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Amyloid of neurofibrillary tangles of Guamanian parkinsonism-dementia and Alzheimer disease share identical amino acid sequence

(cerebrovascular amyloid/amyloid plaques/paired helical filaments/Down syndrome)

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ABSTRACT **The presence of abundant intraneuronal amyloid in the form of neurofibrillary tangles (NFT) in the brains of Guamanian parkinsonism-dementia patients and the absence of extraneuronal amyloid in the form of vascular amyloid deposits or senile plaques permit the purification of NFT without contamination with extraneuronal amyloid. Thus, we have isolated and determined the amino acid sequence of the polypeptide subunit of the amyloid fibrils of these NFT and describe their ultrastructure. The NFT, which consist of single and paired helical filaments, similar to those of Alzheimer disease, and occasionally triple helical filaments, are composed of multimeric aggregates of a polypeptide of 42 amino acids (A_4 protein). The relative molecular mass of the subunit protein, 4.0–4.5 kDa, is the same as the molecular mass of the amyloid of NFT, of the amyloid plaque cores, and of vascular amyloid deposits in Alzheimer disease and Down syndrome; the sequence of 15 amino acid residues at the N-terminus of the amyloid fibrils in the NFT of Guamanian parkinsonism-dementia is identical to that of the amyloid of NFT, amyloid plaque cores, and cerebrovascular deposits in Alzheimer disease and Down syndrome. Furthermore, the heterogeneity, or variation in polypeptide length, of the N-terminus of the amyloid of Guamanian parkinsonism-dementia is the same as in Alzheimer disease and Down syndrome. Our observations indicate that the brain amyloids of these diseases have a common subunit protein, which would also indicate a common pathogenesis.**

We report here the ultrastructure of purified neurofibrillary tangles (NFT) of Guamanian parkinsonism-dementia (PD), its subunit molecular mass, subunit amino acid composition, and the partial amino acid sequence of the N-terminus. In Guamanian PD extracellular amyloid deposits in the form of senile plaques, and deposits of amyloid in vascular walls are absent, whereas typical NFT are abundant in many brain areas (1–4). The absence of senile plaques and vascular amyloid deposits, which could contaminate the purification procedure for NFT, makes Guamanian PD brain tissue an excellent preparation for the study of NFT free from other forms of brain amyloid.

Previous studies indicate a close ultrastructural similarity between the NFT of Alzheimer disease (AD) and that of Guamanian PD (2, 3, 5, 6). Purified NFT of AD are composed of amyloid filaments measuring 10 nm in diameter that are mostly paired and helically wound with a periodicity of 80 nm, although some single filaments are seen (7). Earlier observations by Hirano (3) described the ultrastructural neurofibrillary changes in embedded tissues of Guamanian PD as collections of very dense parallel 10-nm to 30-nm fibrils displaying a regular characteristic constriction at 80-nm intervals that resemble NFT described for AD by Terry (8), Kidd (9), and Wisniewski *et al.* (6).

Masters and coworkers (10–12) have shown that in both AD and Down syndrome (DS) (*i*) the intraneuronal paired helical filaments (PHF) forming NFT, (*ii*) the extracellular amyloid fibrils of amyloid plaque cores, and (*iii*) the vascular amyloid deposits are all composed of an amyloid subunit protein (A_4) of 4.0–4.5 kDa that contains similar amino acids and has an identical 42-amino acid sequence. The heterogeneity, or variation in polypeptide length, at the N terminus in the intraneuronal amyloid fibrils of NFT is common to both diseases. This variation is considerably less in the extracellular amyloid fibrils of amyloid plaque cores (10–12), and Glenner and Wong found no N-terminal heterogeneity in vascular amyloid (13, 14, 22). There has been reluctance on the part of many neuropathologists to accept a common subunit composition for all three structurally different forms of brain amyloid deposition.

MATERIALS AND METHODS

Frozen brain tissues from five Guamanian Chamorros (four PD patients and one control) were studied. The PD cases were two females, 69 and 70 years old, and two males, both 62 years of age; brains were removed 2.5 hr, 7 hr, 4 hr, and 5 hr postmortem, respectively. The control tissue was from a male Chamorro 48 years of age, who was autopsied 27 hr postmortem; this particular specimen was selected as being neuropathologically normal—i.e., free of NFT or other neuropathologic changes. The larger blood vessels, meninges, and most of the white matter were dissected from the brain tissue, and the remaining grey matter was finely minced before processing.

Purification Procedures. The initial procedure was done using the method of Ihara *et al.* (15). The second purification procedure was done according to the method described by Masters *et al.* (12) and modified for NFT isolation (10). The third procedure was a modification of the method of Masters *et al.* (10–12), using 0.07% β-mercaptoethanol in all major steps. The purified sample was stained with Congo red for

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Abbreviations: PD, parkinsonism-dementia; AD, Alzheimer disease; NFT, neurofibrillary tangles; DS, Down syndrome; PHF, paired helical filaments.

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amyloid, and the presence of NFT was confirmed by electron microscopy after each procedure.

Negative Contrast Electron Microscopy. Three-microliter aliquots of each sample were applied to freshly glow-discharged, 400-mesh carbon-coated copper grids for 1 min. Excess sample was drained from the grids, and the grids were washed several times with deionized water. Grids were then stained with 2% (wt/vol) aqueous uranyl acetate for 30 sec, dried, and examined by a Philips EM201 electron microscope at 80 kV.

PAGE. NFT were dissolved in formic acid for 60 min at ambient temperature and centrifuged at 10,000 × *g* for 20 min. The soluble fraction contained 96.5% of the total protein as determined by quantitative amino acid analyses of total NFT. The soluble fraction was then lyophilized, dissolved in a buffer of 6 M freshly deionized urea/1% sodium dodecyl sulfate/1% β-mercaptoethanol/bromphenol blue dye and heated for 30 min at 45°C before loading on a 15% (wt/vol) polyacrylamide gel, 1 mm thick and 7.5 × 7.5 cm that was made from urea-free stock solutions. No differences in resolution were seen between urea-containing and urea-free polyacrylamide gels. Electrophoresis was done at 28-mA constant current and continued until incorporated bromphenol blue dye reached the anode. The protein bands were stained with Coomassie brilliant blue R.

HPLC. Protein components of NFT (1–3 μg total) were dissolved in formic acid and separated from the residue as described for PAGE. The soluble fraction was lyophilized and redissolved in 50 μl of HPLC buffer (0.1% $NaDodSO_4$/200 mM Na_3PO_4, pH 6.8). Chromatography was done on two analytical GF-250 columns (Du Pont) in tandem arrangement with a guard column (2 × 30 mm) filled with pellicular ether phase (Du Pont). The flow rate was 0.2 ml per min, and the protein peaks were detected by absorbance at 214 nm and 280 nm. For amino acid sequencing, proteins were precipitated with acetone [acetone/buffer, 19:1 (vol/vol)] to remove Na_3PO_4 and excess detergent.

Amino Acid Analysis. NFT proteins (0.1–1.0 μg) were dissolved in formic acid as described for PAGE. The soluble fraction was hydrolyzed in 6 M HCl for 24 hr at 110°C under vacuum. Total NFT was hydrolyzed directly in 6 M HCl to quantitate the total protein content of NFT isolates. Analysis was done on an automated amino acid analyzer (LC5001, Biotronik, Frankfurt) at 0.2 absorbance unit at full scale.

Protein Sequence Analysis. Samples were dissolved in 30 μl of formic acid and dried on glass fiber discs of a gas/liquid solid-phase protein sequencer equipped with on-line phenylthiohydantoin-detection (model 470A and model 120A, Applied Biosystems, Foster City, CA). The filters were washed in anhydrous trifluoroacetic acid, dried and preloaded with 1.5 mg of preconditioned polybrene (Aldrich) (10–12). The NFT proteins sequenced were: (*i*) 9-kDa NFT fractions, (*ii*) total NFT, (*iii*) formic acid-soluble NFT proteins, (*iv*) the fraction of NFT proteins obtained by formic acid extraction, which was soluble in HPLC buffer ("collagen-free" NFT proteins), and (*v*) protein of 4.5-kDa relative mass electro-eluted from $NaDodSO_4$/PAGE.

RESULTS

Neuropathological findings confirmed the clinical diagnosis of Guamanian PD in the four patients (1, 16, 17). Congo red staining of neurons and isolated NFT revealed typical green birefringence for these four specimens and was negative for the control tissue. NFT were found only in the four PD cases, and no amyloid filaments were found in the control tissue.

Ultrastructure. Our purified preparations of NFT from Guamanian PD examined by electron microscopy with negative-contrast stain showed aggregates of NFT that were composed of long bundles of filaments (Fig. 1*A*) at low magnification. Closer examination at higher magnification showed paired, helically coiled filaments; these paired helical filaments had an average diameter of 20 nm at its widest point (Fig. 1*B*). Each pair of helical filaments was composed of two filaments 10 nm in diameter that had an average crossover periodicity of ≈180 nm. This periodicity differed significantly from the 80-nm periodicity of PHF observed in AD. Previously, Merz *et al.* (5), who purified PHF from Guamanian PD patients, described them as similar to those of AD. The differences in the crossover periodicity between the PHF of Guamanian PD and AD could have resulted from differences in the purification procedure.

Occasionally, twisted filaments were observed to contain three filaments (Fig. 2 *A* and *B*). Each component filament was ≈10 nm in diameter and morphologically similar to those seen in the paired helical filaments. The resulting twisted triplets were too short to allow measurement of the crossover points. These triplets were found on two occasions in specimens from the same PD case.

Isolated, single filaments with a diameter of 5–15 nm were also observed (Fig. 3*A*). These single filaments appeared to be composed of three to four subfilaments, each having a diameter of 2–5 nm (Fig. 3*B*), and these single filaments are unlikely to be components of PHF, because such subfilaments were not found in the PHF unit filaments.

Protein Studies. NFT used for amino acid sequencing studies were purified from the brains of two 62-year-old Guamanian males with PD. For purifying NFT the method of Masters *et al.*, modified by the addition of β-mercaptoethanol was used. Neuropathologic examination of the hippocampus, frontal cortex, temporal cortex with amygdala (including the nucleus basalis of Meynert), basal ganglia, midbrain, pons and cerebellum at the level of the trigeminal nerve (including the locus ceruleus), medulla oblongata, and upper cervical cord (C1–C2) confirmed the diagnosis of Guamanian PD. There was no evidence of extracellular amyloid in the form

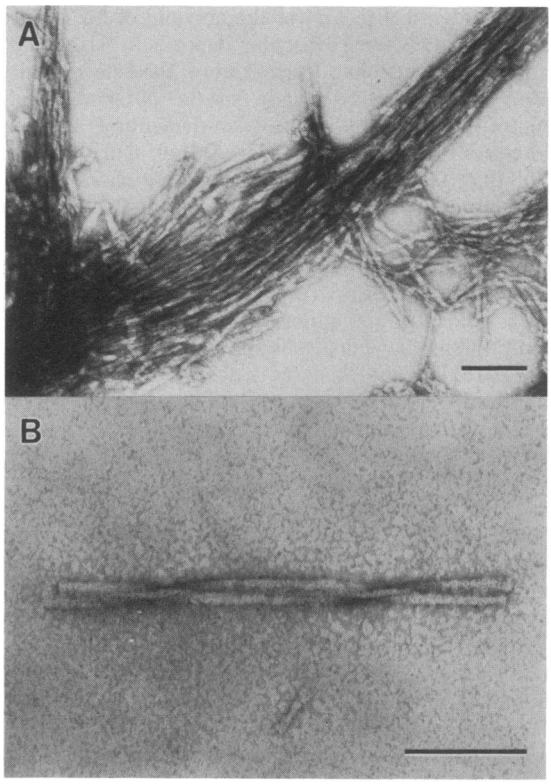

FIG. 1. Electron micrographs of purified NFT from Guamanian PD patient stained with uranyl acetate. Dense bundles of NFT (*A*) and a PHF (*B*) are shown. Bar = 100 nm.

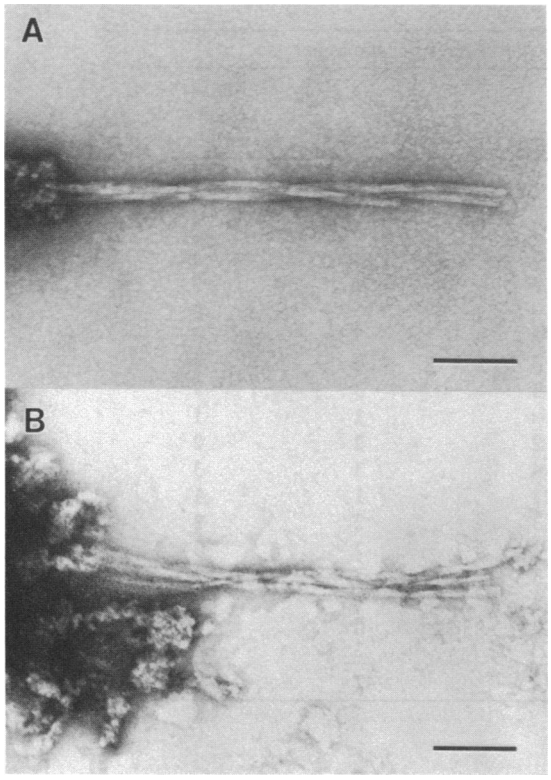

FIG. 2. Electron micrographs of triple helical filaments (*A* and *B*) from purified NFT of Guamanian PD. Bar = 100 nm.

of senile plaques or of vascular amyloid deposits. Immunocytochemical studies on paraffin-embedded sections using monoclonal antibodies raised against the PHF of AD recognized the NFT of Guamanian PD, and polyclonal antibodies raised against the amyloid plaque core of AD failed to demonstrate any extracellular amyloid in Guamanian PD. Most antibodies that reacted with the NFT of AD also reacted with the NFT of Guamanian PD (S. Shankar, personal communication).

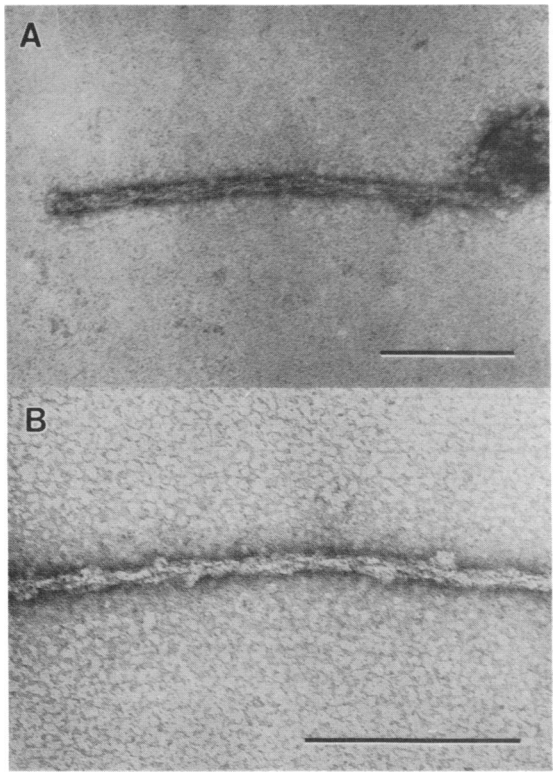

FIG. 3. Electron micrographs showing single unpaired filaments from purified preparation of Guamanian PD. These appear to be composed of three to four subfilaments (Fig. 3*B*). Bar = 100 nm.

Molecular Mass. The isolated NFT were pelleted, and the pellet was extracted with formic acid yielding 0.0039 g of insoluble protein per μl (3.5% of total) and 0.109 μg of soluble protein per μl (96.5% of total protein), as determined by quantitative amino acid analysis. The relative molecular mass of the soluble protein was determined by $NaDodSO_4$/PAGE without urea and stained with Coomassie brilliant blue R-250. The NFT of Guamanian PD and of AD, the synthetic polypeptide of the subunit protein made up of 42 amino acid residues (A_4 protein) (Fig. 4), and the amyloid plaque core of AD (data not shown) showed exactly the same protein band corresponding to a relative subunit molecular mass using $NaDodSO_4$/PAGE.

Amino Acid Composition. No differences were found between the subunit amino acid composition of the formic acid-soluble and insoluble NFT of Guamanian PD (Table 1). Furthermore, the amino acid composition of the NFT of Guamanian PD was similar to that NFT of AD and that of the synthetic polypeptide of 42 amino acid residues of AD (Table 1).

Sequence Analyses. The amino acid sequences of the formic acid-soluble NFT of two Guamanian PD cases were identical to that of the NFT of AD (Table 2), and neither was homologous with any of the known components of normal neurofilaments. The sequence of the first 15 amino acids of

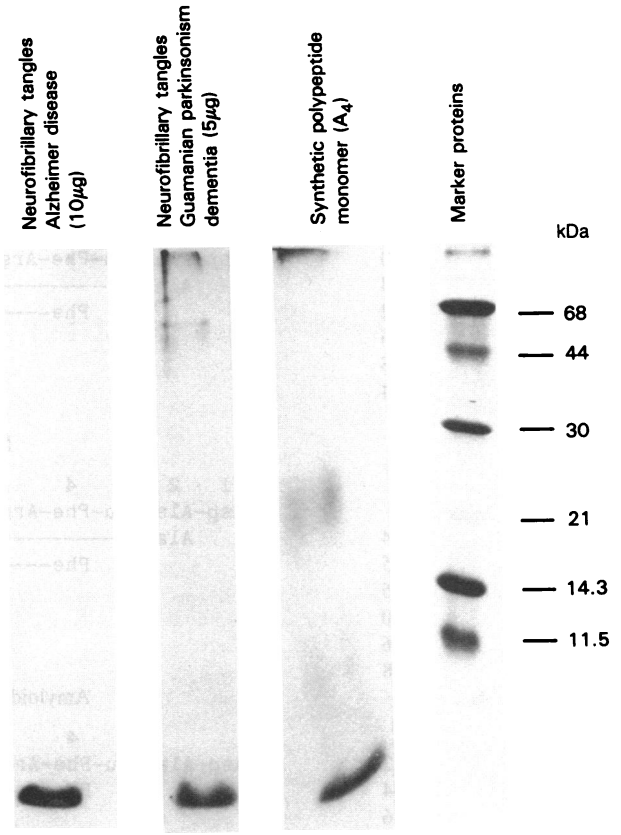

FIG. 4. $NaDodSO_4$/PAGE of the NFT protein from an AD patient (10 μg) (*A*), Guamanian PD patient (5μg) (*B*), and a 42-amino acid synthetic polypeptide A_4 protein; (*C*) proteins were dissolved and loaded in sample buffer containing 6 M urea and 1% $NaDodSO_4$, stained with Coomassie brilliant blue R. Marker proteins are in the right lane.

Table 1. Amino acid composition of NFT of Guamanian PD

Amino acid	Formic acid, mol %		Residues per monomer, no.		
	Soluble (96.5%)	Insoluble (3.5%)	Guamanian PD	AD	Synthetic polypeptide monomer
Asp	7.65	8.02	3	4	4
Thr	5.20	5.53	2	1	0
Ser	8.42	8.58	3	3	2
Glu	11.32	12.03	5	5	4
Pro	5.43	6.64	3	1	0
Cys	+	+	+	+	0
Gly	10.71	12.31	6	7	5
Ala	6.35	6.36	3	3	4
Val	5.20	4.98	3	3	7
Met	−	−	+	1	1
Ile	4.59	4.56	2	2	2
Leu	10.10	7.88	4	3	3
Tyr	3.14	2.63	1	1	1
Phe	6.81	6.09	3	2	3
His	2.30	2.21	1	2	3
Lys	7.96	8.16	3	3	2
Arg	4.82	4.01	2	1	1
Trp	+	+	+	0	0
Total			44	42	42

+, trace amounts; −, not detectable.

the various isolates, subjected to microsequencing, showed a pattern identical to that obtained for both the amyloid of NFT and the amyloid of plaque cores from AD and DS (10–12). The formic acid-soluble fraction, the isolated 4.0- to 4.5-kDa proteins, the 9-kDa HPLC fractions, and the soluble NFT proteins obtained by formic acid extraction and subsequent solubilization with HPLC buffer (figures not shown) gave the amino acid sequence of Table 2. The other HPLC peaks, proteins eluting before the 9-kDA fraction showed release of proline and glycine derivatives in all degradation steps and in

Table 2. N-terminal amino acid sequences of the 4.0- to 4.5-kDa amyloid subunit of the NFT of two Guamanian parkinsonism-dementia cases

% chain	Sequences
	NFT of Guamanian PD
	1 2 4 8 9 10 12 15
60	Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln
4	Ala----------
12	Phe----------
6	Ser----------
8	Gly----------
4	Tyr----------
	Val----------
	NFT of AD
	1 2 4 8 9 10 12 15
	Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln
14	Ala----------
25	Phe----------
15	Ser----------
20	Gly----------
16	Tyr----------
8	Val----------
	Amyloid plaque core of AD
	4 8 9
12	Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln
64	Phe----------
16	Ser----------
8	Gly----------
	Amyloid congophilic angiopathy of AD
100	Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Gln-Val-His-His-Gln

Most of the Guamanian PD polypeptide consisted of the longest chain. Data for the 4.0- to 4.5-kDa amyloid subunit of the NFT and the amyloid plaque core of AD are taken from Masters *et al.* (10, 12), and the meningeal vascular amyloid of AD sequence is taken from Glenner and Wong (13).

some steps also release of leucine and lysine derivatives in addition to those shown in this table. In contrast to the NFT of AD and DS, the NFT of Guamanian PD consisted primarily of full-length chains. These chains, starting at the aspartic acid residue, have been found only in trace amounts for NFT of AD and DS, but these chains occur in greater abundance in vascular amyloid deposits in both AD and DS (14). Smaller species were also detected in less number, 12% of which started with the phenylalanine residue; these shorter chains may all have been produced by proteolysis. The heterogeneity of the N-terminus of the subunit protein of the intracellular amyloid in the form of the NFT of Guamanian PD is also shared by the NFT of AD (Table 2).

DISCUSSION

Intraneuronal amyloid in the form of NFT in PD is composed of single, paired helical, and occasionally triple helical filaments. This pleomorphism of fibril types may indicate a different time sequence in the formation of NFT than in AD and DS, but the subunit amyloid protein from which the filaments are formed is always the same.

That the intraneuronal deposition of insoluble amyloid originates from a precursor protein of extraneuronal origin (from microglia or from serum) is hard to conceive. A more tenable hypothesis is that a gene specifying the precursor protein of intraneuronal amyloid may be derepressed or altered, or that the normally expressed precursor protein may be post-translationally modified. The cDNA coding for the brain amyloid in AD and DS has been isolated, cloned, sequenced, and localized in chromosome 21 by Kang *et al.* (18) and Goldgaber *et al.* (19). This nucleic acid sequence codes for the amyloid of NFT, which appears at an early age in some people living in the high-incidence foci of PD and amyotrophic lateral sclerosis of the Western Pacific, as well as coding for the brain amyloid in AD, DS, and aging.

Studies are needed to determine mechanisms that derepress this gene, alter its expression, or modify its normal product to form amyloid. Environmental factors that lead to the deposition of calcium, aluminum, and silicon in the brains of PD and amyotrophic lateral sclerosis patients (20, 21) in the high-incidence foci of the Western Pacific may provide direction in the task of unraveling this biosynthetic or post-translational modifying process.

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1. Hirano, A., Malamud, N. & Kurland, L. T. (1961) *Brain* **84,** 662–679.
2. Hirano, A. (1965) in *Slow, Latent and Temperate Virus Infection*, Monograph No. 2, eds. Gajdusek, D. C., Gibbs, C. J., Jr., & Alpers, M. (Natl. Inst. Neurol. Dis. Blindness, Natl. Inst. Health, GPO, Washington, DC), pp. 23–27.
3. Hirano, A. (1973) *Prog. Neuropathol.* **2,** 181–215.
4. Hirano, A. (1986) *Prog. Neuropathol.* **6,** 17–31.
5. Merz, P. A., Rohwer, R. G., Kascsak, R., Wisniewski, H. M., Sommerville, R. A., Gibbs, C. J., Jr., & Gajdusek, D. C. (1984) *Science* **225,** 437–440.
6. Wisniewski, H. M., Narang, H. K. & Terry, R. D. (1976) *J. Neurol. Sci.* **27,** 173–181.
7. Wisniewski, H. M., Merz, G. S., Merz, P. A., Wen, G. Y. & Iqbal, K. (1983) *Neurofilaments*, ed. Marotta, C. A. (Univ. Minnesota Press, Minneapolis, MN), pp. 196–221.
8. Terry, R. D. (1963) *J. Neuropathol. Exp. Neurol.* **22,** 629–642.
9. Kidd, M. (1964) *Brain* **87,** 307–320.
10. Masters, C. L., Multhaup, G., Simms, G., Pottgiesser, J., Martins, R. N. & Beyreuther, K. (1985) *EMBO J.* **4,** 2757–2763.
11. Beyreuther, K., Multhaup, G., Simms, G., Pottgiesser, J., Schroeder, W., Martins, R. N. & Masters, C. L. (1986) *Disc. Neurosci.* **3,** 68–79.
12. Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., Macdonald, B. L. & Beyreuther, K. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4245–4249.
13. Glenner, G. G. & Wong, C. W. (1984) *Biochem. Biophys. Res. Commun.* **120,** 885–890.
14. Glenner, G. G. & Wong, C. W. (1984) *Biochem. Biophys. Res. Commun.* **122,** 1131–1135.
15. Ihara, Y., Abraham, C. & Selkoe, D. J. (1983) *Nature (London)* **304,** 727–730.
16. Hirano, A., Kurland, L. T., Krooth, R. S. & Lessell, S. (1961) *Brain* **84,** 642–661.
17. Rodgers-Johnson, P., Garruto, R. M., Yanagihara, R., Chen, K. M., Gajdusek, D. C. & Gibbs, C. J., Jr. (1986) *Neurology* **36,** 7–13.
18. Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K. & Müller-Hill, B. (1987) *Nature (London)* **325,** 733.
19. Goldgaber, D., Lerman, M., McBride, W., Saffiotti, U. & Gajdusek, D. C. (1987) *Science* **235,** 877–880.
20. Garruto, R. M. & Gajdusek, D. C. (1985) *Normal aging, Alzheimer's disease and senile dementia. Aspects on etiology, pathogenesis, diagnosis, and treatment* (Editions de L'Universite de Bruxelles, Belgium), pp. 69–82.
21. Garruto, R. M., Swyt, C., Yanagihara, R., Fiori, C. E. & Gajdusek, D. C. (1986) *N. Engl. J. Med.* **315,** 711–712.
22. Wong, C. W., Quaranta, V. & Glenner, G. G. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8729–8732.