Alzheimer's Disease: Insolubility of Partially Purified Paired Helical Filaments in Sodium Dodecyl Sulfate and Urea

Abstract. *A method is described for the partial purification of the paired helical filaments that accumulate progressively in human neurons in Alzheimer's disease (senile dementia). Paired helical filaments have unusual solubility characteristics, including insolubility in sodium dodecyl sulfate, urea, reducing agent, and guanidine, which prevent analysis of their molecular composition by gel electrophoresis. The paired helical filaments appear to contain covalent bonds other than disulfide, which cross-link individual filaments into a rigid intracellular polymer. Thus, paired helical filaments appear to represent an example in neurons of an insoluble cross-linked protein. Covalently cross-linked protein polymers occur in lens senile cataracts and in terminally differentiated skin keratinocytes, suggesting that there may be a common mechanism for remodeling some structural proteins during cell aging.*

Alzheimer's disease (AD) is a selective neuronal degeneration with an estimated prevalence of more than 600 persons per 100,000 (*1*). It represents the most common neuropathological change in the brains of aged humans with progressive intellectual failure. The importance to public health of understanding the molecular changes leading to neuronal degeneration in AD has recently been emphasized (*2*). Two characteristic neuronal lesions in the cerebral cortex of patients with AD are the neuritic (or senile) plaque and the neurofibrillary tangle (*3*). Small numbers of these lesions can occur in the hippocampus of aged individuals without dementia, and the frequency of occurrence of such lesions in normal persons increases with age (*4*). The density of plaques and tangles in the cortex of patients with AD appears to be correlated with the degree of dementia (*4*, *5*) and also with a deficiency of cholinergic synthetic activity (*6*). Electron microscopy of the neurofibrillary tangle and the altered neurites in the senile plaque shows that both structures contain pairs of abnormal, helically wound intermediate filaments with a periodicity of 80 nm (*3*). These neuronal organelles have not been demonstrated in the brains of aged nonhuman primates or lower mammals, nor in animal models of neurofibrillary degeneration. Despite considerable interest in the molecular pathogenesis of paired helical filaments (PHF), attempts to characterize their composition by analytical protein chemistry have been few and have yielded conflicting results (*7*, *8*). We now report a method of partial purification of PHF from postmortem human cortex. Furthermore, we demonstrate unusual solubility characteristics of PHF, including the unexpected finding that these filaments are highly insoluble in all of the reagents used to solubilize proteins for gel electrophoretic separation (*9*).

Cerebral hemispheres from three patients with AD and two age-matched normal patients were frozen (−70°C) at autopsy (*10*). For purification of PHF, 2.0 g of the frozen cerebral cortex was dissected from areas judged to have many neurofibrillary tangles on the basis of histology of the contralateral cortex. The cortex was homogenized (eight to ten strokes, glass-on-glass) in ice-cold buffer (5 ml/g) containing 0.32*M* sucrose, 1 m*M* $MgCl_2$, and 5 m*M* KH_2PO_4, *p*H 7.0, and centrifuged at 500*g* (maximum) for 10 minutes. The pellet was washed with 10 ml of buffer, and the combined wash and supernatant [total protein content, 30 to 40 mg (*11*)] was centrifuged at 93,500*g* for 20 minutes. The resultant supernatant, containing approximately 50 percent of the original protein, was adjusted to 2 percent in Triton X-100, incubated on ice for 45 minutes, and centrifuged at 110,000*g* for 60 minutes. Recovery of protein in this final pellet was 1 to 2 percent of the starting protein (after centrifugation at 500*g*). The pellet was either fixed for electron microscopy in buffered 2.5 percent glutaraldehyde or prepared for one- or two-dimensional gel electrophoresis (*9*).

Electron microscopy of multiple sections through the final pellets of numerous AD preparations revealed abundant PHF fragments, which were found throughout 70 to 80 percent of the cross-sectional area of the pellet (Fig. 1A). The lengths of the PHF fragments ranged from approximately 160 nm (two turns) to 800 nm (ten turns). The background consisted of lightly stained amorphous material, small dense granules that appeared to be ribosomes, and occasional larger dense bodies that resembled lipofuscin. Almost no membranous profiles were seen. Twenty to 30 percent of the cross-sectional area of the pellet consisted of a zone of fine, dense granules, free of PHF, located at an outer edge of the section. We estimate that 30 to 40 percent of the total visualized material in pellets from numerous AD preparations represented clearly identifiable PHF. No PHF were found in identically prepared pellets from normal cortex.

Two-dimensional gel electrophoresis (*p*H gradient, 4.5 to 6.5) with ultrasensitive silver staining (*9*) was used to compare PHF fractions from AD cortex with identically prepared fractions from age- and area-matched normal cortex (Fig. 2, A and B). Careful comparison revealed highly similar patterns of the more than

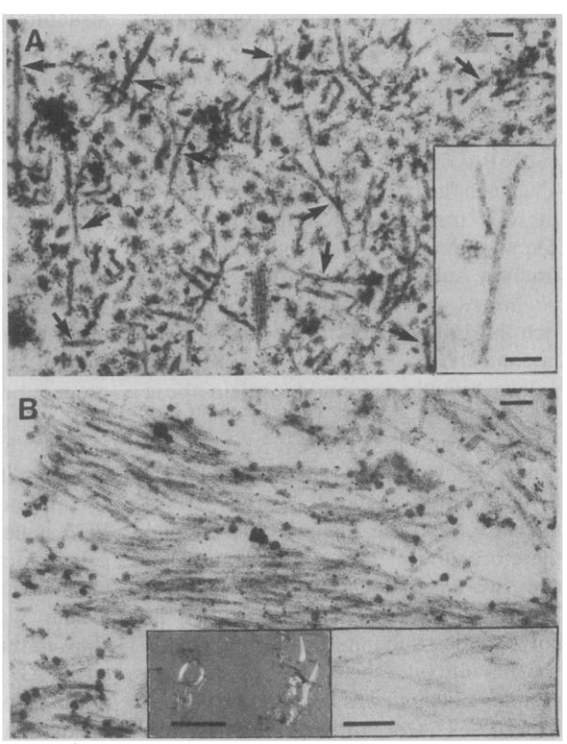

Fig. 1. (A) Electron micrograph of Triton-extracted PHF-rich pellet prepared from cortex of patient with Alzheimer's disease. Fragments of PHF, some of which are indicated by arrows, are scattered throughout the pellet. Inset: higher magnification (scale bars, 100 nm). (B) Electron micrograph of PHF fraction after it was heated in SDS-BME, showing mass of intact PHF (scale bar, 100 nm). Right inset: preservation of helical conformation of PHF (scale bar, 100 nm); left inset: light micrograph of a pellet after it was heated in SDS-BME, showing preservation of birefringent Congo red–stained neurofibrillary tangles, including one with circular (perinuclear) configuration (scale bar, 30 μm).

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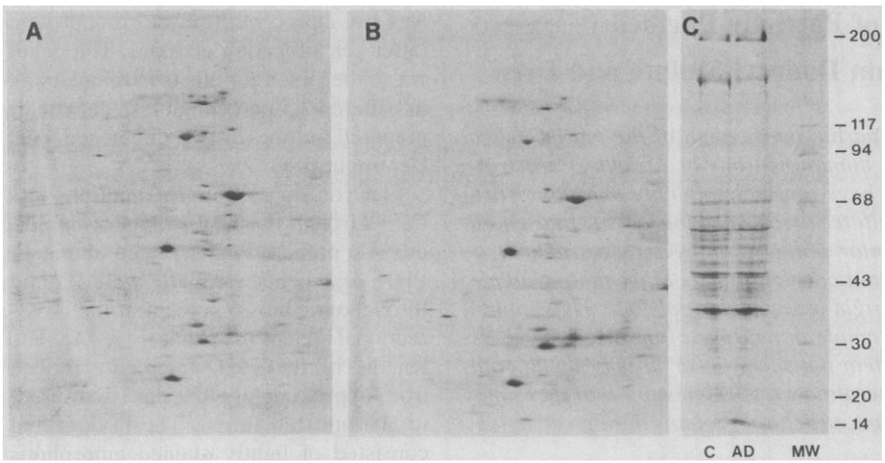

Fig. 2. Two-dimensional, silver-stained gel electrophoretograms of (A) control pellet containing no PHF; (B) identically prepared PHF pellet from the cortex of patient with Alzheimer's disease; and (C) one-dimensional SDS-PAGE (5 to 15 percent linear acrylamide gradient) of control (*C*) and AD pellets; *MW*, molecular weight markers ($\times 10^3$).

200 visualized spots, with very little difference in intensity of major spots and no consistently increased or extra polypeptides in the PHF fractions. Coomassie blue–stained two-dimensional gels showed the same result. The top of the first dimension (isoelectric focusing) gel and the buffer overlying this gel, representing material that did not migrate into the gel, were collected, heated to 100°C for 2 minutes in a sample buffer containing 2 percent sodium dodecyl sulfate (SDS), 5 percent β-mercaptoethanol (BME), 10 percent glycerol, and 35 m*M* tris, *p*H 6.8, and subjected to electrophoresis. The protein patterns of this excluded material were highly similar to those of material that had fully entered the gel; no differences between AD and control samples were seen. Next, AD and control pellets were heated for 2 minutes in sample buffer and examined by one-dimensional SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Again, no consistent change in the polypeptide composition of the AD fraction was found (Fig. 2C). Electron microscopy of a portion of each fraction subjected to protein analysis confirmed that the AD fractions were rich in PHF and that there was no PHF in controls.

Two hypotheses could explain these results. (i) The PHF remain insoluble after exposure to 2 percent SDS, 5 percent BME, 9.5*M* urea, 1 percent NP40, 2 percent Triton X-100, 0.2 percent ribonuclease, and heating to 100°C, and therefore do not enter conventional one- and two-dimensional gel systems. (ii) The PHF are principally composed of polypeptide subunits with molecular weights outside the range of our gel system (12,000 to 220,000). To test these hypotheses, we purified PHF by the above method and divided the final Triton-extracted fraction into two pellets, one receiving no further treatment, and the other heated to 100°C for 2 minutes in a solution of 2 percent SDS and 1 percent BME (SDS-BME) and centrifuged at 110,000*g* for 60 minutes. Electron microscopy revealed abundant PHF fragments throughout the SDS-BME–treated pellet; the helical conformation of the PHF appeared unchanged (Fig. 1B).

Extraction with SDS-BME resulted in considerable further enrichment of PHF in comparison with untreated pellets (Fig. 1, A and B). Electron microscopy of the extracted pellets showed that, although the PHF were the most abundant recognizable structures, other structures insoluble in SDS-BME were present, many resembling poorly preserved lipofuscin granules. The amount of protein recovered in the SDS-BME–insoluble pellet was 0.1 to 0.3 percent of the starting protein and was approximately 10 percent of the protein in the Triton-extracted pellet before SDS-BME treatment. These values, which were highly reproducible, were the results of an assay employing Coomassie blue protein binding (*11*) after thorough dispersion of the pellet in buffer. Identically prepared control pellets (shown by electron microscopy to be PHF-free) were smaller than PHF pellets and had approximately half their protein yield. Electrophoresis of the SDS-extracted PHF pellets on 2 percent agarose tube gels, which allow visualization of high molecular weight polypeptides (to a molecular weight of at least 2 million), showed that no protein entered the gel in either AD or control fractions, thus confirming the absence of soluble proteins in the extracted pellets. A band of excluded material was seen at the top of the gel in the AD sample. Since no quantitative assay for PHF in intact cortex or aqueous samples currently exists, we cannot estimate the precise recovery of PHF after SDS-BME extraction; it is possible that a small portion of the PHF was solubilized. However, the marked enrichment of PHF in the final pellet and the preservation of the characteristic helical configuration suggest that most, if not all, of the PHF are highly insoluble in SDS-BME.

The solubility characteristics of PHF were further assessed by extraction of the Triton-treated pellets in 6*M* guanidine hydrochloride, 1 percent BME, and 0.1*M* tris, *p*H 7.6, at room temperature for 4 hours, followed by centrifugation at 110,000*g* for 60 minutes. Electron microscopy showed that the PHF remained well preserved in guanidine hydrochloride. Analysis of this material by SDS-PAGE after extensive dialysis against urea and then SDS did not demonstrate any protein entering the gel. Electrophoresis of guanidine-extracted samples on 2 percent agarose gels revealed a single band that was identical in AD and controls; a band of excluded material was again seen at the top of the AD gel.

We also purified PHF by a method involving serial nylon mesh sieving of chopped cortex, followed by Dounce homogenization, centrifugation through 1.0*M* sucrose (100,000*g* for 30 minutes), and extraction in 1 percent Triton, 0.6*M* NaCl, 15 m*M* $MgCl_2$, 5 m*M* EDTA, 0.2 mg/ml deoxyribonuclease I, and 20 m*M* tris-HCl, *p*H 7.6. This procedure resulted in a final pellet containing many apparently intact neurofibrillary tangles (bundles of PHF), as judged by the light microscopic appearance of the characteristic birefringence of Congo red–stained neurofibrillary tangles under polarized light. The tangles retained their Congo-red birefringence after they were heated in SDS-BME (Fig. 1B, inset); electron microscopy showed preservation of the PHF, signifying that both the helical conformation of the individual filaments and the forces keeping PHF together in neurofibrillary tangles were not demonstrably altered. Manual counts of the neurofibrillary tangles before and after SDS-BME indicated no quantitative loss of tangles. Also, the isolated tangles remained intact after extraction with either 0.2*N* HCl or 0.2N NaOH. Such neurofibrillary tangle–enriched fractions, when examined by SDS-PAGE after being heated in SDS-BME, showed no protein differences from identically prepared control fractions.

Our results indicate that the PHF that

accumulate progressively in selected neurons during aging of the human brain, particularly in senile dementia of the Alzheimer type, are highly insoluble complexes whose bonds are not broken by SDS-BME, 9.5*M* urea, 2 percent Triton X-100, 1 percent NP40, 6*M* guanidine hydrochloride, and 0.2*N* HCl or NaOH. This unexpected finding may explain our earlier report of no differences in the amount of neurofilament proteins or other neuronal cytoskeletal proteins between electrophoretograms of AD cortical fractions rich in neurofibrillary tangles or PHF and electrophoretograms of control fractions (*8*). It suggests that the established gel electrophoretic techniques used in previous attempts to characterize PHF composition did not allow entry into the gels of the principal polypeptides making up the PHF. This observation provides a caveat regarding the use of gel electrophoresis in the search for protein abnormalities in diseased or aged brain tissue.

The finding of the insolubility of PHF after heating in detergents and reducing agents strongly suggests that covalent bonds other than disulfide (*12*) cross-link specific amino acids of the individual filaments into a rigid intracellular polymer. Precedent for this type of cross-linking of structural proteins exists in descriptions of unusual, covalently bound insoluble polymers in human erythrocytes, skin keratinocytes, and senile cataracts (*12, 13*). Our data provide evidence that such high molecular weight, covalently cross-linked proteins can occur in nerve cells. Different protein polymers in senile cataracts, terminally differentiated epidermal cells, and red blood cells are covalently cross-linked by γ-glutamyl-ε-lysine side-chain bridges (*13, 14*). In each case, the presence of ε-(γ-glutamyl)lysine cross-links was postulated on the basis of insolubility in SDS and reducing agents and was subsequently confirmed. These cross-links are believed to be assembled under the catalytic influence of a Ca^{2+}-dependent transglutaminase (*12–14*). Activation of transglutaminase by an increase in cytoplasmic availability of Ca^{2+} and subsequent protein cross-linking may play a general role in some remodeling reactions during cell aging (*13*). We recently demonstrated that transglutaminase is present in the human brain and can cross-link normal human neurofilament proteins into an insoluble high molecular weight filamentous polymer (*15*). On the basis of these precedents and our findings, we propose a testable model for age-related fibrillary degeneration of human neurons that involves cross-linking of neuronal intermediate filaments [perhaps by ε-(γ-glutamyl)lysine bonds formed by transglutaminase] and consequent accumulation of permanent, highly rigid fibrous polymers that may irreversibly alter the dynamics of the neuronal cytoskeleton and the maintenance of axons and dendrites.

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Autoantibodies to Nucleosomal Proteins: Antibodies to HMG-17 in Autoimmune Diseases

Abstract. *The relative amounts of autoantibodies against defined nucleosomal proteins present in serums from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and mixed connective tissue disease (MCTD) have been examined by an enzyme-linked immunoassay. Autoantibodies to nucleosomal proteins were detected in 45 percent of the patients with SLE, 18 percent of the MCTD patients, and none of the RA patients. The results suggest that, in SLE, antibodies are formed against a subset of nucleosomes which contain protein HMG-17.*

Serums from patients with the clinically distinct systemic rheumatic diseases—systemic lupus erythematosus (SLE), progressive systemic sclerosis, Sjögren's syndrome, rheumatoid arthritis (RA), and mixed connective tissue disease (MCTD)—contain autoantibodies to a variety of nuclear macromolecules. Nuclear molecules recognized as antigens in these autoimmune diseases include single- and double-stranded DNA, RNA, ribonucleoproteins, deoxyribonucleoproteins, nucleosomes, histones, and nonhistone proteins (*1*). One of the central questions in studies of antibodies to nuclear antigens is whether the serological specificities can aid in the clinical diagnoses of systemic rheumatic diseases. Definition of the specificities of the serum antibodies in these patients by accurate, sensitive, and convenient immunoassays could aid in answering the above question.

We have used an enzyme-linked immunoassay (ELISA) (*2*) technique to quantify the relative amounts of antibodies against the four nucleosomal histones, and against nucleosome associated nonhistone chromosomal proteins HMG-1, HMG-2, and HMG-17 in serums from patients with SLE, RA, and MCTD. We find that (i) antibodies to some of these proteins are present in both SLE and MCTD but not in RA. (ii) Antibodies to protein HMG-17 are significantly more prevalent than antibodies to

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