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Alzheimer's disease: A new evidence for common epitopes between microtubule associated protein Tau and paired helical filaments (PHF): Demonstration at the electron microscope level by a double immunogold labelling

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Summary. Paired helical filaments (PHF) are neuronal landmarks of Alzheimer's disease. These pathological filaments are antigenically related to proteins present in the normal cytoskeleton, particularly to microtubule associated protein Tau. The evidence for these common epitopes was studied on sections of cortex from Alzheimer brains after Araldite embedding. Two rabbit immunsera were used: one was raised against PHF isolated from Alzheimer cortex; the other against Tau proteins extracted from bovine cortex. The comparison of adjacent semi-thin sections alternatively treated with anti-PHF and anti-Tau immunesera reveals that both stained degenerating neurofibrils in pyramidal perikaria and in neurites surrounding senile plaques. On ultra-thin sections, double immunogold labelling of PHF was obtained. These results are in accordance with the hypothesis that Tau proteins are major antigenic components of PHF.

Key words: Alzheimer's disease – Paired helical filaments – Tau proteins – Post-embedding immunolabelling – Cytoskeleton

Introduction

Paired helical filaments (PHF) are ultrastructural elements observed in Alzheimer cortex. They are bundles of degenerating neurofibrils (DNF) in pyramidal perikaria and in neuritic endings which surround senile plaques. PHF appear as pairs of 10 nm filaments wound into a double helix showing a regular 80 nm periodicity (Kidd 1963) and their chemical nature and the origin are still controversial. Using immunological methods (immunoblotting and immunohistochemistry), numerous authors have reported the presence of common epitopes between PHF and the normal cytoskeleton, mainly neurofilaments and microtubule-associated proteins (MAP) (reviewed by Selkoe 1986). MAP Tau (a set of 65–45 KDa proteins that promote microtubule assembly) may be the major components of PHF (Grundke-Iqbal et al. 1986a; Kosik et al. 1986; Wood et al. 1986; Delacourte and Defossez 1986) and may be abnormally phosphorylated in PHF (Grundke-Iqbal et al. 1986b; Ihara et al. 1986; Nukina et al. 1987). At the ultrastructural level, immunostaining of PHF was described by Brion et al. (1985) with an anti-PHF and an anti-MAP Tau. Immunolabelling of PHF was also obtained with anti-neurofilaments and anti-MAP immunesera (Perry et al. 1985) and with an anti-MAP Tau immuneserum (Wood et al. 1986). Perry et al. (1986), using enriched PHF extracts applied on grids, demonstrated the existence of a periodicity of labelling by anti-PHF. In a recent study, simultaneous immunogold labelling of PHF was performed directly on ultra-thin sections (post-embedding), by Bancher et al. (1987) with a mouse monoclonal anti-PHF and a rabbit polyclonal anti-Tau.

We have raised an anti-PHF immuneserum which specifically stains DNF on paraffin sections (Persuy et al. 1986) and which is also able to immunolabel DNF on semi-thin sections and PHF on ultra-thin sections (Défossez et al. 1987) and an anti-Tau, raised against bovine MAP Tau, which also immunostains DNF on paraffin sections (Delacourte and Defossez 1986). The aim of this paper was to compare labelling obtained with both immunesera, first on adjacent semi-thin sections and second, on ultra-thin sections of Alzheimer cortex. For comparison at the ultrastructural level, we used a double immunogold staining

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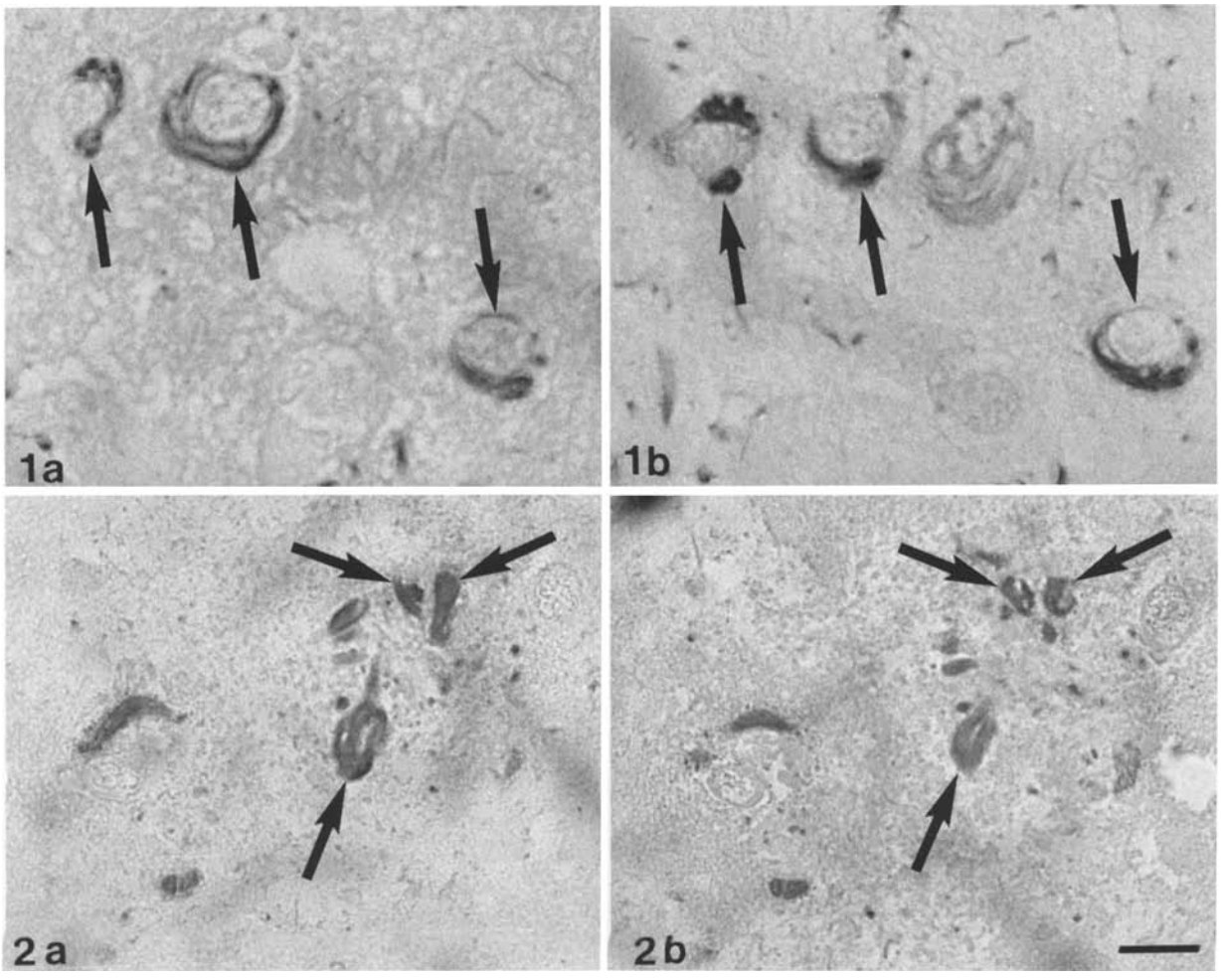

Fig. 1. Light micrograph of two serial semi-thin sections of Alzheimer temporal cortex immunostained by anti-PHF **a** and anti-Tau **b.** Note that the same pyramidal perikaria showing DNF are labelled (*arrows*). Original magnification ×1000. Bar = 10 μm

Fig. 2. Same procedure applied on two other sections. Note the neurites of senile plaques immunostained by both antisera (*arrows*). Original magnification ×1000. Bar = 10 μm

on the same section with two different sizes of gold particles, according to a method previously described by Bendayan (1982).

Materials and methods

Material was obtained from the brains of two Alzheimer patients autopsied within 6 h of death (diagnosis of disease established according to NINCDS-ADRDA classification: Kahnn et al. 1984). Pieces of temporal cortex were fixed for 2 h in the following mixture: glutaraldehyde 0.05%, paraformaldehyde 4%, picric acid 0.2%, phosphate buffer 0.1 M and postfixed for 2 h in osmium tetroxide 1%. After embedding in Araldite, semi-thin sections and adjacent ultra-thin sections were performed.

In order to prepare Tau proteins microtubules were obtained from fresh bovine brain by two cycles of in vitro microtubule assembly and disassembly, according to the procedure of Shelanski et al. (1973). The heat stable fraction of microtubules was mainly composed of Tau proteins and was prepared according to the procedure of Fellous et al. (1978). The microtubule pellets were resuspended in 0.1 M MES buffer, pH 6.5, containing 1 mM GTP mixed in 0.75 M NaCl, boiled for 5 min and centrifuged at 100000 × *g* for 30 min at 4° C. The supernatant was dialysed for 3 h against MES buffer and for one night against phosphate buffer saline. The purification of the Tau preparation was performed by preparative polyacrylamide gel electrophoreses.

PHF preparations were performed using an Alzheimer frontal cortex, according to the different steps first described by Ihara et al. (1983) and modified by Persuy et al. (1985). The insoluble material in sodium dodecyl sulfate was collected by centrifugation, resuspended and recentrifuged on a sucrose gradient; the material recovered at the 1,8–2 M sucrose layer interface was composed of PHF, as revealed by electron-microscopy.

The polyclonal immuneserum against PHF was prepared by Persuy et al. (1985). The sensivity and the specificity of this serum have been described previously in light- and electron-microscopic studies (Defossez et al. 1986; Defossez et al. 1987). The specificity of immuneserum against Tau was checked by

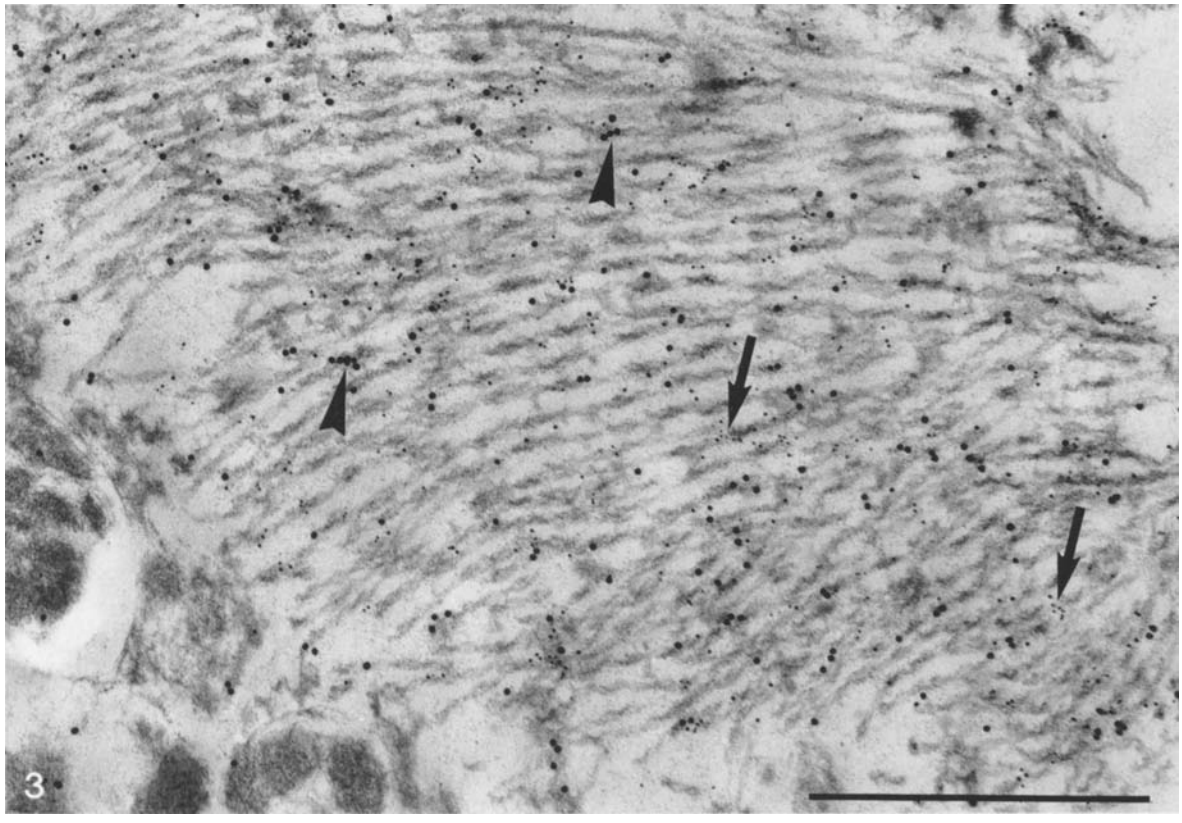

Fig. 3. Electron micrograph of an ultra-thin section of Alzheimer temporal cortex showing double immunogold labelled PHF by anti PHF (gold particles of 5 nm in diameter, *arrows*) and by anti Tau (gold particles of 20 nm in diameter, *arrowheads*). Original magnification ×24000. Bar = 0.2 μm

immunoblotting studies. On a microtubule preparation enriched with Tau proteins electrophoresed and transferred on nitrocellulose sheet, anti-Tau detected the set of 65–50 kDa proteins corresponding to Tau proteins. For specificity controls, both immunesera were immunoabsorbed by their corresponding antigens making them unavailable on histological sections.

Indirect post-embedding immunohistochemistry was performed on adjacent semi-thin sections after removal of Araldite according to Mayor et al. (1961): In brief, the sections were treated consecutively by sodium metoxide (3 min), alcohol-benzene (3 min × 2), acetone (3 min × 2), distilled water (3 min), H_2O_2 10% (10 min) and Coons buffer, pH 7.2 (12 h). Then the immunoreactions were made on adjacent sections using anti-PHF ($^1/_{500}$) and anti-Tau ($^1/_{400}$) alternatively. The sections were incubated for 12 h. After washing in Coons buffer, the sections were treated with conjugated sheep anti-rabbit immunoglobulin ($^1/_{100}$) for 1 h and peroxidase was revealed by diaminobenzidine (5 mg DAB, 10 μl H_2O_2 30%, 10 ml TRIS buffer 0.1 M, pH 7.6).

Using the procedure of Beauvillain et al. (1984), derived from the method of Bendayan (1982), the same ultra-thin section was consecutively treated by the two specific antibodies for immuno-gold studies.

A first immunoreaction was made on one face of the section for PHF detection, and a second on the opposite side for Tau detection. In both cases, an immunogold method was used but with different sizes of gold particle: 5 nm diameter were used for the anti-PHF demonstration and 20 nm diameter for the identification of anti-Tau. After a preliminary treatment with H_2O_2 (10%, 8 min) the grids were floated on the following reagents and washing solutions: (1) anti-PHF or anti-Tau in Coons buffer, pH 7.4, containing 0.1% sodium azide and 2% tween 20, for 12 h at 4° C; (2) phosphate buffer to remove excess antibodies (3 × 10 min); (3) colloidal gold-labelled anti-rabbit IgG ($^1/_{100}$) for 1 h; (4) phosphate buffer (3 × 10 min); (5) distilled water (2 × 10 min).

The sections were finally counterstained with uranyl acetate and lead citrate before electron microscopic observation.

Results

Using semi-thin sections (1 μm) it was easy to compare the same elements on several consecutive sections treated alternatively with both immunesera, anti-PHF and anti-Tau. These antibodies labelled the same pathological structures on adjacent semi-thin sections: DNF on pyramidal perikaria (Figs. 1 a, b) and neurites at the periphery of senile plaques (Figs. 2 a, b). The normal nervous tissue was never immunostained.

After immunogold labelling, PHF were decorated by both gold particles, 5 nm and 20 nm in diameter (Fig. 3). The particles of each size were often grouped, constituting small clusters (3 to 6 particles) fixed on PHF, but no periodicity of labelling was observed. In the majority of cases

where larger particles corresponding to the staining of Tau proteins were observed, smaller particles (anti-PHF labelling) were also present in close proximity. The other cytoplasmic structures were not labelled, especially the amyloid deposits observed in Alzheimer's cortex. The anti-PHF immuneserum (revealed by the gold particles of 5 nm in diameter) presented a high specificity; the hyaloplasm never showed immunoreactivity. Some rare gold particles of 20 nm in diameter, corresponding to the anti-Tau staining, were observed outside the PHF.

Discussion

On semi-thin sections, as well as on ultra-thin sections, both antisera stain DNF specifically. The antigenicities are preserved in spite of fixation and the treatment used to remove the Araldite. In fact, it seems that whatever the fixative and embedding techniques used, it is possible to immunostain the DNF. These observations are consistent with previous reports which have established the stability of antigenic sites of PHF after other fixatives such as Carnoy's, Zamboni's mixtures or formalin (Delacourte and Defossez 1986). These antigenic sites of PHF, recognized by anti-Tau in Alzheimer cortex, may represent stable aberrant phosphorylated Tau proteins, as suggested by Wood et al. (1986). Actually, the anti-Tau labelling of PHF was obtained by Bancher et al. (1987) only after predigestion of the tissue sections with phosphatase. The stability of PHF is in contrast with the fragility of microtubules which are not preserved in autopsy material.

The helical periodicity of PHF structure might be correlated with an identical periodicity of the immunolabelling; this was indeed observed by Perry et al. (1986) with an anti-PHF which immunogold-stained enriched extracts of PHF when applied on grids. However, we did not observe such a periodicity using the post-embedding technique, neither did Brion et al. (1985). Therefore, it is possible that this approach masks certain epitopes. In contrast to Perry et al. (1985), our immunesera raised against the three neurofilament subunits have never labelled the PHF; only anti-PHF and anti-Tau immunesera immunostained this pathological skeleton. However, we have already shown (Delacourte and Defossez 1986) that the anti-PHF was totally absorbed with Alzheimer cortex extracts but partially absorbed with normal cortex extracts. In contrast, normal cortex extracts saturated the anti-Tau completely. Thus, the anti-PHF is probably raised against Tau proteins and also against conformational steric sites of PHF.

In conclusion, our results from both semi-thin and ultra-thin sections demonstrate the high affinity and specificity of anti-PHF and anti-Tau immunesera for PHF structure. After double immunostaining of DNF on histological sections and after the detection of Tau proteins on Western blots by anti-PHF, this result is a new demonstration that Tau proteins are the major antigenic components of Alzheimer PHF.

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