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A68 proteins in Alzheimer's disease are composed of several tau isoforms in a phosphorylated state which affects their electrophoretic mobilities

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The tau-immunoreactive A68 polypeptides found in brains from patients with Alzheimer's disease have been studied by Western blotting using (1) antibodies to synthetic peptides corresponding to sequences that span the complete human tau molecule, and (2) antibodies specific for inserts 1 and 2 found towards the *N*-terminus of some tau isoforms. The three major A68 polypeptides were labelled by all of the antibodies to sequences common to all tau isoforms, but the faster-migrating A68 polypeptide was not labelled by either of the two antibodies specific for inserts 1 and 2. Treatment with alkaline phosphatase of non-solubilized A68 did not change its electrophoretic mobility on SDS/PAGE under the conditions described here. However, A68 that was solubilized before treating it with alkaline phosphatase was found to move faster on SDS/PAGE than untreated A68, to a position similar to that of normal tau. We also confirmed that A68 preparations contain numerous paired helical filaments (PHF). These PHF were labelled by all anti-tau antibodies, including insert-specific antibodies. Our results further support the notion that PHF contain abnormally phosphorylated tau in an aggregated state, and indicate that these abnormally phosphorylated tau forms are composed of several tau isoforms and that the full length of the tau molecule is present in these polypeptides.

INTRODUCTION

Abnormal filaments with a characteristic ultrastructure [the so-called paired helical filaments (PHF)] accumulate in neurons in Alzheimer's disease to form the neurofibrillary tangle, a characteristic neuropathological lesion of the disease. The number of neurofibrillary tangles is correlated with the severity of dementia. Although the mechanisms of formation of these abnormal filaments have still to be elucidated, something of their biochemical composition is known. Immunological (Brion *et al.*, 1985*b*, 1991; Delacourte & Defossez, 1986; Grundke-Iqbal *et al.*, 1986*a*; Kosik *et al.*, 1986; Nukina & Ihara, 1986) and biochemical (Wischik *et al.*, 1988*b*; Kondo *et al.*, 1988; Greenberg & Davies, 1990) studies have shown that the microtubule-associated protein tau is a component of PHF.

Tau proteins are a group of developmentally regulated phosphoproteins generated by alternative splicing of a primary transcript originating from a single gene (Couchie & Nunez, 1985; Goedert *et al.*, 1988, 1989*a*,*b*; Himmler *et al.*, 1989; Himmler, 1989; Kosik *et al.*, 1989; Lee, 1990). Tau proteins are known to induce the polymerization of microtubules and to stabilize microtubules *in vivo* (Cleveland *et al.*, 1977; Kanai *et al.*, 1989; Caceres & Kosik, 1990). Six human tau isoforms are known (Goedert *et al.*, 1989*a*), differing by the presence or absence of two types of inserts in the *N*-terminal half of the molecule (inserts 1 and 2) and each with three or four tandem tubulin-binding repeats in the *C* terminal domain (Goedert & Jakes, 1990).

Abnormalities of tau in Alzheimer's disease might explain the collapse of the microtubule network observed in tangle-bearing neurons in the disease (Dustin & Flament-Durand, 1982). Tau in Alzheimer's disease has been reported to be abnormally phosphorylated (Grundke-Iqbal *et al.*, 1986*b*; Iqbal *et al.*, 1989; Uéda *et al.*, 1990) and abnormal forms of tau, e.g. with slower mobility on SDS/PAGE than normal tau, have been described in Alzheimer's disease (Flament *et al.*, 1989; Gache *et al.*, 1990; Hanger *et al.*, 1991). These slower-migrating tau species in Alzheimer's disease have also been reported to be abnormally phosphorylated (Flament *et al.*, 1989; Hanger *et al.*, 1991). The abnormal phosphorylation of tau in Alzheimer's disease might be pathophysiologically important, since phosphorylated tau has been observed to be less efficient than dephosphorylated tau in promoting tubulin polymerization (Lindwall & Cole, 1984).

Several polypeptides in the molecular mass range 60–68 kDa, termed A68 polypeptides, have been described in Alzheimer's disease brain tissue but are absent from control brains; they were initially identified with the Alz50 monoclonal antibody and are thought to be a component of PHF (Wolozin *et al.*, 1986; Ksiezak-Reding *et al.*, 1990). Alz50 cross-reacts with tau, and A68 polypeptides are labelled by most anti-tau antibodies (Nukina *et al.*, 1987; Ksiezak-Reding *et al.*, 1988, 1990; Uéda *et al.*, 1990). Nevertheless, A68 differs in some physico-chemical properties from normal tau, and the relationship between A68 and tau has been a matter of debate. The recent work of Lee *et al.* (1991) has demonstrated by sequence analysis of some peptides derived from A68 that A68 is derived from tau and in fact appears to be an abnormally phosphorylated form of tau that migrates like tau after treatment with alkaline phosphatase. In other studies it was, however, reported that treatment of A68 with alkaline phosphatase failed to change its electrophoretic mobility (Ksiezak-Reding *et al.*, 1990; Vincent & Davies, 1990).

In order to probe further the relationship between A68 and tau, we have used a panel of antibodies specific for a range of epitopes that span the complete tau molecule, and two antibodies specific for particular tau isoforms. We have also investigated the discrepancy between reports on the effects of alkaline phosphatase on the mobility of A68 in SDS/PAGE. Our findings demonstrate that intact rather than fragments of tau constitute the A68 protein and that A68 is an aggregated and apparently

Abbreviation used: PHF, paired helical filaments.
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hyperphosphorylated form of tau, accounting for its apparent slower mobility in SDS/PAGE.

EXPERIMENTAL

Antibodies

The preparation of antisera to bovine (B19) tau proteins has been described previously (Brion *et al.*, 1991; Hanger *et al.*, 1991). Anti-TP10, -TP20, -TP30, -TP40, -TP50, and -TP60 antisera were prepared against synthetic peptides corresponding to sequences in human tau and have been previously described (Brion *et al.*, 1991). These peptides correspond to the following amino acid residues in the longest human tau isoform (Fig. 1) (Goedert *et al.*, 1989*a*): 9–18 (TP10), 32–41 (TP20), 199–126 (TP30), 212–221 (TP40), 244–253 (TP50) and 387–394 (TP60). BR133 and BR134 antisera were raised to synthetic peptides corresponding to the most *N*-terminal (amino acids 1–16) and the most *C*-terminal (amino acids 428–441) residues of human tau respectively (Goedert *et al.*, 1989*a*). BR200 and BR189 were raised to synthetic peptides corresponding to amino acid residues 61–72 and 76–87 of tau respectively, and are specific for inserts 1 and 2 found in the *N*-terminal half of some human tau isoforms (Fig. 1) (Goedert *et al.*, 1989*a*). Tau-1 is a monoclonal antibody to tau that labels neurofilbrillary tangles strongly only after alkaline phosphatase treatment (Grundke-Iqbal *et al.*, 1986*b*). Alz50 is a monoclonal antibody labelling a 68 kDa species (A68) abundant in Alzheimer's disease brain tissue (Wolozin *et al.*, 1986). The anti-PHF serum (B4) was raised to fractions enriched in PHF (Brion *et al.*, 1985*a*).

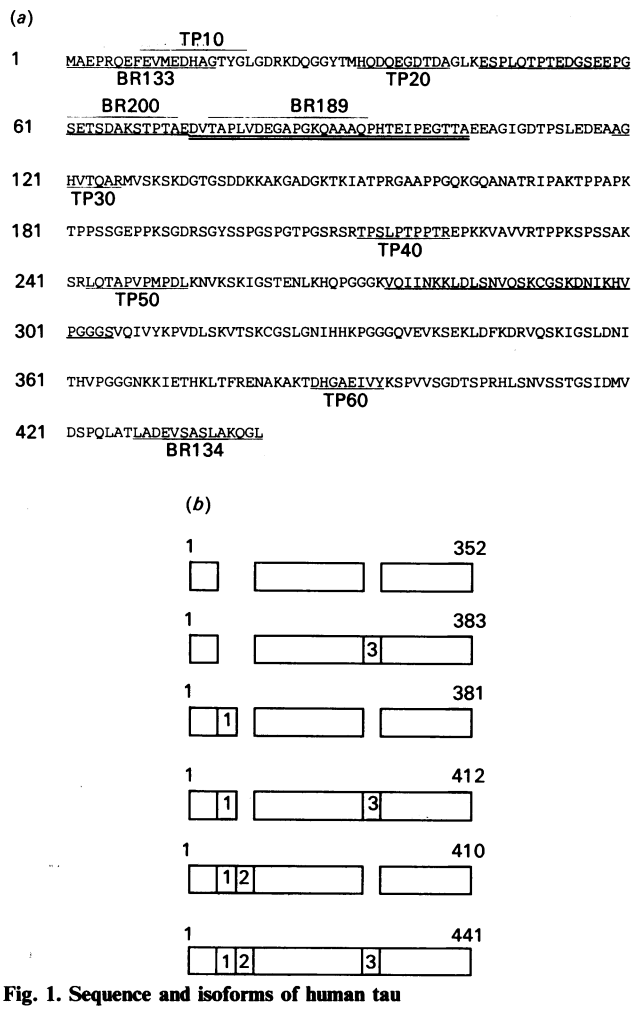

Fig. 1. Sequence and isoforms of human tau

(*a*) Human tau sequence (longest tau isoform; Goedert *et al.*, 1989*a*) showing the sequences corresponding to the synthetic peptides (stippled lines) used to raise antisera. The underlined sequences in the *N*-terminal half correspond to inserts 1 (single underline) and 2 (double underline). The underlined sequence in the *C*-terminal half corresponds to the fourth tubulin-binding repeat. (*b*) Schematic drawing of the six human tau isoforms with and without inserts 1 and 2 in the *N*-terminal domain and insert 3 (fourth repeat) in the *C*-terminal half domain (Goedert & Jakes, 1990).

Preparation and dephosphorylation of tau and A68 fractions

A68 fractions were prepared essentially by the procedure of Ksiezak-Reding *et al.* (1990) from brain tissue taken *post mortem* in four normal subjects (aged 61, 65, 66 and 72 years) and from five Alzheimer subjects (aged 60, 70, 81, 82 and 89 years). Brain tissue from the temporal cortex was homogenized (1 g/10 ml) on ice in 10 mM-Tris/HCl (pH 7.4/0.8 M-NaCl/1 mM-EDTA/10% (w/v) sucrose. The homogenate was centrifuged for 20 min at 15000 $g_{av.}$ and the supernatant was retained. The pellet was rehomogenized in the same volume and centrifuged as above. The two supernatants were combined and treated with 1% (w/v) Sarkosyl for 30 min at room temperature. This fraction was then centrifuged for 30 min at 60000 $g_{av.}$. The supernatant was used as a source of soluble tau proteins and the pellet, constituting the A68 fraction, was washed and resuspended in 50 mM-Tris/HCl, pH 7.5 (0.5 ml/g of starting tissue).

Portions of the A68 preparations were treated for 20 min at room temperature with SDS (2%, w/v, final) and centrifuged for 2 h at 20000 $g_{av.}$. The pellet and the supernatant of these SDS-treated A68 preparations were kept for Western blot analysis.

The A68 preparation, the supernatant from the A68 preparation containing normal tau proteins (see above) and the supernatant of SDS-treated A68 preparation were treated directly with calf intestinal alkaline phosphatase (Boehringer, 400 units/ml) in 50 mM-Tris/HCl, pH 8.3, 50 mM-NaCl, 1 mM-$MgCl_2$, 1 mM-$ZnCl_2$, 1 mM-phenylmethanesulphonyl fluoride, 10 μg of leupeptin/ml, for 16 h at 37 °C. After this dephosphorylation, the A68 preparation was centrifuged (60 min at 20000 $g_{av.}$) and the pellet was retained. Controls were performed by adding Na_2HPO_4/NaH_2PO_4, pH 8.3, to a final concentration of 0.2 M in the same incubation solution to inhibit alkaline phosphatase.

The samples were run on SDS/PAGE [10% (w/v) gels] (Laemmli, 1970) and electrophoretically transferred from gels to nitrocellulose membranes (Towbin *et al.*, 1979). Nitrocellulose membranes were blocked in semi-fat dried milk (10%, w/v) for 2 h at room temperature, and incubated for 18 h with the primary antibodies followed by goat anti-rabbit or anti-mouse immunoglobulins conjugated to alkaline phosphatase (Sigma) (Brion *et al.*, 1991).

The presence of PHF in these preparations at each step was checked by examining, by electron microscopy, portions adsorbed on formvar-coated grids and negatively stained with 1% (w/v) sodium/potassium phosphotungstate.

Immunocytochemistry

Isolated PHF present in A68 preparations and in A68 preparations treated with alkaline phosphatase were adsorbed on formvar-coated grids for electron microscopy and immunolabelled, as previously described (Brion *et al.*, 1985*a*, 1991). Briefly, grids were incubated for 18 h with the primary antibody, washed and then incubated for 2 h with anti-rabbit or anti-mouse immunoglobulins conjugated to colloidal gold particles (Janssens, Beerse, Belgium). After negative staining with 1% (w/v) sodium/potassium phosphotungstate, the grids were observed in a Zeiss EM 109 electron microscope at 80 kV.

RESULTS

Separation of normal tau and A68

Fig. 2 illustrates the labelling on a Western blot of normal soluble tau proteins in extracts from the temporal cortex of a control brain (track A) and a typical age-matched Alzheimer's disease brain (track C). The tau immunoreactivity in the A68 fraction, prepared from the same tissue sample as was used for the preparation of soluble tau, comprises three strong bands (track D). The uppermost band migrated more slowly than normal tau, and the other two bands migrated in the region of the upper bands of normal tau; these findings are in accordance with those of Ksiezak-Reding *et al.* (1990). An additional minor band migrating even more slowly than the major bands was also observed in the A68 fractions. The equivalent A68 fraction from a control brain contained very little tau immunoreactivity (track B). An A68 fraction made from the cerebellar cortex of an Alzheimer's disease brain also contained very little tau immunoreactivity (track E). Numerous PHF were found in the

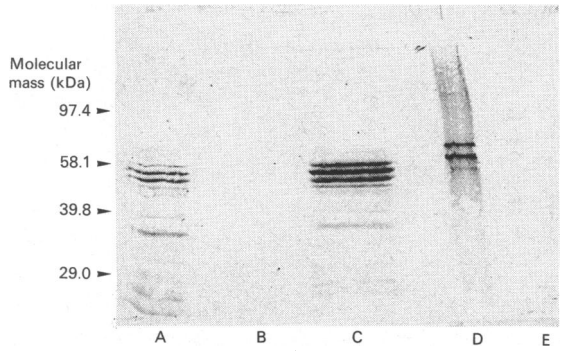

Fig. 2. Western blots with a polyclonal antiserum to tau (B19)

Track A, soluble tau from control human brain (temporal cortex); track B, pellet fraction (equivalent to A68 preparation) from control human brain (temporal cortex); track C, soluble tau from Alzheimer's disease brain (temporal cortex); track D, A68 fraction from Alzheimer's disease brain (temporal cortex), showing the A68 polypeptides; track E, A68 fraction from Alzheimer's disease brain (cerebellum). Tracks A and C show the normal tau bands. The A68 polypeptides are seen only in the A68 fraction from the Alzheimer case (track D). Only a very weak tau immunoreactivity was observed in the A68 fraction prepared from control brain (track B). The A68 polypeptides were not detected in the A68 fraction from Alzheimer's cerebellum (track E). The molecular mass markers used were phosphorylase *b* (97.4 kDa), catalase (58.1 kDa), alcohol dehydrogenase (39.8 kDa) and carbonic anhydrase (29.0 kDa).

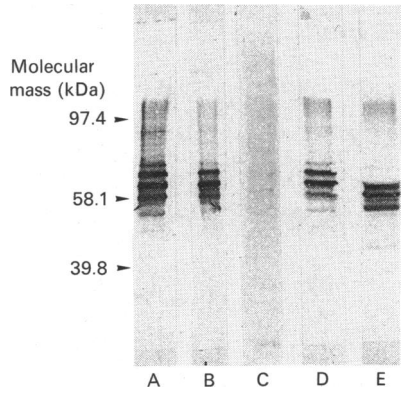

Fig. 4. Western blots with BR133, a polyclonal antiserum to a synthetic tau peptide

Track A, A68 preparation; B, A68 preparation treated with alkaline phosphatase; C, pellet of A68 preparation treated with SDS; D, supernatant of A68 preparation treated with SDS; E, the supernatant of A68 preparation treated with SDS followed by treatment with alkaline phosphatase.

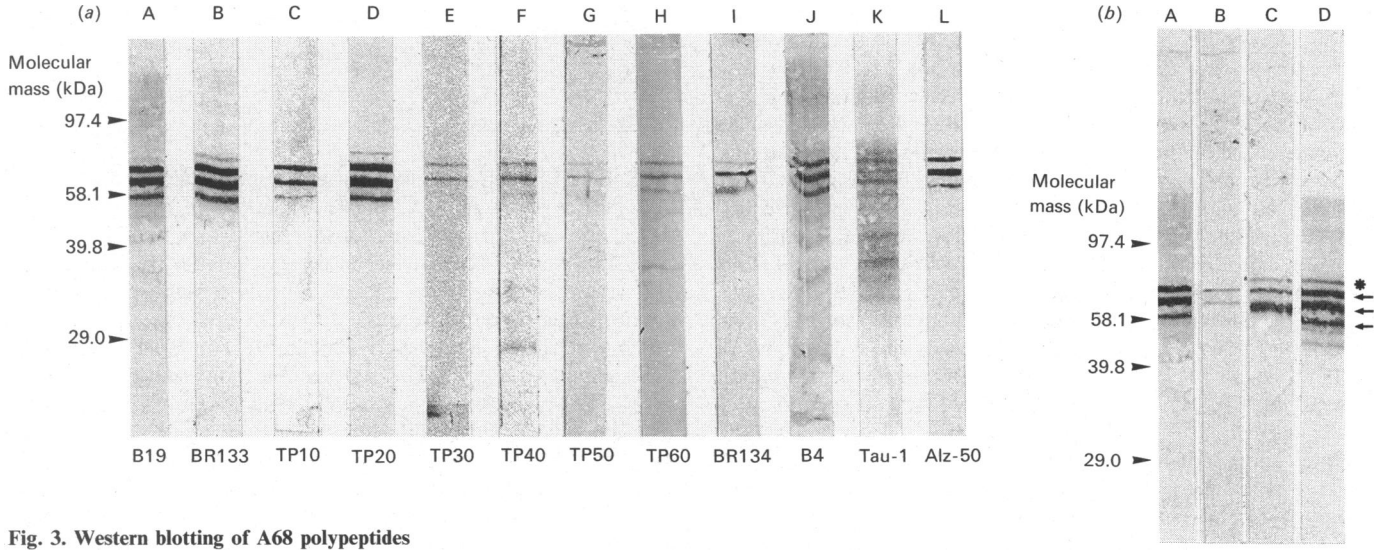

Fig. 3. Western blotting of A68 polypeptides

(*a*) Western blots of A68 preparation with various anti-tau antibodies recognizing the three major A68 polypeptides. For the Tau-1 antibody, the A68 preparation was treated with alkaline phosphatase before subjecting it to SDS/PAGE and blotting, since the Tau-1 antibody does not label A68 without dephosphorylation. Tracks B–I show blots obtained with antibodies to successively sequential epitopes from the *N*-terminus to the *C*-terminus of human tau. Blots A, B, G–J and L were made by transfer from the same gel and blots C–F were transferred from a different gel. (*b*) Western blots of an A68 preparation showing on adjacent tracks the pattern of labelling of A68 polypeptides with the B19 antiserum to tau (track A), the insert-specific antibody BR200 (B), the insert-specific antibody BR189 (C) and the antibody BR133 (D). Arrows point to the three major A68 polypeptides, and the asterisk shows a less intense band of apparent higher molecular mass. Molecular mass markers are as in Fig. 2.

Table 1. Immunolabelling in electron microscopy of isolated PHF present in the A68 fraction

The sequence of antibodies in the list is the sequence of the epitopes from the *N*-terminus to the *C*-terminus of tau. The labelling by Tau-1 required prior treatment with alkaline phosphatase. + + +, strong labelling of many PHF, + +, weaker labelling of many PHF.

Antiserum	PHF labelling
BR133	+ + +
Alz50	+ +
TP10	+ + +
TP20	+ +
BR200	+ +
BR189	+ +
TP30	+ +
Tau-1	+ + +
TP40	+ +
TP50	+ +
TP60	+ +
BR134	+ +

A68-containing fractions from Alzheimer's disease cases, but not in the equivalent fractions from controls. This fractionation and immunolabelling pattern of normal soluble tau and insoluble A68 was consistent for the four control and five Alzheimer's disease cases.

Mapping of tau epitopes in A68

Fig. 1(*a*) shows the amino acid sequences of the six known tau isoforms (Goedert *et al.*, 1989*a*); the sequences that were used for raising the specific anti-(tau peptide) antisera are indicated. Fig. 1(*b*) shows a schematic drawing of the six known human tau isoforms.

The labelling of A68 on Western blots by the various anti-(tau peptide) antisera is shown in Figs. 3(*a*) and 3(*b*). Epitopes that are present in all isoforms of tau and which span the complete tau molecule, including the *N*- and *C*-terminal sequences, are present in all three major A68 species (Fig. 3*a*); the intensity of labelling by some antibodies is weaker than others, but this reflects their known lower titres. The monoclonal antibody Alz50 labelled all three A68 species as described by others (Ksiezak-Reding *et al.*, 1990; Lee *et al.*, 1991). The blots in Fig. 3(*a*) were not all performed on the same SDS gel/polyacrylamide, so the positions of the bands vary slightly from track to track, but they have been arranged so that the corresponding bands are approximately aligned. The labelling of A68 species by the monoclonal antibody Tau-1 required the pretreatment of the A68 fraction with alkaline phosphatase, as reported (Ksiezak-Reding *et al.*, 1990; Lee *et al.*, 1991). The antisera specific for particular tau isoforms, determined by inserts 1 and 2 located towards the *N*-terminus of tau (BR189 and BR200), selectively labelled the upper two major A68 species (Fig. 3*b*). The lowermost major A68 polypeptide was not labelled by either of these antibodies. The species that migrated more slowly than the uppermost member of the A68 triplet was labelled, but usually only weakly, by antibodies recognizing all tau isoforms and also by BR189; antibody BR200 was not a high-titre reagent, and this probably explains why it did not label this band.

Dephosphorylation of A68 and tau

The A68 fractions were treated with alkaline phosphatase and centrifuged. The pellet contained PHF and the A68 polypeptides. Under the conditions described here, this treatment of A68 fractions with alkaline phosphatase before subjecting them to SDS/PAGE failed to change the electrophoretic mobility of the A68 polypeptides (Fig. 4, tracks A and B) in four Alzheimer cases, and induced only a minor increase of this mobility in one case. This may be because A68 is an aggregated form of tau, with restricted accessibility of the alkaline phosphatase. The A68 fraction was therefore treated with SDS and centrifuged. The pellet was shown by Western blotting to contain little, if any, A68 (Fig. 4, track C). The supernatant, containing solubilized A68 (Fig. 4, track D), was treated with alkaline phosphatase. This solubilized and alkaline phosphatase-treated A68 was found to migrate faster than untreated solubilized A68 (Fig. 4, tracks D and E). Treatment of solubilized A68 with alkaline phosphatase in the presence of 0.2 M-phosphate did not induce this faster migration of A68 (results not shown). Soluble tau from Alzheimer or control brains that was treated with alkaline phosphatase before subjecting it to SDS/PAGE migrated faster than untreated tau (results not shown), as noted earlier (Lindwall & Cole, 1984).

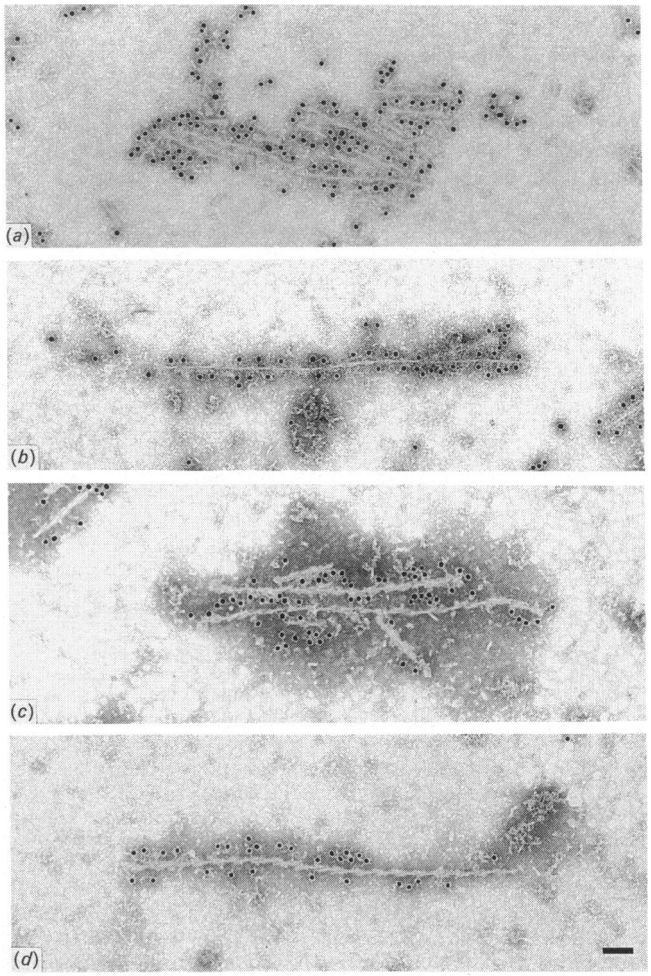

Fig. 5. Immunolabelling in electron microscopy of isolated PHF present in the A68 fraction

Labelling was carried out with (*a*) the TP10 antibody to tau peptide containing residues 9–18, (*b*) the BR134 antibody to tau peptide 428–411 (the most *C*-terminal amino acids), (*c*) the BR200 antibody to tau peptide 61–72 (specific for insert 1), and (*d*) the BR189 antibody to tau peptide 76–87 (specific for insert 2). Scale bar: 100 nm.

Immunoelectron microscopy of PHF in the A68 fraction

The panel of antibodies was used to label in the electron microscope the isolated PHF present in the A68 fraction (Fig. 5).

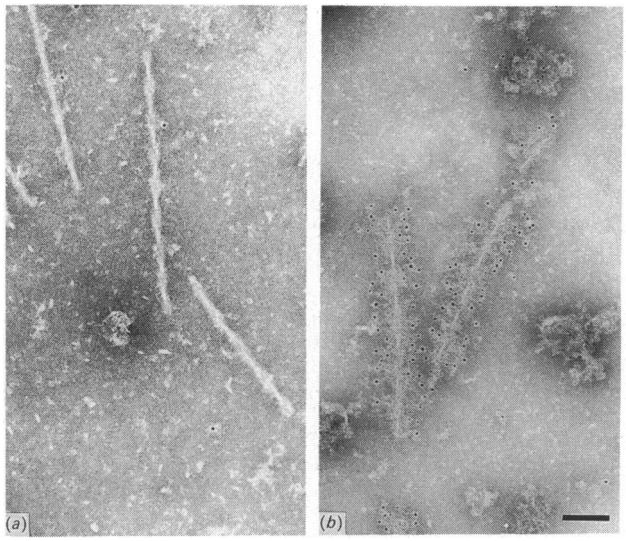

Fig. 6. Immunolabelling in electron microscopy antibody of isolated PHF with the monoclonal Tau-1

(*a*) A68 preparations, (*b*) A68 preparations treated with alkaline phosphatase. Scale bar: 100 nm.

The PHF were labelled as they were isolated or after pretreatment with alkaline phosphatase. All of the antibodies labelled the PHF, but with some variations in the intensity of labelling (Fig. 5 and Table 1). The insert-specific antibodies BR189 and BR200 gave weaker labelling than other antibodies to epitopes in the *N*-terminal half of tau (e.g. TP10) that recognize all tau isoforms. The labelling by Tau-1 was greatly enhanced by alkaline phosphatase treatment (Fig. 6).

DISCUSSION

A68 polypeptides are found in brains of subjects with Alzheimer's and some other diseases, but not in control brains (Wolozin *et al.*, 1986; Ksiezak-Reding *et al.*, 1990). Highly purified A68 preparations were recently observed to contain abundant PHF (Lee *et al.*, 1991), implying that A68 is a major constituent of PHF. Distinct tau proteins have also been shown to be a constituent of PHF (Greenberg & Davies, 1990), but despite immunological cross-reactivity between tau and A68, there has been some debate as to whether or not A68 is an abnormal form of tau (Nukina *et al.*, 1987; Ksiezak-Reding *et al.*, 1988; Vincent & Davies, 1990; Lee *et al.*, 1991). Furthermore, the question of whether the whole of the tau molecule, including all isoforms, is incorporated into PHF has not been answered.

In the present study we have confirmed that preparations of A68 proteins contain abundant PHF. These PHF were decorated by antibodies spanning the whole of the tau sequence, including antibodies specific for inserts 1 and 2. These latter antibodies labelled as many PHF as did other antibodies, indicating that different isoforms of tau are probably not responsible for forming different subpopulations of PHF, but rather that individual PHF contain a mixture of different isoforms.

The A68 proteins migrated on SDS/PAGE as a set of three strongly labelled bands, shown by using antibodies to tau, but there was also a less abundant and slower-migrating form. Bands below the major triplet were weaker and more variable. This pattern is very similar to Western blot patterns described by others (Ksiezak-Reding *et al.*, 1990; Lee *et al.*, 1991). Western blots of A68 have also shown that epitopes spanning the whole of tau are present in the A68 polypeptides and the monoclonal antibody Alz50, labelled A68 in a pattern indistinguishable from that produced by the anti-tau antibodies that label all isoforms of tau. We conclude that A68 is an abnormally migrating (on SDS/PAGE) but aggregated form of tau.

The epitope for antibody BR189 is located in insert 2, which is present in the two largest forms of normal tau (Goedert & Jakes, 1990), and the epitope for antibody BR200 is present in insert 1, which is present in the four largest tau isoforms (Goedert & Jakes, 1990). These two antibodies labelled the two largest major tau forms of A68. The smallest isoform of A68 was not labelled by either of these tau-isoform-specific antibodies.

These data suggest that the different A68 polypeptides are derived from particular tau isoforms. However, it is not yet possible to decide on the basis of the present findings if all six tau isoforms can be modified to produce A68 polypeptides, or if only three tau isoforms are converted to A68. It seems likely, however, that the smallest major A68 band is derived from tau lacking any inserts towards the *N*-terminus (inserts 1 and 2). The two major largest A68 species would contain both inserts 1 and 2 and may differ from each other by the presence either of three or four tubulin-binding sites. Protein sequencing studies have shown that both three- and four-tubulin-binding site forms of tau are present in isolated PHF fractions (Kondo *et al.*, 1988; Wischik *et al.*, 1988*a*), but it cannot be established from these separate studies how the number of tubulin-binding sites is related to tau forms in A68. An additional minor band which migrated more slowly than the major A68 triplet polypeptides was detected with BR189 in A68 preparations, and might correspond to another tau isoform containing insert 2. However, this minor band was not obviously labelled by antibody BR200 as would be predicted from the pattern of insertions in tau, i.e. isoforms containing insert 2 always contain insert 1 (Goedert *et al.*, 1989*a*). The lack of labelling by BR200 is possibly because the staining by this antibody is weaker than that by BR189, rather than indicating a novel splicing of tau in Alzheimer's disease.

Treatment of tau with alkaline phosphatase is known to increase its mobility on SDS/PAGE (Lindwall & Cole, 1984). We and others have shown that abnormal forms of tau in Alzheimer's disease and Down's syndrome brain extracts have lower mobility on SDS/PAGE than normal tau, but this mobility can be shifted into the normal tau mobility range by alkaline phosphatase treatment of the tau following solubilization in SDS (Flament *et al.*, 1989; Hanger *et al.*, 1991). A68 has been reported not to shift on SDS/PAGE following treatment with alkaline phosphatase (Ksiezak-Reding *et al.*, 1990; Vincent & Davies, 1990). We found that the mobility of A68 on SDS/PAGE was increased by alkaline phosphatase treatment under the conditions described here only after the A68 had been solubilized in SDS. This indicates that the lack of effect of alkaline phosphatase on A68 mobility is probably due to the aggregated state of A68.

The labelling of neurofibrillary tangles or PHF by the Tau-1 antibody has been shown to be dependent on prior dephosphorylation (Grundke-Iqbal *et al.*, 1986*b*; Kosik *et al.*, 1988). We observed that treatment of A68 preparations with alkaline phosphatase induced unmasking of the Tau-1 epitope on PHF found in these preparations. This particular treatment of A68 preparations with alkaline phosphatase does not, however, affect A68 mobility on SDS/PAGE, as discussed above, and so the results suggest that dephosphorylation at a site different from the Tau-1 epitope is necessary to change the mobility of A68 on SDS/PAGE.

Apart from the Tau-1 epitope [localized within amino acids 189–207 of tau (Kosik *et al.*, 1988)] and a site close to the *N*-

terminus of tau (Iqbal *et al.*, 1989), Ser^{396} has been recently reported to be phosphorylated in A68 but not in normal human tau (Lee *et al.*, 1991). It is, however, unknown if the abnormal phosphorylation of Ser^{396} accounts for the electrophoretic behaviour of A68 on SDS/PAGE. The phosphorylation of Ser^{416} (human sequence) by a Ca^{2+}/calmodulin-dependent protein kinase has been reported to decrease the electrophoretic mobility of normal tau, although it has not been demonstrated that this shift is sufficient to move tau into the A68 position on SDS/PAGE (Baudier & Cole, 1987; Steiner *et al.*, 1990). Nevertheless, this residue is another potential site of abnormal phosphorylation in A68.

In summary, our results further support the notion that PHF contain abnormally phosphorylated tau but that this abnormal tau is in an aggregated state. The abnormally phosphorylated tau is derived from the intact tau molecules and includes several tau isoforms, rather than a single isoform, in a range of abnormally phosphorylated states.

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