

Alzheimer neurofibrillary tangles contain phosphorylated and hidden neurofilament epitopes

M C HAUGH,* A PROBST,† J ULRICH,† J KAHN,‡ B H ANDERTON*

From the Department of Immunology, St George's Hospital Medical School, London, UK, Institut für Pathologie der Universität, Basel, Switzerland,† Department of Neuropathology, Institute of Psychiatry,‡ London, UK*

SUMMARY Three monoclonal antibodies to neurofilaments (RT97, BF10 and 147), two of which also recognised neurofibrillary tangles (RT97 and BF10), have all been shown to be specific for phosphorylated epitopes. Treatment of histological sections with alkaline phosphatase prior to immunostaining resulted in reduction of axonal neurofilament staining with all three whilst the neurofibrillary tangles staining with BF10 was unaffected. Antibody 147 was found to recognise weakly some neurofibrillary tangles following alkaline phosphatase treatment. The results presented confirm the presence of structurally abnormal but phosphorylated neurofilaments in neurofibrillary tangles.

Neurofibrillary tangles are a pathological hallmark of senile dementia of the Alzheimer type (SDAT). Neurofibrillary tangles present in the perikaryon of affected neurons comprise bundles of 8-13 nm filaments helically twisted about each other in pairs with a cross-over roughly every 80 nm (paired helical filaments¹⁻³). A correlation between the number of neurofibrillary tangles, the extent of choline acetyltransferase loss and the severity of dementia has been observed.⁴

The physical properties of paired helical filaments are somewhat unusual in that many are highly insoluble⁵ but contain some extractable proteins with molecular weights in the range 57 000 to 62 000.⁶ Immunochemical studies indicate that neurofibrillary tangles may contain microtubule-associated antigens,⁷⁻¹² vimentin antigens¹³ and neurofilament antigens.¹⁴⁻²³ Using paired helical filaments as immunogen, three groups have found that polyclonal and monoclonal antibodies recognising the insoluble paired helical filaments either fail to detect cross-reactive material in normal brain or indicate that normal brain contains very small amounts of such antigens, the nature of which remain to be determined. These anti-paired helical filaments antibodies do not

react with any characterised cytoskeletal elements.^{11 24-26}

Previously we have described the specificities of several monoclonal antibodies (Mabs) to neurofilaments which were produced using two different immunogens, either Triton X-100-insoluble rat brain protein (Mabs RT97 and 147) or a crude soluble protein fraction from two pooled hippocampi from SDAT cases (Mab BF10; 14). RT97 and 147 react with the 210 000 (210K) mol wt and BF10 with the 155 000 (155K) mol wt polypeptides of human neurofilaments. Two of these Mabs, RT97 and BF10 also react with neurofibrillary tangles in paraffin sections of brain from SDAT cases. 147 and some other anti-neurofilament Mabs produced by us do not react with neurofibrillary tangles.¹⁴ The lack of neurofibrillary tangles staining by some antisera to neurofilaments has been noted before.^{8 18 27-29}

Sternberger and Sternberger³⁰ have reported that treatment of sections from brain with alkaline phosphatase can alter the staining pattern obtained with different neurofilament monoclonal antibodies, depending on whether they recognised phosphorylated epitopes or not. We now show that Mabs RT97, BF10 and 147 are all specific for phosphorylated neurofilament epitopes but that staining of neurofilaments and neurofibrillary tangles are differentially affected by alkaline phosphatase indicating further that the neurofilamentous contribution to neurofibrillary tangles is from structurally abnormal but phosphorylated neurofilaments. Positive staining with Mab 147 of some neurofibrillary tangles

Address for reprint requests: Dr BH Anderton, Department of Immunology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK.

Received 23 August 1985.
Accepted 20 October 1985

was apparently induced by alkaline phosphatase, suggesting that neurofibrillary tangles additionally contain masked neurofilament epitopes. Trypsin treatment of the highly insoluble isolated neurofibrillary tangles enhanced staining by Mab RT97 when compared with untreated isolated neurofibrillary tangles,²² again indicating the presence of masked neurofilament epitopes.

Materials and methods

All chemicals used were AR grade. Alkaline phosphatase (type VII-S; orthophosphoric-monoester phosphohydrolase; EC 3.1.3.1) and trypsin (type XIII; TPCK treated; EC 3.4.21.4) were obtained from Sigma (England). Purified alkaline phosphatase was a kind gift from Dr PAM Eagles. Primary monoclonal antibodies, produced as described previously,¹⁴ were used as ascites fluids at the dilutions indicated. Immunocytochemistry was carried out using the Vectastain ABC Kit (Sera-Lab Ltd, Sussex, England). Phosphate buffered saline (PBS; 8.1 mM NaH₂PO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, 0.14 M NaCl) and Tris buffered saline (TBS; 50 mM Tris-HCl pH 7.6, 0.15 M NaCl) were used where indicated either with or without sodium azide (0.02% w/v).

Western blotting

A total human brain homogenate was prepared from unfixed frozen "normal" cerebral cortex. A 10% (w/v) homogenate in 5% (w/v) SDS in PBS-azide containing 2 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM p-chloromercuribenzoic acid (PCMB) and 10 mM EDTA was made and diluted with an equal volume of SDS-sample buffer (0.125 M Tris-HCl pH 6.8 containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 2% (v/v) 2-mercaptoethanol). The homogenate was stored in aliquots at -20°C. Electrophoresis on 8% w/v SDS-polyacrylamide gels was performed³¹ and the protein transferred onto nitrocellulose paper (BA85; Anderman & Co Ltd, Surrey, England) essentially as described by Towbin *et al.*³² Before enzyme treatment the nitrocellulose sheet was incubated with 3% (w/v) haemoglobin (bovine, Type II, Sigma) in TBS-azide for at least 2 h to block excess protein binding sites. The nitrocellulose paper was then washed, cut into strips and incubated at 37°C for 18 h in either 0.1 M Tris-HCl pH 8.0 or 0.1 M Tris-HCl pH 8.0 containing alkaline phosphatase (12.5 µg/ml) or 0.1 M sodium phosphate buffer pH 8.0 containing alkaline phosphatase (12.5 µg/ml). After a brief wash the strips were incubated with the primary antibody (diluted in 3% (w/v) haemoglobin in TBS-azide) for one hour at room temperature followed by ¹²⁵I-labelled rabbit anti-mouse immunoglobulins. The autoradiographs were produced by exposure to x-ray film (Fuji, England) of the washed and dried strips.

Immunocytochemical studies of SDAT brain sections

Paraffin wax sections were dewaxed and rehydrated just prior to use. Both the frozen sections and the paraffin wax sections were pretreated with hydrogen peroxide (0.3% v/v) in methanol before enzyme treatment. Alkaline phosphatase treatment (100 µg/ml alkaline phosphatase in 0.1 M Tris-

HCl pH 8.0) was carried out at 37°C for 2.5 h or longer as indicated. Also alkaline phosphatase was applied to some sections in 0.1 M sodium phosphate at the same concentration, or the sections were incubated with buffer only. After incubation with enzyme or buffer the samples were incubated with 20% (v/v) horse serum (Gibco) in TBS-azide for 30 mins followed by primary antibody (diluted as indicated in 1% (v/v) horse serum in TBS-azide) overnight at 37°C. The samples were then stained using the Vectastain procedure except that incubations were carried out for 1 h and washing was done for 30 min (2 × 15 mins). The sections were counterstained with haematoxylin followed by dehydration and mounting.

Immunocytochemical studies of isolated neurofibrillary tangle preparations

Neurofibrillary tangles were isolated essentially as described by Rasool *et al.*²² using the SDS extraction procedure (SDS-NFT). The final homogenates were air-dried (2 µl aliquots) onto teflon-coated multipoint microscope slides (CA Hendley, Essex, England) and stored at 4°C. The samples were either incubated with enzyme in buffer as indicated or in buffer only. Trypsin treatment (40 µg/ml trypsin in 0.05 M Tris-HCl, pH 7.6, 0.3 M sodium chloride and 0.02 M calcium chloride) was carried out at 37°C for 10 mins. Immunocytochemistry was carried out as described above except the samples were not counterstained with haematoxylin.

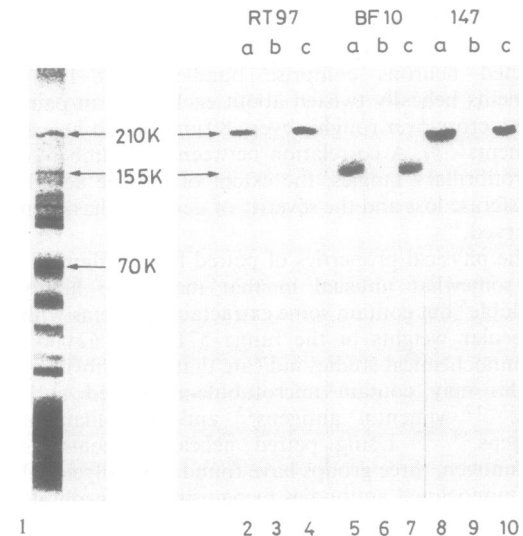


Fig 1 Western blot of total human cerebral cortex protein. Lane 1 shows a strip of polyacrylamide gel, after electrophoresis, stained with Coomassie blue. The bands corresponding to the neurofilament polypeptides are indicated by their M_r s. The remaining lanes correspond to nitrocellulose strips stained with the antibodies shown (dilution 1:10³) following incubation with (a) 0.1 M Tris-HCl pH 8.0; (b) 0.1 M Tris-HCl pH 8.0 containing alkaline phosphatase; (c) 0.1 M sodium phosphate pH 8.0 containing alkaline phosphatase as described in Materials and methods.

Table Summary of results: effects of alkaline phosphatase on axonal neurofilaments and neurofibrillary tangles

Treatment	Antibody	Frozen cerebellum NF	Frozen hippocampus		Fixed hippocampus	
			NF	NFT	NF	NFT
Untreated	RT97	+	+	+	+	+
	BF10	+	+	+	+	+
	147	+	+	-	+	-
Alkaline phosphatase (100 µg/ml) 2.5 h at 37°C	RT97	+	↓	↓	↓	↓
	BF10	↓	+	+	+	+
	147	↓	↓↓	-	+	↓
Alkaline phosphatase (100 µg/ml) 18.0 h at 37°C	RT97	↓	↓↓	↓	↓	↓
	BF10	↓	↓	+	↓	+
	147	↓	↓	↓	↓	↓

NF, axonal neurofilaments; NFT, neurofibrillary tangles; + Normal +ve staining; - Normal -ve staining; ↓ Decreased staining; ↓↓ Marked decreased staining; a, Occasional +ve NFT; b, Result shown for 32 h incubation at 37°C.

Results

WESTERN BLOTS

Treatment of Western blots of human brain protein with alkaline phosphatase appears to abolish or markedly reduce the staining by all three neurofilament Mabs (fig 1). Labelling by a polyclonal rabbit anti-neurofilament serum³³ was not affected by a similar treatment (results not shown). Longer exposure of the autoradiograph shows weak staining of the corresponding neurofilament polypeptides by all three Mabs on the alkaline phosphatase-treated strips (results not shown). This could be because either the antibody has a lower affinity for the non-phosphorylated epitope or the enzymatic removal of phosphate is incomplete. Similar results are found on

alkaline phosphatase-treated Western blots of total rat brain protein (data not shown). Control treatment of Western blots with buffer alone or with alkaline phosphatase in 0.1 M sodium phosphate, pH 8.0 (phosphate inhibits alkaline phosphatase) did not alter the normal staining found, substantiating the conclusion that the reduction in staining is due to the removal of phosphate by alkaline phosphatase.

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The results obtained are summarised in the table. Generally axonal neurofilament staining with all three antibodies was reduced by alkaline phosphatase treatment whereas neurofibrillary tangles staining by BF10 was apparently unaffected under similar treatment conditions and weak staining by 147 of occasional neurofibrillary tangles was apparently induced.

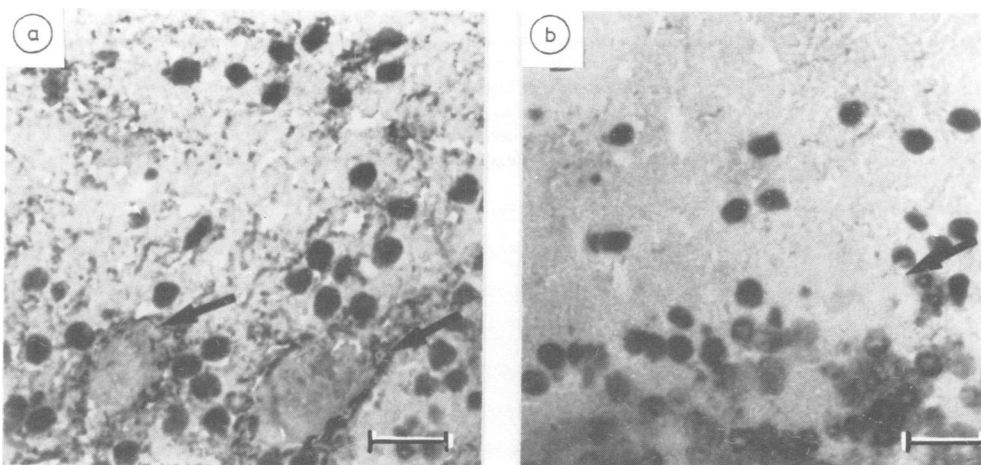


Fig 2 Immunocytochemistry of unfixed human cerebellum. The sections were incubated with (a) 0.1 M Tris-HCl pH 8.0 or (b) 0.1 M Tris-HCl pH 8.0 containing alkaline phosphatase for 18 h at 37°C before immunostaining with RT97 (1:10³) as described in Materials and methods. In a the basket cell fibres are positively stained (as indicated by the arrow) whereas in b, following treatment with alkaline phosphatase there is no staining. (Scale bar 20 µm).

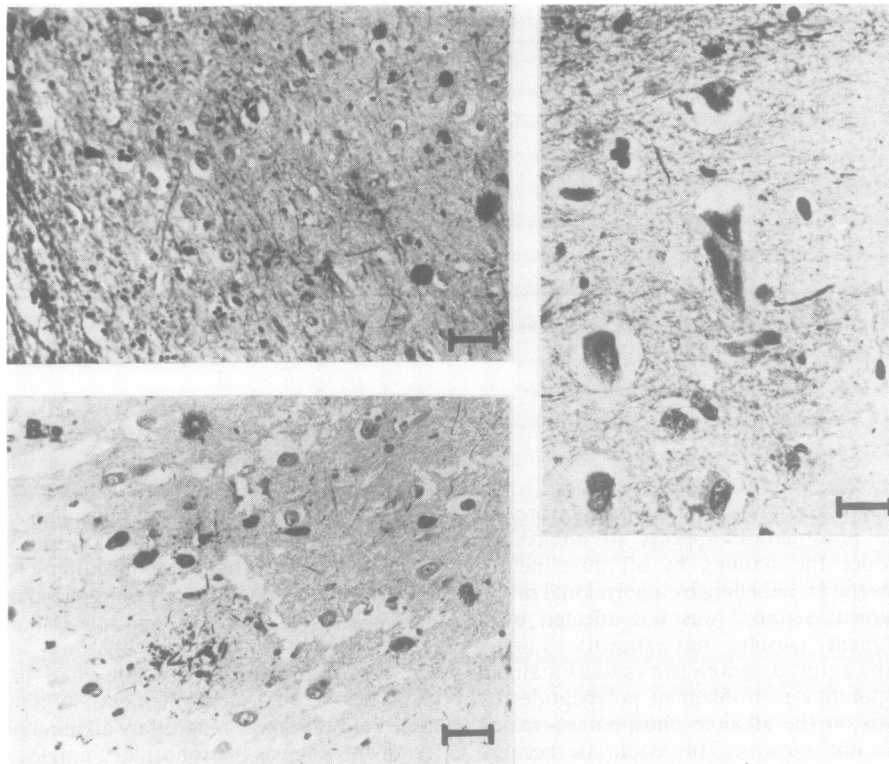


Fig 3 *Immunocytochemistry of SDAT hippocampus.* Fixed SDAT hippocampus sections were incubated with either 0.1 M Tris-HCl pH 8.0 (a) or the same buffer containing alkaline phosphatase (b, c) as described in the methods. Following incubation with Mab BF10 (1:10³; a, b) or Mab 147 (1:10³; c), the reaction was visualised with Vectastain ABC reagents. The result shown in (a) is similar to the untreated control, with axonal neurofilaments positively stained. After incubation with alkaline phosphatase the neurofibrillary tangles and neurites in the plaque periphery remain positively stained whereas the staining of the axonal neurofilaments throughout the whole section is reduced. Although the axonal neurofilament staining by 147 was less susceptible to alkaline phosphatase treatment some neurofibrillary tangles were found to be stained weakly although this antibody does not normally recognise neurofibrillary tangles. Generally no difference was found between unfixed and fixed sections except the unfixed sections showed less well preserved features. (Scale bar a, b 50 μ m; c 20 μ m).

(a) Untreated

The three Mabs have already been shown to react with basket cell axons around Purkinje cells and neuronal fibres in the inner third of the molecular layer of the cerebellum and also with some neuronal fibres in the cerebral cortex and subcortical white matter (fig 2a and ref 14). In addition, Mabs RT97 and BF10 have been shown to stain neurofibrillary tangles in fixed sections of SDAT brain whereas Mab 147 does not stain neurofibrillary tangles.^{14, 28} The former two Mabs also stain small numbers of SDS-isolated tangles but the staining of the majority of these extracted tangles is greatly diminished or abolished by the SDS treatment during isolation.²²

(b) Alkaline phosphatase treatment

Axonal neurofilaments Treatment of frozen sections of human cerebellum with alkaline phosphatase for 2.5 h reduced the axonal staining found with BF10 and 147. Reduction in RT97 staining was found after a longer treatment (18 h; fig 2). The staining of axonal neurofilaments in frozen sections from SDAT hippocampus was reduced by alkaline phosphatase although the three epitopes showed differing susceptibility; 147 and RT97 showed a reduction in staining after a short treatment (2.5 h) although the 147 staining was markedly more reduced whereas reduced staining by BF10 was not found until after a longer treatment (32 h). In fixed sections of SDAT

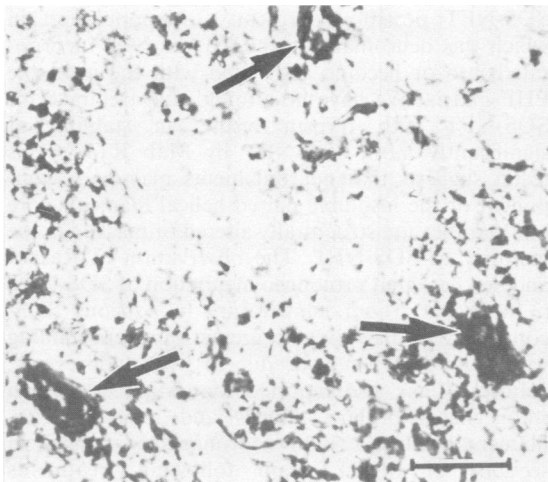


Fig 4 Effects of trypsin treatment of SDS-NFT preparations. The samples were treated with trypsin (40 µg/ml in 0.05 M Tris-HCl pH 7.6; 0.3 M sodium chloride; 0.02 M calcium chloride) or buffer alone for 10 mins at 37°C. Immunostaining with RT97 (1:10²) was carried out as described in the methods. Without trypsin treatment only a few weakly stained neurofibrillary tangles are found (results not shown), whereas following this treatment many more neurofibrillary tangles are stained much more intensely (arrows) (Scale bar 20 µm).

hippocampus the staining of axonal neurofilaments with RT97 was reduced after a short treatment whereas reduction in BF10 and 147 staining was found only after the longer incubation.

Perikaryal neurofibrillary tangles In contrast, neurofibrillary tangles staining and staining of neurites in the senile plaques by BF10 was not affected by similar alkaline phosphatase treatment of unfixed or fixed sections (fig 3a, b). Staining of neurofibrillary tangles by RT97, like the axonal staining, was reduced by prior alkaline phosphatase incubation. The Mab 147, which does not normally stain neurofibrillary tangles, was found to stain weakly occasional tangles in the fixed hippocampus after 2.5 h and in the frozen hippocampus after 18 h treatment with alkaline phosphatase. (fig 3c).

Treatment of the sections with alkaline phosphatase in 0.1 M sodium phosphate buffer, pH 8.0 in place of Tris buffer, resulted in no change in subsequent staining patterns by the Mabs compared with sections treated with buffer alone (phosphate inhibits alkaline phosphatase). To confirm that the observed effects were due to the action of alkaline phosphatase and not due to a contaminating enzymatic activity, some experiments were repeated using a sample of highly purified alkaline phosphatase. Similar results were obtained.

(c) Trypsin treatment of SDS-isolated NFT

Only a minority of neurofibrillary tangles isolated in SDS-containing buffers stain with Mabs RT97 and BF10.²² Following trypsin treatment (40 µg/ml, 10 min at room temperature), significantly more SDS-NFT were stained by Mab RT97 than are found in untreated samples of the same SDS-NFT preparations (fig 4).

Discussion

RT97, BF10 and 147 are directed against phosphorylated epitopes

Alkaline phosphatase treatment of Western blots demonstrated unequivocally that the three Mabs used in this study, RT97, BF10 and 147, are all directed against phosphorylated epitopes present on one or other of the two larger neurofilament polypeptides (fig 1). Binding to neurofilament proteins by a polyclonal antiserum was unaffected by alkaline phosphatase, but since we do not know if all phosphate groups were removed by the enzymatic treatment, it is not possible to conclude that this antiserum contains antibodies recognising only non-phosphorylated epitopes.

Staining of axonal neurofilaments is affected by alkaline phosphatase

The immunocytochemical results also show that staining of axonal neurofilaments by these three Mabs is susceptible to alkaline phosphatase treatment. All three Mabs are similar to the group (ii) antibodies defined by Sternberger and Sternberger,³⁰ since they recognise phosphorylated determinants and have similar staining patterns on fixed histological sections. However, the staining by our Mabs, on histological sections, is affected by alkaline phosphatase treatment, whereas two of the Sternberger's group (ii) Mabs are unaffected by any treatment (either alkaline phosphatase, trypsin or a combination) and the other three are unaffected by alkaline phosphatase treatment alone. However, a reduction in staining with their three group (ii) Mabs was found when the sections were treated with trypsin prior to alkaline phosphatase treatment.

In this study both unfixed and fixed sections from the same SDAT brain were used in order to establish if the fixation process had any effect on results obtained. It was found that the results obtained in both types of section were similar for each antibody, although the degree of effect may have varied (table 2).

From immunocytochemical results it would seem that the RT97, BF10 and 147 epitopes on axonal neurofilaments are not all equally susceptible to alkaline phosphatase, the BF10 epitope being the most

resistant. There was not a corresponding differential response to alkaline phosphatase by the separated neurofilament polypeptides on the Western blots. In the assembled axonal neurofilaments in the tissue sections some phosphorylated amino acids are probably more accessible to alkaline phosphatase so that a differential susceptibility is found. It cannot, therefore, be assumed that alkaline phosphatase treatment, however prolonged, will completely hydrolyse all phosphorylated amino acid side chains since some may be totally sterically blocked with respect to this enzyme.

Staining of NFT by BF10 is unaffected by alkaline phosphatase

Staining of neurofibrillary tangles present in neuronal perikarya and probably of paired helical filaments in the plaque neurites by Mab BF10 was not affected by alkaline phosphatase treatment even though staining of axonal neurofilaments in the same section was greatly diminished. This observation suggests that this epitope in the neurofibrillary tangles is well protected from attack by alkaline phosphatase, whereas the RT97 epitope is less well protected. Possibly neurofilaments associated with neurofibrillary tangles are in a different conformation compared with normal axonal neurofilaments or additional components are closely associated with the neurofilaments or the neurofibrillary tangles. Although the alkaline phosphatase is unable to dephosphorylate the BF10 epitope, the epitope is still obviously accessible to the Mab.

Alkaline phosphatase treatment of neurofibrillary tangles also induces staining, albeit weak, of occasional tangles by the third Mab 147. Presumably, removal of one or more phosphate groups from the neurofibrillary tangles allows access of Mab 147 to some of its epitope. The subsequent weak staining of only some neurofibrillary tangles by Mab 147 may be because this antibody also recognises a phosphorylated epitope and this 147 epitope once exposed could be a substrate for the enzyme, unlike the BF10 epitope in neurofibrillary tangles which is not hydrolysed. Alternatively the 147 epitope may only be partially revealed by the enzyme action and so the subsequent immunostaining would be weak.

Staining of SDS-NFT by RT97 can be enhanced by trypsin treatment

We have previously reported that the majority of neurofibrillary tangles isolated in SDS (SDS-NFT), which enriches for the highly insoluble paired helical filaments core, fail to stain with Mabs RT97 and BF10.²² In these preparations, normal neurofilaments have been completely removed by solubilisation in SDS. The few remaining RT97-positive

SDS-NFT possibly represent a subpopulation in which this neurofilament epitope has been incorporated into or become associated with the insoluble PHF matrix. We have now found that treatment of SDS-NFT with trypsin results in staining of significantly more SDS-NFT by Mab RT97. This result suggests that neurofilaments may be a component of the insoluble paired helical filaments-core but that they are structurally altered or masked in the majority of SDS-NFT. The observation of Rasool and Selkoe²³ that prolonged incubation of SDS-NFT (3 weeks) at room temperature in aqueous SDS-containing media results in greater numbers staining with Mab RT97 may be due to hydrolytic events. Johnson²¹ has also reported that Mab, T4, which stains neurofibrillary tangles and axonal neurofilaments will still stain neurofibrillary tangles in sections of SDAT brain following treatments designed to extract the normal axonal neurofilaments. Further effort is therefore now required in an attempt to break up the insoluble paired helical filaments to test if other cytoskeletal epitopes can be revealed. Possibly "inherent" paired helical filaments antigens are generated by gross modification of cytoskeletal proteins to the extent that anti-paired helical filaments antibodies fail to react with the normal proteins.^{11 24-26} Certainly our findings that neurofibrillary tangles *in situ* can be stained after alkaline phosphatase treatment, by a neurofilament antibody hitherto believed to be unreactive towards neurofibrillary tangles (Mab 147) and that SDS-NFT can be stained in greater numbers by RT97 after trypsin treatment further supports the idea that neurofibrillary tangles *in situ* are composed, at least in part, of abnormal neurofilaments and that this may also extend to insoluble paired helical filaments. Trypsin treatment of sections is a technique which is often used in immunochemical studies and it is thought that this procedure does not cause false positives. In this particular case the possibility of neurofilament fragments becoming associated with neurofibrillary tangles following trypsin treatment is ruled out since normal neurofilaments have been removed from the preparation by prior solubilisation in SDS. Thus the physical state of the abnormal neurofilaments in neurofibrillary tangles is heterogeneous, a proportion being readily extractable and some remaining SDS-insoluble.

Future work should include a study of neurofilament phosphoprotein metabolism

Sternberger and Sternberger suggest that neurofilaments located in neuronal perikarya are not phosphorylated but are filamentous. Phosphorylation of these filaments may provide a further ordering of structure.³⁰ The epitopes recognised by RT97 and

BF10 have been shown to be phosphorylated and so these antibodies do not recognise perikaryal neurofilaments presumably because their epitopes are unphosphorylated. Since these Mabs recognise neurofibrillary tangles in perikarya an abnormal processing or location of, at least these, neurofilament epitopes must occur. Although our data do not provide evidence for a disturbance of phosphoprotein metabolism, it is worthy of investigation since a change in neurofilament phosphorylation state in response to axonal injury in the optic nerve has been proposed.³⁹ This was based upon the observation that staining with Mab RT97 was altered after induced axonal injury.

Paired helical filament subunit substructure

A recent study has suggested that the paired helical filaments subunit has two domains in a radial direction with an axial extent of < 5 nm. On the basis of the model proposed it is unlikely that paired helical filaments can be formed by a simple collapse and twisting of adjacent neurofilaments.⁴⁰ However, the immunocytochemical results presented here suggest that at least two neurofilament epitopes are associated with the neurofibrillary tangles and it may be that these epitopes are present on proteolytic fragments of the whole neurofilament polypeptides which in themselves, or in association with other as yet undefined components, form paired helical filaments.

This work was supported by the Medical Research Council and The Wellcome Trust. We thank Dr PAM Eagles for his gift of purified alkaline phosphatase. Also we acknowledge the technical assistance of Anita Hossmann, Mia Nebiker and Hansraedi Zysset. Our thanks go also to Charlotte Finucane for typing this manuscript.

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