Virchows Arch [Pathol Anat] (1985) 407:13–23

Virchows Archiv A

Anti-PHF antibodies: an immunohistochemical marker of the lesions of the Alzheimer's disease

Characterization and comparison with Bodian's silver impregnation

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Summary. An immune serum raised against paired helical filaments (PHF) was able to stain senils plaques (SP) and neurofibrillary tangles (NFT) specifically, the two characteristic lesions of the dementia of Alzheimer-type. This polyclonal antibody against PHF was characterized by immunochemistry and also compared with the classical Bodian silver staining. NFT and SP were observed where they were expected: in the fronto-temporal neo-cortex and hippocampus of Alzheimer-type patients, and also in hippocampus of non-demented elderley subjects. The pattern of SP visualized by the two methods was identical whereas NFT were not detected specifically by silver salts, specially in the nervous tissue where NFT were in discrete quantities. Since the preparation of the antigen is very easy and the resulting antibodies are specific, we conclude that this technique will be of considerable interest for routine neuropathological diagnosis. Finally, the properties of our anti-PHF antibody are compared with those reported in the literature. This antibody will probably be a good tool for the identification of the chemical nature of PHF components.

Key words: Alzheimer's disease – Neurofibrillary tangles – Senile plaques – Paired helical filaments

Introduction

Neurofibrillary tangles (NFT) and senile plaques (SP) are the two most characteristic histopathological brain lesions of Alzheimer's disease and senile dementia of the Alzheimer type. These two argentophilic lesions are usually visualized by Bodian's method (Bodian 1936) which shows that

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NFT consist of large perikaryal masses of abnormal cytoplasmic fibers. The SP feature is more complex. It refers to numerous distended neurites which contain a large number of dense bodies, a fibrillar material and altered mitochondria surrounding an extracellular amyloid core with an intense astroglial cellular reaction (Terry et al. 1964).

NFT and SP are found in the hippocampus and neocortex of patients with senile and presenile dementia of the Alzheimer type. They also occur, though in much smaller number, in the normally aged brain (Ball 1978). The density and the location of SP and NFT are useful information for the neuropathologist since the number of plaques tends to correlate strongly with the extent of dementia (Ball 1976). Electron microscopic studies reveal that the structural elements of the fibrillar material found in NFT and SP are bundles of paired helical filaments (PHF). Each filament is about 10–13 nm in diameter and the helix shows a regular 80 nm-periodicity (Kidd 1964).

PHF is therefore the ultrastructural element that characterizes Alzheimer's disease. The chemical nature of PHF is still controversial but recently, Selkoe et al. (1982) demonstrated that these structures are rigid polymers with unusual molecular properties, including insolubility in sodium dodecyl sulfate (SDS) and other denaturing solvents and resistance to protease digestion. These properties were turned to account for the preparation of highly enriched PHF-fractions (Selkoe et al. 1982) and for the preparation of specific antibodies (Selkoe et al. 1982; Ihara et al. 1983). Results obtained by these authors are controversial since Ihara et al. (1983) and Rasool et al. (1984) showed that their anti-PHF antibody (1) labels essentially NFT and extracellular NFT (ghost-NFT), (2) is not saturated by soluble proteins extracted from Alzheimer brains, and (3) does not detect any antigenic protein on immunoblots.

On the other side Grundke-Iqbal et al. (1984) and Wang et al. (1984) demonstrated that (1) some monoclonal and polyclonal antibodies against PHF visualized both SP and NFT, (2) that their antibodies are saturated by soluble proteins extracted from normal and dement brains and (3) that they detect proteins of molecular weight between 65 and 40 K. Thus, the chemical composition of PHF is still controversial and it is of interest that additional independant data should be brought in order to complete the characterization of PHF by immunohistochemistry.

We have isolated PHF from the frontal cortex of a woman who died from a juvenile form of the Alzheimer's disease and raised specific anti-PHF antibodies. We report here a critical comparison on the visualization of NFT and SP revealed by immunohistochemistry and by Bodian silver staining. These results are discussed and compared to those reported in the literature.

Materials and methods

Clinical data (Table 1)

MAL, 37 year-old woman: died after a 7 years evolution of a juvenile form of Alzheimer's disease. The cause of death (respiratory infection) was a complication of cachexia.

Table 1. Clinico-pathological findings in the four cases examined

Case	Age of death	Condition	Duration of illness	Neuropathology	
				Hippocampus	Neocortex
MAL	37	Ad juvenile form	7 years	NFT + + SP + + +	NFT + + SP + + +
RUB	68	Ad presenile form	8 years	NFT + SP 0	NFT + SP 0
ABR	80	Control	–	NFT + + SP 0	NFT 0 SP 0
JOS	50	Control	–	NFT 0 SP 0	NFT 0 SP 0

– RUB 68 year-old man: died from urinary and lung infection with an atypical Alzheimer's disease that had begun in the presenium.
– ABR: 80 year-old woman. Coronary thrombosis, no dementia.
– JOS: 50 year-old man: Sudden death. No neurological disease.

The three first autopsies were performed within 3 h after death; the last one only 8 h after death, the body having been stored in a cold room.

Preparation of insoluble material

This was performed using the frontal cortex of the first patient (MAL) following the principles expressed by Selkoe et al. (1982). Seventeen grams of the frozen tissue were finely choped and homogenized in 10 vol of 0.1 M TRIS pH 7.5, 2% SDS and 0.2% B-ME (beta-mercapto-ethanol). The homogenate was centrifuged at 106,000 *g* for 120 min at 4° C. The resultant pellet was resuspended in the same buffer and centrifuged in the same conditions. The resultant pellet was layered on a gradient composed of 1.2, 1.8 and 2 M sucrose layers in 50 mM Tris, 1% SDS and centrifuged at 106,000 *g* for 120 min at 4° C. The material recovered at the 1.8–2 M interface was diluted in 0.1 M TRIS with 2% SDS and pelleted (106,000 *g*, 60 min). The pellet was used for biochemical and immunological investigations. The biochemical analysis revealed that it was an insoluble material of high molecular weight since it was excluded from a 3% acrylamide reticulation SDS gel, according of the method of Laemmli (1970).

Immunization of rabbit

Aliquots corresponding to the tenth of the purified PHF pellet were emulsified in 500 µl of incomplete Freund's adjuvant and 500 µl of physiological serum. Thirty to fourty intradermal injections were performed with additional injections of a dosis of BCG and a dosis of anti-whooping cough vaccine in order to stimulate the immunological reactions. Two weeks later, then every month, the animal received booster injections with the complete Freund's adjuvant. The first bleeding was performed 2 months after the beginning of immunisation and then 8 days after each booster.

Immunohistochemistry

Blocks of hippocampus and frontal cortex of every subject were fixed for 24 h in Carnoy's solution (ethanol, chloroform, acetic acid: 6/3/1), embedded into paraffin and cut in 5 µm thick sections. Immunoperoxidase reactions were performed according to the indirect method, using the specific antibody at 1/1,000 dilution in the first step (12 h) and a 1/40 dilution of anti-rabbit Ig sheep gamma globulin conjugated with peroxidase (purchased from Pasteur Institute, Paris) in the second step (1 h). For visualization of peroxidase, the sections were-reacted with diaminobenzidine (25 mg/100 ml of a 0.1 M Tris buffer pH 7.6, 0.001% H_2O_2).

Bodian's staining

Hippocampus and frontal cortex blocks were fixed and embedded in paraffin. Sections were hydrated and incubated at 37° C in a 1% silver albuminate solution with copper, following a kinetic time from 90 min to 24 h. Reaction was revealed in 5% hydroquinone, turned in 1% gold chloride, reinforced by a wash in 2% oxalic acid and finally fixed in 5% sodium hyposulfite.

Results

Immunochemical reaction

This anti-PHF antiserum identified the 2 typical lesions of Alzheimer's disease (Fig. 1).

In case MAL, numerous SP and NFT were detected in hippocampus and frontal cortex (Fig. 1A). In the cortex, only the large and middle-sized pyramidal cells layers were concerned. SP were visualized as circular lesions of 60 μm in diameter with 2 different appearances (Fig. 2B): the first ones were homogeneously colored sometimes with a central reticular aspect and a crown of dark granulations; the others, which represent the majority, possessed a granular periphery with a non-colored core.

In case ABR, a non demented old woman, some NFT were found only in the pyramidal layer of the hippocampus and none in the frontal neo-cortex (Fig. 2C). No other nervous structures (fibers, granular cells) were stained with the anti-PHF. NFT, as revealed by our antiserum, corresponded to an accumulation of a dark fibrillar material surrounding the cell nucleus and projecting forehead into the proximal part of the apical dendrite (Fig. 2D).

In the frontal neo-cortex of RUB, there was only a small amount of NFT and no senile plaque was detected (Fig. 2E and F).

No staining was noted in the hippocampus and neo-cortex of JOS.

Bodian's staining

Up to now, Bodian's method was considered to be the most specific technique for diagnosis in Alzheimer's disease. However, numerous variables can affect the quality and the specificity of the staining, especially the time of incubation with silver albuminate and the quality of the chemical products. Thus, in order to determine the best experimental conditions for optimal staining of both SP and NFT, we have critically compared the quality of the staining obtained after different reaction times (from 1H30 to 22 H) with silver albuminate (Table 2).

We observed that the cellular bodies were stained very early (at 2 H 30), first just very near the nucleus, then in the whole perikaryon and so the pyramidal cells were recognized in 3 H 30. The visualization of fibers appeared to be effective after 5H30 of incubation time. After that time, the background turned dark. After 20 H, the nuclei, the vascular collagen fibers and the ependymal cell were strongly stained.

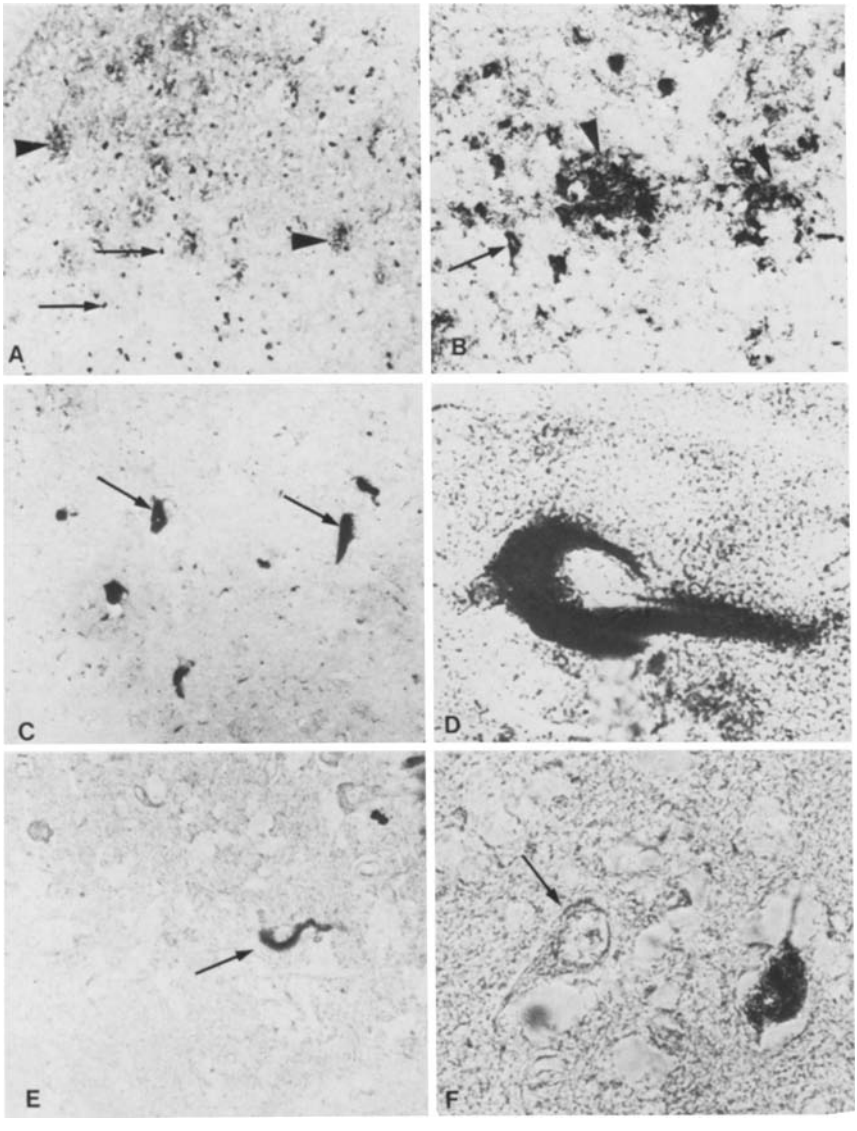

Fig. 1. A, B Paraffin sections of Mal frontal cortex immunostained with anti-PHF serum. In **A**, numerous tangles (*arrows*) and senile plaques (*arrowheads*) are immunolabelled (× 100). In **B**, a higher magnification allows to observe neurofibrillary tangles (*arrow*) and mature and immature senile plaques (*arrowheads*) (× 350). **C, D** Paraffin sections of ABR hippocampus immunostained with anti-PHF serum. In **C**, several tangles (*arrows*) are immunolabelled (× 400); **D**, neurofibrillary tangle with dark fibrillar material in the perikaryon and the apical dendrite (× 2,000). **E, F** Paraffin sections of RUB frontal cortex immunostained with anti-PHF serum. In **E**, a lonely tangle is immunolabelled (*arrow*) (× 600); in **F**, a tangle is immunolabelled whereas an another neuron remains unstained (*arrow*) (× 1,000)

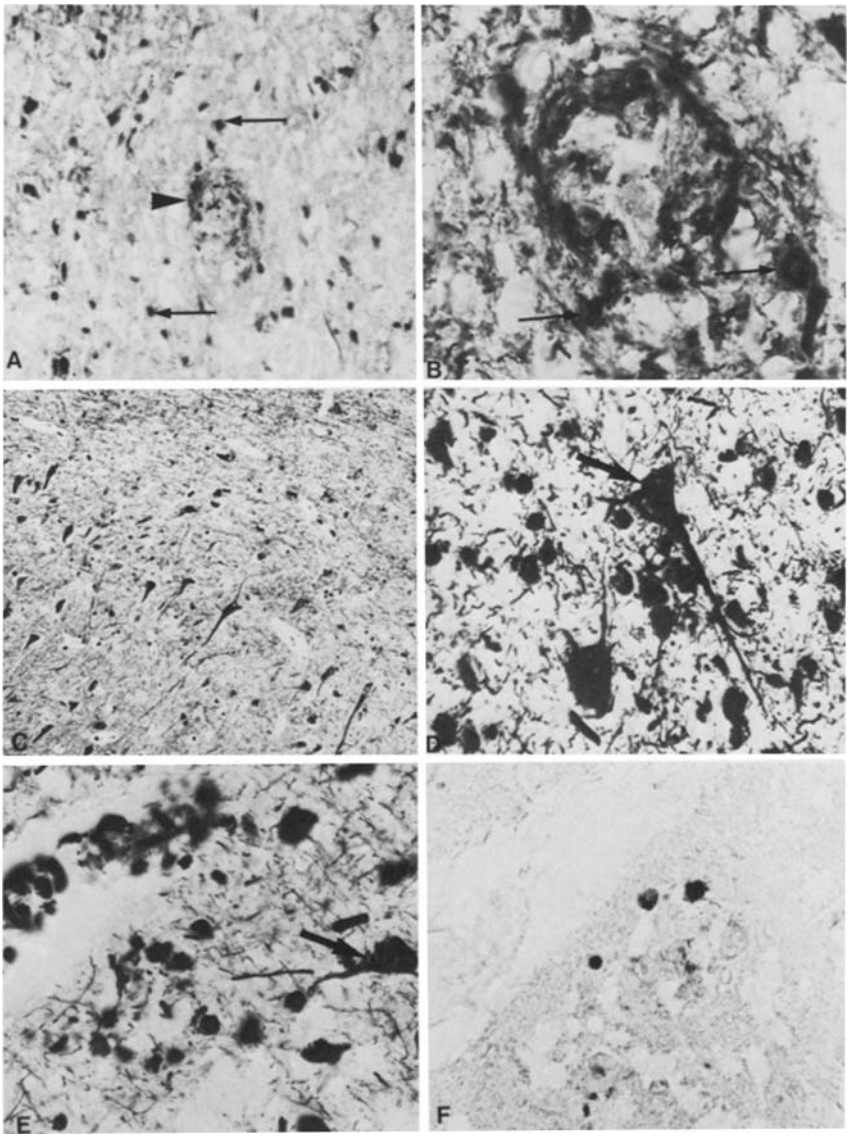

Fig. 2. **A, B** Paraffin sections of MAL frontal cortex stained by Bodian's method. In **A** numerous tangles (*arrows*) and senile plaque (*arrowhead*) are stained (× 300). In **B**, mature senile plaque surrounded by fibers and neurons (*arrows*) are stained (× 1,000). **C, D** Paraffin sections of ABR hippocampus stained by Bodian's method. In **C**, numerous neurons and fibers are stained (× 100). In **D**, a higher magnification allows to observe a neuron presumed to contain neurofibrillary tangles (*arrow*) (× 600). **E, F** Adjacent paraffin sections of Rub cortex stained by Bodian's method **E** or immunostained with anti PHF serum **F** (× 400). A pyramidal cell stained by Bodian's method was suspected to contain NFT **E** (*arrow*) but was not labelled with anti-PHF serum **F**. Reversely, three small cells immunostained with anti-PHF serum **F** are not detect by Bodian's staining

Table 2. Kinetic study of Bodian's method on paraffin sections of MAL frontal cortex

Time	Background	Nervous fibers	Cellular soma	NFT	SP
1.5 h	0	+−	0	0	0
2.5 h	0	+−	+	+−	0
3.5 h	0	+−	++	+−	+
4.5 h	+	+	++	+−	++
5.5 h	+	++	++	++	++
22 h	+	+++	+++	+	+

SP were easily detected after 3 H 30 of reaction, because of their characteristics structure. But it was more difficult to detect NFT among the other cells and the optimal incubation time for their visualization was estimated at about 5 H 30, when the background was still clear. Therefore, in our hands, 5 H 30 seemed to be the optimal incubation time for the visualization of SP and NFT. For example, in case MAL (Fig. 2A), numerous NFT and senile plaques were observed. Most of the SP possessed a central uncoloured core surrounded by peripheral well-stained granulations (Fig. 2B). Moreover, no SP was found in the hippocampus of ABR, but a high number of pyramidal cells were stained (Fig. 2C). Among them, numerous neurons seemed normal, and few neurons distinguishable by their intense staining were observed. According to their aspect, these elements seemed to be NFT-containing neurons due to their intense darkness, with sometimes a fibrillar organization. The outline of these cells appeared irregular and stiff with sometimes curly deformations of the segment of the apical dendrite (Fig. 2D).

Comparison of the two methods

Since it was difficult to detect and quantify NFT, particularly on tissue sections where these lesions were in small quantities (ABR, RUB), we performed both techniques on serial sections of the same tissue in case RUB. Near a blood vessel, where endothelial cells were stained by silver salts, we focused our interest on a typical degenerating neuron suspected to contain some fibrillar material (Fig. 2E). On adjacent sections, immunochemical staining was performed which revealed that this characteristic neuron was not stained by the anti-PHF antibody. In addition, this antibody revealed a group of 3 neurons which were not detected as abnormal neurons after the silver staining (Fig. 2F). However, since these 3 neurons have a small size, we cannot ascertain that they were present on the two adjacent sections.

Discussion

Immunohistochemistry

The antiserum was raised against a preparation of SDS-insoluble PHF found in characteristic lesions of a juvenile form of the Alzheimer's disease

and found specific for NFT and SP Alzheimer lesions for the following reasons:

The anti-PHF antibody detected the Alzheimer lesions where they were expected: senile plaques and NFT were found in the neocortex in the juvenile form of the disease (MAL), NFT were only detected in the neo-cortex of the senile form of the disease (RUB) and in the hippocampus of the old age control (ABR). In RUB case, the use of the specific antiserum was able to give evidence of the presence of PHF in the brain of this demented man, whereas clinical and neuropathological data were atypical.

The antibody labelled perfectly well SP and visualized two different aspects of the senile plaques. They probably correspond to different states of maturation: (i) the mature senile plaque, when the core is composed of an amyloid deposit, the antibody stained only the periphery of SP where PHF are present and did not stained the central core amyloid plaque. In MAL, these lesions were numerous. (ii) the immature senile plaques when the lesion is entirely coloured. Here PHF are distributed homogeneously in the entire plaque.

This labelling of NFT and structures located in SP is in good agreement with electron microscopic observations showing that PHF are present in both lesions (Terry 1964).

Moreover, no immunostaining was obtained in the cortex of an old patient without dementia and on the cerebral tissue of a 50 years old normal control. Besides, the anti-PHF antibody did not stain normal nervous structures which are rich in neurofilaments (basket cell fibers of the cerebellum, spinal axons or peripheral nerves).

Properties of this antiserum which revealed to be specific for PHF-containing structures are similar to those of previous reports of Ihara et al. (1984); Grundke-Iqbal et al. (1984) who demonstrated that some polyclonal(3–34) and monoclonal antibodies are able to stain NFT and SP. The undesirable cross reactions observed by these authors (labelling of endothelial cells, the important background or the particular labelling of white matter) were not observed with our antiserum.

The anti-PHF antibody was also characterized biochemically in order to compare its properties with those reported by Ihara et al. (1982) and Grundke-Iqbal et al. (1984). The main properties of this anti-PHF are (manuscript in preparation):

(1) immunoblotting techniques reveal that the anti-PHF detected proteins with molecular weight in the range 65K-40k on blots of resolved protein extract of brain cortex and that neurofilament proteins were not detected.

(2) The anti-PHF antibody was saturated by a preparation of NFT and also by low quantities of soluble proteins extracted from dement cortex and large quantities of soluble proteins from normal cortex (approximatively 100 times when compared to the dement cortex extract), as described by Grundke-Iqbal et al. 1984.

(3) The saturation was characterized by a parallel disappearance of the labelling of both SP and NFT on tissue section and on immunoblots by the suppression of the labelling of proteins between 65-40K.

Thus, our anti-PHF antibody possesses properties that are very similar to those reported by Grundke-Iqbal et al. (1984) and consistently different from the reports of Selkoe et al. (1982). These differences of labelling may be explained by the purity of the antigen but there are no accurate techniques to determine precisely the quality and the purity of the preparation since the chemical nature of PHF is still unknown. The physical nature of the antigen is probably also important. The boosted antigens may be more or less dissociated PHF or a more or less denaturated material (Rasool et al. 1984b). However, it remains clear that anti-PHF antibodies elaborated by the method presented here are a good tool for the quantitification of NFT and SP lesions by immunohistochemistry.

Bodian silverstaining

This staining is based upon a reduction by copper of a silver salt that preferentially stains the neurofibrillary material. Historically, this method allowed the identification of Alzheimer's disease, at the beginning of this century. The staining probably occurs on neuro-cytoskeleton proteins (neurofilaments and neurotubules) (Potter 1971). Gambetti et al. (1981) demonstrated with the blotting method that neurofilament subunits possess a strong affinity for silver, suggesting indirectly that PHF are made up of neurofilaments. However, our kinetic study of the Bodian staining shows that neuronal structures normally rich in neurofilaments such as axons are less coloured than the perikaryons of normal neurons where neurofilaments are in small amounts (Peters et al. 1976) and than the neuron nucleus and the vascular elements where neurofilaments are absent.

Mature or young SP were quickly detected (3H 30) by the silver impregnation whereas the staining of NFT appeared later (5H30) and concomitantly with the visualization of some normal cells. Therefore, it was difficult to quantify the presence of NFT. The only criterion to detect NFT is the distorted shape of the neurons in which an accumulation of a neurofibrillary material is observed. The fibrillar material accumulated in pyramidal cells of Alzheimer nervous tissue was detected by both methods. However, interpretation of Bodian staining for quantification of NFT is difficult, as revealed by our observations on RUB nervous tissue where a distorted cell, which was suspected to be a degenerative neuron, was not stained by the anti-PHF antibody.

Other methods of staining have been described that seem to be more specific for the labelling of NFT (Cross 1982). But since neuropathological laboratories are dealing with different or modified methods with very likely different labelling specificities, it is also of interest to persue the comparison of anti-PHF antibodies with the different silver salt impregnation techniques in order to standardize the silver technique.

In conclusion we suggest that Bodian's method, which is not based upon biochemical grounds does not appear to be the best way for studying NFT, specially if these structures are not widely distributed. Moreover, silver staining techniques are time consuming and poorly reproducible. However, this method remains of interest for studying senile plaques which are very well

recognized, because of their characteristic size and shape. The immunochemical study, which is easily reproducible, appears to be a more precise and specific method. The method described by Selkoe et al. (1982) for the preparation of the antigens (and slightly modified by us) is very easy to undertake and we show here that the resulting antibodies are very specific for Alzheimer lesions. Thus, we think that this immunological method will be of considerable interest for neuropathologists.

The possibility to precisely visualize lesions of the Alzheimer-type is very important because it allowed us: firstly, to diagnose Alzheimer's disease, even in atypical forms (RUB); secondly, to quantify these lesions, thirdly, to confirm the presence of PHF both in NFT and in senile plaques; fourthly, to corroborate the fact that the central core amyloid plaque does not share antigenic determinants with PHF; fifthly, to demonstrate the chemical identity of certain lesions (NFT) found in senile dementia of the Alzheimer type and also in normal senescence, and finally to point out the absence of relationship between neurofilaments and PHF.

Furthermore, the use of specific antibodies against PHF will be of interest in studying early and transitional forms of both lesions and in establishing links between lesions found in other neurodegenerative diseases with late occuring dementia (Down's syndrome, Pick's disease, Parkinson's disease). The immunohistochemical method can also be used to identify the chemical nature of PHF precisely.

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Accepted March 11, 1985