Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8045–8049, October 1989
Medical Sciences

Abnormal processing of multiple proteins in Alzheimer disease

(neurofilaments/tau/amyloid β-protein/antibody affinity/monoclonal peroxidase–antiperoxidase)

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Communicated by Berta Scharrer, June 1, 1989

ABSTRACT **Cerebrovascular amyloid is the main constituent of the perivascular and neuritic plaques typical of Alzheimer disease, whereas neurofilaments and microtubule-associated tau protein have been considered primary contributors to the formation of the characteristic Alzheimer tangles. Plaques and tangles and their constituents have at times been ascribed a role in pathogenesis of the disease. Normally, neurofilaments become phosphorylated only upon axonal entry. In many neurologic disorders, neurofilament phosphorylation, as detected by any of the available monoclonal antibodies (mAbs) to neurofilament phosphorylated epitopes is shifted from an axonal to a cell-body location. An exception is provided by Alzheimer disease, where tangles (which are neuronal cell-body-derived structures) exhibit only one phosphorylated epitope. However, the very presence of neurofilaments in tangles and plaques has been questioned because of a reported cross-reaction of mAbs to phosphorylated neurofilaments with tau protein. On reinvestigating this cross-reactivity we found that four of five mAbs to phosphorylated neurofilaments and four of five mAbs to nonphosphorylated neurofilaments failed to react with tau protein. A fifth mAb (07-5) to phosphorylated neurofilament cross-reacted with partially denatured tau protein at an affinity 1/1700th of that for denatured neurofilaments; nondenatured tau protein in tissue sections did not cross-react. A fifth mAb (02-40) to nonphosphorylated neurofilament also cross-reacted weakly. In Alzheimer disease normal-appearing axons were revealed with all the mAbs to phosphorylated neurofilaments, but tangles were revealed with only one of them (mAb 07-5). mAb to tau protein did not stain or did so indistinctly. Four of five mAbs to nonphosphorylated neurofilaments failed to reveal axons. Upon dephosphorylation of tissue, staining by mAbs to phosphorylated neurofilaments disappeared, and axons were revealed with the mAb to tau protein and all mAbs to the nonphosphorylated neurofilaments. Tangles became stained with tau mAb and one mAb to the nonphosphorylated neurofilaments (mAb 10-1). Quantitative evaluation of immunocytochemical staining intensities and immunoblot cross-reactivity showed that neurofilaments are, indeed, constituents of tangles—apparently exceeding the concentration of tau protein 17-fold. Contribution of both conformation and primary structure to IgG specificity may explain the lack of any cross-reaction of mAbs to neurofilaments with tau protein in intact tissue and the appearance of cross-reaction in immunoblots where conformation specificity may be largely lost. The present data extend earlier findings of abnormal processing of neurofilaments and tau protein in Alzheimer disease and, together with reported abnormal processing of cerebrovascular amyloid β-protein, suggest that inhibition of the processing of multiple proteins is basic to the pathogenesis of Alzheimer disease, whereas formation of plaques and tangles could be merely the most striking histologic result.**

Abundance of tangles and plaques are diagnostic features of Alzheimer disease (AD). Amyloid β-protein is the most prevalent constituent of plaques (1), whereas neurofilaments (2–6) and microtubule-associated tau protein (7, 8) have been detected immunocytochemically in tangles. We have developed a series of monoclonal antibodies (mAbs) that distinguish various degrees of phosphorylation in the heavy (200-kDa) and middle (160-kDa) protein of neurofilaments (9). In normal brain mAbs to phosphorylated neurofilaments (αPNFs) reacted exclusively with axons, whereas neuronal cell bodies and dendrites were the exclusive domain of reaction with mAbs to nonphosphorylated neurofilaments (αNPNFs). Phosphorylation was shown to occur only upon entry of neurofilaments into the axon. However, in several neurologic disorders (for review, see ref. 10) neurofilament phosphorylation was shifted to cell-body locations in the neuron. Phosphorylation was detected in these locations by any of the αPNFs, and, therefore, might be considered a physiologic reaction to injury. AD was an exception in that only one phosphorylated epitope had been regularly detected in tangles, which are cell-body-derived structures, thus leading to the concept of abnormal neurofilament processing in this disorder (3, 5). However, the very presence of neurofilaments in tangles has been questioned because of reported cross-reaction of neurofilament phosphorylated epitopes with tau protein (11, 12). Because abnormal phosphorylation of tau protein is another feature of tangles (7), the abnormal phosphorylation of neurofilaments could be questioned as well. We have, therefore, quantitatively evaluated the cross-reaction of mAbs to neurofilaments and tau protein in immunoblots and intact tissue and examined the effects of phosphorylation on the reaction of these mAbs with tissues of patients with AD and of age-matched and young controls. The data, together with reports of abnormal processing of amyloid β-protein (1), led us to conclude that AD may be a processing disorder of multiple proteins.

MATERIALS AND METHODS

Seven brains from demented individuals, ages 72–92 yr, five brains from nondemented individuals, ages 70–88 yr, and four brains from neurologically normal-appearing individuals, ages 24–42 yr were obtained 6–40 hr after death. For immunocytochemistry, formalin-fixed vibratome sections were pretreated overnight at 32°C with either 0.1 M Tris hydrochloride, pH 8.0/0.002 M phenylmethylsulfonyl fluoride or with the same buffer containing *Escherichia coli*

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Abbreviations: AD, Alzheimer disease; ClonoPAP, monoclonal peroxidase–antiperoxidase; mAb, monoclonal antibody; αPNF, mAb to phosphorylated neurofilaments; αNPNF, mAb to nonphosphorylated neurofilaments; αtau, mAb to tau protein.

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phosphatase at 66 μg/ml. The primary antibodies selected for immunocytochemistry from a large library of mAbs are listed in Table 1. Immunocytochemical staining was developed by the unlabeled antibody method (13) by using peroxidase–antiperoxidase complex made from mAb to peroxidase (ClonoPAP, Sternberger-Meyer Immunocytochemicals, Jarrettsville, MD) (9).

Immunoblots were done on cytoskeletal (14) and tau protein (15) preparations and analyzed with ClonoPAP after reaction with the mAbs with and without prior dephosphorylation. Gels were loaded with tau protein or cytoskeletal preparations in 6-μg amounts for the six major Coomassie blue-stained PAGE bands of the former and the 200-kDa band of the latter, as determined by standardization with bovine serum albumin in an LKB laser densitometer.

Immunocytochemical staining of tangles was expressed as the average of their median staining intensities in each section of hippocampus (16).

RESULTS

In denaturating gels, we could see a cross-reaction of tau protein with only one (07-5) of five αPNFs and only one (02-40) of five αNPNFs. However, abnormally high concentrations of mAbs (dilutions of 1:400) were required, and even at these concentrations, cross-reaction was weak (Fig. 1). mAb 07-5 had to be diluted 700,000 times to yield with 6 μg of the heavy neurofilament protein a staining intensity equal to that of its cross-reaction at a 400-fold dilution with 6 μg of tau protein (Fig. 2). Hence, the cross-reaction with tau protein was only 1/1700th of its specific reaction with heavy neurofilament on immunoblots.

In intact tissue, we could not confirm this cross-reaction, even at abnormally high concentrations of mAb. In normal cerebellum, αPNF 07-5 stained basket-cell fibers and axons in the granular layer, leaving the rest of the molecular layer reaction-free (Fig. 3). In contrast, αtau stained parallel fibers in the molecular layer, whereas basket-cell axons and structures in the granular layer remained unstained. The reaction of αtau in normal tissue was unaffected by dephosphorylation.

All cases of AD were replete with tangles by Bielschowsky staining. In the age-matched controls tangles were rare. Without dephosphorylation, αtau stained normal axons and tangles in Alzheimer tissue irregularly or not at all (Figs. 4 and 5; Table 1). However, in contrast to normal tissue, dephosphorylation in AD had a strong effect on the reaction with αtau. Abundant tangles were stained, and axonal profiles, though sparse, were enhanced. When the tissue had not been dephosphorylated, all αPNFs stained normal axons in all AD cases. However, only one of these αPNFs, 07-5, stained tangles (Figs. 4 and 6; Table 1). Staining of individual tangles was strong, but the *number* of tangles appeared less than those revealed with αtau. Without dephosphorylation of

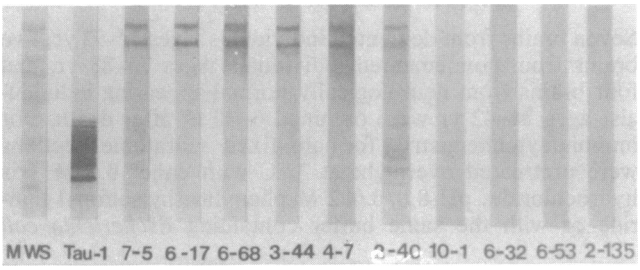

FIG. 1. Immunoblot of bovine tau protein stained with the listed mAbs. Lane 2, antibody dilution 1:2000; remaining lanes, antibody dilution 1:400. Standards (MWS, top to bottom), 200, 116, 92, 66, 45, and 31 kDa.

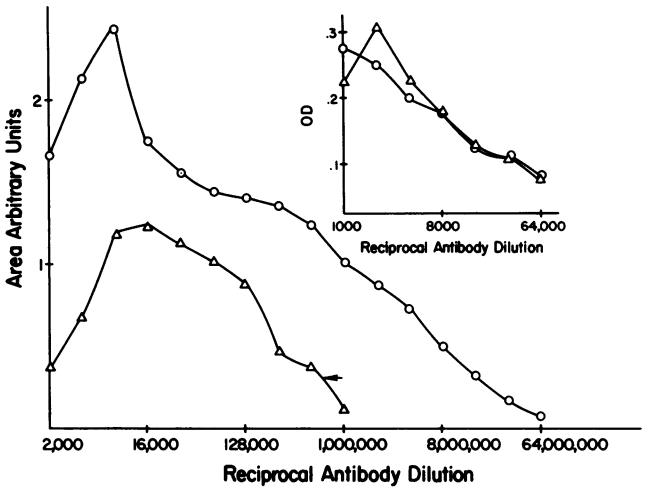

FIG. 2. Integrated staining intensity (arbitrary units) of immunoblots of 6 μg of tau protein stained with various dilutions of mAb to tau protein (αtau) (○) and of 6 μg of heavy neurofilament stained with various dilutions of αPNF 07-5 (△). (*Inset*) Averages of median staining intensity (OD) of Alzheimer tangles in vibratome sections stained with various dilutions of αtau (○) and αPNF 07-5 (△). ←, Staining intensity of 6 μg of immunoreactive tau protein bands with αPNF 07-5 diluted 1:400.

tissue, phosphorylation-dependent αNPNFs (9, 13) failed to stain axons or tangles in any case (Figs. 4 and 7; Table 1). Upon dephosphorylation, however, staining appeared in axons with all these αNPNFs, but only αNPNF 10-1 stained occasional tangles in every case.

Age-matched controls revealed identical selectivity of staining as for cases of AD, except for fewer tangles. Tissues from young normal controls had more abundant immunostained axons and cell bodies than those of older individuals. Thick and thin axons were stained with all αPNFs and cell bodies and dendrites with all αNPNFs. Some proximal and

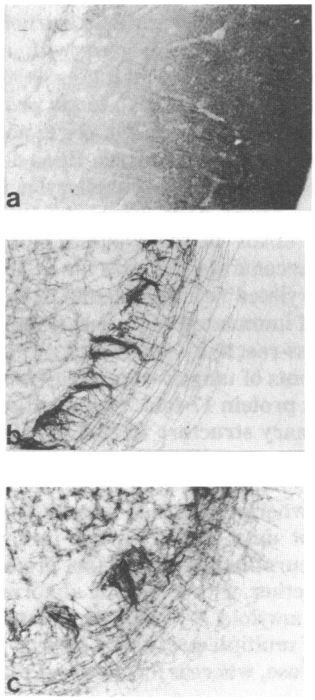

FIG. 3. Cerebellum from 24-yr-old accident victim stained with αtau (*a*) diluted 1:8000, αPNF 07-5 (*b*) diluted 1:8000, and αPNF 07-5 (*c*) diluted 1:400. (ClonoPAP; ×130.)

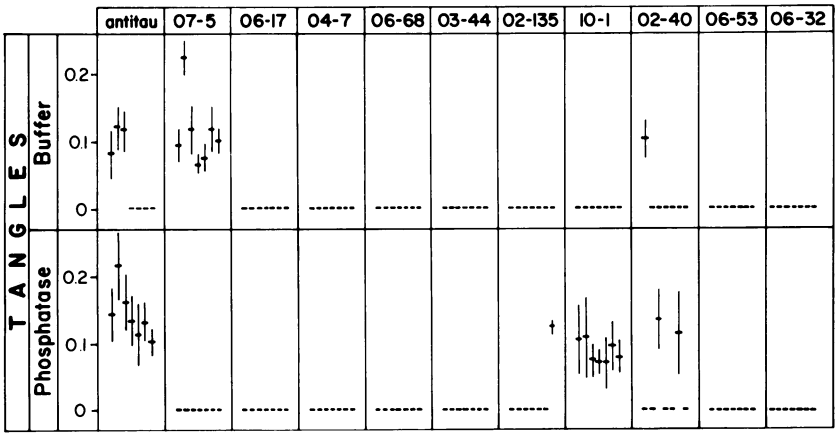

FIG. 4. Averages of median OD of tangles from seven cases of AD. Vibratome sections pretreated with buffer or phosphatase.

thick axons were also stained with the αNPNFs. Upon dephosphorylation axonal staining became more widespread with any of the phosphorylation-dependent αNPNFs, whereas staining with αPNFs had disappeared.

The relative amounts, per tangle, of tau protein and of the neurofilament component revealed by αPNF 07-5, were evaluated after comparing the staining intensities of αtau and αPNF 07-5 on immunoblots with tau protein and heavy neurofilament (6 μg of each) (Fig. 2). Immunoblot data were identical whether blots were unfixed or fixed in formaldehyde before immunostaining. On blots, αtau reacted 17 times stronger than αPNF 07-5. In tangles, on the other hand, reaction with αtau was only equal to that of αPNF 07-5. Thus, concentration of the heavy neurofilament component in tangles exceeded that of tau protein 17-fold. In addition, reduction of staining intensity at very high concentrations of mAb [Eng–Bigbee effect (18, 19)] seen with αPNF 07-5, but not with the more potent αtau, independently indicated a higher concentration of neurofilament than tau protein in tangles.

DISCUSSION

Existence of a strong cross-reaction of a mAb, such as mAb to neurofilament, with an unrelated protein, such as tau protein, would invalidate the use of immunologic methods for establishing the contents of Alzheimer tangles. In previous studies we have shown that αPNFs are strictly specific in intact adult tissue (13, 20). However, two other groups (11, 12) reported a cross-reaction between several αPNFs and αtau on immunoblots when abnormally high concentrations of mAb were used. Cork *et al.* (10) and Gambetti *et al.* (21) were unable to observe this cross-reaction. Our findings confirm the cross-reaction of at least one αPNF (07-5), but the cross-reaction was weak, being only 1/1700 of the specific reaction with neurofilaments. Thus, if the reaction of αPNF 07-5 with tangles was due to a cross-reaction with tau protein, the concentration of tau protein in tangles should exceed that of neurofilament 1700 times. In fact, the concentration of neurofilament exceeded that of tau protein, and this excess was by a factor of 17.

Phosphorylation-dependent αNPNF epitopes (Table 1) have previously been shown to be strictly specific for neuronal perikarya, dendrites, and some axons, especially proximal axons (9). In our work and also in that of Kiezak–Reding *et al.* (12), phosphorylation-dependent αNPNFs failed to cross-react with tau protein on immunoblots, even at high concentrations. However, mAb 02-40, a phosphorylation-independent αNPNF, did cross-react with tau protein, albeit weakly so. This antibody also cross-reacted weakly with macrophages and some liver cells (20). These findings show that weak cross-reaction of αNPNF with tau protein, when seen, is not necessarily due to phosphate groups as surmised (11, 12).

Specificity of the reaction of IgG antibodies is contributed by both the conformation and sequence of epitopes (22–26). Upon denaturation, such as occurs in PAGE immunoblots, the contribution of conformation is partially lost, and specificity rests largely upon primary sequence. Geysen *et al.* (27) have shown that in a hexapeptide sequence critical to an antibody-binding site, usually not more than three amino acids contribute to specificity. Substitution of any amino acid

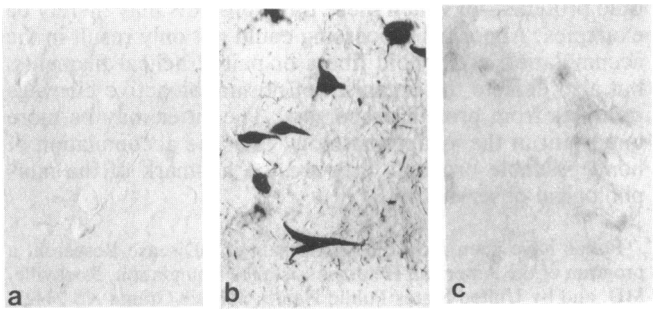

FIG. 5. AD. Hippocampus stained with αtau diluted 1:8000. (*a*) Lesion area, buffer pretreatment. (*b*) Tangles, phosphatase pretreatment. (*c*) Normal-appearing axons, phosphatase pretreatment. (ClonoPAP; ×130.)

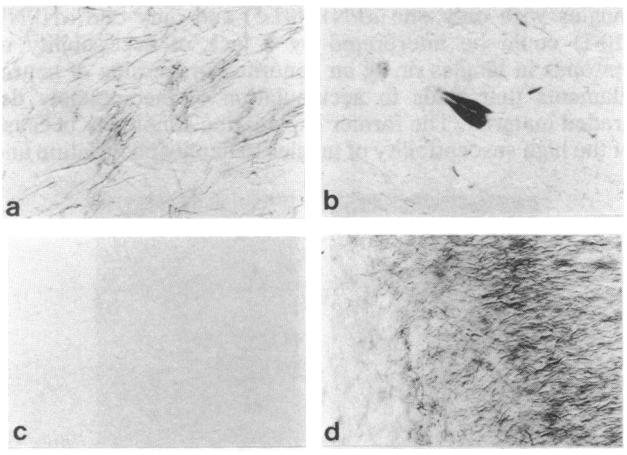

FIG. 6. AD. Hippocampus stained with αPNFs diluted 1:8000. (*a*) Normal-appearing axons, buffer pretreatment, αPNF 07-5. (*b*) Tangles, buffer pretreatment, αPNF 07-5. (*c*) Lesion area, phosphatase pretreatment, αPNF 07-5. (*d*) Normal-appearing axons, buffer pretreatment, αPNF 06-17. (ClonoPAP; ×130.)

Table 1. Immunostaining of intact and enzymatically dephosphorylated brain tissue with monoclonal antibodies

Specificity of antibody	Antibody	Normal				Alzheimer			
		Axon		Cell body		Axon		Tangle	
		I	ED	I	ED	I	ED	I	ED
Extensively phosphorylated neurofilament	06-17	+	−	−	−	+	−	−	−
	07-5	+	−	−	−	+	−	+	−
	04-7	+	−	−	−	+	−	−	−
	06-68	+	−	−	−	+	−	−	−
Hypophosphorylated neurofilament	03-44	+	−	−	−	+	−	−	−
Nonphosphorylated neurofilament (phosphorylation-dependent)*	02-135	−†	+	+	+	−	+	−	−‡
	06-32	−†	+	+	+	−	+	−	−
	06-53	−†	+	+	+	−	+	−	−
	10-1	−†	+	+	+	−	+	−	+
Nonphosphorylated neurofilament (phosphorylation-independent)§	02-40	+	+	+	+	+	+	−‡	−‡
tau	antitau (17)¶	+	+	−	−	−‡	+	−‡	+

I, intact; ED, enzymatically dephosphorylated.
*Nonreactive when neurofilaments are phosphorylated.
†Except for proximal and thick axons.
‡With occasional + exceptions.
§Reactive independently of neurofilament phosphorylation.
¶Axons stained by αtau and mAb to neurofilaments did not overlap.

for each of three specificity-contributing amino acids would not allow for $>20^3$ variations and, therefore, not >8000 different antibodies that can distinguish primary structure. Thus, limitation of antibody reactivities to primary structure alone would imply rather poor specificity for antibodies. The actual existence of a near infinite number of antibodies with different specificities is due to the contribution of conformation to specificity in addition to that of primary structure. It is, therefore, not surprising that on immunoblots of partially denatured proteins occasional mAbs to neurofilaments exhibit a weak cross-reaction with tau protein that is not detectable in intact tissue. The lack of significance of any cross-reaction of mAb to neurofilament with tau protein permits extension, in this paper, of previous work by Cork *et al.* (5) and ourselves (3) on abnormally incomplete phosphorylation of neurofilaments in AD, with only one neurofilament phosphorylated epitope reactive in tangles.

Some tangles also contain material generally reactive, after dephosphorylation, with only a single αNPNF (10-1). Again, this situation contrasts with normal neuronal cell bodies that stain with any of the αNPNFs. The restricted reaction of tangles with only one αPNF (07-5) and only one αNPNF (10-1) could be interpreted by a lack of accessibility of epitopes in tangles or by an abnormal processing of neurofilaments that leads to accumulation of incompletely degraded material. The former explanation is unlikely because of the high susceptibility of tangles to dephosphorylation and even more so because of the ready accessibility of αNPNF 10-1 to its epitope, once the masking due to phosphorylation is removed.

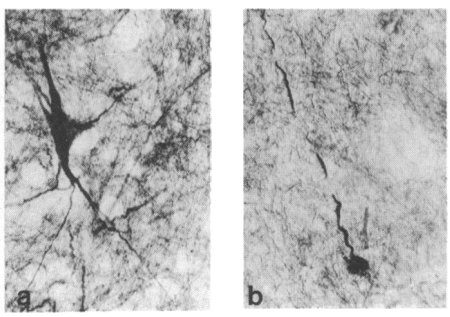

FIG. 7. AD. Hippocampus stained with αNPNF 10-1 diluted 1:8000. Phosphatase pretreatment. (*a*) Normal-appearing area. (*b*) Tangle. (ClonoPAP; ×130.)

The present work also confirms previous data of Grundke-Iqbal *et al.* (7) on abnormal phosphorylation of tau protein in tangles. Here, phosphorylation may have masked accessibility or changed conformation in tangles so as to prevent reaction with αtau. Dephosphorylation was necessary for staining of tangles, whereas axons in young normal controls were stained with αtau independently of dephosphorylation. Conclusions that could be drawn from these findings would include the existence of an abnormally high extent of phosphorylation of tau protein in tangles or an unusual conformational change effected in tangles by phosphorylation.

The 43-amino acid amyloid β-protein of plaques (1) is derived from a 695-amino-acid precursor (28). The significance of this protein as a normal precursor was elucidated by the work of Allsop *et al.* (29), who showed that a peptide consisting of residues 8–17 of the amyloid β-protein acted as a ligand to a specific membrane receptor in normal tissues. The data suggested that amyloid β-protein in senile plaques results from the deposition of an abnormally processed protein.

The conjoined appearance in AD of such seemingly unrelated lesions as plaques and tangles and the abnormal processing of such apparently unrelated proteins as amyloid β-proteins, neurofilament, and tau protein suggest that basic to the disease pathology is an abnormal processing of multiple proteins—of which these three proteins may merely be examples. Abnormal processing could not only result in the accumulation of amyloid fibers or paired helical filaments, but also deplete the organism of normal bioactive cleavage products from protein precursors. The latter may be more important in the symptomatology than the accumulation of nondegradable products that are the hallmark of the morphological observations.

Funds have been provided by Alzheimer's Disease Research, a program of the American Health Assistance Foundation, Rockville, MD, and by United States Public Health Service Grants NS 24423, NS 26583, and NS 24185 and National Science Foundation Grant BNS 8696090.

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