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Monitoring pathological assembly of tau and β-amyloid proteins in Alzheimer's disease

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Abstract This double-labelling confocal microscopy study of the neuropathology of Alzheimer's disease (AD) reports the use of a fluorescent dye, thiazin red, which has staining properties similar to thioflavin-S. Thiazin red fluorescence can be visualised selectively in the red channel, and we have used this property to compare it with the labelling seen using monoclonal antibody (mAb) 423, which detects tau protein C-terminally truncated at Glu-391, and mAb 4G8, which detects β-amyloid protein. Thiazin red is shown to recognized the typical histopathological deposits associated with both proteins. However, not all deposits containing these proteins are stained. Specifically, diffuse β-amyloid plaques and severely degraded extracellular tangles are unlabelled. Likewise a characteristic mAb 423-reactive granular plaque-like structure, typically present in cases with abundant extracellular tangels, is unlabelled by thiazin red. Such plaques can be shown to be continuous with the basal dendrites of degraded tangle-bearing pyramidal cells. These findings suggest that paired helical filaments (PHFs) continue to undergo degradation in the extracellular space, which is associated with loss of thiazin red binding sites, but preservation of mAb 423 immunoreactivity. This epitope appears to be characteristic of a stable core element of the PHF which is highly resistant to proteolysis. Compounds such as thiazin red with high affinity for β-pleated protein structures can be used to monitor the state of pathological assembly of amyloidogenic protein species found in AD.

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Introduction

Neurofibrillary tangles (NFTs) and senile plaques (SPs) are the major neuropathological features of Alzheimer's disease (AD) brains and their presence in large amounts usually correlates with dementia. Although heterogenous in composition, all subtypes of SP contain the so-called β/A4-amyloid protein [10]. Molecular cloning studies have shown that β/A4 derives from a larger precursor protein [5]. The classical SP is a complex lesion containing a central core of β/A4-amyloid surrounded by dystrophic neuritic processes and glial components. NFTs represent dense accumulations of insoluble abnormal filaments referred to as paired helical filaments (PHFs) [7]. The microtubule-associated protein tau is an integral structural constituent of the PHFs [9, 20, 21]. The monoclonal antibody (mAb) 423 recognises a specific C-terminal truncation at Glu-391 of tau protein which is within PHFs [13].

In a previous immunohistochemical study we reported on the progressive accumulation of C-terminally truncated tau protein in AD brain tissues using mAb 423 [11]. We found that a feature of AD cases with extensive extracellular neurofibrillary pathology was the presence of plaque-like structures with granular mAb 423 immunoreactivity. Such granular plaques appear to be distinct in appearance from SPs of both the classical and primitive types. In formalin-fixed brain tissue the presence of this type of plaque was always found to be associated with an abundance of ghost tangles present in the same area. Typically these plaques were found in layers II and III of the neocortex and in the CA1/subicular area of the hippocampus [11, 12]. Moreover, these structures share similar immunoreactive properties with tangles and thread-like structures stained with mAb 423, i.e. trypsin and phosphatase resistance.

We have used a laser scanning confocal microscope for serial reconstruction and double labelling to investigate

the relationship between granular mAb 423 immunoreactivity and β-amyloid plaques. To permit comparison between differentially labelled structures, we have used a fluorescent dye, thiazin red, which labels β-pleated protein structures and has histological staining properties identical to those of thioflavin-S. Thiazin red is more homogeneous chemically [15, 19] and emits fluorescence which can be visualised selectively in the red channel, thereby permitting comparison with fluorescein-conjugated second antibodies.

We report that the granular mAb 423-immunoreactive plaque is derived from extensive degradation of neuritic aggregates which, at least in some cases, represent sites of pathological arborisation of the basal dendrites of pyramidal cells. The processing of PHFs in the extracellular space involves loss not only of the N-terminal half of the tau molecule located in the fuzzy coat of the PHF, as reported previously [2], but also loss of binding sites for molecules such as thiazin red. This dye was also found to distinguish between amorphous and fibrillar β-amyloid-deposits. Thus, molecules such as thiazin red can be used to monitor the state of assembly of amyloidogenic protein species found in AD brain tissues.

Materials and methods

Frontal cortex and hippocampi from 18 AD cases with dementia duration of 10 years or more (mean age 75.5 ± 15 years) and 8 non-AD control cases (mean 77.8 ± 9.2 years) were used in this study. The brain tissue were fixed in either 4% paraformaldehyde solution or a mixture of 4% paraformaldehyde/10% formalin (0.1 M PBS buffer) for 3 days at 4° C. Specimens were double labelled with thiazin red and either mAb 423 or mAb 4G8. mAb 423 recognises a truncation at the Glu-391 of the PHF-associated tau protein [13]. mAb 4G8 recognises amino acids 17–23 of mature β-amyloid [8]. Thiazin red (Fluka, Busch) is a red fluorescing dye which labels plaques and tangles in AD [15, 19]. Double labelling with thiazin red and mAb 423 or mAb 4G8 was performed on free-floating 50- to 60- μm-thick vibratome sections. Sections were placed in PBS-Triton X-100 (0.2%) and then incubated with the primary mAbs (423, dilution 1:20 or 4G8, dilution 1:30), overnight at 4° C. This was followed by incubation in FITC-tagged goat anti-mouse IgG (Vector laboratories, Burlingame; dilution 1/40). After several rinses with PBS-Triton X-100, the sections were counterstained with thiazin red (diluted 1/100 in water), for 5 min. Prior to the incubation with the mAb 4G8, sections were treated with 98% formic acid for 2 min followed by extensive washing with PBS. Likewise, selected sections were pretreated with formic acid prior to the mAb 423 immunostaining. The double-labelled sections were transferred to gelatine-coated slides and mounted in anti-quenching media (Vectashield, Vector Labs.). Additional sections were single immunostained with 423 and anti-mouse/anti-horseradish peroxidase (HRP) mAb, McC10 [6] following previously described protocol [11].

Specimens were viewed with a 60x (N.A.1.4) oil immersion objective on a Nikon microscope with attached confocal system (Bio-Rad MRC 600, Watford, UK). From each region 5–25 serial optical Z-sections (0.5–2.0 μm thick) were collected using the dual channel imaging system with a green exciter filter (514 nm) and a blue exciter filter (418 nm). The resulting optical sections were fully projected onto the two-dimensional plane. Data was stored on 1000 or 200 Mbytes rewritable optical disk cartridges (Panasonic or IBM 3363, respectively).

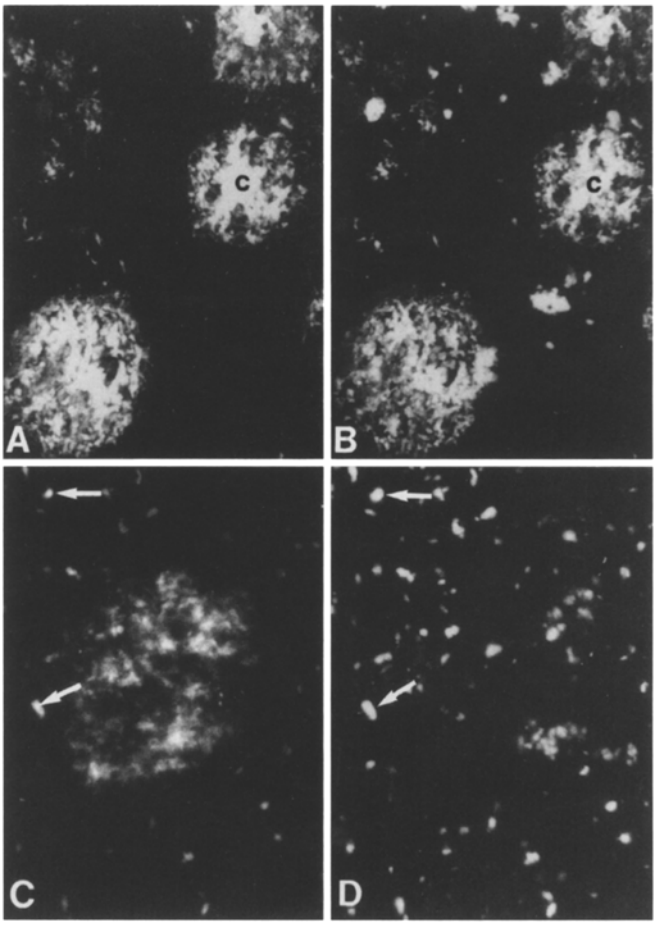

Fig. 1 Double labelling with 4G8 (green channel, **A, C**) and thiazin red (red channel, **B, D**) in formic acid pre treated sections. **A, B** Classical plaques present in the field display labelling with the two markers. **C** A diffuse plaque is identified by monoclonal antibody (mAb) 4G8 but is not labelled with thiazin red **(D)**. Lipofuscin granules are fluorescing in both the channels *(arrows)*. (*c* compact core of the classical plaque). **A, B** × 480; **C, D** × 600

Results

Labelling of AD pathology by thiazin red

Thioflavin-S is widely used to label the neuropathological lesions seen in AD brain tissues, including NFTs and amyloid plaques [1, 3, 16, 22, 26]. We have found that another dye, thiazin red produces similar labelling in histological sections [15, 19], but can be visualised at wavelengths above 520 nm when excited in the vicinity of 418 nm, the "rhodamine filter" configuration commonly used in fluorescence microscopy. Although autofluorescent lipofuscin granules are also seen in the red channel, these can be differentiated from the thiazin red-labelled structures both by characteristic morphology and by the autofluorescence which can be seen in both channels in the absence of thiazin red staining (Fig. 1C, D, arrows). The specific utility of thiazin red consists in providing a stain which can be visualised selectively in the red channel, permitting compar-

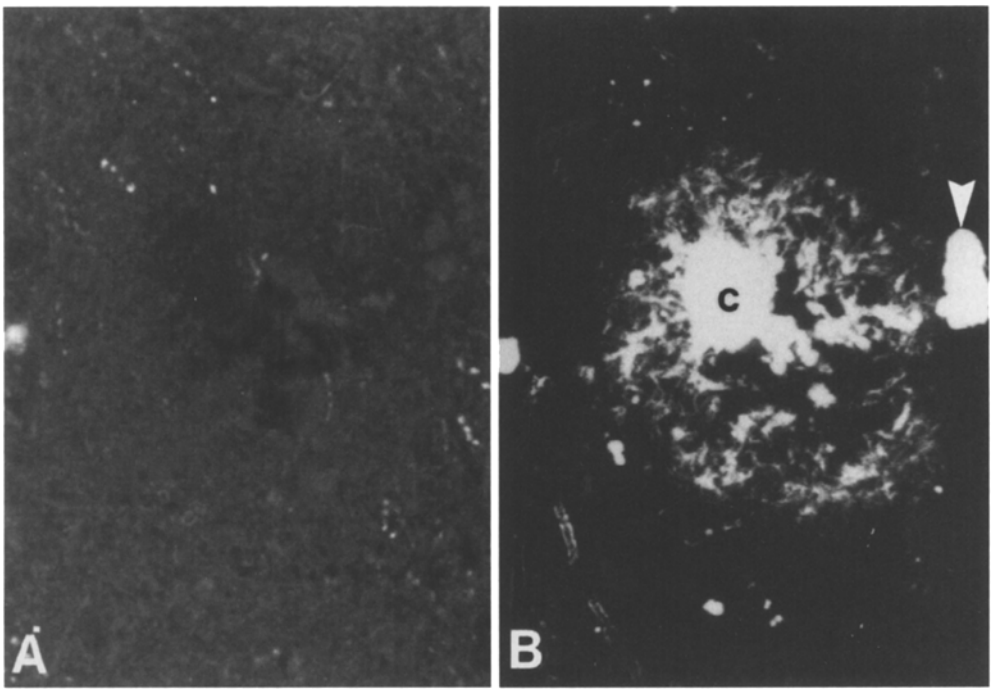

Fig. 2 Hippocampus of a non-demented normal elderly control case double-labelled with **A** mAb 423 and **B** thiazin red. The thiazin red-labelled classical plaque (**B**) is not stained by mAb 423 (**A**). A cluster of lipofucsin granules display strong red fluorescence in the vicinity of the amyloid deposit *(arrowheads)* (*c* compacted core of the classical plaque) **A, B** × 880

ison with immunohistochemical labelling that can be visualised in the green channel using FITC-conjugated second antibodies. Thin layer chromatography also shows that thiazin red is chemically more homogeneous than the complex mixture sold as thioflavin-S (C.M. Wischik and H-C. Thogersen, unpublished observation).

Double labelling of classical plaques with thiazin red and a β-amyloid antibody

Figure 1 illustrates the comparative labelling of classical amyloid plaques with thiazin red and the anti-β-amyloid mAb 4G8 [8]. The latter typically requires formic acid treatment of the histological section. When the two are compared by double-labelling confocal microscopy, both markers have very similar patterns of labelling, including the dense core of the plaque (c in Fig. 1A, B) and the more diffuse periphery (Fig. 1A, B). Formic acid treatment also reveals diffuse deposits of β-amyloid not associated with classical plaques (Fig. 1C). Such plaques are not labelled by thiazin red (Fig. 1D). Thus, although the β-amyloid protein is present in these diffuse deposits [23, 24], they do not contain the binding sites required for thiazin red staining.

Double labelling of plaques in brain tissues from non-demented aged controls with thiazin red and mAb 423

β-amyloid deposits are frequently found in brain tissues from non-demented elderly controls. As expected, such plaques are stained by thiazin red (Fig. 2B). mAb 423 was not found to label plaques or any other pathological structure in such cases (Fig. 2A).

Double labelling of classical plaques with thiazin red and mAb 423

SPs can also be labelled with anti-tau antibodies, such as mAb 423, because they contain PHFs [18]. A comparison of thiazin red and mAb 423 labelling shows that the β-amyloid plaque labelled by thiazin red (Fig. 3B) is not labelled by mAb 423 (Fig. 3A). However, double labelling was observed in some of the neuritic components associated with this plaque (Fig. 3A, B, short arrows). In addition, mAb 423 labels dystrophic neurites scattered throughout the neuropil, some of which are also labelled by thiazin red (Fig. 3A, B, arrowheads). Furthermore, some dystrophic neurites were labelled by thiazin red but not by mAb 423 (Fig. 3B, long arrows) or by mAb 423 and not thiazin red (Fig. 3A, open arrows). In general, the neuropil threads labelled by mAb 423 did not show any particular clustering in the vicinity of the amyloid plaque visualised with thiazin red (Fig. 3A, B). Thus, in addition to staining classical β-amyloid plaques, thiazin red also stains some neuritic structures which can also be labelled with a PHF tau-specific antibody.

We have found that formic acid pretreatment of the section very much enhances the immunoreactivity seen with mAb 423. This is shown in Fig. 3C, where strong labelling of neurites is seen throughout the field. This enhancement of labelling did not extend to labelling by mAb 423 of the classical plaque labelled by thiazin red in the same field (Fig. 3D), and again there was no evidence of clustering of formic acid enhanced neurite labelling in the

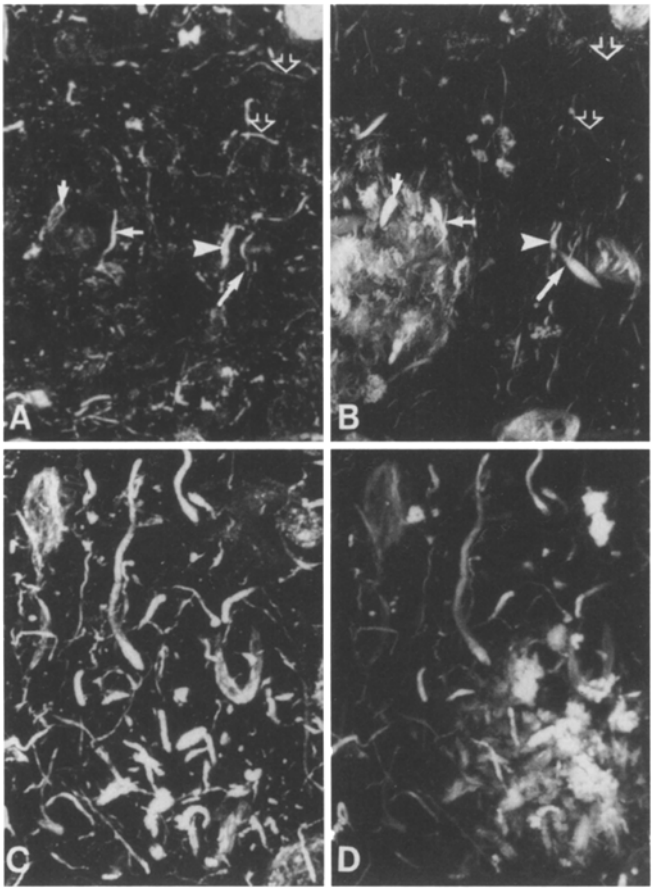

Fig. 3A–D mAb 423/thiazin red double-labelled sections. **A** mAb 423 does not stain the classical plaque which is labelled by thiazin red **(B)**. Some plaque-associated dystrophic neurites are double-labelled *(short arrows)*. However, some other are either labelled by mAb 423 (**A**, *open arrows*) or thiazin red (**B**, *long arrow*). Formic acid pretreatment enhances mAb 423-labelled neuritic elements **(C)** but does not affect the labelling of the amyloid plaque that is in the red channel and stained with thiazin red **(D)**. Projection of 14 optical sections at every 1.0 μm **A, B** × 720; **C, D** × 880

vicinity of such plaques. As shown by comparison with Fig. 3C and D, mAb 423 labelling of neurites generally coincided with thiazin red labelling, although there was a tendency for the latter to be reduced in sections which had been treated with formic acid.

Double labelling of granular plaques with mAb 423 and thiazin red

We have previously reported that in some AD cases, typically of long duration and containing abundant extracellular NFTs, plaques of granular appearance are seen which can be labelled by mAb 423 [11]. These plaque-like structures differ somewhat in appearance from either classical or diffuse β-amyloid plaques (Fig. 1A, C).

In view of the properties of thiazin red labelling described above, it is of some interest to compare the mAb 423-labelled granular plaques with the thiazin red labelling by confocal microscopy. Figure 4A shows a typical granular plaque labelled by mAb 423 and visualised in the green channel. When the same field was examined in the red channel, thiazin red labelling was seen to be restricted to the tangles and swollen neurites adjacent to the granular plaque (Fig. 4B). None of the granular material labelled by mAb 423 was labelled by thiazin red. Formic acid pretreatment of the sections did not affect this mutually exclusive pattern of labelling, Thus, although mAb 423 immunoreactivity is typically associated with PHFs, which are also labelled by thiazin red, the deposits which characterise the granular plaque contain tau protein immunoreactive with mAb 423, but without thiazin red binding sites.

Topographical relationship between granular plaques and tangles

It has been noted above that the mAb 423-reactive granular plaques are typically found in cases with abundant extracellular tangles. In the material used in the present study, it was possible on occasion to visualise both types of structures in close proximity to each other (Fig. 5A). Using the confocal microscope, a given histological field can be examined either at single optical sections of 1 μm, or as a summed projection image from a large number of optical sections. Figure 5A shows a projection image after mAb 423 labelling. The two granular plaques appear to be linked to the adjacent extracellular NFT by the two large "ghost" basal dendrites of the latter (Fig. 5A, arrows). This is further supported by examination of single optical sections at higher magnification. A direct link between elements of the granular plaque and both basal dendrites can be seen at different optical levels 5 μm apart (Fig. 5 B, C). We conclude from such images that the labelling of granular plaques seen with mAb 423 may derive from neuritic structures which were continuous with tangle-bearing neurons prior to their extrusion into the extracellular space in the course of cell death.

Labelling of extracellular tangles with mAb 423 and thiazin red

The anatomical continuity between mAb 423-reactive structures with and without binding sites for thiazin red implies the existence of distinct configurations of mAb 423-reactive material derived from the same cell. NFTs can be shown to undergo loss of thiazin red binding sites in the course of degradation in the extracellular space. Thus, as shown in Fig. 6, a typical extracellular tangle labelled by mAb 423 (A, green channel) is shown to be labelled only very weakly with thiazin red (B, red channel), in contrast to the strong labelling shown, for example, in Fig. 4B.

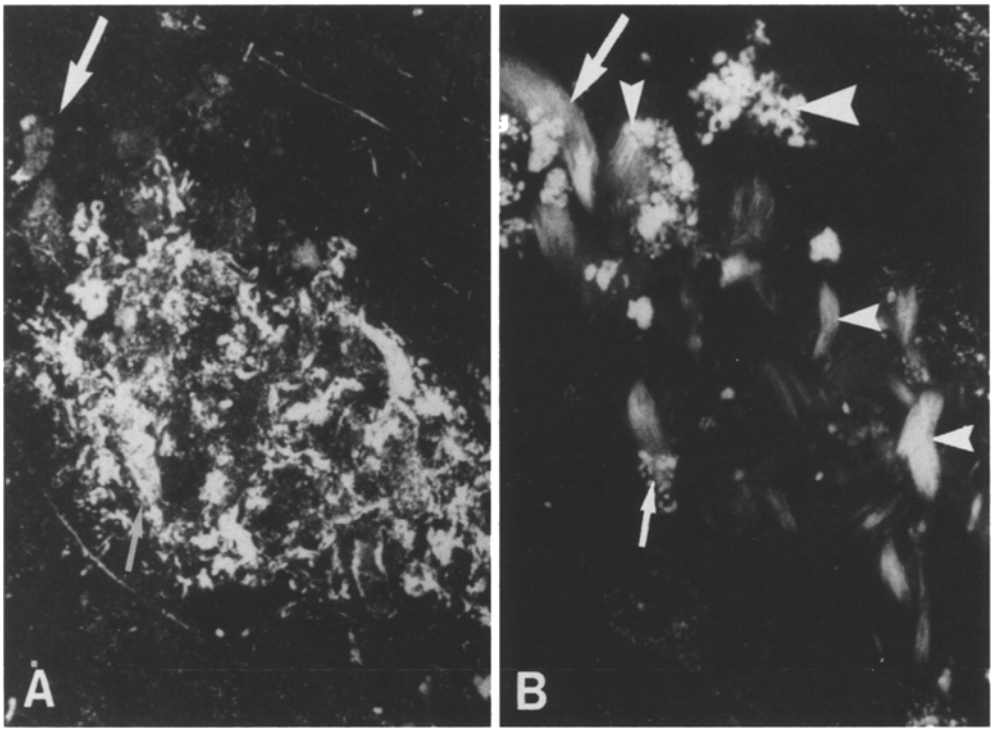

Fig. 4 Double labelling with mAb 423 (**A**) and thiazin red (**B**). **A** The granular plaque fluorescing in green channel is not labelled by thiazin red. **B** Most of the thiazin red labelled enlarged neurites *(small arrowheads)*, and the tangle *(large arrow)* is not labelled by mAb 423. However, some structures are double labelled *(small arrow)*. Lipofucsin strongly fluoresces in red channel *(large arrowhead)*. **A, B** × 880

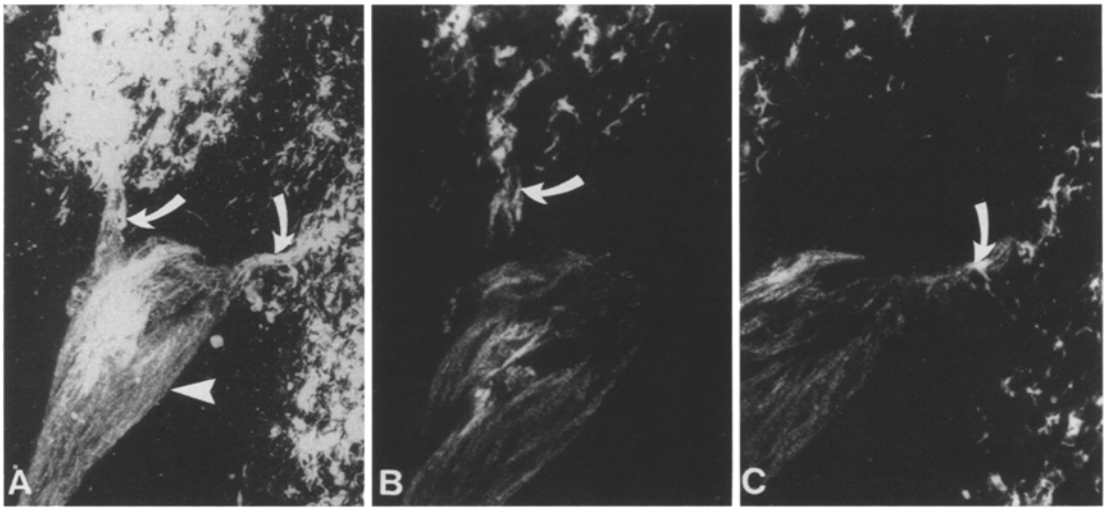

Fig. 5 **A** mAb 423 stains a extracellular neurofibrillary tangle (NFT; *arrowhead)* which shows a granular plaque deposit at both ends of its basal neurites *(arrows)*. Higher magnification of the left (**C**, *arrow*) and right (**C**, *arrow*) end of the mAb 423-immunoreactive tangle shown in **A** to better appreciate the continuity of the end with the ganular components of the plaque. **A** Projection of 24 optical sections each 0.5 μm. × 840; **B, C** single optical sections × 1120

Discussion

This is the first study reporting the use of thiazin red as a fluorescent dye which can be used like thioflavin-S to study the neuropathology of AD. Since dye preparations sold as thioflavin-S are chemically heterogeneous, it is not possible to determine which constituents are useful histologically. Thiazin red has histological staining properties which are very similar to thioflavin-S, but is more homogeneous chemically. In particular, thiazin red is a useful dye in confocal microscopy because its fluorescence can be visualised selectively in the red channel, thereby permitting comparison with immunohistochemical labelling which can be visualised in the green channel by means of fluorescence-conjugated second antibodies, as has been done in the present study.

Fluorescent dyes such as thiazin red have staining properties which both overlap with and differ from antibodies which label amyloid deposits in AD brain tissue. The mAbs 4G8 and 423 used in this study are selective for particular proteins: 4G8 recognising residues 17–24 of the β-amyloid peptide [8], and 423 recognising tau protein C-terminally truncated at Glu-391 [13]. We have shown in

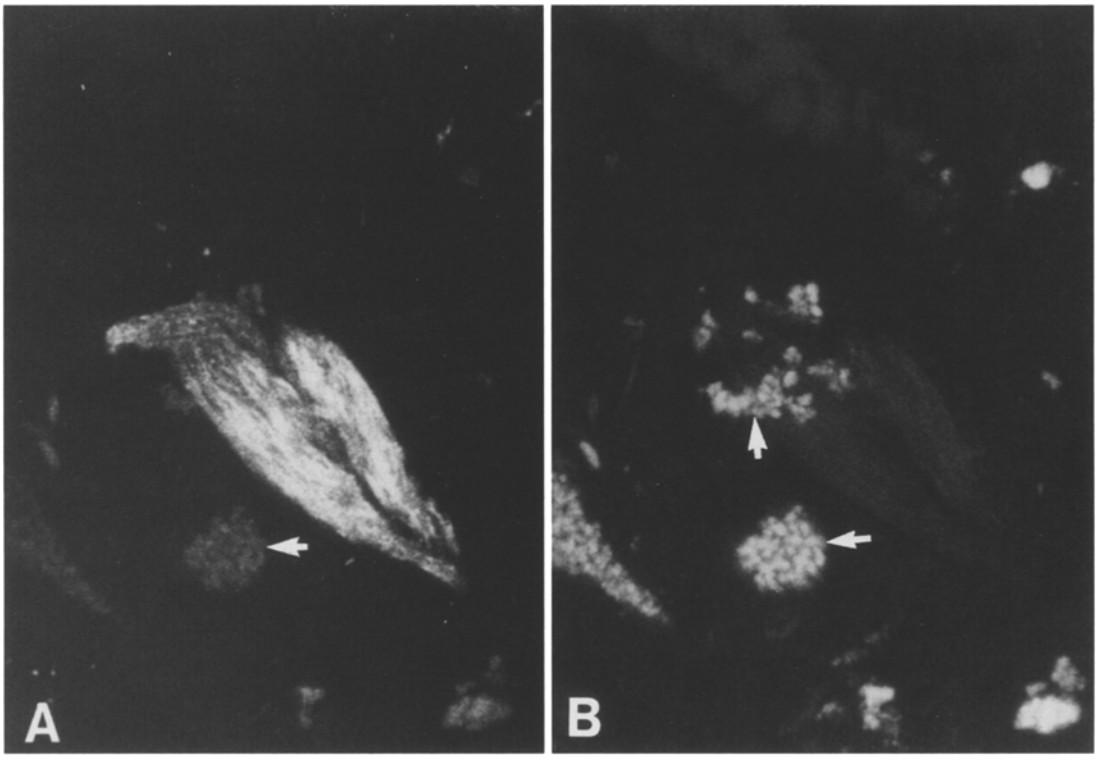

Fig. 6 **A** The extracellular NFT which is strongly labelled by mAb 423 is not stained by thiazin red **(B)**. Clusters of lipofucsin granules area present in the vicinity of the tangle *(arrows)*. **A, B** Projection of seven optical sections each of 1.0 μm; × 1120

the present study that thiazin red recognises the typical histological deposits associated with both these proteins. Thus, thiazin red labels typical β-amyloid plaques, and this labelling coincides with mAb 4G8 in double-labelled preparations (Fig. 1). Thiazin red also labels typical NFTs and dystrophic neurites (Fig. 3B, D), and these too can be shown to coincide with mAb 423 labelling in double-labelled preparations (Fig. 3). Thus, pathological deposits which are distinct at the protein level nevertheless contain similar dye-binding sites.

Conversely, structures that contain the same proteins need not be the same in terms of dye binding. This can be shown both for β-amyloid deposits and for accumulations of PHF-tau. Thus, some of the β-amyloid deposits found in AD brain tissues, specifically those of the diffuse type, are not labelled by thiazin red (Fig. 1C). Similar observations have been reported in relation to thioflavin-S [3, 22, 24]. The difference is thought to reside in the state of aggregation of the β-amyloid peptide, which has been shown to be amorphous in diffuse β-amyloid deposits, and fibrillar in the compact deposits. This suggests that presence or absence of binding sites for thiazin red coincides with presence or absence of a fibrillar state of aggregation of the β-amyloid peptide.

A similar situation exists in relation to the PHF-tau deposits detected by mAb 423. NFTs undergo proteolytic degradation in the extracellular space which involves the loss of epitopes associated with the N-terminal half of the tau molecule located in the fuzzy coat of the PHF [2]. This process is associated with the progressive exposure of the epitope identifed by mAb 423, which has been demonstrated to correspond to Glu-391 [13]. We now report that tangle degradation in the extracellular space progresses to the point of loss of binding sites for thiazin red, which is particularly evident in severely degraded extracellular NFTs (see Fig. 6). By analogy with the diffuse β-amyloid deposits, we presume that the loss of thiazin red binding sites coincides with loss of fibrillar structure of PHFs in the extracellular space. Electron microscopic studies of tangles in the extracellular space are in agreement with this interpretation with highly degraded extracellular PHFs appearing as linear amorphous deposits [17]. This suggests that the ghost tangle is not the final form of the tangle [4], but that it continues to undergo degradation in the extracellular space. In this regard, the preservation of mAb 423 immunoreactivity in structures which have even lost thiazin red binding sites is particularly striking, since the removal of a single amino acid residue at Glu-391 is enough to abolish immunoreactivity [13]. We conclude that this epitope is characteristic of a stable core element of the PHF which is highly resistant to proteolysis.

These properties make it possible to understand the observations relating to the granular plaques labelled by mAb 423. In the case shown in Fig. 5, two such plaques are continuous with structures which appear to be derived from the two basal dendrites of the NFT-bearing pyramidal cell. Their continuity with the NFT via the basal dendrites suggests that the whole complex is the residue of extensive PHF accumulation occupying the somatodendritic compartment of a pyramidal cell. The absence of

thiazin red labelling of the granular plaque is consistent with extensive degradation in the extracellular space, with preservation of mAb 423 immunoreactivity, as observed in highly degraded extracellular tangles. Presumably extracellular proteolytic activity leads to the formation of compact granular deposits of modified tau fragments which retain mAb 423 immunoreactivity after the disintegration of PHFs and loss of thiazin red binding sites.

The present study demonstrates that in AD, deposits of β-amyloid and C-terminally truncated tau exist in forms with differential binding affinity for a compound (thiazin red) which has high affinity for β-pleated sheet protein structures [15]. We interpret this differential binding to be due to the state of assembly of these proteins, rather than the particular protein species present. Silk, for example, binds thiazin red with high affinity [15]. In AD, diffuse plaques which are thought to contain non-fibrillar β-amyloid protein have low affinity for thiazin red, in contrast to the high-affinity binding seen in classical plaques. Likewise, ghost tangles which have undergone extensive degradation in the extracellular space have low affinity for thiazin red binding, in contrast with the high-affinity binding seen in intracellular tangles. This latter is the opposite of what would be expected if thiazin red binding to tangles were due to the presence of associated β-amyloid, which is largerly associated with extracellular tangles [2, 14, 17, 25]. We conclude that compounds such as thiazin red can be used to monitor the state of pathological assembly of amyloidogenic protein species found in AD.

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