

# Ultrastructural Studies of the Cells Forming Amyloid Fibers in Classical Plaques

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**ABSTRACT:** Three-dimensional reconstruction and ultrastructural studies of classical plaques from the cortex of patients with Alzheimer's disease showed that microglial cells of the plaques are the amyloid-forming cells. The amyloid star of the single plaque represents the product of five or six microglial cells covering about 80% of the amyloid star surface. The amyloid fibers appear to be formed within altered cisterns of the endoplasmic reticulum. Distended cisterns form channels filled with amyloid fibers. Numerous vesicles derived from the Golgi apparatus appear to be attached to or fused with the amyloid-filled channels. Reconstruction of the amyloid star and the microglia cell pole that forms the amyloid star reveals three different zones of distribution of cytoplasmic organelles and amyloid deposits. The peripheral zone comprises channels filled with loosely packed amyloid fibers arranged in a parallel manner. The transient zone consists of a mixture of fusing amyloid channels and products of disintegration of cytoplasmic pockets, dense bodies and fragments of cellular membranes. The core of the amyloid star is composed of condensed, densely packed amyloid fibers that are free of cellular debris. Formation of the three zones supports the idea that the microglia/macrophages are not phagocytes but instead are the cells manufacturing the amyloid fibers.

**RÉSUMÉ:** Études ultrastructurales de cellules formant des fibres amyloïdes dans les plaques classiques La reconstruction en trois dimensions et des études ultrastructurales des plaques classiques provenant du cortex de patients atteints de la maladie d'Alzheimer ont montré que les cellules microgliales des plaques sont probablement les cellules qui forment la substance amyloïde. L'étoile amyloïde d'une seule plaque est le produit de cinq ou six cellules microgliales couvrant à peu près 80% de la surface de l'étoile amyloïde. Les fibres amyloïdes semblent être formés à l'intérieur de citernes anormales du réticulum endoplasmique. Des citernes distendues forment des canaux remplis de fibres amyloïdes. De nombreuses vésicules formées à partir de l'appareil de Golgi semblent être attachées ou fusionnées aux canaux remplis de substance amyloïde. Une reconstruction de l'étoile amyloïde et du pôle cellulaire microglial qui forme l'étoile amyloïde révèle trois zones différentes de distribution des organelles cytoplasmiques et des dépôts amyloïdes. La zone périphérique comprend des canaux remplis de fibres amyloïdes en amas étalés, disposés de façon parallèle. La zone de transition consiste en un mélange de canaux amyloïdes se fusionnant et de produits de la désintégration de pochettes cytoplasmiques, de corps denses et de fragments de membranes cellulaires. Le coeur de l'étoile amyloïde est composé de fibres amyloïdes condensées, en amas serrés, qui sont exempts de débris cellulaires. La formation des trois zones supporte l'idée que les cellules microgliales/macrophages ne sont pas des phagocytes mais bien des cellules fabriquant des fibres amyloïdes.

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Microglial cells were originally recognized as a cell population distinct from other glial cells by Del Rio-Hortega, who used silver impregnation methods for their identification.<sup>1</sup> Acceptance of microglia as a distinct cell population was hindered by difficulties encountered in identifying them by means of electron microscopy.<sup>2-4</sup> The combination of silver staining and close correlation with light microscopy subsequently yielded a definitive identification of the microglial cell group.<sup>5-8</sup>

Microglial cells were characterized as small oval cells with a variable number of branching processes and scant cytoplasm which usually contained dense bodies.<sup>5-7,9,10</sup>

Four different forms of microglia have been identified: (1) resting or ramified, (2) reactive, (3) amoeboid which is recognized as a transient cell form during development and (4) round cell which is restricted to the early postnatal period. Ramified microglia, which are derived from the amoeboid form and have a lower level of phagocytic activity, are considered to be quiescent precursors of the reactive cell.<sup>11,12</sup>

Ultrastructural studies of neuritic (senile) plaques in humans and animals<sup>12,13</sup> have described cells that are associated with deposits of amyloid fibers. These cells have the appearance of reactive microglia and macrophages. Because some have the

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morphological features of macrophages, the question arises whether they function as phagocytes removing the amyloid deposits or engage in production of amyloid. The purpose of this study was to demonstrate, on the basis of serial semithin and ultrathin sections and computer-aided reconstruction of classical plaques, that the microglia/macrophages are the cells in which amyloid fibers are manufactured.

#### METHODS AND MATERIALS

Biopsy material from the cortex of a 72-year-old woman with Alzheimer's disease was used for this study. Tissue was fixed in 3% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 and postfixed in 1% osmium tetroxide in Sorensen buffer, pH 7.4. After dehydration, material was embedded in Epon.

Areas rich in plaques were chosen using sections stained with toluidine blue. Two punctures made in this region in the Epon block served as reference points for three-dimensional reconstruction. Tissue blocks were cut serially in semithin (0.3  $\mu\text{m}$ ) and ultrathin (0.06  $\mu\text{m}$ ) sections alternately. Ultrathin sections were stained with uranyl acetate and lead citrate. Three classical plaques were reconstructed from 22, 34 and 48 cross sections. Each cross section was represented by 12 to 30 electron micrographs. Final magnification of the prints used for preparation of the montages was 9,882X (original magnification 3,294X).

In 16 reconstructed microglial cells, participating in formation of three classical plaques, the following parameters were calculated: the volume and surface of the cell and the volume of its nucleus, cytoplasm and organelles: rough endoplasmic reticulum (RER), mitochondria and dense bodies. Measurements of the area of the cross sections of the investigated structures were made using semiautomatic image analyzer Videoplan (C. Zeiss).

Volume of the cell was calculated from area of cell cross section on serially reconstructed montages and distance of investigated sections. Volumetric density and volume of cellular organelles were calculated according to Weibel.<sup>14</sup>

#### RESULTS

In the three reconstructed plaques, about 80% of the surface of the amyloid star was in contact with the cell membranes of five to six microglial cells. The size of the microglial cells varied. The average volume of microglial cells in the plaques was 597  $\mu\text{m}^3$  (SD 261  $\mu\text{m}^3$ ). The volume of the smallest reconstructed cell was 403  $\mu\text{m}^3$ . The cells in which cytoplasm was filled with dense bodies were the largest.

The ratio of nucleic to cytoplasmic volume was variable. The nucleus consisted of 6.7 to 17.9% of the total volume of the cell, the average being 12.3% (SD 4.1). The volume of the cell nucleus varied from 52 to 101  $\mu\text{m}^3$ , the average being 68  $\mu\text{m}^3$  (SD 4.1). Three-dimensional reconstruction highlighted a very rich sculptured surface of the nucleus.

The most prominent features of these cells are their polymorphism and specific polarity. Their shapes vary from ellipsoid to very irregular multiprocesses cells. However, regarding their relationship to the amyloid star, all microglial cells demonstrate a clear polarity. The polarity of cells is characterized by the specific distribution of cellular organelles and infoldings of the cellular membrane which appear to be connected with a labyrinth of channels filled with the amyloid fibers.

The cell pole nearer to the amyloid star forms a well-developed system of RER, large Golgi complex, numerous vacuoles, smooth and coated vesicles and mitochondria. Centrioles are also present in this cell. The cell pole farther from amyloid deposits is poorer in cytoplasmic organelles. In some cells, this region is filled with electron-dense storage material.

#### Rough Endoplasmic Reticulum

In microglial cells the volume of RER is from 15 to 31  $\mu\text{m}^3$ , averaging 23  $\mu\text{m}^3$  (SD 6.2  $\mu\text{m}^3$ ). RER comprises from 2.5 to 8.7% of the total volume of the microglial cytoplasm.

RER forms channels with lengths of 1 to 3  $\mu\text{m}$ . Locally it appears as a branching system of channels. This form is commonly observed at the "amyloid pole" of the cell close to the Golgi complex (Figures 1, 2). Continuum of RER with the smooth ER (SER) membranes was frequently observed (Figures 2, 4). In the dilated lumen of the SER, amorphous material with medium electron density was observed. Within these distended cisterns amyloid fibers were also seen. It appears that as the cisterns enlarge, they form a system of channels filled with the amyloid fibers (Figures 2, 3). There appears to be a continuum between the membranes of the channels filled with the amyloid deposits and the cell membrane.

#### Golgi Apparatus

The Golgi apparatus is made up of three to five complexes of cisterns and vacuoles that form an irregularly shaped ring that is usually located at the amyloid pole of the cell (Figure 4). Microglial cells are relatively rich in numerous vacuoles and vesicles found near the Golgi complex. Some of the vesicles are attached to or fused with the cisterns or channels filled with amyloid fibers (Figures 3, 4).

#### Microglial Cell Interface with Amyloid Star

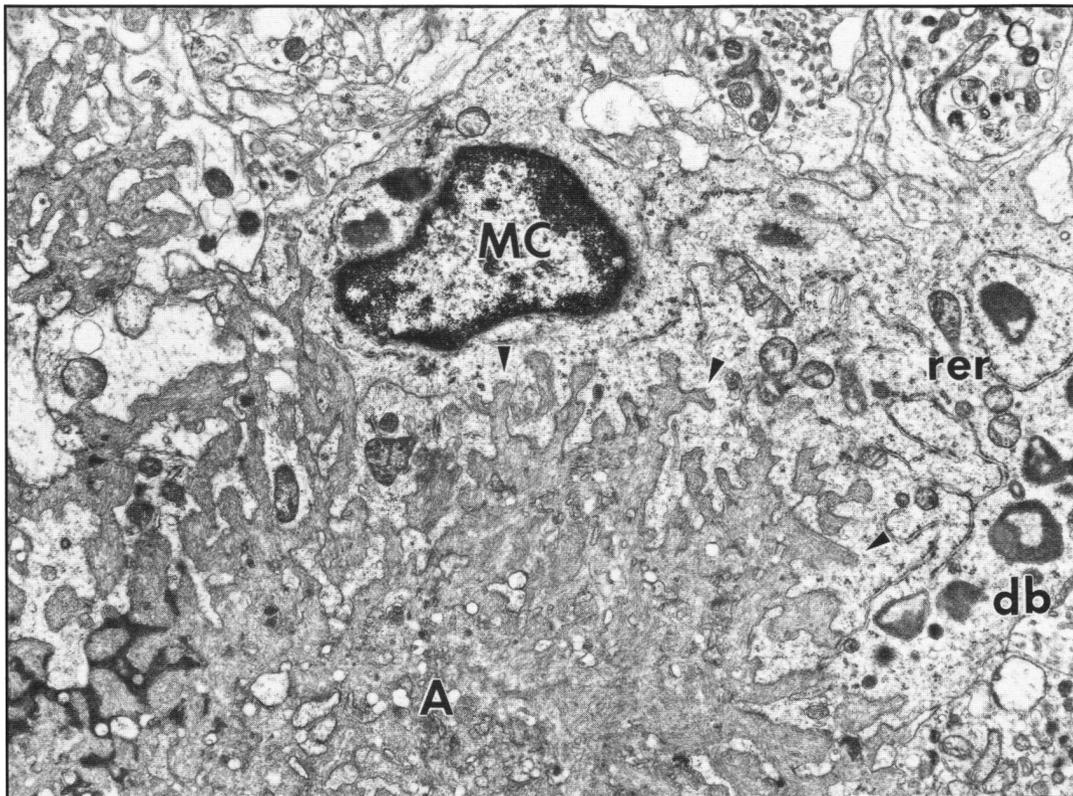
From 13 to 40% of the cell surface (average approximately 26%) borders on amyloid deposits. In this area, the membranes of the amyloid-filled channels appear to form the cell membrane (Figures 1-5). The channels are approximately 0.5  $\mu\text{m}$  wide and 2  $\mu\text{m}$  long. On random cross sections, especially on cross sections adjacent to the periphery of the amyloid star, these channels form a complicated labyrinth, resulting from interconnections of channels, ER membranes, vacuoles and vesicles.

Fusion of vesicles, especially of coated vesicles produced by Golgi apparatus, with the walls of channels is observable. All stages of fusion are evident (Figure 4).

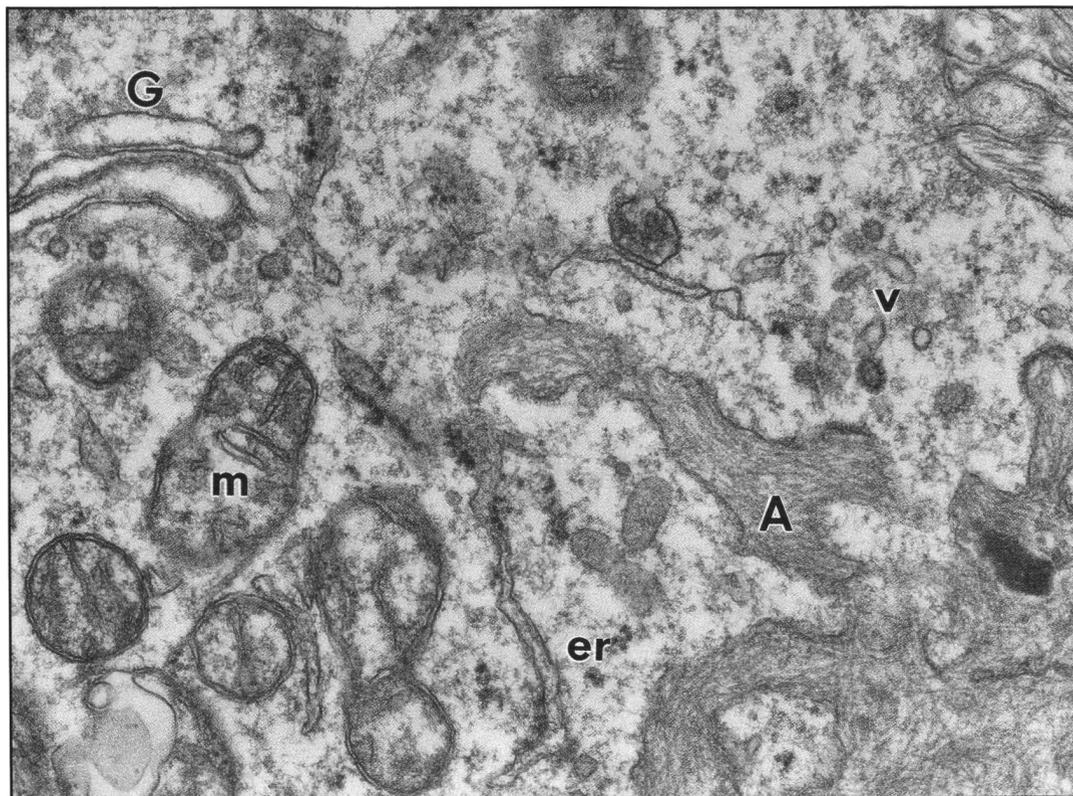
#### Amyloid Star

The periphery of the amyloid star consists of multiple channels filled with amyloid fibers in a parallel fashion. As indicated above, these channels appear to be in continuity with the endoplasmic reticulum cisterns and the cell membrane. Between the amyloid-filled channels are pockets of cytoplasm containing coated and smooth vesicles, aggregates of free ribosomes, and scattered dense bodies (Figures 5, 6). In the transient, deeper zone the pockets of trapped cytoplasm in between the amyloid deposits show evidence of degenerating cytoplasmic organelles (Figures 5, 6).

In the center of the star, the amyloid fibers retain the parallel arrangement seen at the periphery of the plaque; however, in comparison to the periphery, the aggregates of the amyloid fibers are packed more densely at the center. The length and



1



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Figure 1 — Microglia cell (MC) round amyloid star (A). Channels filled with amyloid fibers (arrowheads). Cytoplasm rich in rough endoplasmic reticulum (rer). Clusters of dense bodies (db). 12,000X

Figure 2 — Amyloid pole of microglia cell with mitochondria (m), rough endoplasmic reticulum (er), Golgi apparatus (G), vesicles and vacuoles (v). Accumulation of granular and fibrillar material in distended, endoplasmic reticulum. Relatively regular, parallel arrangement of amyloid fibers in intracytoplasmic channels (A). 55,000X

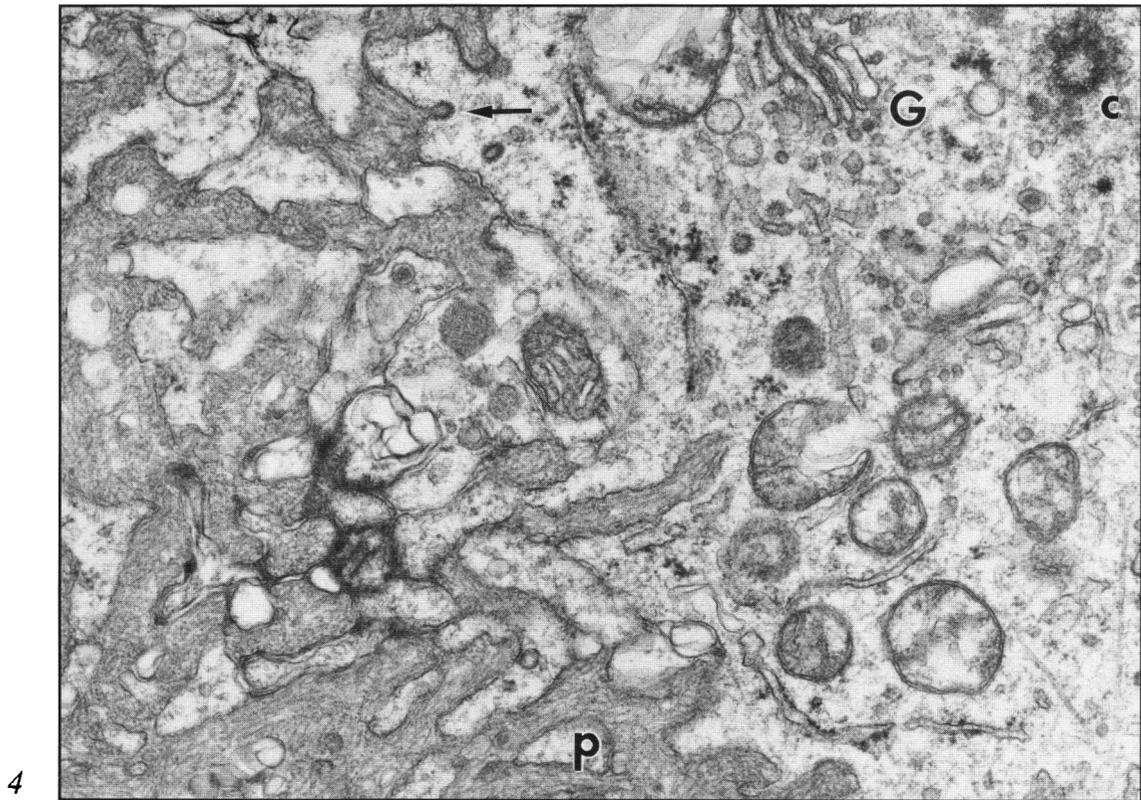
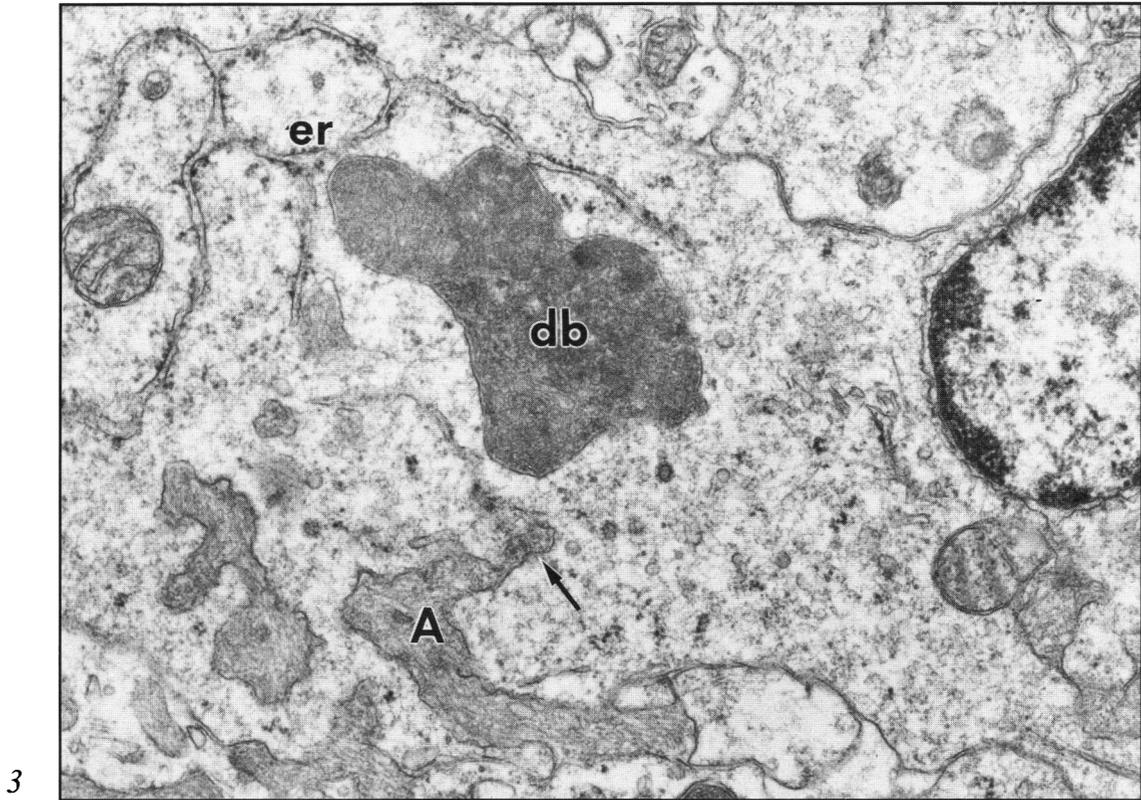


Figure 3 — Branched system of amyloid channels (A) resembles the cellular system of endoplasmic reticulum (er). Direct communication of ER and amyloid channels (arrow). Dense body (db). 33,000X

Figure 4 — Labyrinth of amyloid channels interspersed with cytoplasmic pockets (p). Absence of ER in cytoplasm trapped between amyloid channels. Region of the Golgi apparatus (G) rich in vacuoles and vesicles. Fusion of coated vesicles with amyloid channels walls (arrow). Centriole (c). 33,000X

width of the amyloid packages in the core of the star are smaller than those at the periphery (Figure 6). The central part of the amyloid star is more homogeneous. This region is almost completely free of dense bodies, vacuoles, vesicles and membranaceous formations. In the center of the star the bundles of amyloid fibers retain the arrangements of the amyloid fibers seen in the channels at the periphery of the plaque (Figure 6).

### Mitochondria

Cross sections of the mitochondria from microglial cells were oval and their maximum diameter ranged between 0.5 and 1.5  $\mu\text{m}$ . Sporadically, the cross sections are V-shaped or ramified. The mitochondrial cristae are located transversely to the long axis of the mitochondria. Approximately 20% of mitochondria revealed focal rarefaction of the matrix, and deformation and focal disintegration of the cristae (Figures 2-4). The volume of the mitochondria comprised from 1.4 to 3.1% of the total volume of the cytoplasm (SD 1.5%).

### Dense Bodies

The quantity of dense bodies and their shape, size and composition are variable in microglial cells of classical plaques. The total volume of the structures varied from 1.2 to 431  $\mu\text{m}^3$ , that is, from 0.3 to 44% of the cytoplasmic volume of 16 reconstructed cells. On the average, those structures made up approximately 30% of the cytoplasmic volume (SD 18%). In each plaque, there are one or two cells that are filled with osmiophilic bodies. In the other cells, dense bodies comprised only a small percentage of the total volume of cytoplasm.

Dense bodies are very heterogeneous. Most of them appeared to be composed of osmiophilic granular material (Figures 1, 3). They also contain vacuoles that appear as lipid droplets with low electron density and with diameters ranging from 0.2 to 1.5  $\mu\text{m}$ . Such vacuoles make up approximately 9.5% of the volume of the dense bodies. The third components are osmiophilic lamellar aggregates. The same cells also contain phagosomes containing remnants of the cellular structures in different stages of disintegration, segregation and condensation.

### Centriole

In reconstructed cells, the centriole is usually located in the vicinity of the nucleus, in the indentation between its lobes. Sometimes they are found near the Golgi complex (Figure 4). Centrioles contain numerous medium-length and long microtubules radiating from the active center. Usually the cellular centriole is located between the nucleus and the interface of the cells with the amyloid star.

## DISCUSSION

The serial cross sections and three-dimensional reconstruction of the classical plaque showed that five to six cells are closely associated with the amyloid star. These cells cover over 80% of the surface of the amyloid star. Ultrastructurally, they show clear polarization, with specific reorganization of the cytoplasm and the cellular membrane on the border with amyloid deposits. The first amyloid fibers appear in the distended cisterns of smooth ER. With increasing accumulation of amyloid, the altered ER forms a complex labyrinth of channels whose membranes appear to fuse with the cell membrane. Generally,

from 13 to 40% of the individual cell surface is associated with the deposits of amyloid fibers.

The other pole of the cell contains the nucleus, other cytoplasmic organelles and electron-dense bodies. Between the nucleus and the amyloid-producing pole is a large Golgi complex and well developed system of RER and numerous vacuoles, as well as smooth and coated vesicles. In some cells, centrioles were found. Frequently, the smooth and coated vesicles were observed to fuse with membranes of amyloid-containing channels. Reconstruction of the amyloid star and the microglial cell pole that forms the amyloid star reveals three different zones of distribution of cytoplasmic organelles and amyloid deposits. The peripheral zone is composed of fingerlike aggregates of amyloid-filled channels. Newly formed deposits of the amyloid fibers are loosely packed. Numerous coated and smooth vesicles are present in this region, many of which attach to and fuse with the membranes of the amyloid-containing channels. Pockets of the cytoplasm that occur between channels filled with newly formed amyloid appear normal. The second zone, called the transient zone, is made up of fingerlike aggregates of amyloid and debris of degenerating cytoplasm trapped between amyloid aggregates. The peripheral and transient zones of the amyloid star are rich in nucleoside diphosphatase.<sup>15</sup>

The core of the amyloid star is made up of densely packed amyloid fibers, which usually retain their parallel arrangement. The core is usually free of dense bodies and degenerating cytoplasmic organelles that are present in the transient zone of the star. The absence of cellular debris in this area suggests that the matrix of the amyloid star contains enzymes that are able to degrade degenerating cytoplasmic organelles.

On the basis of the morphology and histochemical data<sup>15</sup> that demonstrated nucleoside diphosphatase activity in the endoplasmic reticulum and the cisterns filled with amyloid, we concluded that these cells are the brain microglia/macrophage cells. Because the amyloid fibers are first seen in the distended cisterns of the SER system, we assumed that these cells are engaged in the formation, not the phagocytosis, of the amyloid fibers. We hypothesize that the microglia/macrophage cells synthesize and secrete the beta-peptide (they are probably both the producer and processor cells), either as an overexpressed, truncated gene product or an aberrant peptide resulting from abnormal post-translational processing.

The same configuration of cellular membranes on the border with amyloid deposits was observed in microglial cells in brains of aged dogs and in brains of mice with scrapie.<sup>16-18</sup> It is also observable in other types of cells that produce amyloid deposits in experimental amyloidosis of mice: fixed reticular cells and sinus lining cells in the spleen, Kupffer cells in the liver, kidney mesangial cells and endothelial cells.<sup>19-23</sup> According to the current concepts based on studies of systemic amyloidosis, the formation of amyloid fibers is a two-step process consisting of the production of amyloidogenic protein, which is further processed, either locally or at distant sites, to yield amyloid fibers. In most forms of amyloidosis, one type of cell produces the amyloidogenic proteins, for example, hepatocytes in secondary amyloidosis.<sup>24-26</sup>

As indicated above, we hypothesized that the microglia/macrophage is both the producer and the processor cell. However, Glenner and Wong suggested that the precursor pro-

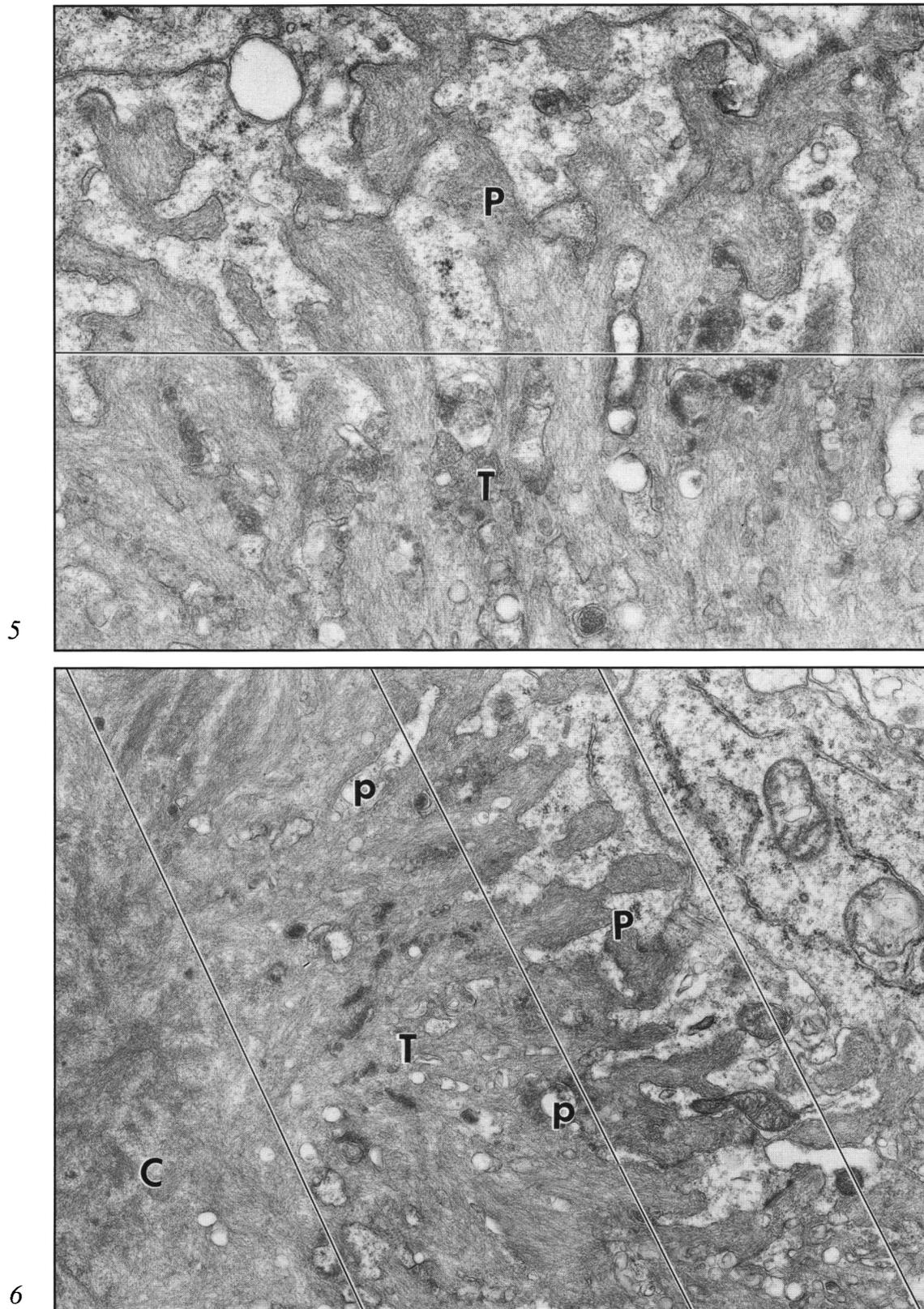


Figure 5 — Periphery (P) of the amyloid star formed by channels filled with amyloid fibers. Transient zone (T) is a mixture of trapped fragments of cytoplasm isolated between amyloid clusters. Degeneration of trapped cytoplasmic fragments, coalescence of amyloid-filled channels. 55,000X

Figure 6 — Peripheral (P), transient (T) and central (C) zone of the amyloid star. Regular arrangement of newly synthesized amyloid fibers in amyloid channels in peripheral zone. Mixture of amyloid clusters with products of disintegration of cytoplasmic pockets (p) trapped in transient zone. Condensation of amyloid deposits in the plaque center (C). 33,000X

tein is derived from blood and is processed in the brain.<sup>27,28</sup> Other investigators suggested that the amyloid protein precursor comes from neurons<sup>29-34</sup> or cells associated with the vessels.<sup>35</sup>

Many aspects of the process by which the 33-kd apolipoprotein E (ApoE) is secreted by macrophages spread on an immune complex-coated surface<sup>36</sup> are similar to morphological changes seen in microglial cells involved in the synthesis of amyloid fibers. In the secretion of ApoE, there is macrophage polarization with (1) redistribution of cell organelles such as Golgi apparatus, coated vesicles and secretory granules, (2) transformation of cellular membranes with formation of deep invaginations on the adherent surface, and (3) fast secretion of the ApoE. This polypeptide is found throughout the secretory pathway. It is found in lower concentrations in the endoplasmic reticulum and stacks of the Golgi complexes and in higher concentrations in the secretory granules. ApoE is also seen intracellularly in a more dilute form in large vacuoles that are contiguous with the basal cell surface.<sup>36-38</sup>

Recently while studying the metabolism of heparan sulfate proteoglycan, Iozzo<sup>39</sup> showed a progressive movement of the newly synthesized proteoglycan from the Golgi to the cell surface, where it became closely associated with the plasma membrane. Cell-surface and extracellular proteoglycans are internalized by the cell and converted into a series of progressively smaller pieces by proteolysis of the core protein and endoglycosidic cleavage of the heparan sulfate side chains. The final degradation products are then released from the cell. The deglycosylation of the small peptide could lead to its precipitation.<sup>39</sup> Many studies of microglial cells emphasize the enormous metamorphic capacity of microglial cells and their functional plasticity. Microglial cells may function in astrocytic differentiation and in mediation of immunological processes,<sup>40,41</sup> or as antigen-presenting cells, thus representing effector cells.<sup>41</sup> Involvement of the immune system in the pathogenesis of systemic amyloidosis is well documented.<sup>42,43</sup> Recent studies by Rogers and colleagues<sup>44</sup> and McGeer and co-workers<sup>45</sup> demonstrate the presence in AD brain of all elements of immune-competent cells. The presence of these cells in the brain is of great importance because, on proper antigenic stimulation by as yet undetermined antigen(s), these cells can initiate the same cascade of reactions that leads to amyloid formation in systemic amyloidosis, by macrophages, Kupffer cells, or hepatocytes.<sup>19-23</sup> The fact that in AD beta peptide amyloid deposits are found only in the brain underscores the importance of organ-specific conditions needed for amyloid deposits.

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