

Comparative study of commercially available anti- α -synuclein antibodies

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Abstract

Immunohistochemistry for alpha-synuclein has become the histological technique of choice for the diagnosis for Parkinson's disease, Dementia with Lewy bodies and Multiple System Atrophy (www.ICDNS.org). Nevertheless, no standardised protocol has been proposed. We have reviewed 242 of the 270 studies published until June 2005 that mentioned immunohistochemistry for anti-alpha synuclein on human tissue and we found that only 75 (31%) used commercial antibodies. We also noted that protocols, particularly dilution and antigen unmasking, varied between studies, even when the same antibody was employed. In order to establish a standardised protocol for alpha-synuclein immunohistochemistry, which can be applied in diagnostic neuropathology we tested seven commercial monoclonal antibodies in brains of subjects with Parkinson's disease, dementia with Lewy bodies, multiple system atrophy, multiple sclerosis with incidental Lewy bodies and aged-matched normal brain and determined for each antibody the best suited protocol for antigen unmasking. We evaluated the intensity of immunolabelling in Lewy bodies, neuropil threads, dendrites, pre-synaptic terminals, granular cytoplasmic positivity, peri-axonal positivity, glial inclusions and non-specific immunolabelling. Although our results showed that all the antibodies detected alpha-synuclein inclusions, differences were noted between antibodies, particularly with regard to the detection of glial inclusions. From our study, the best antibodies of the seven tested appeared to be those directed against amino acids 116-131 and 15-123 and we suggest them to be used in routine diagnostic practice for alpha-synucleinopathies.

Keywords : alpha-synuclein ; immunohistochemistry

Alpha-synuclein (α SN) is a highly conserved, natively unfolded 18 kDa protein [1] which represents the major component of Lewy bodies (LBs) [2], and the glial cytoplasmic inclusions (GCIs) associated with Multiple System Atrophy (MSA) [3]. The increased sensitivity of α SN immunohistochemistry over conventional histological stains has established the technique as a standard tool in the diagnosis of Parkinson's disease (PD), Dementia with Lewy Bodies (DLB) and MSA [4], and led to new insights regarding the extent and variation of α SN-containing structures and their formation. α SN aggregations vary throughout the brain, with brainstem pathology characterized by compact LBs and neuropil threads. Pigmented neurones of the substantia nigra may also contain diffuse fine or coarse granules, which likely represent the state of aggregation preceding LBs [5,6]. Cortical pathology consists of more diffuse LBs and punctate granular positivity that corresponds to pre-synaptic terminals [7].

Despite the growing frequency with which immunohistochemistry is used as a diagnostic tool for PD, DLB and MSA, a standardized protocol for α SN has not been established. Pretreatment with proteinase K or formic acid has been reported to increase the immunoreactivity of α SN; however, the only studies to investigate this systematically were conducted with non-commercial antibodies [8] or brain tissue unaffected by neurodegenerative disease and devoid of any pathologically associated protein aggregations [9]. To assess the use of commercial vs. non-commercial anti- α SN antibodies and different antigen-unmasking pretreatments in research, we performed a search of the PubMed database updated to 30 June 2005 for 'alpha-synuclein'. We found 1567 citations pertaining to the protein, and after reviewing all of these abstracts, we selected studies that mentioned the use of α SN immunohistochemistry on human tissue (270 overall). We screened the methodology of 242 articles for type of antibody used (commercial or non-commercial), dilution and pretreatment for antigen unmasking. Only 75 studies (31%) used commercial anti- α SN antibodies. Thirty-seven studies (15%) reported using a pretreatment for antigen unmasking and 17 (7%) included no details at all regarding the antibodies used. Also, many studies performing immunohistochemistry with the same commercial anti- α SN antibodies used different dilutions and protocols.

We observed that different anti- α SN antibodies varied in how effectively they identified different types of inclusions. In order to standardize the protocol for a more rigorous and reproducible neuropathological diagnosis of PD, DLB and MSA, we have compared seven commercially available monoclonal anti- α SN antibodies (mAbs) representative of the clones and immunogens currently available. Details of the mAbs' manufacturer, clone, the dilution and antigen-unmasking technique used, and the epitope they recognize or immunogen against which they were raised are summarized in Table 1. According to manufacturers' data sheets, four mAbs are specific to human α SN (A, B, F, G), one recognizes human, mouse and rat isoforms (C), one was untested in species other than human (D), and the cross-reactivity of one was unspecified (E). Using a protein BLAST search, we compared epitopes or immunogens against which the mAbs were raised and found no sequence homology to any other known human proteins. The seven mAbs were tested on tissue from five *post mortem* brains selected on the basis of neuropathological diagnosis. One case each of idiopathic PD, DLB and MSA, and one age-matched control were provided by the Parkinson's Disease Society Tissue Bank (PDSTB). A case of multiple sclerosis with numerous incidental brainstem LBs was provided by the UK Multiple Sclerosis Tissue Bank. Brains were fixed in 10% buffered formalin for at least 2 weeks, and sample blocks taken during the fixed tissue dissection were processed routinely up to paraffin embedding. Ten micron sections from the superior frontal gyrus and the substantia nigra from each case were stained with haematoxylin-eosin and with each of the seven anti- α SN mAbs using the avidin biotin complex/peroxidase method (ABC, Vector, Burlingame, CA, USA). All primary mAbs were incubated overnight at 4°C. We first followed manufacturers' instruction for dilution and pretreatment for antigen unmasking and subsequently tested different dilutions and pretreatments to optimize the protocols. For antigen unmasking, we tested incubation in 100% formic acid for 5 min, and heating in a microwave oven at 350 W for 20 min using 10 mM citrate buffer at pH 6 and 1 mM ethylenediamine tetra-acetic acid (EDTA) at pH 8. Immunoreactions with omission of the primary mAb were performed for every case as controls. All immunoreactions were evaluated independently by two authors (E.C. and F.R.). We rated LBs, cytoplasmic granular positivity neuropil threads, presynaptic terminals, periaxonal deposition and GCIs using a four-point system from - (no reactivity) to +++ (intense staining). A rating of +/- was also added to our assessment on GCIs to indicate that, regardless of the intensity of staining, the mAb failed to identify nearly as many GCIs as were observed with other mAbs on adjacent sections, suggesting only a partial affinity for this form of α SN aggregate. We also assessed nonspecific background staining of the neuropil, rating it either none, low (light non-specific staining, generally confined to white matter tracts) or high (non-specific staining throughout the neuropil, dark enough to compete with specific labelling). The immunostaining profiles for each antibody are outlined in Table 2.

Table 1. Details of the seven monoclonal anti- α SN antibodies used in this study

Manufacturer [clone]	Immunogen [epitope]	Dilution	Pretreatment
A. Abeam [Syn204]	Human recombinant α SN [aa 87-110 (12)]	1:50	100% FA 5 min
B. Alexis [15G 7]	Synthetic peptide aa 116-131	1:10	100% FA 5 min
C. Becton-Dickinson[42]	Rat synuclein aa 15-123	1:300	100% FA 5 min
D. Chemicon [7B 2,12]	Human recombinant α SN	1:400	100% FA 5 min
E. NovoCastra[KM51]	Human recombinant α SN	1:40	H-T EDTA
F. SantaCruz[Syn211]	Human recombinant α SN [aa 121-125 (12)]	1:100	100% FA 5 min
G. Zymed [LB509]	Purified LBs [aa 115-122 (13)]	1:200	H-T citrate

LBs, Lewy bodies; FA, formic acid; H-T, high temperature (20 min in microwave at 350 W); α SN, alpha-synuclein; EDTA, ethylenediamine tetra-acetic acid.

Table 2. Immunostaining profiles for each monoclonal anti- α SN antibody (mAb)

mAb	LBs	Neuropil threads	Dendrites	Terminals	Granular cytoplasmic	Peri-axonal	GCIs	Non-specific
A	++	++	+++	++	++	-	+/-	High
B	+++	++	++	+++	+++	+	+++	None
C	+++	+++	++	+	+++	+	+++	None
D	+	+	-	+	++	-	+/-	High
E	++	++	-	+++	++	++	+	None
F	+++	+++	+	+++	+++	+	+++	High
G	++	++	+	+	+	++	++	None

LBs, Lewy bodies; GCI, glial cytoplasmic inclusion; α SN, alpha-synuclein.

Our results showed that immunostains using mAbs A, C, D and F were not significantly affected by pretreatments for antigen unmasking. In contrast, mAbs E and G showed far intenser immunostaining after microwaving in EDTA and citrate buffer, respectively. All mAbs demonstrated good results in detecting LBs (both brainstem and cortical), neuropil threads and cytoplasmic granular deposition as well as the diffuse, granular deposition characteristic of cortical grey matter (Figures 1 and 2). However, a broad range of staining intensity was observed across the mAbs. The intensity of immunolabelling of large neuropil threads paralleled that observed in LBs. Smaller threads likely corresponding to dendrites rather than axons were only clearly highlighted by three mAbs (A, B and C). Two mAbs (B and F) demonstrated intra-axonal, neurite-like α SN deposition in the cortical white matter of the DLB case. Four mAbs (B, E, F and G) showed finely granular periaxonal positivity in the white matter of the PD and DLB cases, which appeared denser and more evenly distributed than that observed in the cortical grey matter. Double immunolabelling with one of these anti- α SN mAbs (G) and anti-myelin basic protein (polyclonal, DAKO, Carpinteria, CA, USA, 1 : 200, no pretreatment), suggests that this type of α SN deposition may co-localize with the myelin sheath (data not shown).

The most distinct, and diagnostically relevant, variability between the mAbs evaluated was observed in their labelling of GCIs. Three mAbs (A, D and E) barely labelled these, while the other four labelled them clearly (Figure 3). Chemical differences have been reported between α SN aggregates in MSA and PD [10], which may explain the marked differences in reactivity we observed across our panel of mAbs. An earlier immunohistochemical study of MSA tissue suggests that immunoreactivity for GCIs is maximized by antibodies directed to the carboxyl-terminal of α SN, but that immunoreactivity can be improved for other antibodies after formic acid pretreatment [11]. Three of the mAbs in the present study (A, F and G) are identical to non-commercial clones used in earlier study [12,13], and, with respect to these three mAbs and mAb B, our findings support the observations of Duda *et al.* [11] in that our immunostaining appeared progressively intenser as epitopes moved progressively closer to the carboxyl terminus. However, the other clones raised against full-length recombinant α SN (D, E) showed only weak immunoreactivity, and mAb C, raised against amino acids 15-123 of rat synuclein, demonstrated excellent results. Because the specific epitopes recognized by these last mAbs have not been reported, we cannot generalize staining intensity as a factor of proximity to the carboxyl-terminus.

As a result of this comparative study, we now preferentially use mAbs that recognize amino acids 116-131 or 15-123 (A and C) for diagnostic purposes based on their strong immunoreactivity for a variety of α SN-containing structures, with the dilutions and pretreatment recommended in Table 1. Depending on the experimental design, other mAbs may be preferable. For example, although it is not a primary concern in studies of *post mortem* human tissue, cross-reactivity with mouse synuclein would be a major concern for investigators studying human α SN expression in transgenic mice.

The wide variety of staining patterns observed across the seven antibodies emphasize the need for standardization prior to the interpretation of immunohistochemical results. Previous analyses of glial fibrillary acidic protein [14] and prion protein [15] have demonstrated that variability across antibodies could account for discrepancies between research studies and affect diagnostic accuracy. All mAbs in this study adequately labelled LBs and are suitable for diagnosis of idiopathic PD or use in research; however, as the neuropathological nuances differentiating alpha-synucleinopathies become increasingly refined, the use of a panel of antibodies, or one which is known to label multiple structures of diagnostic relevance, may become necessary.

Figure 1. *Substantia nigra of the case of idiopathic PD contains intensely stained Lewy bodies (a, $\times 400$) and neuropil threads (b, $\times 200$). Diffuse and finely granular immunolabelling corresponds to presynaptic terminals (mAb C). PD, Parkinson's disease; mAb, monoclonal anti- α -synuclein antibody*

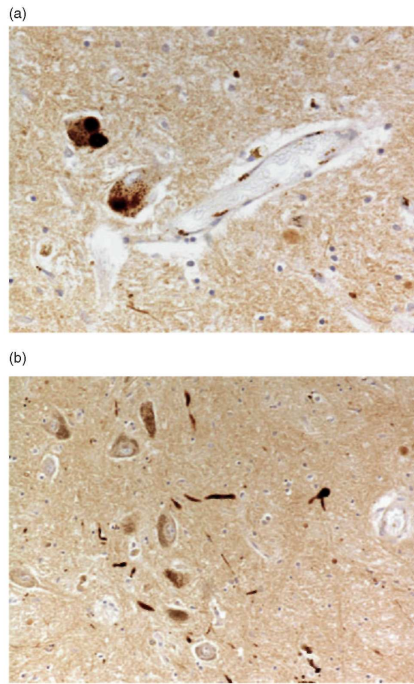


Figure 2. Diffuse cortical α SN immunolabelling is present in the superior frontal gyrus of the case of DLB (a, whole mount). Many neurones are seen to contain LBs (b, $\times 100$) (mAb B). DLB, Dementia with Lewy bodies; α SN, alpha-synuclein.

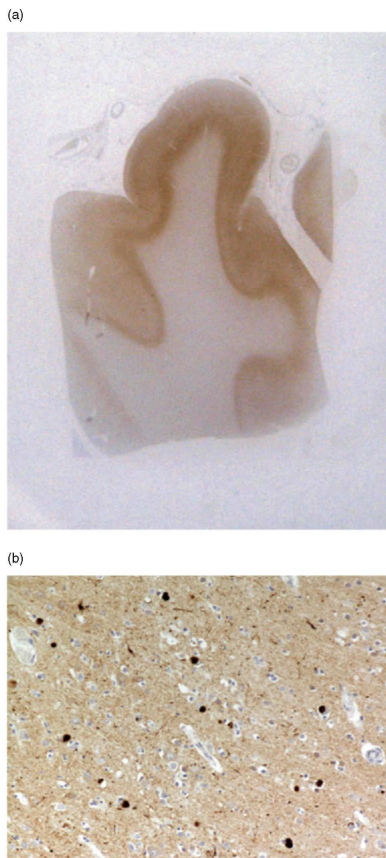
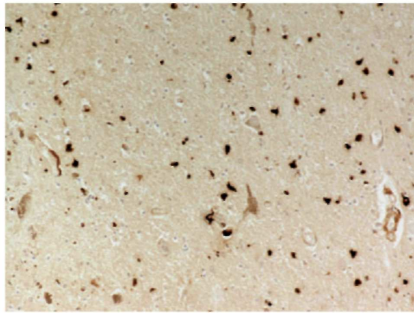


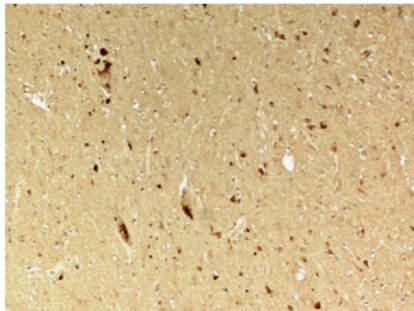
Figure 3. The monoclonal antibody raised against amino acids 15-123 (mAb C) strongly stains oligodendroglial inclusions in the case of MSA (a, $\times 100$). In the same region, the antibody directed against amino acids 87-110 stains consistently fewer GCIs (mAb A) (b, $\times 100$). mAb, monoclonal anti- α SN antibody; α SN, alpha-synuclein;

MSA, Multiple System Atrophy; GCI, glial cytoplasmic inclusion.

(a)



(b)



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