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Alzheimer Paired Helical Filaments: Cross-reacting Polypeptide/s Normally Present in Brain *,**

I. Grundke-Iqbal, K. Iqbal, Y. C. Tung, G. P. Wang, and H. M. Wisniewski

New York State Office of Mental Retardation and Developmental Disabilities, Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road; Staten Island, NY 10314, USA

Summary. Antisera to microtubule-enriched fraction from normal human brain (anti-MT sera) label neurofibrillary tangles and neurites of neuritic (senile) plaques in brain sections of cases with Alzheimer disease/senile dementia of the Alzheimer type (AD/SDAT); the plaque core amyloid is not labeled. These anti-MT sera label both tangles in tissue sections and smears of isolated tangles which had been extracted with sodium dodecyl sulfate (SDS) to remove impurities trapped in between the paired helical filaments (PHF). The tangle labeling of anti-MT sera is eliminated on their absorption both with microtubule-enriched fractions from human and animal brain and with the isolated PHF. Neurofilament triplet, actin, myosin, keratin, or fibroblasts do not absorb the tangles staining antibodies. Furthermore, antisera containing antibodies to tubulin, microtubule-associated high mol. wt. polypeptides (MAPS), neurofilament triplet, and the 50,000 mol. wt. contaminant of CNS neurofilament preparations do not label tangles. On immunoblots of SDS-polyacrylamide gels of isolated PHF anti-MT sera label some of the same polypeptides identified with antisera to PHF; affinity-purified antibodies to tubulin used as a control do not label any PHF polypeptide on the immunoblots. The anti-MT sera, when preabsorbed with the PHF polypeptides eluted from SDS-polyacrylamide gels, do not label tangles. These studies demonstrate that a polypeptide/s cross-reactive with Alzheimer PHF is indeed normally present in brain and that it is different from tubulin, neurofilament triplet, actin, myosin, vimentin, and keratin.

Key words: Alzheimer disease – Senile dementia – Paired helical filaments – Neurofibrillary tangles – Cytoskeletal proteins – Microtubules

Introduction

A major lesion in Alzheimer disease and senile dementia of the Alzheimer type (AD/SDAT) is the presence of numerous intraneuronal argentophilic fibrillary tangles which are composed of paired helical filaments (PHF). A few of the tangles contain 15-nm and/or 10-nm straight filaments admixed with PHF (Oyanagei 1979). PHF are morphologically unlike any of the normal neurofibers, i.e., neurotubules, neurofilaments, and microfilaments (Iqbal et al. 1977). In addition to the Alzheimer neurofibrillary tangles, bundles of PHF are found in the neurites of the senile (neuritic) plaques, another major lesion of the Alzheimer brain, and less frequently as individual fibers in myelinated axons (Wisniewski et al. 1981). The concentration of the tangles and the plaques, both of which are also present in small numbers in normal aged humans strongly correlates to the degree of dementia (Roth et al. 1966; Tomlinson et al. 1970). The origin of the PHF is as yet unknown.

Immunocytochemical labeling of tangles has been observed both with antisera against the isolated 50 kilo dalton (kDa) polypeptide (Grundke-Iqbal et al. 1979a) identified in tangle-containing isolated neuronal cell bodies (Iqbal et al. 1974) and with monoclonal antibodies (Wang et al. 1984) and antisera to isolated PHF (Grundke-Iqbal et al. 1981, 1984; Ihara et al. 1983). The antigen/s these latter antisera are directed against is inherent to the PHF (Grundke-Iqbal et al. 1984). Both biochemical (Iqbal et al. 1984) and immunochemical (Grundke-Iqbal et al. 1984) studies

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Offprint requests to: Dr. Inge Grundke-Iqbal (adress see above)

strongly indicate that this PHF antigen/s might be a polypeptide which forms oligomers of multiple molecular weights. This polypeptide/s, although immunochemically not cross-reactive with neurofilament or microtubule major polypeptides, might not be unique to PHF since absorption experiments indicate antigenic cross-reactivity with an antigen/s present in small amounts in normal human and animal brain (Grundke-Iqbal et al. 1984).

Immunostaining of tangles has also been demonstrated with some antisera to preparations enriched in normal brain microtubules (Grundke-Iqbal et al. 1979b; Iqbal et al. 1980a,b; Yen et al. 1981) and with some antisera and monoclonal antibodies to neurofilaments (Ishii et al. 1979; Gambetti et al. 1980, 1983; Dahl et al. 1982; Anderton et al. 1982). Studies with both monoclonal antibodies and antisera to neurofilaments have suggested that the tangle-cross-reacting antigen/s in the neurofilament preparations are the neurofilament triplet polypeptides (Gambetti et al. 1983; Anderton et al. 1982). However, it remains to be determined whether these tangle-cross-reacting neurofilament antigens are adventitious or inherent to PHF (Rasool et al. 1983). In contrast to the neurofilament antibodies, absorption studies with the tangle-reactive antimicrotubule sera have indicated that the tangle-cross-reacting antigen/s co-purifies with microtubules, but is different from tubulin and high-molecular-weight microtubule-associated proteins and neurofilament major polypeptides (Iqbal et al. 1980a,b; Yen et al. 1981). The relationship of this tangle-cross-reacting antigen/s to the PHF is not known. The present study was undertaken to identify immunochemically the PHF antigen/s recognized with antimicrotubule sera and compare it with that recognized by antisera to PHF.

Materials and Methods

Antigens for Immunization and Absorption

Microtubule enriched fractions were prepared from fresh human or bovine brain by two cycles of in vitro assembly and disassembly (Shelanski et al. 1973). These preparations contained about 85% – 90% tubulin. The remainder of the protein consisted of microtubule-associated proteins (MAPS) and as contaminants several proteins including neurofilament triplet polypeptides.

Tangles of PHF were isolated from homogenates of autopsied cerebral cortex of cases with AD/SDAT using the long procedure of Iqbal et al. (1984). PHF prepared by this method are highly purified, ultrastructurally well preserved, and free from normal neurofibrils; the 15-nm and the 10-nm filaments seen in a few tangles in tissue sections are lost by the SDS treatment employed for the isolation of PHF.

Neurofilaments were prepared from the $75{,}000 \times g$ supernatant of bovine or rabbit spinal cord by assembly in 4 M glycerol at 4° C (Iqbal et al. 1981).

Actin-myosin filaments were prepared from chicken gizzard by the method of Malik (1978).

Keratin was prepared from epidermal shavings of the hands of the investigators using the method of Steinert and Idler (1975).

Cultured human skin fibroblasts F 700 were scraped from the tissue culture flasks into 0.02% EDTA in phosphate-buffered saline, disrupted by sonication, and separated into a pellet and a supernatant by centrifugation at $100{,}000 \times g$ for 30 min. Alternatively, the fibroblasts were lysed with 0.5% Triton X–100 in phosphate-buffered saline and separated into Triton-soluble and -insoluble fractions by centrifugation at $8{,}000 \times g$ for 10 min.

PHF and neurofilament polypeptides were extracted from unfixed and unstained SDS-polyacrylamide gels (5%-22% linear acrylamide gradient) as described previousy (Iqbal et al. 1984).

Antisera

Antisera to in vitro assembled microtubules and PHF were raised in rabbits as described previously (Grundke-Iqbal et al. 1979a). Anti-PHF serum was the same as employed for a previous study (Grundke-Iqbal et al. 1984). Affinity-purified antiserum to 6S tubulin from bovine brain was purchased from CAABCO Inc., Houston, TX, USA. Antisera and antigens used for this study are described in Table 1.

Table 1. Antisera employed and their immunochemical analysis on paperblots[a]

Antiserum	Antigen	Source of antigen	Polypeptides in MT and NF preparations labeled by antisera
Anti-PHF[b,e]	isolated PHF	AD brain, 56 yr	52-kDa
Anti-MT I[b,f]	MT, 2 cycles	human brain, 28 yr non-neurologic	tubulin[c], MAPS[d], P70, P150, P200, 50-kDa
Anti-MT II[b]	MT, 2 cycles	human brain, 74 yr non-neurologic	tubulin[c], MAPS, P70, P150, P200, 50-kDa
Anti-MT III	MT, 2 cycles	same as for anti-MT II	tubulin[c], MAPS, P70, P150, P200, 50-kDa
Anti-MT IV	MT, 2 cycles	same as for anti-MT II	tubulin[c], MAPS, P50, P150, P200, 50-kDa
Anti-MT V	MT, 1 cycle	calf brain	tubulin[c], MAPS, P70, P150, P200
Anti-tubulin	6 S tubulin	beef brain	tubulin[a]

[a] The antisera were analyzed by immunobinding on paperblots of SDS-polyacrylamide gels of microtubule (MT) and neurofilament (NF) preparations
[b] These antisera labeled Alzheimer neurofibrillary tangles
[c] Strongly labeled polypeptides
[d] MAPS, high molecular weight microtubule-associated polypeptides; neurofilament triplet polypeptides are abbreviated as P70, P150, and P200
[e] Grundke-Iqbal et al. (1984)
[f] Grundke-Iqbal et al. (1979b)

Absorption of the Antisera

Immunoabsorption was carried out at a tenfold higher antiserum concentration than the final concentration used for immunocytochemistry (Grundke-Iqbal et al. 1984).

Tissue for Immunocytochemistry

Paraffin blocks of autopsied hippocampus from 17 cases with clinically diagnosed and histologically confirmed AD/SDAT were used for immunocytochemistry. The tissue was fixed in 10% neutral formalin for an average of 1 week prior to embedding in paraffin; pieces of the hippocampus of one additional case with SDAT were kept in formalin for about 3 months. Six micrometer serial sections were cut and mounted on gelatine-coated slides.

Unfixed blocks of hippocampus from two SDAT cases and a rabbit cerebellum were quick-frozen in a slurry of isopentane and liquid nitrogen (Grundke-Iqbal et al. 1980) and sectioned with a cryostat.

SDS-treated Tangles for Immunocytochemistry

Isolated tangles were suspended in 0.32M sucrose containing 2% SDS and heated in a boiling water bath for 5 min. Those tangles which were not dissolved by this treatment were pelleted at $2,500 \times g$ for re-extraction with SDS as described above. After each heating step an aliquot containing about 100 tangles was dried into the wells of gelatin-coated *Toxoplasmosis* slides (Bellco Glass, Vineland, NJ, USA) for immunocytochemistry.

Immunocytochemistry:

Immunocytochemical studies on deparaffinized and rehydrated sections were performed using the peroxidase-antiperoxidase (PAP) technique (Sternberger et al. 1970) with minor modifictaions. The primary antisera at dilutions ranging from 1:250 to 1:5,000 were incubated with the tissue at 4°C for about 18h. This was followed by 1-h incubation steps at room temperature with the "bridge" antiserum, goat anti-rabbit IgG (Sternberger and Mayer, Jarrettsville, MD, USA) at 1:120 dilution and with rabbit PAP complexes (Cappel, Malvern, PA, USA) at 1:50 dilution. In between each step the slides were washed in phosphate-buffered saline (10mM sodium phosphate buffer, pH7.2, 0.15M NaCl). At the end, the slides were stained with 0.05% diaminobenzidine and 0.01% H_2O_2 in 50mM Tris buffer, pH7.6. Primary antiserum and PAP were diluted in phosphate-buffered saline containing 2% bovine serum albumin and 1% normal goat serum. The normal goat serum was not added to dilutions of the bridge serum. Pre-immune serum and a hyperimmune serum to bacteriophage ΦX were used as controls. Generally, phosphate-buffered saline was used for dilution of the antiserum and washes in between each incubation step. In some tissues with certain antisera, the neuronal cytoplasm stained so darkly that it was difficult to distinguish the immunostained tangles. In such cases, reduction of the background staining using high salt buffers (Sternberger et al. 1978) facilitated better visualization of the immunostained tangles. PAP staining on cryostat sections was performed according to the modification of Moeller et al. (1978).

Every antiserum was tested on cryostat sections of two SDAT cases and of normal rabbit cerebellum. On paraffin sections anti-MT I–III and anti-PHF sera were tested on 17 AD/SDAT cases. Anti-MT IV and V sera were tested on four of those cases in which tangle staining was detected with anti-MT I and II sera. Tangles were counted in areas of $0.14 mm^2$ at a magnification of × 195 using a Zeiss microscope equipped with a drawing tube.

Immunolabeling on Paperblots

PHF preparations were sonicated for 30min at output control setting no. 1 and 10% pulse (output about 10W) with 4-s on/off cycles, using a Branson Model 200 sonifier equipped with a tapering microprobe, and then heated in a boiling waterbath for 3min with SDS and β-mercaptoethanol each at 1% final concentration. Microtubules were solubilized like the PHF but without prior sonication. The samples were electrophoresed on a 0.75-mm slab gel using 5%–30% linear acrylamide gradient and the Laemmli (1970) buffer system. Electrophoretic transfer of protein from gels to nitrocellulose paper (0.20μm, Schleicher and Schuell) and immunoblots were carried out according to Towbin et al. (1979) at 100V for 60 min using tap water cooling. Some of the paperblots were stained with Amido Black (Schaffner and Weissman 1973). For immunolabeling, the unstained blots were postfixed in transfer buffer containing 40% methanol and 10% acetic acid for 30min. Protein binding sites were blocked by incubation with 3% bovine serum albumin for 1h at room temperature. The blots were incubated with antiserum diluted in phosphate-buffered saline containing 0.1% sodium azide and 20mg/ml bovine serum albumin for about 18h at room temperature. Incubation was carried out in plastic lids of 96-well microtiter plates (Flow Laboratories Inc., McLean, VA, USA) placed in a humid chamber. The paperblots were then washed in phosphate-buffered saline for 10min, and the bound immunoglobulin was visualized using the PAP method as described above.

Protein Concentrations

Protein concentrations were determined by the method of Lowry et al. (1951). PHF preparations were incubated with 4N sodium hydroxide overnight at room temperature prior to the protein determination (Iqbal et. a. 1984).

Results

Two of five antisera raised against microtubule preparations from normal human brains stained tangles and some neurites of the neuritic plaques in tissue sections; plaque core amyloid was not labeled. Staining of tissue sections with pre-immune sera revealed only weak background staining. Both tangle-reactive antimicrotubule sera also labeled smears of isolated tangles which remained after extraction with 2% SDS at 100°C in addition to treatment with SDS at room temperature which is part of the routine isolation procedure (Fig. 1).

The tangle-positive antimicrotubule sera (anti-MT I and anti-MT II sera) were raised against preparations from two non-neurologic cases. No tangle labeling in either cryostat or paraffin sections was observed with an antiserum against microtubules prepared from calf brain or with the other two antisera against the human microtubules, one of which (anti MT III serum) had been raised in a parallel experiment with the same antigen-adjuvant mixture used for the production of the anti-MT II serum (Table 1).

Analysis of anti-MT I-V sera on paperblots of brain microtubule and neurofilament preparations

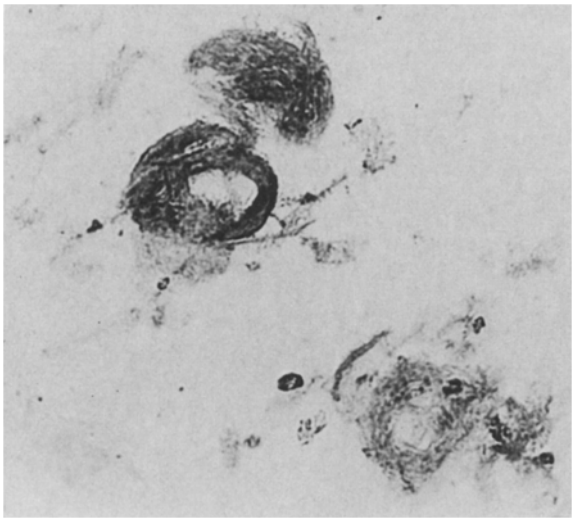

Fig. 1. Isolated Alzheimer neurofibrillary tangles immunostained with anti-MT I serum (1:1,000) using PAP technique. These tangles were twice extracted with 2% SDS at 100°C before making the smear. ×1,000

Table 2. Frequency of the labeled Alzheimer neurofibrillary tangles

Antiserum	No. of cases with tangles immunolabeled/ total no. of cases	% tangles immunolabeled[a]
Anti-PHF	17/17	95–100 (4 cases)
Anti-MT I	9/17	30– 63 (3 cases)
Anti-MT II	7/17	14– 86 (3 cases)

[a] In comparison with the number of tangles stained with Bodian in parallel sections

showed that each of these sera contained antibodies to several polypeptides including tubulin, microtubule-associated high-mol.-wt. proteins, neurofilament triplet polypeptides, and the 50-kDa polypeptide which is a common contaminant in brain neurofilament preparations (Table 1). No coincidence between the occurrence of any one of these antibodies and the immunostaining of tangles was detected.

Anti-MT I and II sera, like the antiserum which was raised against isolated PHF (anti-PHF serum, Grundke-Iqbal et al. 1984), immunostained tangles and neurites of the neuritic plaques in both AD and SDAT cases. Immunolabeling of tangles and plaques was seen even in a SDAT brain which had been removed from the body 5 days postmortem. No decrease in labeling efficiency was apparent whether tissue from a case was kept in formalin for 3 days or for 2 weeks. However, no immunostaining of tangles or any other

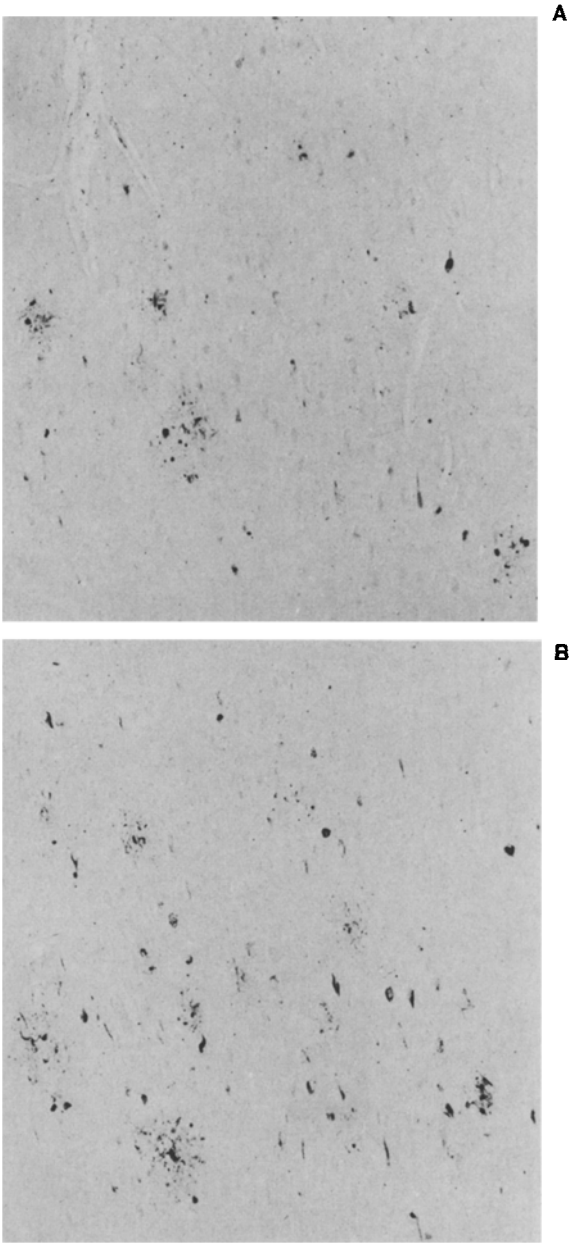

Fig. 2A, B. Alzheimer neurofibrillary tangles and neuritic (senile) plaques stained with (*A*) anti-MT I serum (1:1,000 dilution) and (*B*) anti-PHF serum (1:1,000 dilution) in neighboring parallel sections of a SDAT hippocampus. Anti-MT I serum immunostains only a fraction of the tangles labeled by anti-PHF serum. PAP technique. ×90

tissue component by these antisera was observed in tissue from a SDAT case which had been stored in formalin for more than 3 months.

Anti-MT II serum appeared to immunostain mainly tangles and plaque neurites. Anti-MT I serum also strongly stained white matter axons and in most of the cases neuronal cytoplasm and the apical dendrites

Table 3. Absorption of tangle-staining antibodies with different antigens: Comparsion between anti-MT and anti-PHF sera

Antigens	Anti-MT I and II sera[a]		Anti-PHF sera[a]	
	(μg antigen)	Tangle staining	(μg antigen)	Tangle staining
PHF	5	–	2.5	–
Microtubules	5	–	5–100	+
Neurofilaments	5–100	+	5–100	+
Actin-myosin	5–1,000	+	5–1,000	+
Keratin	50–1,000	+	50–1,000	+
Fibroblast fractions:				
Triton X-100 insoluble	40	+	40	+
Triton X-100 soluble	40	+	40	+
100,000 × *g* supernatant	200	+	200	+
100,00 × *g* pellet	200	+	200	+

[a] Absorption was carried out using 100 μl of each antiserum, anti-MT I (1:100), anti-MT II (1:300), or anti-PHF (1:100). After absorption the antisera were further diluted ten fold and tested for reactivity with tangles on paraffin sections using PAP technique

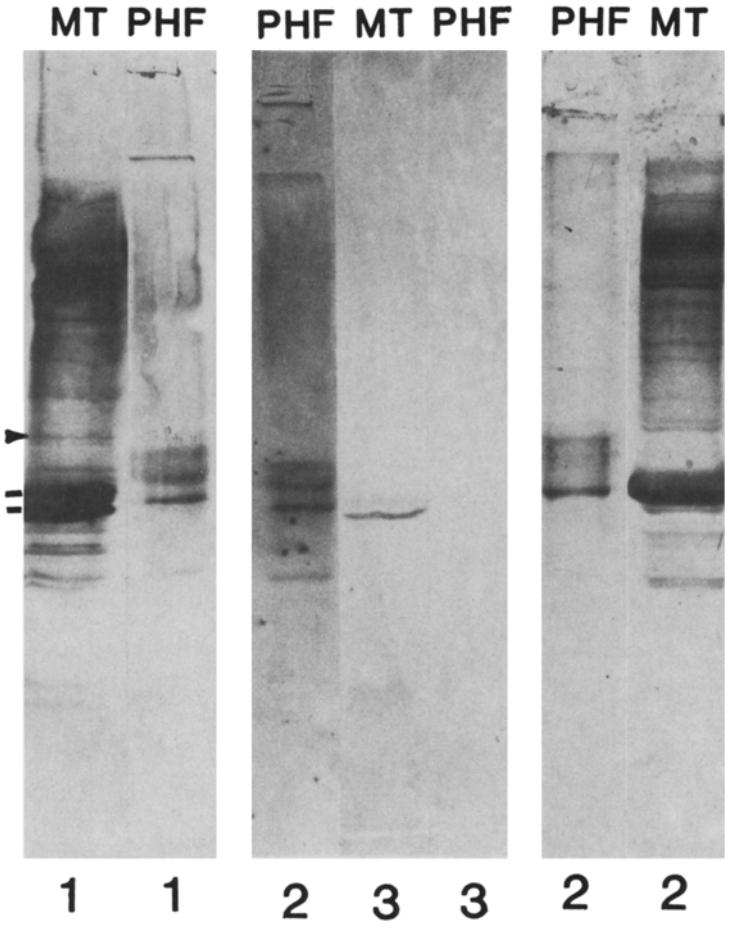

Fig. 3. Paperblots of SDS-polyacrylamide gels of PHF and brain microtubule (*MT*) preparations stained by the PAP technique with (*1*) anti-MT I serum, 1:1,000 dilution; (*2*) anti-MT II serum, 1:1,000 dilution; (*3*) anti-tubulin antibodies, affinity purified, 1 μg/ml. The bottom end of the figure represents the anodic end of the gel. The position of neurofilament polypeptide P70 and tubulin dimer is indicated with *arrowhead* and *dashes*, respectively. The PHF preparation in the right panel which showed mostly labeling of the fastet moving one of the three polypeptides was different but from the same Alzheimer brain as the PHF preparation in the other two panels

of hippocampal pyramidal cells, which sometimes interfered with the identification of the tangles. However, this difficulty was largely overcome by the use of high salt buffer (Sternberger et al. 1970) for the immunocytochemical reaction.

When anti-MT I and II sera were tested on several AD/SDAT brains it was detected that these antisera did not label tangles in all the Alzheimer cases. A comparative study on hippocampal paraffin sections of 17 AD/SDAT cases revealed that anti-MT I and

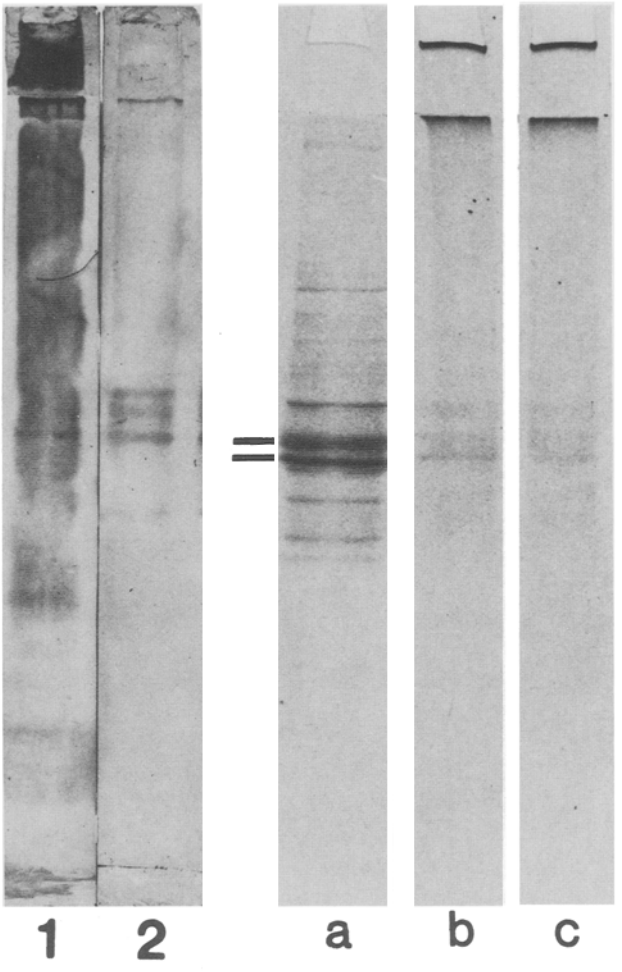

Fig. 4. **A** Paperblots of PHF polypeptides which had been transferred from adjacent lanes of a SDS-polyacrylamide slab gel to the same nitrocellulose paper: (*1*) anti-PHF serum, 1:1,000 dilution, (*2*) anti-MT II serum, 1:1,000 dilution. The rest of the details are the same as in Fig. 3. **B** Protein pattern of (*a*) microtubule and (*b,c*) PHF preparations on SDS-polyacrylamide gel which remained after transfer to nitrocellulose paper; the gel was stained with Coomassie blue. Position of the tubulin dimer is indicated in lane (*a*). Immunoblots of PHF polypeptides from lanes *b* and *c* are shown in **A**

anti-MT II sera stained tangles in nine and seven cases, respectively (Table 2). In contrast, anti-PHF serum immunolabeled tangles of all the 17 AD/SDAT cases. Furthermore, comparsion of the immunolabeled, tangle-positive tissue sections with silver-stained, parallel sections revealed that, whereas anti-PHF serum appeared to immunolabel every tangle, a smaller number of tangles were stained by the anti-MT sera (Fig. 2). The percentage of the tangles that were labeled by the anti-MT sera varied widely from case to case. This was most remarkable with the anti-MT II serum, which labeled only 14% in one case, but almost all of the tangles in another (Table 2). No increase in the number of immunolabeled tangles was observed when anti-MT II serum was used at tenfold higher concentration (1:300) instead of the optimal dilution of 1:3,000. Although no quantitative studies were carried out on the labeling of plaques neurites with the anti-MT sera, the staining of plaques appeared to parallel that of the tangles.

The anti-MT I and II sera (100 µl of 1:100 and 1:300 dilution, respectively) when preabsorbed with small amounts of isolated PHF (5 µg) from different brains as well as with microtubule preparations (5 µg, 2 cycles of assembly and disassembly) from human or even bovine brain did not stain tangles (Table 3). In contrast, tangles staining antibodies of the anti-PHF serum (100 µl of 1:100 dilution) were only removed with small amounts of isolated PHF (2.5 µg) but not with microtubule preparations. Similarly, tangles and plaques staining with either anti-MT or anti-PHF sera were not eliminated when these were employed after absorption with as much as 100 µg neurofilament, 1,000 µg actin-myosin, 40 µg Triton X-100 soluble or insoluble fractions of human skin fibroblasts, or 200 µg fibroblast 100,000 $\times g$ pellet or supernatant.

On immunoblots of SDS-polyacrylamide gels of isolated PHF strong labeling of three polypeptides in the 50-kDa to 70 kDa area was observed with both anti-MT I and anti-MT II sera (Fig. 3). The electrophoretic mobility of the fastest moving PHF polypeptide labeled was in between that of α and β tubulin, the mobility of the intermediate polypeptide was slightly slower than that of tubulin, and the slowest moving polypeptide was slightly ahead of neurofilament triplet polypeptide P70. In addition, both antisera bound to a polypeptide of about 40 kDa; in some immunoblots a weak staining of a background smear was also observed. Neither changes in the dilution of the antiserum (anti-MT II from 1:250 to 1:3,000) nor substitution of phosphate-buffered saline with tris-buffered saline produced any changes in the immunolabeling pattern and in the intensity of the background smear. Immunoblots with anti-MT sera on PHF preparations from five additional Alzheimer brains revealed similar labeling patterns. Immunostaining of PHF polypeptides with affinity-purified antibodies to tubulin was negative.

Similar to the anti-MT sera, anti-PHF serum also always labeled in all six PHF preparations polypeptides in the 50-kDa to 70-kDa area. However, the anti-PHF serum strongly labeled the background smear which often made the immunolabeled 50-kDa to 70-kDa polypeptide bands barely discernible. In addition, some of the polypeptides below 50-kDa recognized with anti-PHF serum were different from those labeled with anti-MT I or II sera. Figure 4 shows a direct

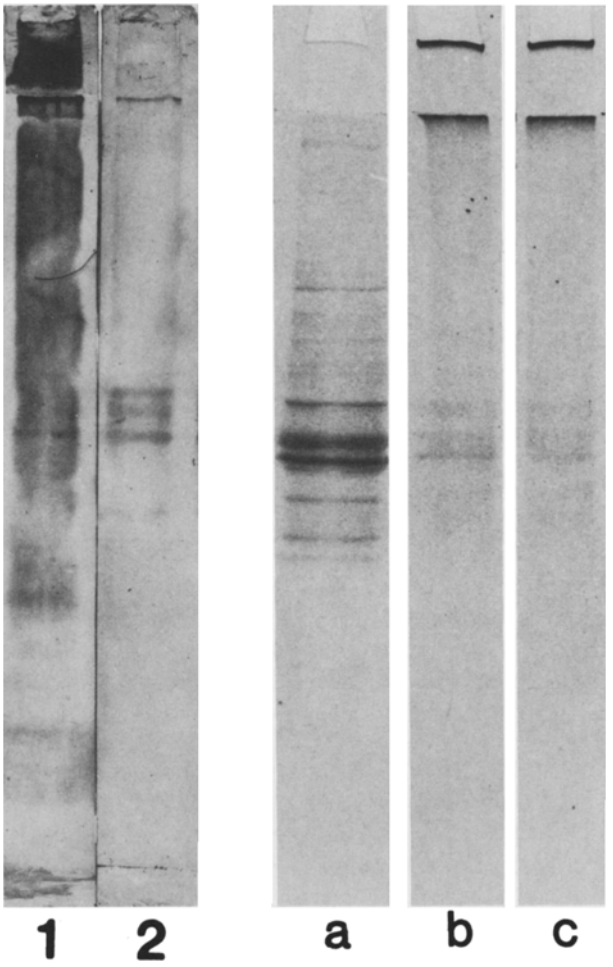

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Table 4. Absorption of tangle-staining antibodies with PHF and control polypeptides extracted from SDS-polycrylamide gels: Comparsion between anti-MT and anti-PHF sera

Antigens	Anti-MT II (100 µl 1:300)[a]		Anti-PHF (100 µl 1:100)[a]	
	(µg antigen)	Tangle staining	(µg antigen)	Tangle staining
PHF polypeptides:				
> 400 kDa[b]	1–2	–	1–2	–
50–70 kDa	1–2	–	1–2	–
35–50 kDa	1–2	–	1–2	–
15–35 kDa	1–2	–	1–2	–
Control polypeptides:				
Bovine serum albumin	25	+	25	+
Neurofilament P200	20[c]	+	25	+
Neurofilament P150	30[c]	+	50	+
Neurofilament P70	10[c]	+	50	+

[a] For immunocytochemistry the absorbed antisera were further diluted ten times
[b] Polypeptides which do not enter the gel
[c] After absorption no detectable labeling of respective NF polypeptides with anti-MT II serum on immunoblots was observed

comparison of anti-MT serum (anti-MT II) with anti-PHF serum on immunoblots of PHF polypeptides which had been transferred from adjacent lanes of a slab gel to nitrocellulose paper.

To elucidate whether the immunolabeled polypeptides in the PHF preparations might be keratin contaminants, immunoblots were performed on PHF which had been solubilized with and without 2-mercaptoethanol. Keratin forms protomers with similar electrophoretic mobility as those of PHF, but only upon reduction (Ochs 1983). No differences in the immunolabeling patterns of the reduced and unreduced PHF preparations were observed with either anti-MT II or anti-PHF sera. Furthermore, preabsorption of these antisera with large amounts of keratin filaments (up to 1 mg/100 µl antisera, see Materials and Methods) sufficient to remove the contaminating antibodies to keratin, did not remove the tangle-staining antibodies (Table 3) or change the immunolabeling pattern on immunoblots of PHF (figure not shown).

PHF specificity of the polypeptides immunolabeled on paperblots was tested by absorption of anti-MT II and anti-PHF sera with the various polypeptide fractions extracted from different areas of SDS-polyacrylamide gels of isolated PHF (Table 4). The tangle staining antibodies were not only removed by absorption of anti-MT II serum with the material at top of spacer and resolving gels and with the fraction containing the 50-kDa to 70-kDa polypeptides, but also with the fractions containing the 15-kDa to 35-kDa polypeptides. Likewise, as had been observed previously (Grundke-Iqbal et al. 1984), the anti-PHF serum, when absorbed with each of these fractions, did not stain the tangles or plaques. In contrast, no effect on the tangles or plaque staining was observed when using anti-MT or anti-PHF sera absorbed with neurofilament triplet polypeptide fractions or with bovine serum albumin which had been extracted identically from SDS-polyacrylamide gels.

Discussion

The presence of a tangle-cross-reacting antigen/s in microtubule-enriched fractions from normal brain was reported previously by us (Grundke-Iqbal et al. 1979b) and by Yen et al. (1981). The significance of this finding, however, was not clear since it had not been shown that this cross-reactivity was with PHF and not with some contaminant. In this study, we have demonstrated that this cross-reactivity is indeed most likely with PHF and that the cross-reaction is mainly with the 50-kDa to 70-kDa PHF polypeptides. It is thus possible that PHF might be made up of some normal brain polypeptide/s. PHF might be the product of some post-translational modification of normal brain proteins as a consequence of some error in metabolism. This metabolic error might be caused in genetically susceptible individuals by some environmental factor/s including unconventional transmissible agent/s (Wisniewski and Iqbal 1980).

The reaction of anti-MT I and II sera with tangles that remained after extractions in SDS suggests that the antigen/s recognized by these anti-MT sera like the antigen/s labeled by anti-PHF sera (Grundke-Iqbal et al. 1984) is not loosely trapped in between the fibrils of the tangles but is most likely inherent to the PHF. These conclusions are supported by a recent report of Perry et al. (1984) in which they demonstrated the immunolabeling of PHF at the electron-microscope level by a tangle-reactive anti-MT serum.

The different frequency of tangle labeling in tissue sections observed with antimicrotubule and anti-PHF sera indicates that the antigen/s recognized by anti-PHF serum is apparently common to all tangles, whereas the antigen/s recognized by anti-MT I and II sera is either not present in sufficient quantity or not accessible to the antibodies in all tangles. The tangle antigen/s recognized by the anti-MT sera might be more prone to formalin-induced changes than that recognized by the anti-PHF sera. Alternatively, these data might indicate different stages of maturation of the PHF during which a precursor which is labeled by the antimicrotubule sera undergoes some post-translational changes until it is either not recognized by or accessible to the antibodies. The possibility that this antigen/s might only be fortuitously present in some tangles and not in others seems unlikely because the anti-MT sera label the SDS-treated tangles and on immunoblots some of the same PHF polypeptides stained with anti-PHF sera. That most of the polypeptides in PHF preparations labeled by anti-MT and anti-PHF sera are PHF-specific is indicated by the similar labelling patterns obtained with monoclonal antibodies to PHF (Grundke-Iqbal et al., in preparation).

Comparison of immunoblots on PHF polypeptides developed with anti-MT II and anti-PHF sera shows that anti-PHF serum in addition to the aggregates at the top of the gel and the 50-kDa to 70-kDa polypeptides (Grundke-Iqbal et al. 1984), strongly labels a background smear also observed on the Coomassie blue-stained gels (Iqbal et al. 1984). The presence of significant amounts of antibodies to this material in anti-PHF serum may explain the differences between anti-MT and anti-PHF sera observed in immunocytochemical labeling of tangles in tissue sections and in absorption of the tangle staining antibodies with microtubule fractions.

Re-electrophoresis of PHF polypeptides eluted from different areas of SDS-polyacrylamide gels has suggested that the PHF polypeptide/s forms oligomers and/or aggregates of multiple molecular weights, some of which are too large to enter the gel (Iqbal et al. 1984). This property of PHF polypeptides has recently been also observed by gel filtration studies in aqueous extracts of isolated PHF (Iqbal et al. in preparation). It thus appears that part of the antibodies in the anti-PHF serum might be directed against the PHF polypeptide/s which forms aggregates at the top of the gel and which during electrophoresis continuously keeps undergoing disaggregation and migration forming a smear along the gel. In contrast, the majority of PHF-reactive anitbodies in the anti-MT sera seem to react mainly with the PHF polypeptide bands corresponding to 50-kDa to 70-kDa area. However, although no reaction on immunoblots of anti-MT sera with PHF polypeptides below 40 kDa was observed, when extracted from the SDS-polyacrylamide gels and employed for absorption < 40-kDa PHF polypeptides easily removed the tangle-reactive antibodies from both the anti-MT and the anti-PHF sera. These < 40-kDa PHF polypeptides have been shown upon re-electrophoresis to form oligomers/aggregates of multiple molecular weights including 45–62-kDa polypeptides (Iqbal et al. 1984). Thus, the absorption of the tangle-reactive antibodies of anti-MT serum by the < 40-kDa polypeptides might have been due to the generation of such oligomers during extraction from the gel. The antigenic site/s recognized by the anti-MT sera might not be present or available in the individual polypeptide chains. Alternatively, it is possible that the removal of tangle-reactive antibodies from the anti-MT serum by the < 40-kDa fractions might have been due to proteolytic fragments of 50–70-kDa polypeptides. This possibility is, however, unlikely since unlike anti-PHF sera only very little, if any, polypeptides beyond about 40,000 mol. wt. were recognized by the anti-MT sera on immunoblots.

It is not possible at this stage to know whether the differences in the immunoblots of PHF polypeptides and in the immunostaining of tangles in tissue sections between anti-MT and anti-PHF sera are due to the antibodies elicited against different polypeptides or against different conformational sites of the same polypeptides/s. Isolation and characterization of PHF polypeptide/s, currently underway in our laboratory, will hopefully provide an answer to this problem.

The absorption studies show that the PHF antigens, which are recognized by anti-PHF serum and by anti-MT I and II sera, are present in comparably large amounts in isolated PHF. In normal brain, however, only the PHF cross reacting antigen/s which is recognized by anti-MT sera is present in significant amounts. This antigen/s occurs not only in human but also in animal brain. In contrast, antigen/s recognized by anti-PHF serum only does not seem to co-purity with any of the normal neurofibers. Furthermore, as was demonstrated recently (Grundke-Iqbal et al. 1984), this antigen/s occurs in only small amounts in the normal brain in a fraction prepared identically to the PHF but is at best present in trace amounts in the microtubule- and neurofilament-enriched preparations.

Analysis of anti-MT sera on paperblots of microtubule-enriched fractions reveals no coincidence between the occurrence of antibodies to tubulin, high-mol.-wt., microtubule-associated proteins (MAPS) or neurofilament triplet polypeptides and the labeling of tangles in tissue sections. Furthermore, the lack of absorption of the tangle-staining antibodies in the

anti-MT sera with neurofilaments, neurofilament triplet, actin, myosin, and keratin suggests that the PHF cross-reacting polypeptides/s must differ from these normal fibrous proteins.

Recently, Yen et al. (1983), using brain microtubule preparation as immunogen, generated a tangle-reactive monoclonal antibody which cross reacted with vimentin. However, since in the present study the tangle-reactive antibodies of anti-MT and anti-PHF sera are not absorbed with any of the human fibroblast fractions which contain significant amounts of both vimentin and tubulin, it is unlikely that considerable cross-reactivity might exist between the PHF antigens recognized by these antisera and vimentin or tubulin.

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