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Monoclonal antibodies with selective specificity for Alzheimer Tau are directed against phosphatase-sensitive epitopes

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Summary. A modified form of the microtubule-associated protein Tau is the major component of the paired helical filaments (PHF) found in Alzheimer's disease. The characterization of these posttranslational Tau modifications is hindered by the lack of sufficient PHF-Tau-specific markers. Here we describe several monoclonal antibodies, prepared by immunization with PHF, two of which showed a selective specificity for PHF-Tau without cross-reactivity with normal Tau. Epitope recognition by these two monoclonals was sensitive to alkaline phosphatase treatment. In Western blotting these monoclonal antibodies reacted specifically with the abnormally phosphorylated epitopes on Alzheimer's disease-associated PHF-Tau. One of the new antibodies can be used for the construction of a sandwich enzyme-linked immunosorbent assay for the specific detection of PHF-Tau without cross-reactivity to normal Tau proteins.

Key words: Alzheimer's disease – Tau – Phosphorylation – Monoclonal antibodies

The neurofibrillary tangles (NFT) of Alzheimer's disease are ultrastructurally composed of paired helical filaments (PHF) [16]. A modified form of the microtubule-associated protein Tau is believed to be a major structural component of the PHF [2, 9, 18, 19, 32]. This PHF-Tau shares many epitopes with normal Tau proteins, but also shows several distinct properties such as a lower mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a different isoelectric charge [5, 21, 31]. Several groups have found evidence for excessive or aberrant phosphorylation as being the major modification in the PHF-Tau [5, 11, 14, 15, 23], which is variously known as Tau 64 and 69 [3, 5] or A68/Alz50 [21, 23, 31]. Tau 69 and A68 are apparently the same protein [4]. The differences in phosphorylation between normal and abnormal Tau are still not completely elucidated and the characterization is hindered by a lack of sufficiently specific markers. However, the localization of these phosphorylation sites and the identification of the kinases involved are crucial for a better understanding of the formation of PHF in Alzheimer's disease.

The monoclonal antibodies described in this study confirm the presence of normal, as well as abnormal, Tau epitopes in PHF-Tau. We also report the isolation of two monoclonal antibodies with selective specificity for PHF-Tau without showing cross-reactivity with normal Tau epitopes. These antibodies are directed against alkaline phosphatase-sensitive epitopes and are, therefore, useful tools for the study of abnormal phosphorylation in PHF-Tau.

Materials and methods

Antigen preparation

PHF-Tau was partially purified by a modification of the method of Greenberg and Davies [10]. Briefly, postmortem tissue consisting mostly of gray matter from the frontal and temporal cortex was obtained from histologically confirmed Alzheimer's disease patients. This Alzheimer gray matter brain sample (5–10 g) was homogenized with 10 vol cold buffer H (10 mM TRIS/1 mM EGTA/0.8 M NaCl/10 % sucrose, pH 7.4) in a Teflon/glass Potter S (Braun, Germany) homogenizer. After centrifugation in a 60 Ti MSE rotor at 27,000 *g* for 20 min at 4 °C, the pellet was removed and the supernatant was adjusted to 1 % (wt/vol) *N*-lauroylsarcosine and 1 % (vol/vol) 2-mercaptoethanol and incubated while rotating on a mixer 820 (Swelab, Sweden) for 2.5 h at 37 °C. The supernatant mixture was centrifuged at 108,000 *g* for 35 min at 20 °C. The PHF-containing pellet was gently washed with PBS and finally suspended in 1 ml of the same buffer. The antigen preparation was evaluated in 10 % SDS-PAGE and in immunoblotting with the previously characterized polyclonal rabbit anti-human normal Tau antiserum [24].

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Immunization and fusion

BALB/c mice were primed subcutaneously with 100 μg partially purified PHF-Tau in complete Freund's adjuvant and boosted intraperitoneally three times at 3-week intervals with 100 μg of the same antigen in incomplete Freund's adjuvant. On days 3 and 2 before the fusion, mice were boosted intraperitoneally with 100 μg PHF-Tau in saline. Mouse spleen cells were fused with SP2/0 cells by a modified procedure of Köhler and Milstein [17]. The wells were screened after 12 days for specific antibody production and positive cells were subcloned immediately. Hybridomas were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum, sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and nonessential amino acids. All products were purchased from Gibco, Paisley, UK.

Antibody characterization

Sandwich ELISA for antibody screening. The screening ELISA used for the detection of anti-Tau monoclonal antibodies was a sandwich ELISA system with polyclonal rabbit anti-human Tau antibodies in the coating phase. For this purpose polyclonal rabbit anti-human Tau serum was affinity purified. Purified human normal Tau, prepared as described [24], was used for the preparation of an immuno-affinity column using cyanogen bromide-activated Sepharose (Pharmacia, Uppsala, Sweden). The affinity-bound anti-Tau fraction was eluted from this column with a 0.1 M citric acid buffered solution at pH 2.5. After neutralization, the anti-Tau-containing fractions were pooled and coated overnight at 1 μg/ml at 4 °C on high-binding microtiter plates (Nunc, Gibco) in coating buffer (10 mM TRIS, 10 mM NaCl, 10 mM NaN_3, pH 8.5). After overcoating for 30 min with 125 μl 10%-saturated casein in PBS to reduce nonspecific binding, the plates were incubated with 100 μl of a PHF-Tau preparation at 500 ng/ml and incubated for 60 min at 37 °C. The plates were washed three times with PBS-0.05% Tween 20 (v/v), and 100 μl hybridoma supernatant was added and incubated for 1 h at 37 °C. After washing, the bound monoclonal antibodies were detected with peroxidase-conjugated rabbit anti-mouse serum (Dakopatts, Glostrup, Denmark). All reagents were diluted in PBS with 10% casein. After final washing, 100 μl 0.42 mM 3,5,3′,5′-tetramethylbenzidine containing 0.003% H_2O_2 v/v in 100 mM citric acid, 100 mM disodium hydrogen phosphate, pH 4.3, was added as peroxidase substrate. The reaction was stopped with 50 μl 2 M H_2SO_4. Absorbance was read in a Titertek Multiscan (Flow Laboratories, Eflab, Oy, Finland) at 450 nm.

The cross-reactivity of the monoclonal antibodies with normal Tau in ELISA was tested in a sandwich ELISA identical to the screening assay, except that affinity-purified normal Tau was used instead of PHF-Tau.

Immunoblots. After 10% SDS-PAGE according to Laemmli [22], purified normal human Tau and PHF-Tau were transferred to nitrocellulose (Hybond-C, Amersham, Brussels, Belgium) in 10 mM $NaHCO_3$, 3 mM Na_2CO_3, pH 9.9 for 120 min at 55 V with cooling. After blotting, the nitrocellulose was equilibrated with PBS, and protein binding sites were blocked with blot buffer (PBS supplemented with 5% w/v skimmed dried milk and 10% v/v newborn calf serum). Blotted proteins were incubated with primary antibody overnight at 4 °C and detected with horseradish peroxidase-labeled rabbit anti-mouse immunoglobulins (Dakopatts). Diaminobenzidine was used as substrate.

Phosphatase treatment. ELISA detection with and without prior dephosphorylation and phosphatase treatment of PHF-Tau on immunoblot and tissue sections were performed using the method of Sternberger and Sternberger [29]. For ELISA and Western blot, dephosphorylation of the antigen was performed using 20 U/ml bovine intestinal mucosa alkaline phosphatase type VII-L (Sigma, Poole, UK) for 60 min at 37 °C in 10 mM TRIS, 0.5 mM $MgCl_2$. For immunocytochemistry, sections were pretreated with 400 μg/ml trypsin in 0.05 M TRIS buffer pH 7.6 containing 0.03 M NaCl and 0.02 M $CaCl_2$ for 10 min at 37 °C. Subsequently they were treated for 24 h with 400 μg/ml bovine intestinal mucosa alkaline phosphatase type VII-L (Sigma) in 0.1 M TRIS buffer pH 8.0 containing 0.01 M phenylmethylsulfonylfluoride. After incubation with the appropriate AT antibody they were immunostained using the avidin-biotin complex (ABC) method [12].

Immunocytochemistry. Paraffin sections of formalin-fixed brain tissue from neocortex, hippocampus, cerebellum, pons, and spinal cord of several Alzheimer's disease patients and age-matched controls were prepared, as well as were sections of peripheral nerve from one control. Cryostat sections from Alzheimer and age-matched control brain were post-fixed in acetone for 10 min at room temperature. Tissues were immunostained with the ABC technique [12] using Amersham (UK) reagents as described previously [28].

Human brain homogenates for PHF-Tau detection

Postmortem brain tissue from frontal cortex, derived from histopathologically confirmed Alzheimer's disease patients and from age-matched control patients, was homogenized 1/5 (w/v) in buffer A as described [24] and stored at −70 °C. The homogenates were further diluted in blot buffer to a final dilution of 1/200 and 1/1000 before use in the PHF-Tau specific ELISA.

ELISA for PHF-Tau detection

This sandwich ELISA was performed as described for normal Tau detection [24], except that the PHF-Tau specific AT8 monoclonal antibody instead of the Tau-specific BT2 monoclonal antibody was used for coating. The polyclonal rabbit anti-Tau antiserum produced against affinity-purified human Tau [24] was used as detecting antibody and horseradish peroxidase-conjugated donkey anti-rabbit serum as conjugate. As incubation buffer to reduce non-specific binding and for diluting reagents and brain homogenates, we used blot buffer instead of the 10%-saturated casein in PBS as described [24].

Other methods

Human neurofilament proteins for use in solid-phase ELISA were purified by the method of Hui et al. [13].

Results

Characterization of the antigen and reactivity of the isolated monoclonal antibodies in ELISA and Western blot

SDS-PAGE analysis of the PHF-enriched fractions used for immunization mainly showed the presence of three proteins of apparent molecular masses ranging between 57 and 68 kDa. Their molecular masses and reactivities with a polyclonal anti-Tau serum characterizes these proteins as PHF-Tau (Fig. 1). Minor contamination with other proteins, probably not PHF-associated, was present in both low and high molecular weight ranges. Because of the Tau specificity of the screening assay, these proteins did not interfere with the detection of

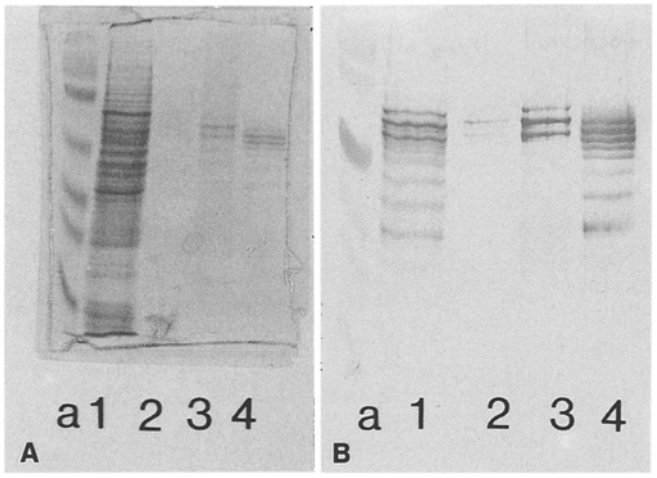

Fig. 1A,B. Evaluation in 10% SDS-PAGE of the paired helical filament (PHF)-Tau preparation used for immunization. **A** Coomassie blue staining of the gel. **B** Immunoblot with polyclonal rabbit anti-human normal Tau antiserum. *Lane a:* Prestained molecular weight markers. From top to bottom: 106, 80, 50, 32.5, and 27.5 kDa. *Lane 1:* Supernatant of the final centrifugation in the preparation of PHF-Tau. *Lane 2:* Wash of the PHF-Tau pellet. *Lane 3:* PHF-Tau pellet after resuspension. *Lane 4:* Affinity-purified normal human Tau. Note the differences in apparent molecular masses of the PHF-Tau (*lane 3*) and the normal human Tau isoforms (*lane 4*)

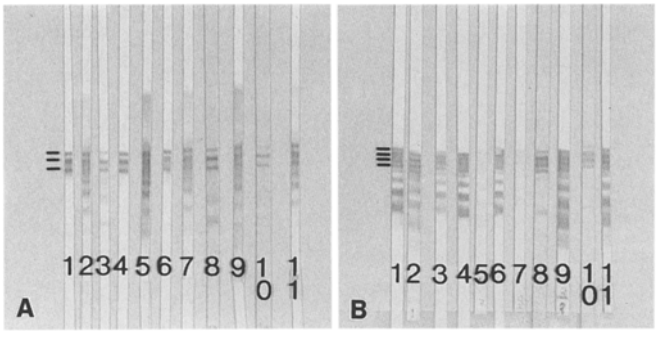

Fig. 2. Immunoblot of PHF-Tau (**A**) and normal human Tau (**B**) with AT monoclonal antibodies. *Lanes 1 to 11* are, respectively, stained with the antibodies AT1, AT3, AT4, AT6, AT8, AT9, AT10, AT11, AT12, AT13 and AT14. Note the absence of staining with AT8 (**B,** *lane 5*) and AT10 (**B,** *lane 7*) of normal Tau isoforms

Table 1. Immunocytochemistry results on paraffin sections for pathological and normal structures

Antibody	Alzheimer NFT	Alzheimer plaque neurites	Control normal axons
AT 3	+++	++	+++
AT 8	++++	++++	–
AT 10	++++	++++	++
AT 11	++	+	–
AT 12	+++	++	–
AT 13	+	+	–
AT 14	++	+	–

NFT, Neurofibrillary tangles

anti-PHF-Tau monoclonal antibodies. After screening with a Tau-specific sandwich ELISA, 11 hybridomas were selected, all belonging to the IgG1 κ subclass, which reacted with the PHF-Tau triplet in Western blotting (Fig. 2A). The supernatants of these cultures were also tested for reactivity with normal Tau in ELISA and Western blot (Fig. 2B). In ELISA, seven antibodies (AT1, AT4, AT6, AT9, AT11, AT12, and AT14) reacted with both normal and PHF-Tau. The antibodies AT3, AT13, AT8 and AT10 reacted only weakly or negatively when tested against normal Tau in ELISA (data not shown). Of these, AT3 and AT13 show a clear reaction with normal Tau in Western blotting experiments, leaving only AT8 and AT10 as truly PHF-Tau-specific monoclonal antibodies both in Western blot and in ELISA.

Immunocytochemical characterization

All antibodies were tested for in situ reactivity on paraffin and cryostat sections of formalin-fixed brain tissue from Alzheimer's disease patients and age-matched controls. Four antibodies showed no reaction on Alzheimer or control brain tissue, suggesting they were directed against fixation-sensitive epitopes. The other seven produced clearly positive immunostaining on tissue sections (Table 1).

On the basis of these results the antibodies AT3, AT8 and AT10 were selected for further study. AT3 recognized both pathological and normal structures. Normal axons were strongly labeled in many parts of the CNS (Fig. 3B) as well as in peripheral nerves (data not shown), conforming to the normal distribution of Tau as previously described [1, 30]. In Alzheimer tissue, NFT and senile plaque (SP) neurites were labeled (Fig. 3A), indicating that the AT3 epitope was also present in pathological structures.

Fig. 3A–D. Immunocytochemistry micrographs of paraffin sections. **A** Section through hippocampus of an Alzheimer's disease patient showing positive neurofibrillary tangles (NFT) and senile plaque neurites demonstrated with AT3. **B** Nuclei pontis of a non-Alzheimer control showing AT3-positive axons in transversal and longitudinal section. **C** Hippocampus of an Alzheimer patient, showing AT10-positive NFT (*arrow*), neuropil threads, granulovacuolar degeneration (*solid arrowhead*) and perinuclear staining (*hollow arrowhead*). **D** Nuclei pontis of a non-Alzheimer control showing AT10-positive axons in transversal and longitudinal section. **A,B,D** × 195, **C** × 245

Fig. 4.A–D. Immunocytochemistry micrographs of paraffin sections. Section through hippocampus of an Alzheimer's disease patient stained with AT8. **A** General view with positive NFT and senile plaques. Note the contrast between white matter (*right*) and gray matter (*left*), due to abundant positive neuropil threads in gray matter. **B** Higher magnification showing positive NFT and senile plaque neurites. **C** Close-up of a neuron bearing a positive NFT. **D** Close-up of a senile plaque showing the negative amyloid core surrounded by positive neurites. **A** × 98, **B** × 637, **C,D** × 987

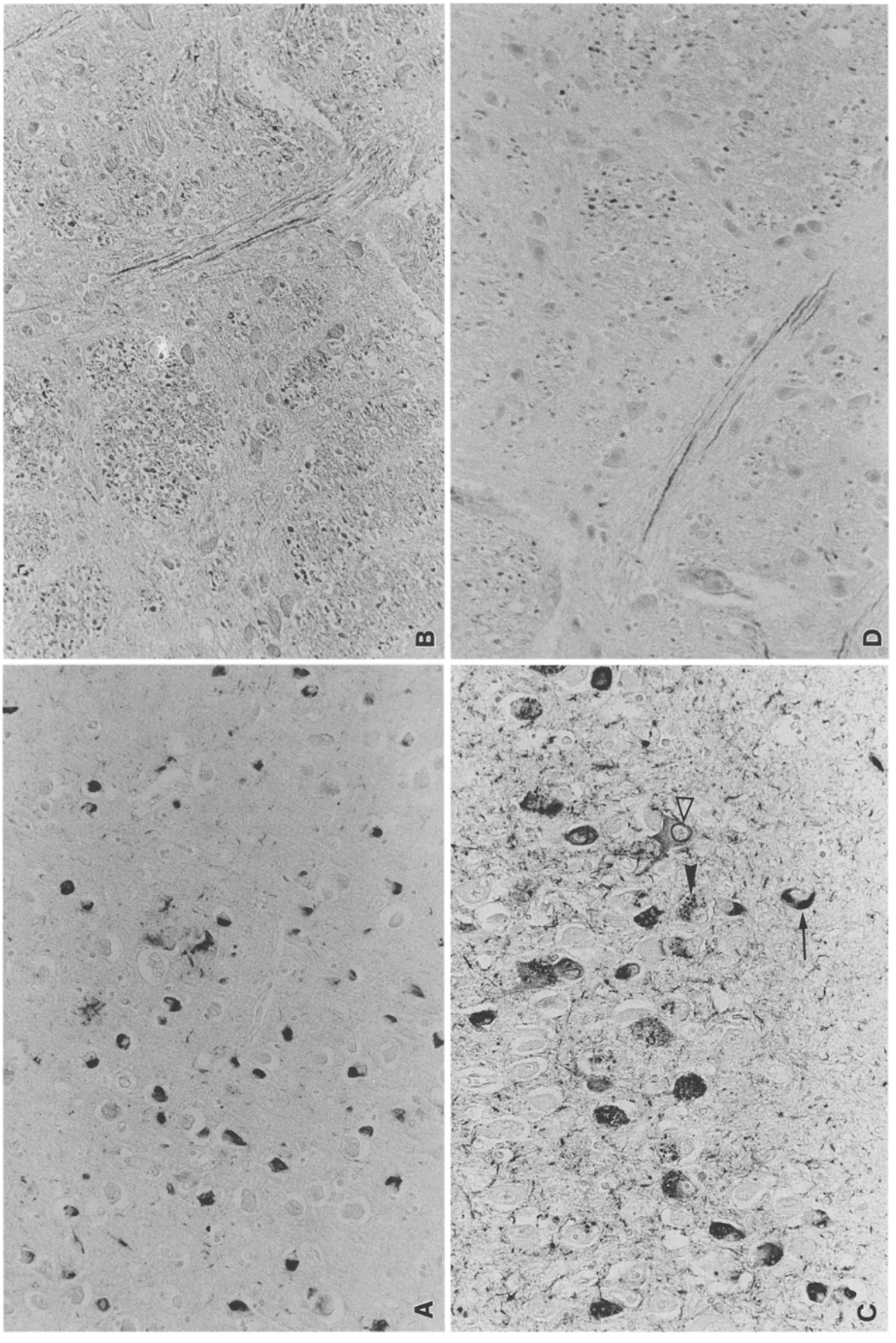

Fig. 3

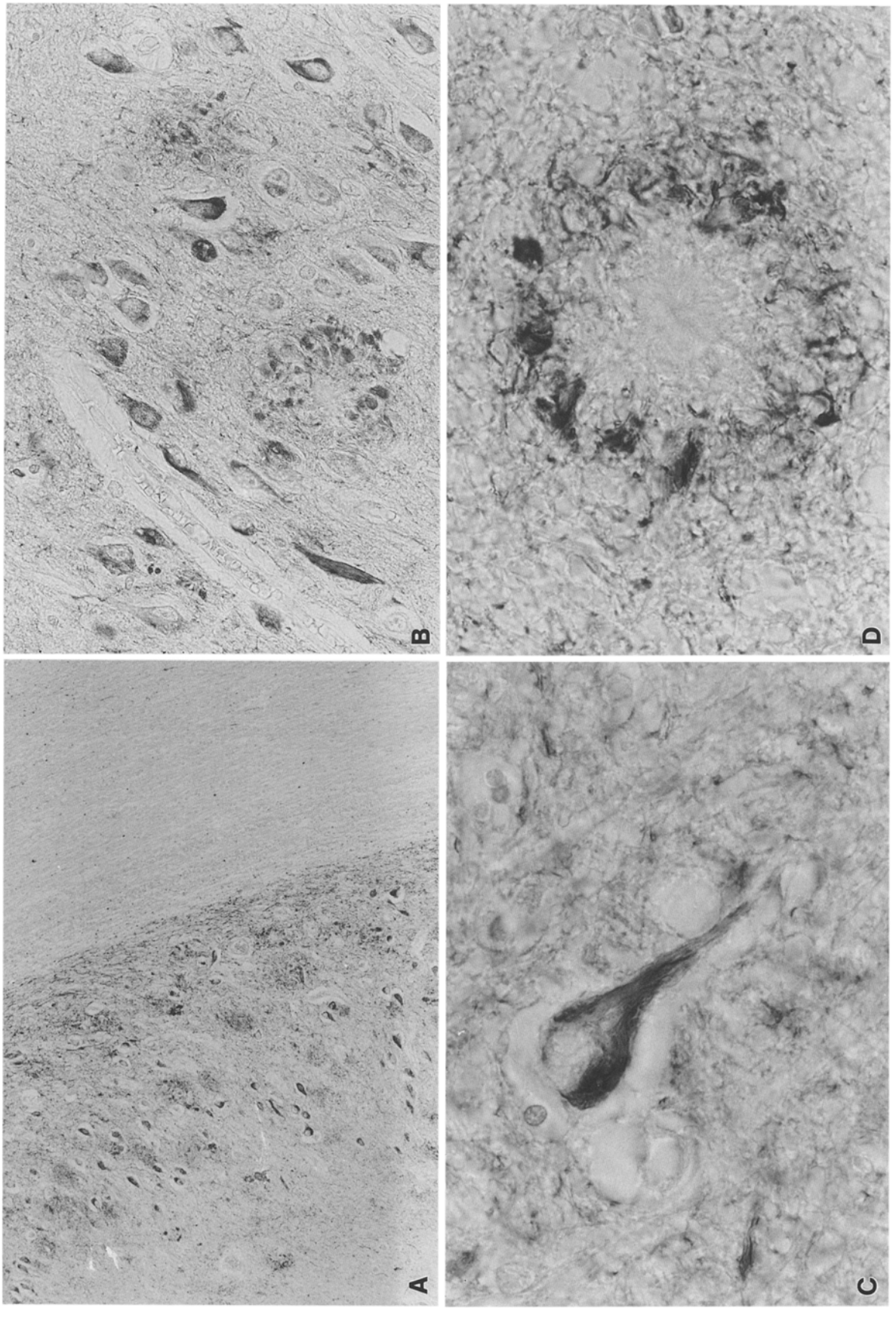

Fig. 4 (for legend see next page)

Using AT8 no normal structures could be visualized, but abundant staining could be shown of Alzheimer-specific pathology, including NFT and neurites, both in SP and dispersed in the neuropil ('neuropil threads') (Fig. 4A–D). The amyloid core of SP remained unstained (Fig. 4D). Cortical neurons often exhibited a strong perinuclear staining (not shown). Granulovacuolar degeneration (GVD) in hippocampal neurons, however, was negative (data not shown).

AT10 reacted with normal axons in formalin-fixed control sections (Fig. 3D). On cryostat sections some nuclei were stained, as well as some microglial cells. It is not clear whether this represents an aspecific reaction (data not shown). In Alzheimer brain NFT, SP neurites and neuropil threads were labeled as with AT8, and the same perinuclear staining was observed (Fig. 3C). Contrary to AT8, however, GVD in hippocampal neurons was strongly positive (Fig. 3C), reminiscent of the reactivity of our previously described anti-PHF antibody NFT200 [8].

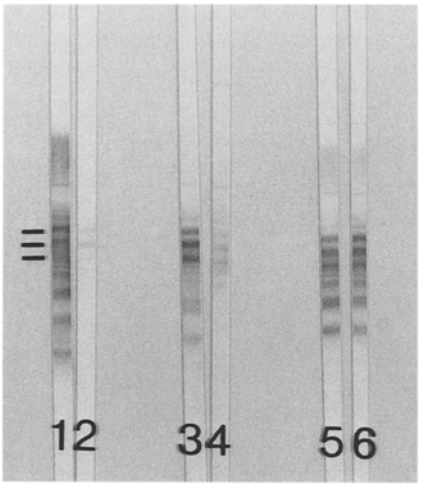

Fig. 6. Immunoblot of PHF-Tau with the monoclonal antibodies AT8, AT10 and AT14 without (*lanes 1,3 and 5*) and with (*lanes 2,4 and 6*) phosphatase treatment of the antigen after blotting

Phosphatase treatment

All antibodies were tested for the phosphatase sensitivity of their epitopes in ELISA, Western blotting, and immunocytochemistry. The signals of AT8 and AT10 were almost completely abolished after alkaline phosphatase treatment of the antigen in ELISA (Fig. 5), in Western blot (Fig. 6) and on tissue sections (data not shown). The reactivity of the other antibodies was insensitive to phosphatase treatment (data not shown).

Cross-reaction with human neurofilament proteins

All AT antibodies were examined for their reaction in Western blot with homogenates of Alzheimer brain cortex to investigate their reactivity with other brain proteins. They consistently showed a specific reaction with the PHF-Tau triplet, but normal Tau detection was weak or absent (data not shown). No other brain proteins were detected except by the AT3 antibody which, in addition to the PHF-Tau triplet, also stained a protein of higher molecular mass. This band is in the M_r range of the 145-kDa medium-sized neurofilament subunit. Therefore, we investigated the possibility of cross-reaction with purified human neurofilament proteins of all AT antibodies in a solid-phase ELISA. Only the AT3 antibody gave a positive reaction in this ELISA. The AT3 band seen in Western blots of brain homogenates is, thus, probably due to a cross-reaction of this antibody with the medium-sized neurofilament subunit.

Sandwich ELISA for the detection of PHF-Tau in brain homogenates

The AT8 antibody was selected as the coating antibody for the construction of a PHF-Tau-specific sandwich ELISA. This ELISA was able to discriminate brain homogenates of histopathologically confirmed Alzheimer patients from aged-matched controls. In the four Alzheimer brain homogenates we tested, the PHF-Tau could easily be detected with this ELISA up to a final dilution of 1/1000. The three control brain homogenates showed no signal even at the lower dilution of 1/200 (Fig. 7).

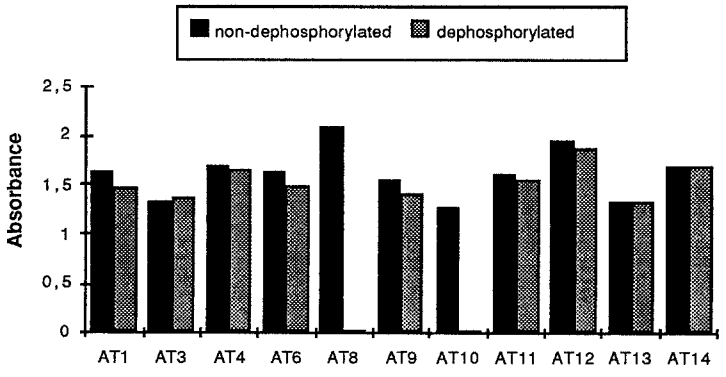

Fig. 5. Reaction in ELISA of the AT monoclonal antibodies with dephosphorylated and untreated PHF-Tau. All antibodies were tested in duplicate and the data presented as absorbance represent the mean of two values

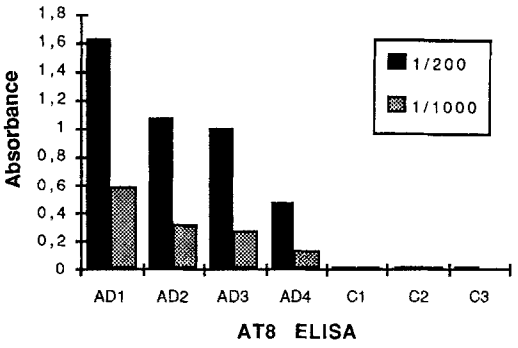

Fig. 7. Reaction of different dilutions (1/200 and 1/1000) of brain homogenates from Alzheimer's disease patients (*AD*) and age-matched controls (*C*) in the AT8-based PHF-Tau-specific ELISA. All dilutions are tested in duplicate and the data presented as absorbance represent the mean of two values

Discussion

Evidence is accumulating that hyperphosphorylation is a major posttranslational modification of the Tau proteins incorporated in PHF [5, 11, 14, 15, 23]. So far, only a few monoclonal antibodies have been described that recognize unique PHF epitopes, but none of these are directed against phosphatase-sensitive epitopes [25, 33]. Their usefulness for the localization of the abnormal phosphorylation sites is, therefore, limited. Results obtained with polyclonal antibodies against synthetic Tau peptides [23] suffer from the inherent difficulty of batch variation in the polyclonal antisera, and their polyclonal nature entails reactivity against several distinct epitopes, thereby increasing the risk of cross-reaction with normal Tau epitopes. In this study we describe two monoclonal antibodies, AT8 and AT10, that show no cross-reactivity with normal Tau in Western blot and ELISA; only AT8 shows complete specificity in situ by immunocytochemistry. Both antibodies are directed against phosphatase-sensitive epitopes which provides additional evidence that abnormal phosphorylation is a major modification in the Tau proteins incorporated in PHF.

Immunocytochemical evaluation of anti-Tau antibodies is often difficult. Most normal Tau epitopes are destroyed by fixation except when incorporated in PHF [27]. Most antibodies described in this study thus fail to show a normal Tau reaction in control brain tissue, although many react with normal Tau as well as with PHF-Tau in Western blot. AT3 and, surprisingly, also AT10, are the only antibodies that show reactivity on normal axons. Cross-reactivity with a human neurofilament epitope is a possible explanation for AT3. The reactivity of AT10 is more puzzling, since this antibody shows no cross-reaction with normal Tau proteins nor with neurofilament proteins in ELISA and Western blot experiments. The similarity between the hippocampal staining pattern of AT10 and that of NFT200, a PHF-specific antibody thought to be associated with neurofilament proteins [8] suggests a possible role of neurofilament proteins, perhaps due to modification of a phosphorylated neurofilament epitope by fixation into an AT10-like epitope. The cross-reaction between certain phosphorylated neurofilament epitopes and PHF-Tau epitopes is indeed well documented [20, 26]. Thus, while the observed reaction of AT3 and AT10 with normal axons conforms to the described distribution of normal Tau in the CNS [1, 30], the possibility that this reaction is due to neurofilament cross-reactivity of these antibodies cannot be dismissed.

Using a sandwich ELISA system based on the selective reaction of AT8 with pathological Tau, we were able to distinguish Alzheimer brain homogenates from control brain homogenates. The specificity of this enzyme immunoassay is comparable with the sandwich ELISA based on Alz50 developed by Ghanbari et al. [6, 7]. However, because of the cross-reaction of Alz50 with normal Tau, the specificity of the latter depends entirely on the specificity and reproducibility of the polyclonal antibody. In our assay the coating antibody ensures the desired specificity while detection is carried out either with other monoclonal antibodies directed against other Tau epitopes or with polyclonal anti-Tau antibodies raised against normal human Tau [24]. In view of the strong correlation between abnormally phosphorylated PHF-Tau-reactive NFT tangles and clinical dementia of the Alzheimer type, AT8 may provide the basis for an Alzheimer's disease diagnostic test.

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