubiquitin-positive NFTs in these areas. LBs in the substantia nigra were not seen in the majority of the new LB cases detected with AS IHC. In none of these cases were LBs detected only in the brainstem, and the number of LBs in the amygdala always exceeded those seen in the substantia nigra.

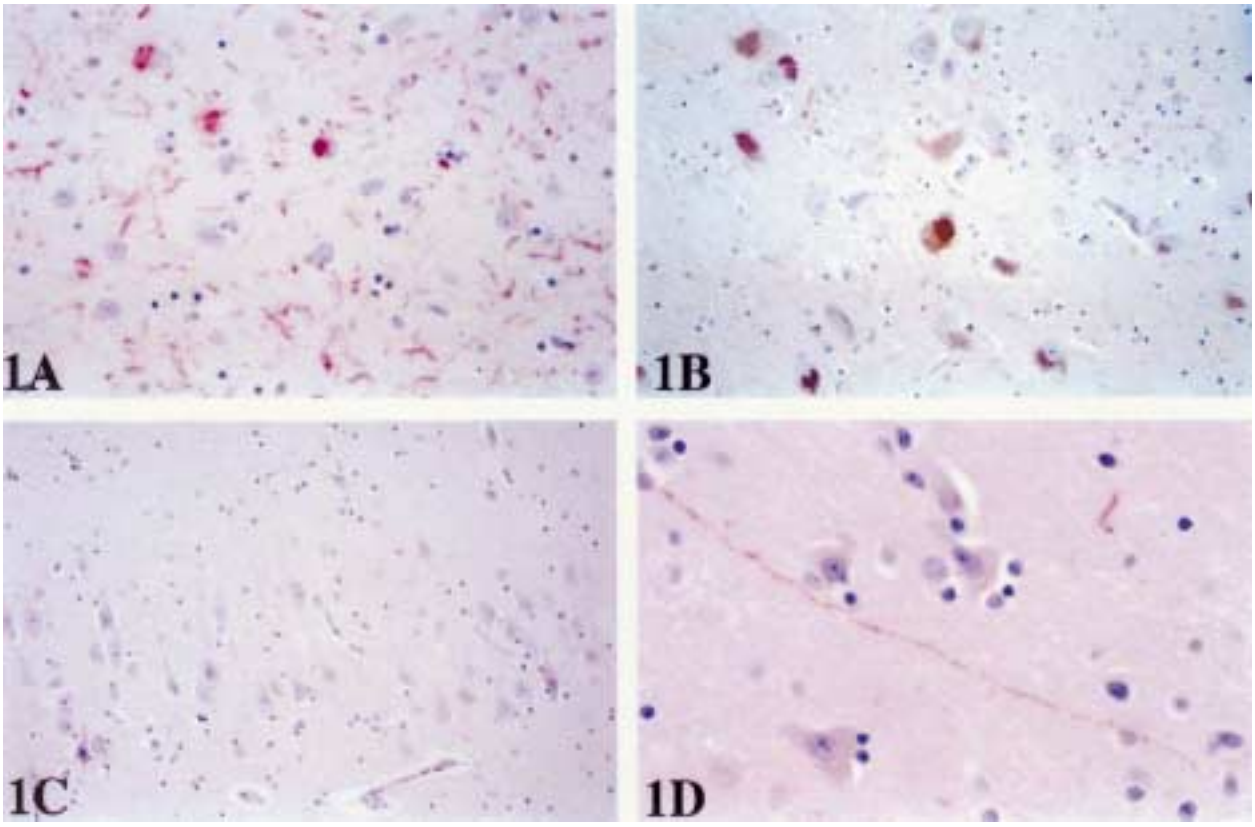

Figure 1. a-Synuclein immunohistochemistry (AS IHC) in cases with LBs predominantly in the amygdala. Some cases of AD+LB detected by AS IHC had relatively abundant LBs in the amygdala, while few or no LBs were found in the brainstem. **A** (×400) demonstrates the numerous LBs and frequent thread-like Lewy neurites (LN) in the amygdala of one such case. In the substantia nigra of this case, no classic LBs were detected on H&E stained sections and only a single neuron with cytoplasmic staining was seen with AS IHC, although widely scattered LN could be seen (**B**, ×400). Other areas commonly affected in LB cases such as the CA-2/3 region of hippocampus (**C**, ×200) were consistently negative in such cases, however, other areas would occasionally show rare AS-positive thread-like LN in the cortical neuropil although no LBs were present, as seen in this section of insular cortex from this same case (**D**, ×600).

LBs and Braak stage. Previous studies in dementia cases with combined AD pathology and LBs have noted a relative paucity of NFT formation in LB cases (6, 12, 16, 22). This has led to considerable and sometimes heated debate as to whether such cases are a "plaque-only" type of AD, and best designated as LB variant of AD (LBVAD) (8, 11) or whether they should be considered as primarily a LB disorder with "pathologic aging" (5, 7). The importance of NFT formation has been emphasized in the recent National Institute on Aging and Reagan Institute (NIA-RI) criteria for the diagnosis of AD (1). While CERAD criteria only require the presence of a specific age-related plaque score, NIA-RI criteria also require a Braak stage of 5-6 to ensure that there is a "high likelihood" that the dementia is due to AD pathology. The cohort in this study had 95 cases of AD that were Braak stage 5-6, thus fulfilling the more stringent NIA-RI criteria for the diagnosis of AD and 54/95 (56.8%) of these cases were found to have LBs by AS IHC. The average Braak stage of the 88 AD cases with LB was 4.8 ± 1.2. Although this was slightly lower than the average Braak stage of the "pure" AD cases (5.1 ± 0.75), the difference between the two groups was not statistically significant ($p=0.094$, student's t-test). Thus, LBs can be found in the majority of AD cases whether or not numerous neocortical NFTs are required for the diagnosis.

Discussion

The use of AS IHC to sensitively and specifically label LBs will now allow for confident evaluation of LB pathology in autopsy brains. Both clinical and pathologic criteria for cases with LB pathology continue to evolve.

Our findings demonstrate that LBs can be detected in the majority of sporadic AD cases using AS IHC. In this study we examined 145 cases clinically and pathologically diagnosed with AD and using AS IHC found that 60% of these cases have LBs. AS IHC has also detected LBs in the amygdala of over 60% of familial AD (FAD) cases (24) and in over 50% of Down's syndrome cases with AD pathology (25). Taken together, these findings indicate that LBs frequently accompany AD pathology in both genetic and sporadic cases.

As in the cases of Down's syndrome and familial AD, this study found that the amygdala is consistently involved and this study also confirms previous observations that in some cases the amygdala may be the only area with LBs (24, 25). Using a slightly modified version of the LB scoring system proposed by the Consortium on dementia with Lewy Bodies (28), we found that 26/88 (30%) of cases with LBs had a LB score of 0-2. Using the terminology of that system, these cases would be designated as "brainstem predominant," however, in none of these cases were LBs in brainstem structures (such as substantia nigra or locus ceruleus) more frequent than in the amygdala and adjacent entorhinal cortex, and in most AD cases with a LB score of 0-2, LBs in the substantia nigra were extremely rare even with AS IHC. The susceptibility of the amygdala to LB formation has been noted in other studies. Studies by Rezaie and colleagues demonstrated that LB density is greatest in the amygdala in dementia with LBs (33) and Kosaka (21) has emphasized that there is a cortical LB disease which does not necessarily involve brainstem LBs. Studies in Parkinson's Disease (PD) have also shown that in addition to the nearly universal presence of LBs in cortical areas (15, 34), the amygdala routinely shows LB formation (4). Other studies using AS IHC have noted that there is little correlation between the number of LBs in the substantia nigra and in the cortex (10). Although some investigators contend that all LB cases will have nigral LBs if enough sections are examined (18), it is clear that, at least in AD, LB are readily and consistently identified by AS IHC in the section of amygdala in the majority of cases.

The clinical significance of LBs in AD remains an open question. Clinical symptoms associated with LBs include hallucinations, sensitivity to neuroleptics, fluctuating clinical course, unexplained falls and extra-pyramidal signs (27, 28). In the presence of numerous cortical LBs and little or no AD pathology, these clinical symptoms may be able to identify many cases of dementia with LBs (27). In one recent study of 25 patients with dementia with LBs (DLB), there was no difference in the clinical symptoms between those with and without concomitant AD pathology (10). In one study examining 15 AD patients, extra-pyramidal signs predicted those with many LBs (30). On the other hand, in a large cohort of 135 AD patients, no differences in clinical symptoms could be detected between those with LBs and those with AD alone (26). AD cases with LBs have also been reported to show a faster cognitive decline and accelerated mortality compared to patients without LB (32). It remains to be seen if the increased sensitivity afforded by AS IHC will clarify these issues.

In conclusion, AS IHC will detect LBs in the majority of sporadic AD cases, whether these are diagnosed using CERAD criteria, or with the more stringent NIA-RI criteria. The amygdala appears to be the most consistent area of LB formation in AD. The amygdala can be used as a screen with AS IHC to determine if further sections need to be stained with AS IHC to determine the extent of LB pathology.

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