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Ultrastructural heterogeneity in cerebral amyloid of Alzheimer's disease

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Summary. Cerebral amyloid deposits from five patients with presenile or senile cerebral disease of the Alzheimer type were stained with uranyl acetate and lead citrate or with periodic acid-thiocarbohydrazide-silver proteinate, and examined with traditional high-resolution electron microscopy and with a goniometer tilting stage. In addition to a carbohydrate-rich matrix, we also consistently found local cell-derived vesicles within plaque and dyshoric amyloid. The most likely source for these vesicles appeared to be degenerate neurites. Amyloid fibrils were intimately associated with plasmalemmata, particularly those of degenerate neurites, which supported a neuronal origin for the amyloid fibril of Alzheimer's disease.

Key words: Amyloid – Alzheimer's disease – Carbohydrate – Senile Brain – Vesicle

Amyloid has been defined traditionally as an abnormal, *extracellular*, hyaline substance which binds certain dyes (e.g., Congo red), displays green birefringence in polarized light and has a fibrillary ultrastructure [6–8]. Several different types of amyloid exist; these can be distinguished from each other by their distribution and by the chemical composition of their fibrillar proteins [12, 13, 17]. Other substances, assumed to be secondary components, have been identified in amyloid deposits, such as glycosaminoglycans [43] and glycoproteins [6–8]. *Intracellular* paired helical filaments, by definition, should not be considered a form of amyloid and, consequently, are not included in this discussion.

The origin and genesis of amyloid within neuritic plaques and cerebral blood vessels in patients with Alzheimer's disease (AD) have been a subject of debate ever since it was described [5, 9, 25, 33]. Two major origins for the amyloid protein have been postulated: local, probably neuronal [5], and serum [25]. Histochemical [24, 40, 42, 50] and immunohistochemical [19, 22, 41, 42] studies provided some clues as to the chemical composition of AD amyloid and indicated that it, too, consisted of protein, glycosaminoglycan and glycoprotein. Until recently, biochemical data was restricted to a solitary study by Nikaido et al. [35], in which they found that AD amyloid differed from systemic amyloid (AA); this was later supported by histochemical [40, 41, 44] and immunohistochemical [41, 42] data. As in systemic amyloidosis, the biochemical search for the nature and origin of AD amyloid has focused on its fibrillar protein, the predominant and distinctive chemical moiety. Recent efforts have resulted in major breakthroughs. Several independent laboratories have isolated a 4.2–4.5-kDa protein, termed β or A_4 protein, from both cerebrovascular [14] and plaque amyloid [26], which appears to be biochemically unique and clearly differs from other known amyloids [1, 14, 26, 60]. Its determinant gene has been mapped to the 21st chromosome [16], near the familial Alzheimer disease locus [51]. Most recently, a tentative precursor to the A_4 protein has been isolated and found to have the characteristics of a cell surface receptor [20] and β protein messenger RNA has been identified in central neurons [4].

While these biochemical studies have enhanced our understanding of AD amyloid, they have dealt largely with the composition of its fibrillar protein. The role of minor, non-fibrillar components also must be considered in any pathogenetic scheme. Ultrastructural studies of AD amyloid [21, 29, 34, 53, 59] also have been limited in that most have focused on the fibril itself [29, 34]. Consequently, we initiated the present investigation with two goals: (1) to extend conventional ultrastructural studies of AD amyloid and pro-

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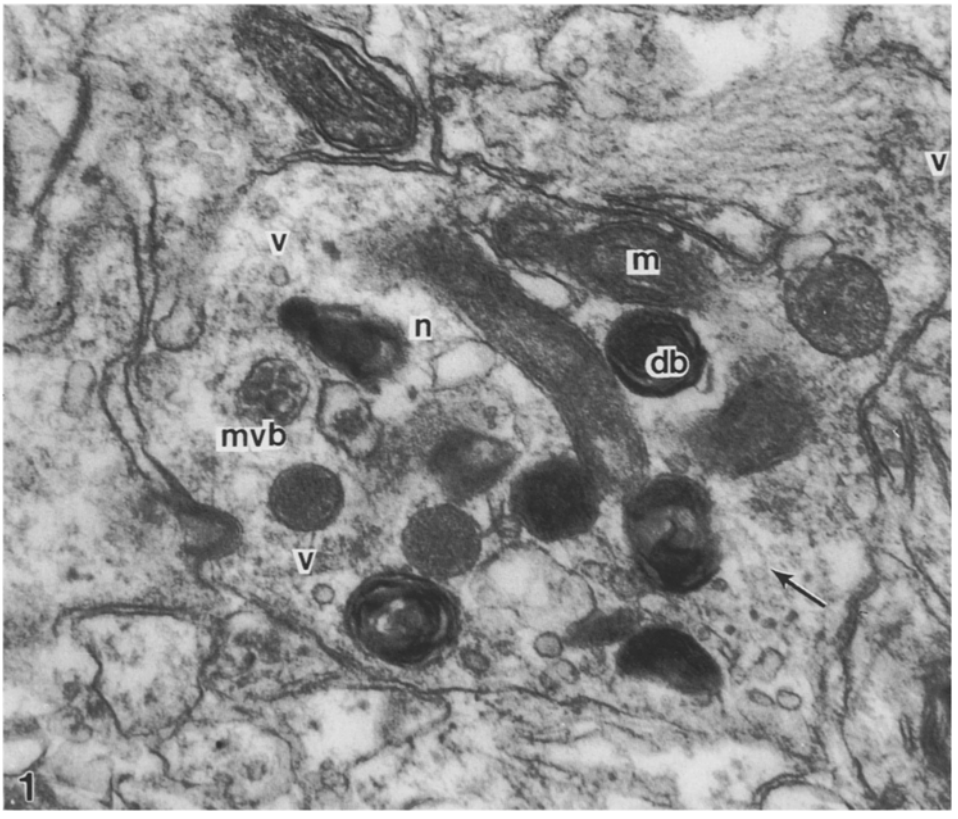

Fig. 1. Biopsy. Degenerate neurite (*N*) contains mitochondria (*m*), dense bodies (*db*), vesicles (*v*), a multivesicular body (*mvb*) and microtubule-vesicle arrays (*arrow*); section (0.25 μm); UA-PbCit; ×60488

vide corroborative morphological information on the origin of the fibril, and (2) to further document and emphasize the presence of non-fibrillar components within amyloid deposits of Alzheimer's disease. A portion of this study has been previously reported [38].

Methods

Epoxy resin blocks of neocortex or hippocampus from three presenile patients (55-year-old female, biopsy; 62-year-old male, biopsy; 62-year-old male, autopsy) and two senile patients (69-year-old male, biopsy; 77-year-old female, autopsy) with clinical dementia and pathological documentation of Alzheimer's disease were used in this study. Immersion fixation was achieved with 4% neutral buffered glutaraldehyde followed by 2% buffered osmium tetroxide. The biopsy samples proved suitable for silver proteinate staining and high-resolution traditional-goniometer tilting stage electron microscopy, while autopsy samples were adequate only for silver proteinate staining. The 0.25-μm sections, stained with uranyl acetate and lead citrate (UA-PbCit) were examined with an Hitachi HK-6 goniometer tilting stage (0° to ± 35° tilt, 0° to 360° azimuth) at 100 kV in a Hitachi HU-12 electron microscope. Thin sections with both plaque and dyshoric amyloid were stained with periodic acid-thiocarbohydrazide-silver proteinate [54] or with UA-PbCit.

The relationship of amyloid fibrils to cell processes intimately associated with them was scrutinized. Electron micrographs of selected tilted images at final magnifications of ×150000 were viewed stereoscopically. Negative plates (×32000) of these same images were subjected to image processing: enlargement, registration and addition in a Zeiss IBAS 2000. Finally, the position of the same amyloid fibrils in relation to plasmalemmata in electron micrographs (×200000) was traced on transparencies. The transparencies were then superimposed to provide a composite picture of these images.

Results

Fibrils

Plaque amyloid fibrils consisted of 4- to 9-nm tubular profiles of variable length. They were located in the extracellular space and organized into stellate bundles or indefinite masses, whose fibrils transected each other randomly. Fibrils were most intimately associated with neurites, which were considered to be degenerate because of enlarged mitochondria with electron-dense matrices and electron-dense cytoplasmic bodies (presumably lysosomes) (Figs. 1 and 2). They were also closely associated with hydropic cellular processes; some contained α particles of glycogen and a few intermediate filaments and were consistent with astrocytes, others displayed only a few vacuoles or microtubules and were consistent with dendrites. The least frequent cell process associated with plaque

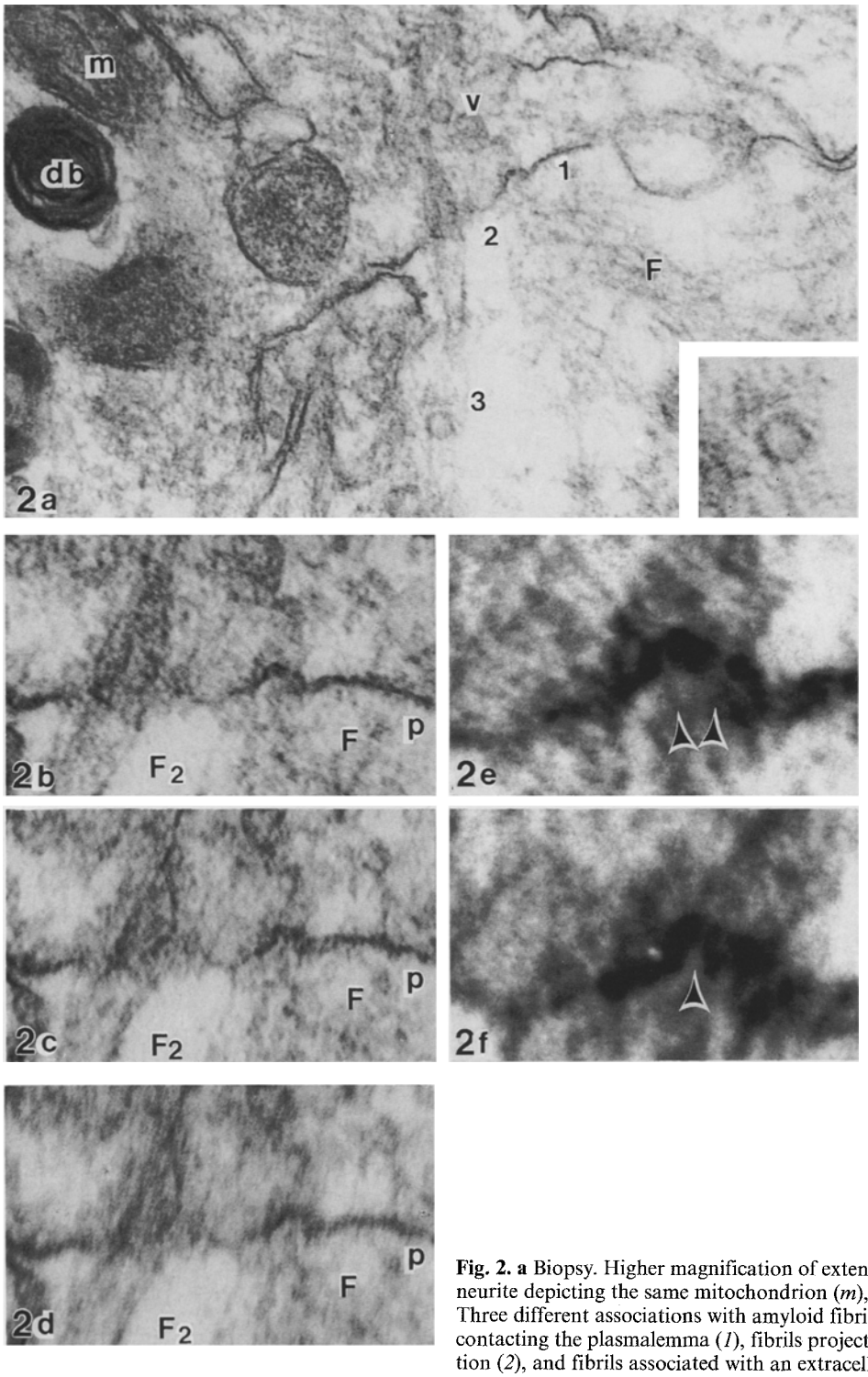

Fig. 2. a Biopsy. Higher magnification of extended *right upper portion* of the same neurite depicting the same mitochondrion (*m*), dense body (*db*) and vesicles (*v*). Three different associations with amyloid fibrils (*F*) are portrayed: fibrils contacting the plasmalemma (*1*), fibrils projecting from, or into, a deep invagination (*2*), and fibrils associated with an extracellular vesicle (*3*). *Inset:* Higher magnification of the extracellular vesicle and amyloid fibrils. **b–f** Biopsy. Tilted specimen of same neurite focuses on the interrelationship between amyloid fibrils (*F*) and the plasmalemma (*p*). Another cluster of fibrils (F_2) is also present. **b** Tilt + 10, azimuth 0; **c** tilt −15, azimuth 240; **d** tilt + 10, azimuth 240; **e, f** (same images as **b** and **c**, but subjected to computer enlargement and maximal contrast enhancement) demonstrate apparent interruption of plasmalemmal integrity (**e**, *right arrowhead*) and deformation of plasmalemma (**e**, *left arrowhead* and **f**, *arrowhead*). Section (0.25 μm); UA-PbCit; **a** × 83170 (inset × 135000); **b, c, d** × 150000; **e, f** × 500000

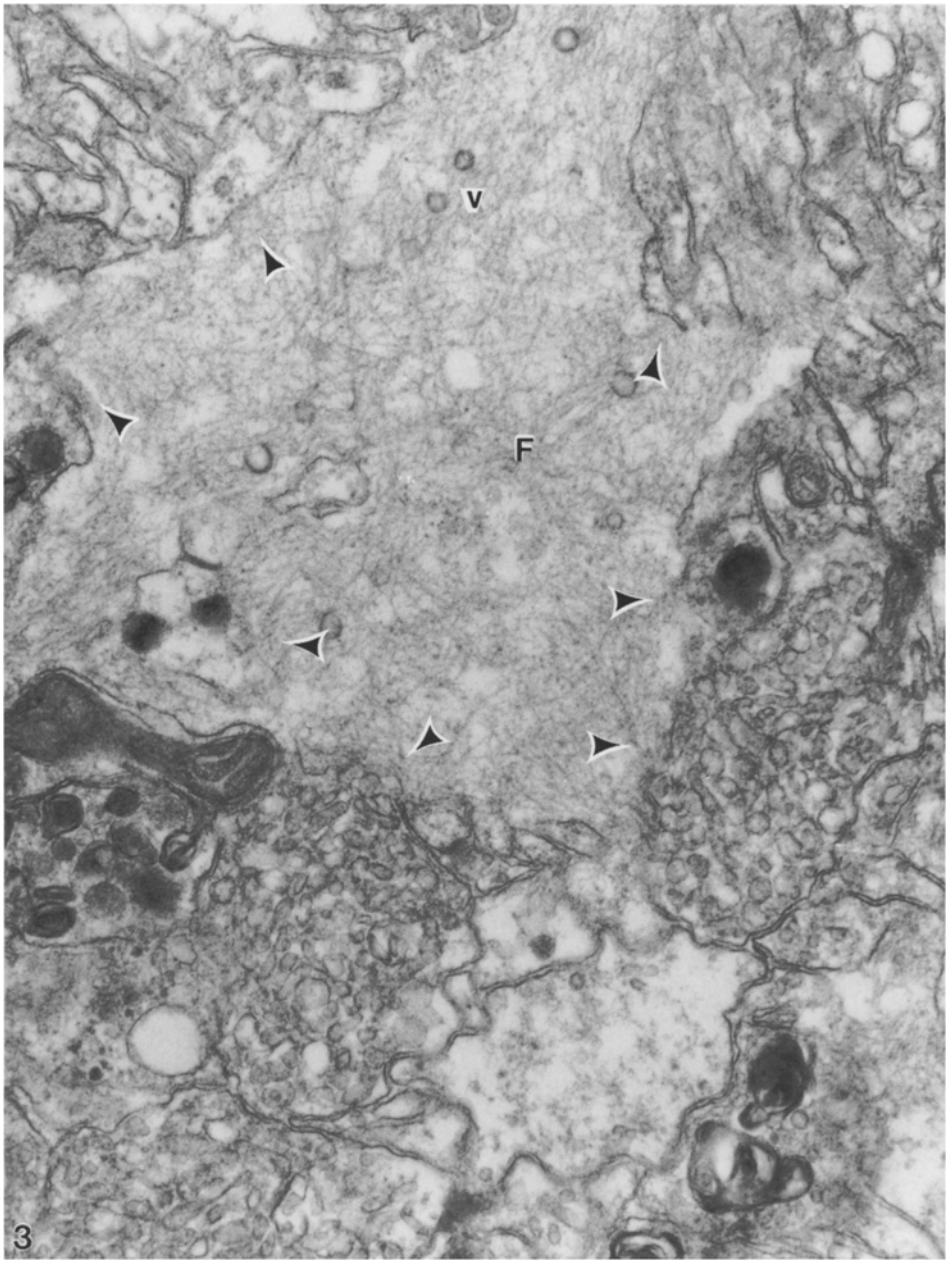

Fig. 3. Biopsy. Several vesicles (*v*) of different sizes are admixed with fibrils (*F*) which are closely apposed to plasmalemmata (*arrowheads*). UA-PbCit; ×45000

amyloid fibrils was more electron dense due to large numbers of ribosomes and other cytoplasmic organelles, such as cytofilaments, microtubules, mitochondria and vesicles. These latter cells were interpreted as microglia. Fibrils often clustered at the plasmalemmata of these three cell types. They often contacted neuritic plasmalemmata in perpendicular or near perpendicular formations. Frequently, plasmalemmata appeared to be focally deficient or blurred adjacent to clusters of amyloid fibrils (Figs. 3–5). Closer examination of these apparent defects in plasmalemmal integrity with the goniometer usually revealed that plasmalemmata were still present and separated amyloid fibrils from cell cytoplasm which did not contain fibrils. Occasional degenerate neuritic processes contained deep invaginations from, or into, which arrays of parallel extracellular fibrils projected. Less frequently, shallow invaginations were intimately associated with lattices of amyloid fibrils (Fig. 2a). Tilting and rotating one such shallow invagination and examining photographic preparations stereoscopically and with computer-assisted image proces-

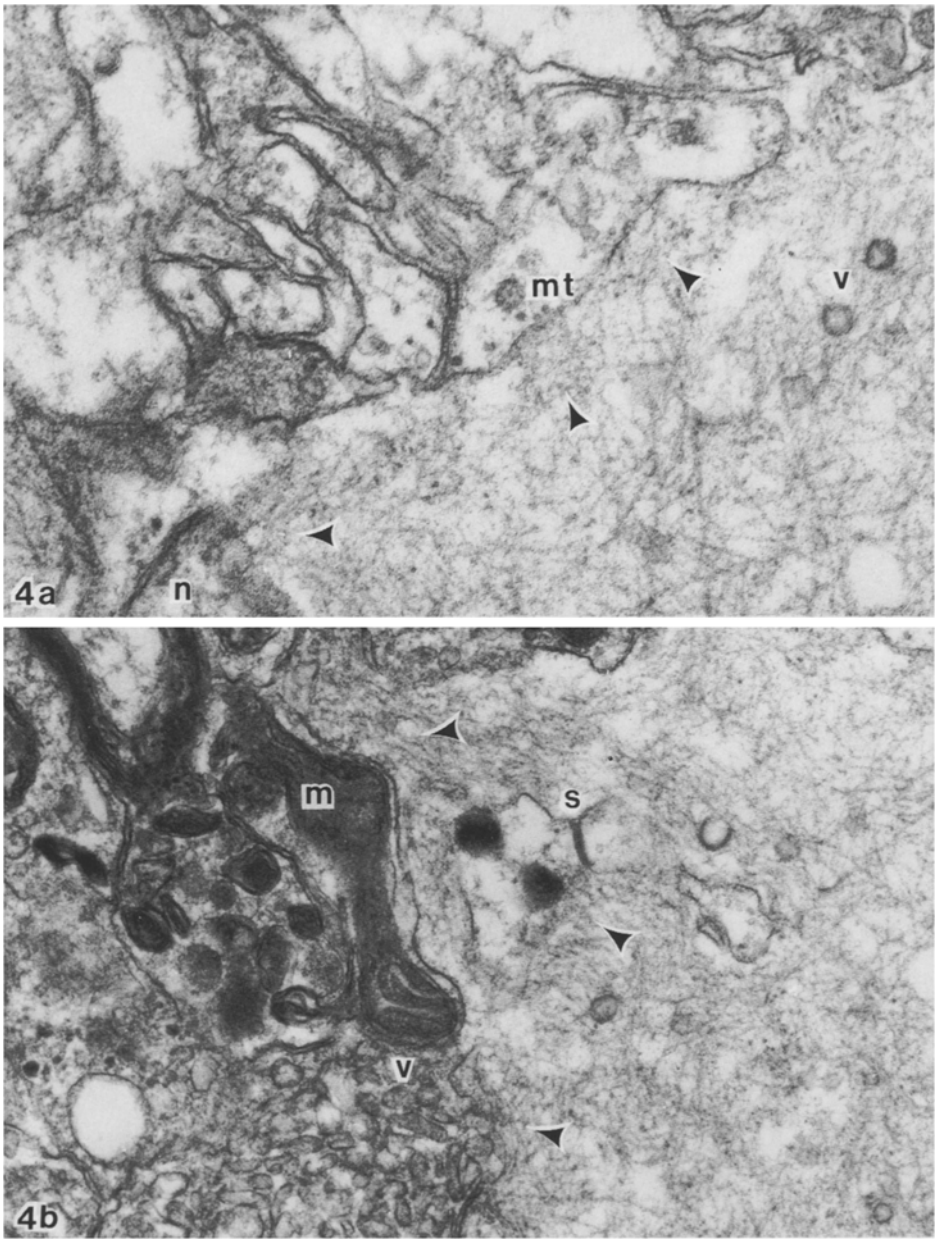

Fig. 4a, b. Biopsy. Higher magnification of two areas of Fig. 3. **a** This illustrates the multifocal and intimate topographical relationship (*arrowheads*) between amyloid fibrils and an intact dendritic process with microtubules (*mt*), a degenerate neurite (*n*) and extracellular vesicles (*v*). Blurring of the plasmalemma is noted beneath the microtubules and along the degenerate neurite. **b** This again portrays the close affinity (*arrowheads*) that amyloid fibrils show for the plasmalemma of a vesicle-enriched process (*v*), a degenerate neurite with an enlarged dense mitochondrion (*m*) and a synaptic complex (*s*). UA-PbCit; **a** × 72000, **b** × 49500

sing suggested that fibrils may arise within neuritic plasmalemmata, as well as in the immediate extracellular space (Fig. 2b–f).

Vesicles

The second structural element in plaque amyloid was a clear to opaque vesicular structure which measured 40–80 nm in diameter (Fig. 3). These vesicles often displayed a vague electron-dense rim in traditional thin sections stained with uranyl acetate and lead citrate. When these vesicles were tilted or when others were viewed without tilting at high magnifications, a limiting unit membrane was observed (Fig. 2a). The thickness of this unit membrane approximated that of plasmalemmata, around 7 nm. The vesicles appeared to be haphazardly distributed within the fibrillar network, but they often contacted amyloid fibrils. Other

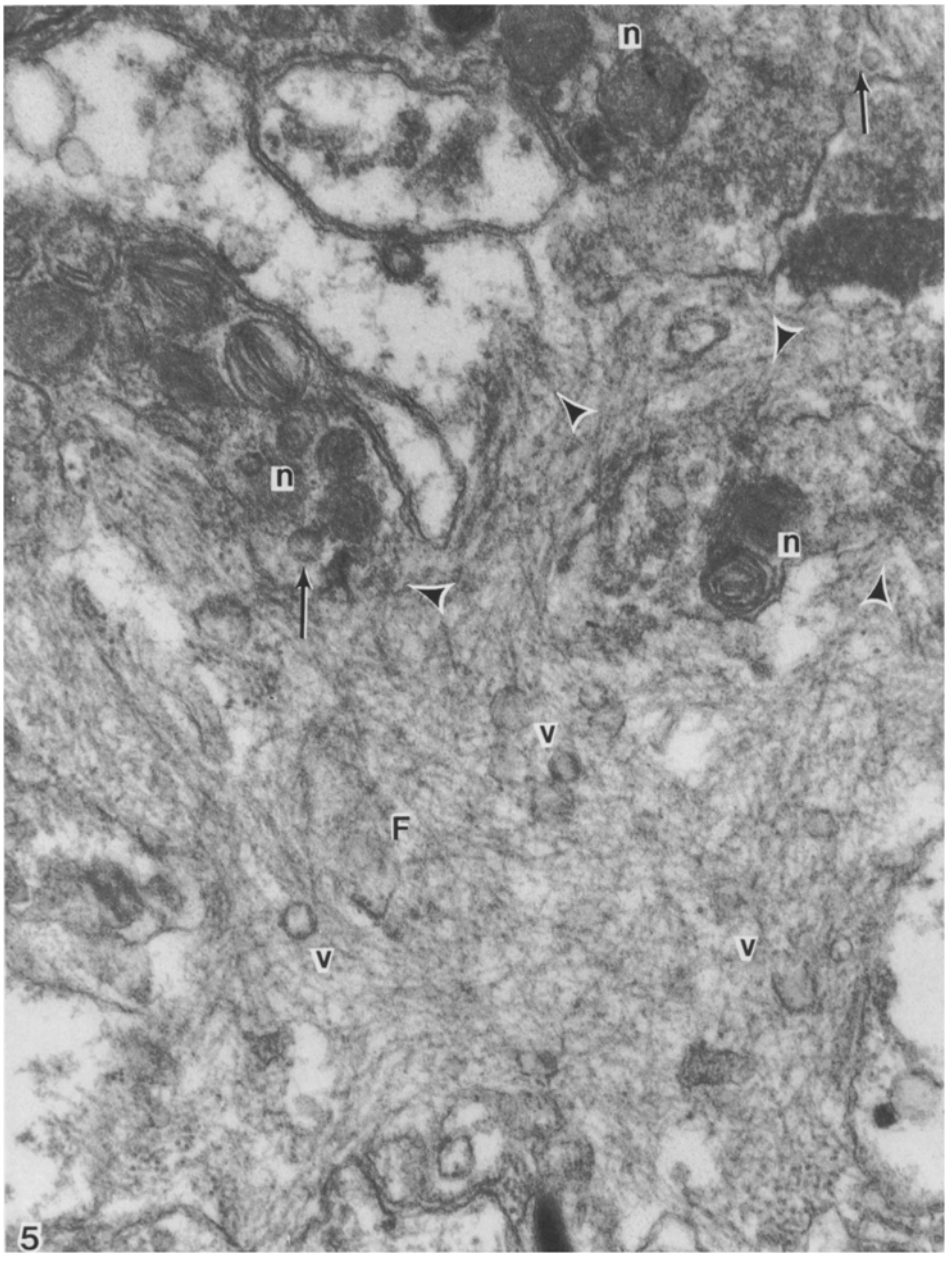

Fig. 5. Biopsy. A variety of vesicular forms (*v*), some ill-defined, are enmeshed in fibrils (*F*) which are intimately associated with degenerate neurites (*n*) and swollen processes of probable dendritic origin (*arrowhead*). Note intracytoplasmic vesicles (*arrows*) in degenerate neurites. UA-PbCit; × 54000

vesicles, similar in size and appearance, were identified in the cytoplasm of several cells: neurons, astrocytes, pericytes, endothelial and presumptive microglial cells (Figs. 1, 3 and 4). Amyloid vesicles were larger than clear, round synaptic vesicles which measured approximately 30–40 nm in diameter. The intracytoplasmic counterparts were uncoated, did not appear to fuse with plasmalemmata, and were most numerous in the cytoplasm of degenerate neurites.

Matrix

Silver proteinate staining disclosed a uniform carbohydrate-rich matrix, which was associated with fibrils and vesicles (Fig. 6). This was observed in both the well-preserved biopsy specimens and, more easily, in the somewhat autolyzed autopsy specimens. Vesicles were particularly striking in these preparations, because they acquired a thick peripheral coat (Fig. 7).

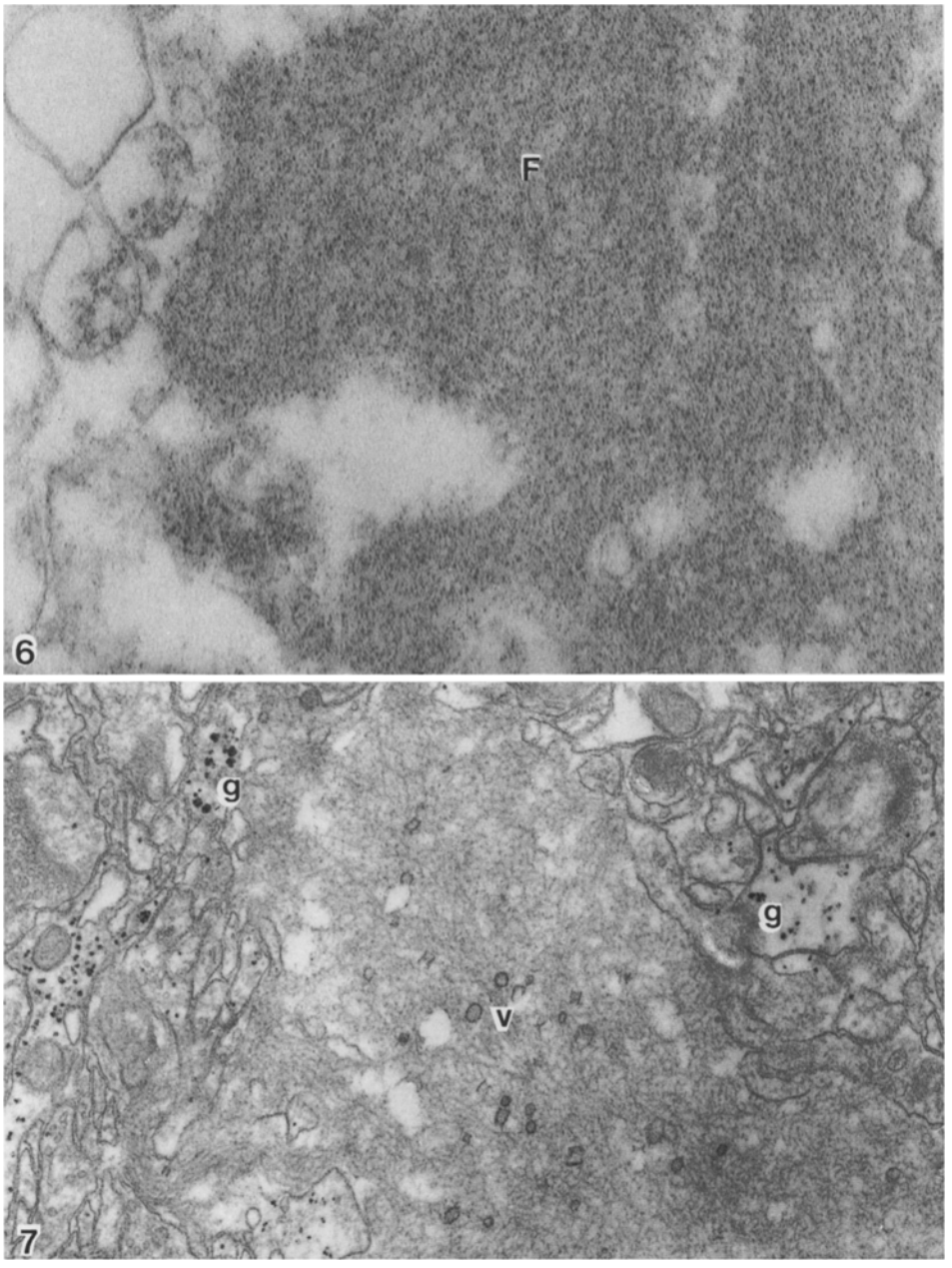

Fig. 6. Autopsy. Silver proteinate staining discloses a carbohydrate-rich matrix which obscures the fibrils (*F*); ×49500

Fig. 7. Biopsy. Silver proteinate staining labels vesicles (*v*) and intracellular glycogen (*g*) probably in astrocytes; ×24000

Silver proteinate-positive vesicles measured approximately 80–100 nm in outside diameters; the thickness of the reactive coat was approximately 20–25 nm. It is noteworthy that neither intracytoplasmic vesicles nor synaptic vesicles ever stained with silver proteinate. Internal positive control staining was observed in glycogen particles and corpora amylacea of astrocytic processes and in glycogen particles of degenerate neurites. Other neuronal processes also contained excessive amounts of β and α glycogen. Several dyshoric deposits of amyloid revealed a conspicuous peripheral localization of silver proteinate-positive vesicles (Fig. 8).

Discussion

The present investigation demonstrates that there is a local cellular contribution to, and a carbohydrate-

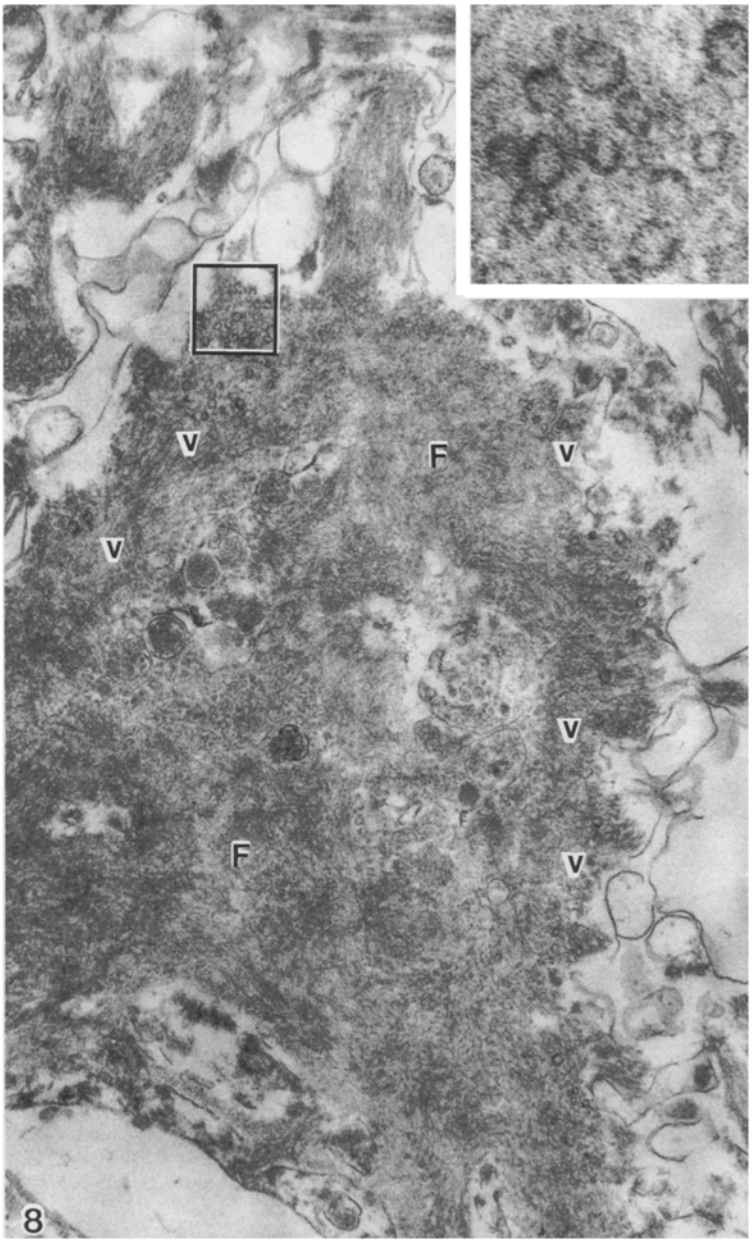

Fig. 8. Autopsy. Silver proteinate-positive vesicles (*v*) are clustered at the edge of the dyshoric amyloid fibrils (*F*). *Inset:* Higher magnification of labeled vesicles. ×22500; *inset* ×91375

rich matrix in, plaque and dyshoric amyloid of AD. Although an ultrastructural origin for the fibril has not been established unequivocally, its close association with plasmalemmata, particularly neuronal, provides morphological support for biochemical data suggesting that it is derived from a cell surface receptor [20], perhaps of neuronal origin [4].

Fibrils

The chemical composition and origin of the amyloid fibril of AD has been a controversial subject for many years [5, 9, 25, 33]. Evidence for both local, probably neuronal, [1, 4, 5, 11, 26, 39–41] and serum [10, 14, 15, 19, 25] origins have been presented. Alternate possibilities, such as microglia [59] and basement membranes [31] have also been entertained. Histochemical data [40, 42, 50] indicate that plaque and dyshoric amyloid of Alzheimer's disease is comparable to the amyloid of medullary carcinoma of the thyroid, and less so to the amyloid of insulinoma and diabetic or senile islets of Langerhans. Other similarities exist between the fibrillar proteins of Alzheimer's disease, medullary carcinoma and pancreatic islets. All appear to be small, low molecular weight proteins (brain: 42 or 43 residues, molecular weight of 4200 to

4500 daltons; thyroid: 53 residues, molecular weight of 5680 daltons; islet: 37 residues, molecular weight of 3850 daltons) [14, 20, 47, 58]. In contrast to systemic amyloids (AL, AA) [6–8, 12, 13, 17], they are highly resistant to suspension in distilled water [1, 14, 58]. Both thyroid and islet amyloids are examples of amyloid limited to endocrine organs (AE); their fibrillar proteins are derived from local parafollicular or islet cells [18, 47, 56–58].

Until recently, this laboratory produced the only morphological (immunohistochemical) evidence for a local brain protein in AD amyloid [39, 41]. Both monospecific antibodies and antibodies to double-electrophoresed neurofilament triplet proteins (NF) disclosed a common antigen in the amyloid of neuritic plaques, dyshoric angiopathy and congophilic angiopathy of Pantelakis [37]. Other immunohistochemical studies failed to confirm the presence of neurofilament epitopes in plaque amyloid [3]. Recent chemical and immunohistochemical [1, 14, 15, 20, 26, 60] analyses have also demonstrated that the same antigen is present in all three types of AD amyloid. Biochemical and in situ hybridization data [4, 20, 26] have supported a neuronal origin for AD amyloid protein [4]. Amino acid sequence data [14, 20, 26], however, are inconsistent with a neurofilamentous origin. Consequently, it appears that our NF antibodies recognized a cross-reacting neuronal protein, a possibility originally considered [41]. Comparison of the presumptive A_4 precursor with NF reveals that both contain domains particularly rich in glutamic and aspartic acids [20]. A computer search, using Fast P program and the Protein Identification Resource as database, reveals a 30% identity in 70 amino acid overlap between the glutamic acid-rich portions of neurofilament protein (β domain of tailpiece) and the presumptive A_4 precursor (M. J. Weise and J. M. Powers, unpublished observations).

AD amyloid fibrils have an intimate relationship with plasmalemmata, particularly those of degenerate neurites. A comparable affinity for plasma membranes of parenchymal cells is also characteristic of AE (amyloids of thyroid and islets) [2, 18, 56]. The contention that amyloid deposition results from focal disruption of plasmalemmata and outpouring of cytoplasmic precursors, as suggested in AE [18, 56], cannot be upheld in AD amyloid. After tilting or rotating suspicious areas, some plasmalemma is usually interposed between cytoplasm and extracellular fibrils. We also cannot support the postulate that extracellular amyloid fibrils in AD amyloid result from the extrusion of intact intracytoplasmic filaments, since fibril- or filament-rich cells are not commonly associated with amyloid fibrils in this material. Our observations are most consistent with the hypothesis that degenerate neurites liberate amyloidogenic proteins into the immediate extracellular space, where they polymerize into fibrils. Rarely, in situ polymerization of amyloidogenic proteins in plasmalemmata of degenerate neurites may occur (see Fig. 2).

Matrix

Histochemical studies [24, 40, 50] readily disclose the presence of carbohydrates and glycosaminoglycans in AD amyloid. The present investigation extends these findings to the ultrastructural level and confirms the presence of a carbohydrate-rich matrix surrounding fibrils. A comparable carbohydrate-rich matrix has been identified in hamster amyloid (AA) [27] and in human systemic amyloid [50]. The silver proteinate stain also demonstrates an apparent increase in glycogen within nerve cell processes in comparison to age-matched controls, which is in agreement with a recent observation [23]. The silver proteinate stain cannot distinguish between various carbohydrate-rich substances in amyloid deposits, but immunohistochemical staining [19, 22, 41] reveals a variety of serum glycoproteins.

The present study has not addressed another conspicuous matrix component noted in histochemical preparations of AD amyloid, glycosaminoglycans (GAGS). Previous studies from this laboratory [40] have established that AD amyloid contains hyaluronic acid and/or chondroitin sulfate, and greater amounts of an hyaluronidase-resistant glycosaminoglycan, such as heparan or dermatan sulfate, or a sialidase-resistant sialoglycoprotein. Our findings have been confirmed recently [48].

Vesicles

Vesicles, similar or identical to those described above, were noted in one of the original ultrastructural studies of AD [21]; the author speculated that they were derived from necrotic cellular material. They were later either described or illustrated, but not discussed, in plaque and dyshoric amyloid [46] and in dyshoric and congophilic angiopathies [36]. Other ultrastructural studies of AD amyloid have illustrated vesicular structures, but have not described or discussed them [32, 52, 59]. Amyloid-associated vesicles are not restricted to AD, and have been reported in both murine and human systemic amyloids [49], but they have been reported most commonly in AE of islet cells [56], insulinoma [2] and pituitary adenoma [45]. All authors recognize the local cellular origin of vesicles. In the amyloid of endocrine origin, vesicles appear to be derived from the same cell which produces the fibrillar

protein: the thyroid parafollicular cell, the pancreatic islet cell and, usually, the pituitary acidophil.

The demonstration of membrane-bound vesicles in AD amyloid documents another local cellular contribution to the amyloid deposit. The source of the vesicle, on the other hand, is a matter of debate. Based on the biochemical, histochemical and ultrastructural similarities between AD amyloid and AE, one would predict that the local source would be a secretory or neurosecretory cell. The vesicles are most prevalent in neurons, especially degenerate neuritic processes. These data implicate the neuron as the most likely source for amyloid vesicles. If we pursue the same line of reasoning, then the brain cell that releases vesicles would also be expected to be the cell of origin for the fibrillar protein.

We have not elucidated the mechanism by which vesicles gain entrance to the extracellular space or their contents. Similar vesicles have been found to be transported along microtubules in squid axoplasm [30] and in presynaptic nerve terminals [28]; the latter may contain calcium. It is conceivable that the vesicles in AD amyloid carry precursor, or unpolymerized, amyloid fibrillar protein.

In summary, the present ultrastructural demonstration of a carbohydrate-rich matrix and vesicles derived from local cells complements previous histochemical, immunohistochemical, biochemical and in situ hybridization data, which have identified a neuronal protein, glycosaminoglycans, glycoproteins, calcium, aluminum and silica in AD amyloid. AD amyloid, as other amyloids, is a heterogeneous mixture of several components. The popular trend to consider only the fibril protein and to equate amyloid with all congophilic proteins possessing a β-pleated sheet conformation understates the complexity of amyloidogenesis [55].

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