

Detection of τ Proteins in Normal and Alzheimer's Disease Cerebrospinal Fluid with a Sensitive Sandwich Enzyme-Linked Immunosorbent Assay

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Abstract: Alzheimer's disease is a progressive degenerative dementia characterized by the abundant presence of neurofibrillary tangles in neurons. This study was designed to test whether the microtubule-associated protein τ , a major component of neurofibrillary tangles, could be detected in CSF. Additionally, we investigated whether CSF τ levels were abnormal in Alzheimer's disease as compared with a large group of control patients. We developed a sensitive sandwich enzyme-linked immunosorbent assay using AT120, a monoclonal antibody directed to human τ , as a capturing antibody. With this technique, the detection limit for τ was less than 5 pg/ml of CSF. Using AT8, which recognizes abnormally phosphorylated serines 199–202 in τ , the detection limit was below 20 pg/ml of CSF. However, with AT8, we found no immunoreactivity in CSF, suggesting that only a small fraction of CSF τ contains the abnormally phosphorylated AT8 epitope. Our results indicate that CSF τ levels are significantly increased in Alzheimer's disease. Also, CSF τ levels in a large group of patients with a diversity of neurological diseases showed overlap with CSF τ levels in Alzheimer's disease. **Key Words:** Abnormally phosphorylated τ —Dementia—Diagnostic marker—Microtubule-associated proteins—Monoclonal antibody—Paired helical filaments— τ protein.

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Alzheimer's disease (AD) is the most common form of dementia in adults and is diagnosed clinically, primarily by exclusion of other forms of dementia. Neuropathologically, the illness is characterized by the presence of senile plaques and neurofibrillary tangles (NFT). NFT are neuronal inclusions that consist of paired helical filaments (PHF), of which the main protein component is a modified form of the microtubule-associated protein τ (Brion et al., 1985). Under normal circumstances, τ promotes microtubule assembly and stability (Weingarten et al., 1975). τ present in PHF, called PHF- τ , is abnormally phosphorylated (Lee et al., 1991). This abnormal phosphoryla-

tion causes a conformational change in τ , probably resulting in self-association and formation of PHF. PHF- τ is phosphorylated at several sites. We recently developed a monoclonal antibody (mAb), called AT8, that specifically recognizes phosphoserines 199 and/or 202, found only in PHF- τ (Biernat et al., 1992; Goedert et al., 1992).

Previously, we reported the development of a τ -specific sandwich enzyme-linked immunosorbent assay (ELISA) with a lower detection limit of 1 ng/ml (Mercken et al., 1992b). By using this ELISA, based on the selective reaction of AT8 with PHF- τ , we were able to distinguish Alzheimer brain homogenates from control brain homogenates (Mercken et al., 1992b). However, the assay using AT8 was not able to detect τ in CSF. Therefore, we developed a more sensitive τ -specific catalyzed reporter deposition (CARD) ELISA with a higher sensitivity, using a new mAb directed to human τ called AT120. CARD involves an analyte-dependent reporter enzyme (horseradish peroxidase), which catalyzes the deposition of additional reporter molecules (biotin-labeled phenols) on the surface in a solid-phase immunoassay. This extremely sensitive assay allows the detection of τ protein in nonconcentrated CSF. We used the

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Abbreviations used: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; CARD, catalyzed reporter deposition; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; NFT, neurofibrillary tangles; OND, other neurological diseases; PBS, phosphate-buffered saline; PHF, paired helical filaments.

CARD ELISA assay to investigate whether the microtubule-associated protein τ could be detected in the CSF of AD and control patients.

MATERIALS AND METHODS

CSF samples

CSF samples from 190 patients at the Department of Neurology of the University Hospital of Antwerp were obtained by lumbar puncture performed for routine diagnostic purposes. In addition, CSF samples from seven patients with AD, three patients suffering from amyotrophic lateral sclerosis (ALS), and seven patients suffering from other neurological diseases (OND) were obtained from Athena Neurosciences Inc. (South San Francisco, CA, U.S.A.). All CSF samples were frozen and kept at -75°C in small aliquots until use.

Basically, patients were divided into three different groups: 27 patients diagnosed with probable AD according to McKhann et al. (1984), 41–88 years of age (mean 67); 51 mentally intact control patients, who underwent lumbar puncture for radiculography, 5–80 years of age (mean 44); and 129 patients suffering from OND, 1 month to 85 years of age (mean 48). The OND group included inflammatory ($n = 55$), vascular ($n = 19$), and other ($n = 31$) diseases. The OND group further contained a group of patients with neurodegenerative diseases ($n = 24$), such as adenoleukodystrophy ($n = 1$), frontal lobe-type dementia ($n = 1$), hereditary cerebellar atrophy ($n = 2$), Pick's disease ($n = 2$), progressive supranuclear palsy ($n = 1$), Parkinson's disease ($n = 2$), olivopontocerebellar atrophy ($n = 1$), ALS ($n = 7$), polyneuropathy ($n = 1$), external ophthalmoplegia ($n = 1$), bulbar palsy ($n = 1$), and miscellaneous others. For each patient, age, sex, and diagnosis were noted. Statistical differences in τ measures between patient groups were verified using an unpaired Student's t test.

Production and characterization of AT120

Purification of PHF- τ and production of mAb AT120, which is produced by a subclone of AT12, were performed as described previously (Mercken et al., 1992b). For characterization, AT120 was tested by western blot on both normal τ and PHF- τ . Reactivity of AT120 with normal τ and PHF- τ was tested in a sandwich ELISA with affinity-purified polyclonal rabbit anti-human τ antiserum as coating antibody, as described by Mercken et al. (1992b).

Normal τ and PHF- τ protein standards

The preparations of affinity-purified normal τ and PHF- τ were described elsewhere (Mercken et al., 1992a,b). Purity of normal τ and PHF- τ standards was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Dephosphorylation was performed as described earlier (Mercken et al., 1992b). The preparations used contained only τ proteins of molecular mass 43–67 kDa. The samples were also analyzed on a 420 A/H amino acid analyzer (Applied Biosystems B.V., Maarssen, The Netherlands) according to the manufacturer's instructions. Both normal τ and PHF- τ showed the expected amino acid compositions. The exact protein concentrations of both affinity-purified normal τ and PHF- τ were determined using an internal standard peptide. The concentrations found were 7.7 $\mu\text{g}/\text{ml}$ and 380 ng/ml for normal τ and PHF- τ , respectively.

AT120 ELISA for τ detection in CSF

AT120, purified from serum-free conditioned medium on a protein G column, was coated overnight at 4°C on high-binding microtiter plates (Nunc, GIBCO, Paisley, U.K.) in coating buffer at 3 $\mu\text{g}/\text{ml}$ (10 mM Tris, 10 mM NaCl, 10 mM NaN_3 , pH 8.5). After overcoating for 30 min with 150 μl of 10% saturated casein in phosphate-buffered saline (PBS) to reduce nonspecific binding, the plates were incubated with 100 μl of an appropriately diluted τ standard, or with unconcentrated CSF samples, supplemented with 5% Tween 20, for 60 min at 37°C . The plates were washed five times with PBS/0.05% Tween 20 (vol/vol), and 100 μl of affinity-purified rabbit anti-normal τ was added at 2 $\mu\text{g}/\text{ml}$ and incubated for 1 h at 37°C . After washing, horseradish peroxidase-conjugated donkey anti-rabbit serum (Amersham, Buckinghamshire, U.K.) was added for 30 min at 37°C . After another washing, the CARD enhancement procedure was done by adding a 100- μl stock dilution of 0.3 M biotin-tyramine (Sigma, Poole, Dorset, U.K.) prepared in dimethyl sulfoxide, according to Bobrow et al. (1989), diluted 1:100 in conjugate diluent (50 mM Tris-HCl, 0.001% H_2O_2 , pH 8), and incubated for 15 min at room temperature. Following washing with PBS/Tween 20, 100 μl of streptavidin-biotinylated horseradish peroxidase complex (Amersham) diluted 1:1,000 was added for 15 min at room temperature. All reagents, except the biotin-tyramine conjugate, were diluted in PBS with 10% casein. After a final washing, 100 μl of 0.42 mM 3,5,3',5'-tetramethylbenzidine, 0.003% (vol/vol) H_2O_2 in 100 mM citric acid, 100 mM Na_2HPO_4 , pH 4.3, were added as peroxidase substrate. The reaction was stopped with 50 μl of a 2 M H_2SO_4 solution. Absorbance was read in a Titertek Multiscan (Flow Laboratories, Eflab Oy, Finland) at 450 nm. Optical density values obtained from the CSF samples were compared with standard curves generated from known quantities of affinity-purified normal human τ .

We found that absorbance values of CSF samples were reproducible. However, frequent thawing and freezing of samples decreased τ concentrations found in ELISA.

AT8 ELISA for PHF- τ detection in CSF

AT8 ELISA for PHF- τ detection in CSF of AD patients and control patients was essentially the same as the AT120 assay, except that AT8 instead of AT120 was applied as coating mAb.

RESULTS

AT120 characterization

On immunoblots, the antibody reacted equally well with both normal τ and PHF- τ , as shown in Fig. 1. In contrast, the Tau-1 antibody (Binder et al., 1985) reacted solely with normal τ and the AT8 antibody (Mercken et al., 1992b) only with PHF- τ .

AT120, AT8, and Tau-1 mAbs were tested for phosphatase sensitivity of their epitopes by ELISA and by western blot on PHF- τ antigen. The reactivity of the AT120 antibody with PHF- τ was not sensitive to phosphatase treatment either in ELISA (Fig. 2) or on western blots (data not shown). The reactivity of AT8 was completely abolished after alkaline phosphatase treatment of the PHF- τ antigen in ELISA. Dephosphorylation of PHF- τ enhanced Tau-1 immu-

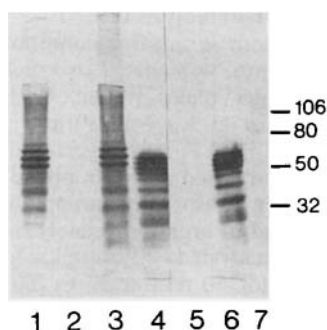


FIG. 1. Western blots of AT8, AT120, and Tau-1 antibodies with equal amounts of PHF- τ and normal τ loaded on a 10% sodium dodecyl sulfate gel. Lanes 1, 3, and 5: PHF- τ ; lanes 2, 4, and 6: affinity-purified normal human τ . Lanes 1 and 2 were incubated with AT8, lanes 3 and 4 with AT120, and lanes 5 and 6 with Tau-1. Lane 7 contains prestained molecular mass markers in kDa.

noreactivity, as described before (Papazosomenos and Binder, 1987).

Both τ standards, i.e., normal τ and PHF- τ , spiked in a CSF pool, were run in the amplified AT120 sandwich ELISA and the amplified AT8 sandwich ELISA. We found no significant differences in detection limits between normal τ and PHF- τ detection for AT120 (Fig. 3A). For AT8, no reaction was shown with normal τ (Fig. 3B), as reported earlier (Mercken et al., 1992b).

AT120 τ detection in unconcentrated CSF samples

A number of CSF samples were tested in the AT120 CARD assay for τ detection. CSF samples of patients were first divided into three different groups, i.e., AD patients, control patients, and patients suffering from OND (Table 1), and then subdivided according to age group (older or younger than 60 years). Table 1 and Fig. 4 clearly show that CSF τ levels were increased significantly in AD patients. If a cutoff value of 1.11 pg/ml is used (mean of control CSF τ levels + 2 standard deviations), 81% (22/27) of all patients clinically

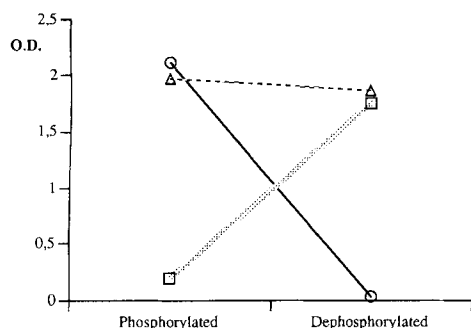


FIG. 2. Reactivity of AT8 (○), AT120 (△), and Tau-1 (□) with untreated (phosphorylated) and dephosphorylated PHF- τ in ELISA. Samples were tested in triplicate and the data presented as optical density (OD) units.

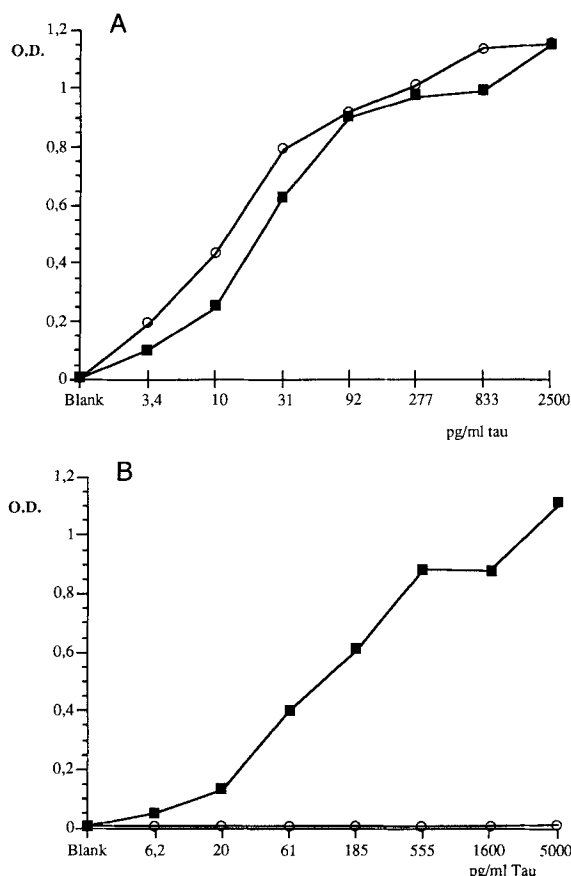


FIG. 3. Titration of normal τ (○) and PHF- τ (■), spiked in a CSF pool in the amplified (CARD) sandwich ELISA with AT120 (A) and AT8 (B). All dilutions were tested in duplicate and the data presented as optical density (OD) units.

diagnosed as AD showed higher CSF τ levels, compared with 36% (47/129) for the group of patients suffering from OND and 4% (2/51) of the control patients. A significant difference was found between the Alzheimer group in toto and the OND group ($p < 0.001$), as seen in Fig. 4. This difference was also found between the OND and the control groups, and between AD and control groups ($p < 0.001$).

When patient groups were examined in more detail, it was found that two control patients showed slightly positive τ values (Fig. 4). However, as one control patient was 77 years old, we cannot exclude the possibility that he suffered from preclinical AD. Another striking observation is that in patients older than 60, τ in CSF was clearly higher in AD than in OND (Table 1), whereas for younger patients, the difference was much less pronounced and did not reach significance ($p > 0.78$). When the group of OND patients was analyzed in greater detail (Table 1 and Fig. 5), we found no diagnostic groups that consistently showed increased CSF τ . Several patients suffering from neurodegenerative diseases showed a strong increase of CSF τ (hereditary cerebellar atrophy, oliv-

TABLE 1. CSF levels of τ in AD and OND

Group (n)	Age (yr)	τ in CSF (pg/ml)		
		All ages	<60 yr	>60 yr
AD (27)	67 $\pm$ 13	10.9 $\pm$ 4.9	4.7 $\pm$ 4.9	14.3 $\pm$ 8.5
Controls (51)	44 $\pm$ 15	0.1 $\pm$ 0.5	0.05 $\pm$ 0.3	0.3 $\pm$ 0.9
OND (129)	48 $\pm$ 18	3.9 $\pm$ 7.4	4.5 $\pm$ 8.3	2.7 $\pm$ 5.1
Degenerative (24) ^a			8.2 $\pm$ 8.7	3.6 $\pm$ 7.3
Inflammatory (55) ^a			3.8 $\pm$ 7	0.7 $\pm$ 1.8
Vascular (19) ^a			4.8 $\pm$ 9.4	5.3 $\pm$ 3.8
Others (31) ^a			4.6 $\pm$ 10.5	0.5 $\pm$ 1.4

All data are given as means $\pm$ SD.

^a Subpopulations of OND group.

pontocerebellar atrophy, ALS, Parkinson's disease). However, this increase was not consistent, as five of seven ALS patients had CSF τ values within the range of the control group. A similar heterogeneity was observed for inflammatory nervous system diseases: four of 20 multiple sclerosis patients, five of 11 Guillain-Barré patients, two of three herpes simplex encephalitis patients, and one of three Lyme disease patients had increased CSF τ . Patients suffering from cerebrovascular diseases and stroke patients showing multiple old and recent ischemic infarcts had the highest CSF τ values in the OND group. More importantly, three patients suffering from multiple infarct dementia and a patient suffering from vascular dementia and pseudobulbar syndrome had increased CSF τ . Single diagnostic groups were too small to determine whether there was a correlation between the severity of CNS damage and the extent of τ elevation. Two patients with hydrocephalus and an infant with sudden infant death syndrome showed extreme increases in CSF τ , the significance of which remains undetermined.

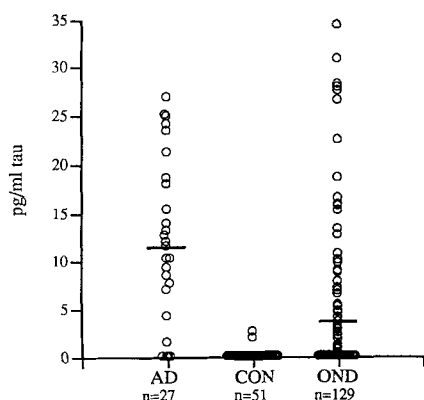


FIG. 4. CSF τ quantification in ELISA (100 μ l/assay) of individual CSF samples, using the AT120 assay. The mean value of each group is indicated by horizontal bars. CSF τ concentrations were based on standard curves with affinity-purified normal human τ . Results are shown as pg of τ /ml. CON, controls.

Unconcentrated and concentrated CSF samples analyzed in the AT8 assay

CSF samples of 20 AD patients, 10 control patients, and 10 OND patients were selected for PHF- τ detection using AT8 as a capturing antibody. AD CSF samples having high τ values in the AT120 assay were selected for PHF- τ detection. However, no PHF- τ was found in these unconcentrated CSF samples, indicating that, in our assay, the sensitivity of 30 pg/ml PHF- τ was not sufficient for CSF detection of PHF- τ .

Moreover, when a pool of AD CSF samples was concentrated 12 times, resulting in a hypothetical sensitivity of less than 3 pg/ml PHF- τ , again no signal was found. Thus, if PHF- τ , as detected by the AT8 immunoassay, is present in CSF, its concentration must probably be below 3 pg/ml. Consequently, either τ proteins found in AD CSF samples with the AT120-based assay are not phosphorylated at the

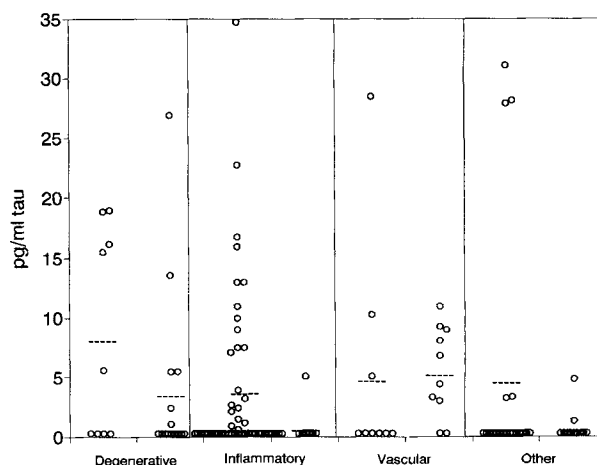


FIG. 5. CSF τ detection in ELISA (100 μ l/assay), using the AT120 assay on CSF of OND patient subgroups (see text). Each group is subdivided according to age (left data points: younger than 60; right data points: older than 60). The mean value of each group is represented by a horizontal bar. CSF τ concentrations were based on standard curves with affinity-purified normal human τ . Results are shown as pg of τ /ml.

AT8 site and could be normal τ or dephosphorylated PHF- τ , or the AT8 region is not present or available for immunoreactivity with the AT8 antibody.

DISCUSSION

AD is diagnosed clinically with a variable degree of certainty by excluding other causes of dementia. This diagnosis can only be confirmed histopathologically, by observing a sufficient number of NFT and senile plaques in the neocortex (Khachaturian, 1985). No protein specific for AD has yet been found. However, PHF and amyloid plaques contain posttranslationally modified normal brain proteins. Because CSF offers the richest potential source of altered proteins in a neurodegenerative disease, we set up a sandwich ELISA that specifically recognized the microtubule-associated protein τ , the principal component of PHF.

Besides τ , other proteins that may play an important role in the pathogenesis of AD, like α_1 -antichymotrypsin, ubiquitin, and amyloid precursor protein (APP), can also be detected in CSF.

Recently, α_1 -antichymotrypsin, a serine protease inhibitor and an acute phase protein found to be specifically present in senile plaques (Abraham et al., 1988), was analyzed in CSF. Studies concerning α_1 -antichymotrypsin CSF levels in AD versus control groups show contradictory results (Matsubara et al., 1990; Delamarche et al., 1991; Vandermeeren, unpublished observations), so it would be premature to argue whether this protease inhibitor represents a discriminative marker for AD.

Other groups investigated the potential usefulness of ubiquitin as an antemortem diagnostic marker for AD. It was found that ubiquitin reactivity of PHF could be measured in CSF (Wang et al., 1991). The authors showed that there was a significant increase in AD ubiquitin CSF levels versus control CSF samples. However, overlaps between the two different patient groups were also reported.

At first, analysis and quantitation of APP in CSF of AD and control patients demonstrated no significant differences (Chong et al., 1990; Henriksson et al., 1991), i.e., only a small decrease in APP CSF levels was found in AD patients as compared with healthy controls. Palmert et al. (1990), using western blot analyses, demonstrated a reduction of soluble APP forms in AD CSF. In addition, their data also suggested an age-dependent reduction in β -APP CSF levels. In a recent report (Van Nostrand et al., 1992), it was shown that β -APP CSF levels were decreased significantly as compared with controls or a group of demented non-AD individuals. One limitation is that this assay does not distinguish between AD and hereditary cerebral hemorrhage with amyloidosis (Dutch type).

PHF contain modified forms of τ as a major protein component (Lee et al., 1991). Therefore, we in-

vestigated the presence of this antigen in CSF. CSF τ concentrations are extremely low, so the τ detection assay had to be very sensitive, a problem resolved by applying CARD amplification and a high-affinity mAb, called AT120. Several authors reported the development of a τ -specific assay. One group clearly showed that PHF- τ could be detected in Alzheimer brain homogenates and not in normal brain homogenates (Ghanbari and Miller, 1990; Ghanbari et al., 1990). Their ELISA was based on a combination of a highly specific polyclonal anti-PHF- τ serum and the mAb Alz-50. The latter mAb, the epitope of which has been mapped to the N-terminal region (Ksiezak et al., 1990; Goedert et al., 1991), had already been described as a powerful tool to detect PHF- τ (A68/ADAP) in some concentrated Alzheimer CSF samples with immunoblotting techniques (Wolozin and Davies, 1987). Unfortunately, these early findings have not been confirmed by other groups. Analysis of unconcentrated CSF samples by western blot with highly specific anti-normal τ and anti-PHF- τ polyclonal antibodies showed that a single 55-kDa band was immunostained, quite different from τ profiles that are observed in brain homogenates (Delacourte et al., 1989). Finally, the development of competitive ELISAs for selectively detecting normal, soluble τ and PHF-associated τ protein in brain tissue has been reported (Harrington et al., 1990, 1991). The authors did not discuss normal τ or PHF- τ CSF detection.

In summary, we developed a highly sensitive sandwich ELISA for the detection of τ in unconcentrated CSF samples. The assay detects normal τ and PHF- τ equally well, i.e., applying affinity-purified normal and pathological τ in the AT120 sandwich assay resulted in similar detection limits. The location of the AT120 epitope outside the repeat region (Vanmechelen et al., unpublished observations) indicates that cross-reactivity with other phosphorylated proteins like MAP2, MAP1, and neurofilaments, which share parts of their sequence with τ protein (Nukina et al., 1987; Lewis et al., 1988), can be excluded. In fact, AT120 showed no reactivity in ELISA when the above-mentioned cytoskeletal proteins were applied in the coating phase (data not shown).

Our results with the AT120-based assay indicate that elevated normal τ levels are found not only in AD, but also in different kinds of neurological diseases where neuronal death or damage occurs. Although some of these OND patients can be discriminated clinically from AD patients, our results show substantial overlap between AD and other forms of dementia, reducing the predictive value of elevated CSF τ . However, especially in patients older than 60, CSF τ is a useful marker for AD.

Because of the exquisite specificity of the AT8 antibody, showing no cross-reaction with normal τ , we set up a sandwich ELISA for the selective detection of PHF- τ proteins in AD CSF samples. Unfortunately, we were not able to demonstrate any PHF- τ antigens

in unconcentrated AD CSF samples, nor in a 12-fold concentrated AD CSF pool. It remains unclear why we did not detect any AT8-reactive τ in CSF. Contrary to our own negative results, it was reported that, in some Alzheimer CSF samples, a positive signal with an assay based on the PHF-1 mAb can be found (Ghanbari et al., 1991). This discrepancy may be due to a different sensitivity of both assays. Also it is possible that the AT8 epitope may be eliminated from PHF- τ that would be present in CSF, either by protease or phosphatase activity. In this respect, it may be significant that the AT8 epitope is more sensitive than PHF-1 to phosphatase treatment (unpublished observations). A selective loss of PHF- τ epitopes from CSF could also be explained by the observation that AT8 does not immunostain "ghost tangles" or extracellular NFT in tissue sections. Extracellular NFT are not composed of full-size τ (Tabaton et al., 1991). Possibly some of the N-terminal epitopes outside the repeat region, including the AT8 epitope, are digested by microglia (Cras et al., 1991).

Nevertheless, in brain extracts of AD patients, higher τ concentrations have been described, and it seems that this increase is due mainly to PHF- τ (Ghanbari et al., 1990; Harrington et al., 1991; Bramblett et al., 1992; Khatoon et al., 1992). These findings should stimulate further research on the development of a highly sensitive PHF- τ -specific assay for the study of CSF.

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