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Diffuse plaques associated with astroglial amyloid β protein, possibly showing a disappearing stage of senile plaques

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Abstract To clarify whether senile plaques disappear, we examined amyloid β protein (Aβ) deposits in non-demented subjects, and found novel diffuse plaques associated with astroglial Aβ. Formalin-fixed paraffin-embedded sections from cortical areas were immunolabeled with a panel of Aβ antibodies, and astroglial and microglial markers. Cerebral Aβ deposition was primarily found as diffuse plaques (DP) in these subjects. A subset of DP was associated with clusters of intensely Aβ-positive small granules. The clusters, which were located just adjacent to astroglial nucleus, had the characteristics of lipofuscin granules and, therefore, were quite different from "small stellate deposits". Substantial amounts of Aβ-positive granules were found inside astrocytes by dual labeling of Aβ and glial fibrillary acid protein, and the majority of astroglial Aβ immunoreactivity was located on lipofuscin granules. Aβ-positive granules lacked immunoreactivity with antisera for the N-terminal region of Aβ. These peculiar DP showed a much weaker staining than ordinary DP. The DP associated with astroglial Aβ were found in about one third of the subjects, although the density varied widely among individuals. From these findings, we propose that DP, which are associated with the N-terminal truncated Aβ in astrocytes, represent the disappearing stage of senile plaques.

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Introduction

β Amyloid is a polymer of the amyloid β protein (Aβ), and is a hydrophobic self-aggregating peptide consisting of 40–42 residues [13]. Aβ is derived from a larger membrane-bound protein, the amyloid β protein precursor (APP). Examination of patients with early-onset familial Alzheimer's disease (AD) revealed point mutations in the APP gene near the Aβ cleavage site. The brains of subjects with these mutations showed typical AD pathology, containing both numerous neurofibrillary tangles and senile plaques (SP) [12]. Studies of the presenilin gene, the causative gene in early-onset familial AD, indicated that mutations in the presenilin gene caused increased production of Aβ42 (for review see [8]). These studies support the idea that Aβ plays a key role in the pathogenesis of AD.

Massive cerebral β amyloid deposition is a histopathological characteristic of AD. However, considerable numbers of β amyloid deposits have been found even in mentally normal subjects [10, 16]. The number of SP in the cerebral cortex, therefore, does not correlate well with the degree of mental decline. A study of non-demented subjects found that the prevalence of cerebral Aβ deposits continued to increase with age, but the density of plaques did not increase [10]. This indicated that plaque density reached a plateau before the expression of clinical dementia. We were interested in this phenomenon and postulated that SP could disappear, and that the dynamic balance between plaque formation and destruction resulted in a maximum plaque density. In this study, we found novel diffuse plaques (DP), which were associated with astroglial Aβ, and discuss the possibility that these plaques represent the disappearing stage.

Table 1 Panel of the used antibodies and immunoreactivity of the astroglial Aβ. Underlining represents epitope (*Aβ* amyloid β protein, *APP* amyloid precursor protein, *GFAP* glial fibrillary acidic protein)

Antibody	Antigen	Glial Aβ	Species	Dilution	Source	Pretreatment
Ordinary anti-Aβ						
G42	1–40	++	Rabbit	1:1,000	Own [20]	Formic acid
β28K	1–28	++	Rabbit	1:1,000	Own [20]	Formic acid
4G8	17–24	++	Monoclonal	1:1,000	Dr. Kim	Formic acid
6E10	1–17	–	Monoclonal	1:2,000	Dr. Kim	Formic acid
6F/3D	8–17	– or +	Monoclonal	4 μg/ml	DAKO	Formic acid
BNT77	11–16	– or +	Monoclonal	1:1,000	[3]	Formic acid
End-specific anti-Aβ						
MBC42	37–42	++	Monoclonal[a]	1:100	Own	Formic acid
BC42	37–42	++	Rabbit	1:1,000	Own [20]	Formic acid
MBC 40	32–40	– or ++	Monoclonal[a]	1:10	Own	Formic acid
BC40	33–40	– or ++	Rabbit	1:1,000	Own [20]	Formic acid
BN1	1–7	–	Rabbit[b]	10 μg/ml	Own	Formic acid
Anti-N3(pE)	3(pE)–7	–	Rabbit[b]	2 μg/ml	[14]	Formic acid
Anti-N17(L)	17–27	–	Rabbit[b]	10 μg/ml	[14]	Formic acid
APP						
63N	18–38	–	Rabbit	1:500	Own [19]	Triton
61C	666–695	–	Rabbit	1:500	Own [19]	Triton
Z31 (preß)	577–596	–	Rabbit	1:500	Own [19]	Triton
Glial marker						
GFAP			Rabbit	1:1,000	Own	
mGFAP			Monoclonal[a]	1:50	IBL	
HLA-DR (CR3/43)			Monoclonal	3 μg/ml	DAKO	Autoclaving
CD11c (Ki-M1p)			Monoclonal	1:50	Seikagaku	
CD68 (KP1)			Monoclonal	8 μg/ml	DAKO	Trypsin

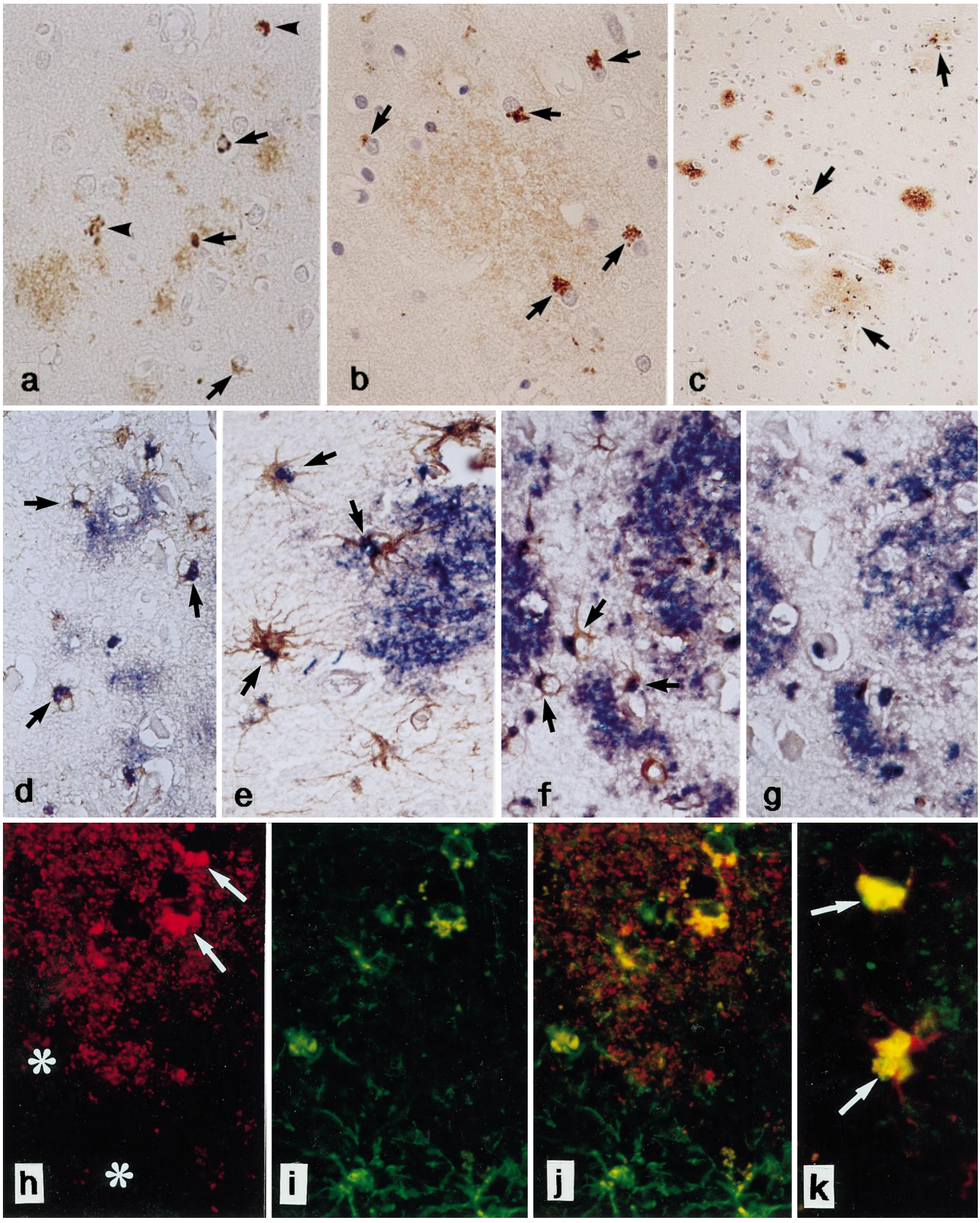
[a] Culture supernatant
[b] Affinity purified

Materials and methods

We had examined 123 brains obtained at autopsy from non-demented patients that had died with malignant neoplasms in their thirties to fifties, and found cerebral β amyloid deposits in 4 subjects in their forties and 14 subjects in their fifties [16]. In addition to these 18 subjects, we examined brains containing Aβ deposits from 20 patients between the ages of 61 and 90 years. None of the patients showed any signs of dementia when examined retrospectively. Whole brains were fixed in 10% formalin for 1–10 days. Tissue samples were taken from two to eight sites in the cerebral cortex: superior frontal gyrus and inferior temporal gyrus including hippocampal area in all subjects, and further samples from central, parietal, occipital and temporal cortices in interesting subjects. Samples were embedded in paraffin, and 4 μm-thick serial sections were prepared. Tissue sections were pretreated, and then reacted with a panel of antisera (Table 1). Immunoreaction was visualized using a Vectastain ABC Elite kit (Vector, USA) with diaminobenzidine and H_2O_2 solution. As controls, Aβ antisera were preabsorbed using commercially available synthetic β peptides (Bachem California, USA).

For double immunolabeling of Aβ and glial markers, tissue sections were first treated with glial marker antibodies (Table 1), and developed with peroxidase avidin-biotin-peroxidase complex (ABC) and the diaminobenzidine system (brown color). Then, the sections were treated with Aβ antisera, which were from a different species to that in which the glial marker antibodies were raised, and alkaline phosphatase-tagged secondary antisera (blue color). Double immunofluorescence staining was also performed, using fluorescein (FITC)-conjugated AffiniPure goat anti-rabbit IgG (40 μm/ml; Jackson, USA) and Texas red-conjugated AffiniPure goat anti-mouse IgG (20 μg/ml; Jackson, USA). Tissue sections were observed under a fluorescence microscope (Olympus BX-FLA, Japan) using WIB, NIBA, and WIG mirror units.

Fig. 1 a, b DP associated with clusters of intensely Aβ-positive granules (*arrows* and *arrowheads*) in the frontal lobe of a 47-year-old female, and in the temporal lobe of a 59-year-old male. The clusters of Aβ-positive granules appearedin the vicinity of the astroglial nucleus (*arrows*); **a, b** G42 staining. **c** DP associated with clusters of Aβ-positive granules (*arrows*) are more weakly labeled than other plaques (G42 staining). **d–g** Double immunolabeling of Aβ and astroglial marker (**d, f** GFAP and MBC42, **e** GFAP and 4G8) or Aβ and microglia marker (**g** Ki-M1p and G42), showing Aβ granules predominantly in astroglial cell bodies (*arrows*). The GFAP labeling of astrocytes is weak in the 48-year-old subject (**d**). **h–k** Double immunofluorescence study. **h–j** Aβ was detected with MBC42 antibody (**h** Texas red, WIG mirror unit), and astrocytes were labeled with GFAP antiserum (**i** FITC, WIB mirror units). Red fluorescence of astroglial Aβ granules (*arrows* in **i**) overlaps with autofluorescence of lipofuscin granules (**i** yellow under the WIB mirror units) and becomes orange by double exposure (**j**), while lipofuscin granules in the astrocytes lacking Aβ granules (*asterisks*) remain yellow. **k** Double labeling with a reverse combination (FITC for G42 and Texas red for mGFAP) shows that astrocytes (*arrows*) have some small Aβ granules (*green*) in addition to the Aβ-positive lipofuscin granules (*yellow*) by double exposure (WIB + WIG). (*DP* Diffuse plaques, *Aβ* amyloid β protein, *GFAP* glial fibrillary acidic protein) **a**, **b**, **d**–**g** × 400; **c** × 100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The nomenclature for plaques was described in the previous report [17].

Results

Novel DP with Aβ-positive granules

In 2 out of the 4 subjects in their forties, we found novel DP associated with clusters of intensely Aβ-positive granules (DP-Aβ granules) (Fig. 1 a). The clusters (5–10 μm in diameter) consisted of small round granules (< 2 μm in diameter). Out of the 14 subjects in their fifties, 6 subjects had DP-Aβ granules (Fig. 1 b). In the 20 subjects between the ages of 61 and 92 years, 5 showed DP-Aβ granules (Fig. 2). The density of the DP-Aβ granules varied widely among individuals, and also varied among sites (lobes) in the same subject. Intensity of the Aβ labeling in the plaque part of the DP-Aβ granules was much weaker than that in the DP without Aβ granules (Fig. 1a–c). Aβ granules appeared both inside and outside plaques, but they were not seen in areas without SP.

Co-localization of Aβ and astroglial marker in the granules

The clusters of Aβ-positive granules tended to be located just adjacent to the oval and pale nuclei, which had characteristics of astrocytes (Fig. 1 a, b; arrows). The clusters resembled lipofuscin in shape, and were quite different from "small stellate deposits", which were common in the AD brain [5]. To determine the exact localization of the intensely Aβ-positive granules, we used double immunostaining. As shown in Fig. 1 d–g, a considerable portion (about ½ to ⅔) of Aβ granules (purple) were seen in the astrocytes (GFAP; brown). They were rarely seen in the microglia (Fig. 1 g). Double immunofluorescence study further showed that most of the astroglial Aβ immunoreactivity was located on the auto-fluorescence of lipofuscin granules after the double exposure (Fig. 1 h–j). Some small Aβ-positive fine granules, similar to lysosomes were also found in addition to the lipofuscin granules (Fig. 1 k).

N-terminal truncated Aβ in the astrocytes

Using a panel of Aβ antisera (Table 1), glial Aβ granules were consistently positive with MBC42 and BC42 (42 end-specific; Fig. 1 d, f, h). They were positive with MBC40 and BC40 (40 end-specific; Fig. 2 a) when DP were positive with the antibodies. Glial Aβ granules were also intensely positive with G42 (1–40; Fig. 1 a–c, g, k), β28K (1–28), and 4G8 (17–24; Figs. 1 e, 2 b), but consistently negative with 6E10 (1–17; Fig. 2 c). The labeling with 6F/3D (8–17) and BNT77 (11–16) varied among subjects; partly positive in some subjects and almost negative in others. Glial Aβ granules were consistently negative with BN1 (1 end-specific; Fig. 2 d), anti-N3(pE) (3pE end-specific; Fig. 2 e) and anti-N17 (L) (17 end-specific), whereas small stellate deposits were positive (Fig. 2 d). The plaque part of DP-Aβ granules were positive with most antisera (Fig. 2 a–e), which indicated that deposited Aβ in the plaque part was longer than the astroglial Aβ. However, anti-N17(L) (end-specific) rarely demonstrated plaques as previously reported [14]. None of the APP antisera stained DP-Aβ granules (Table 1), suggesting that Aβ granules contained neither full-length form of APP nor amyloidogenic C-terminal fragments. In the control study, the labeling of astroglial Aβ disappeared after the preabsorption of the antisera with synthetic peptides. The DP-Aβ granules were not seen with Congo red stain, suggesting non-fibrillar Aβ deposits in the granules.

Discussion

In this study, we showed that astroglial Aβ granules consisted of a shorter form of Aβ than that seen in the plaque part and small stellate deposits. C-terminal residues are preserved at the 42 (40) position. N-terminal residues may have been lost between positions 8 and 17, because granules were infrequently positive with 6F/3D (8–17) and BNT77 (11–16), and always negative with end-specific

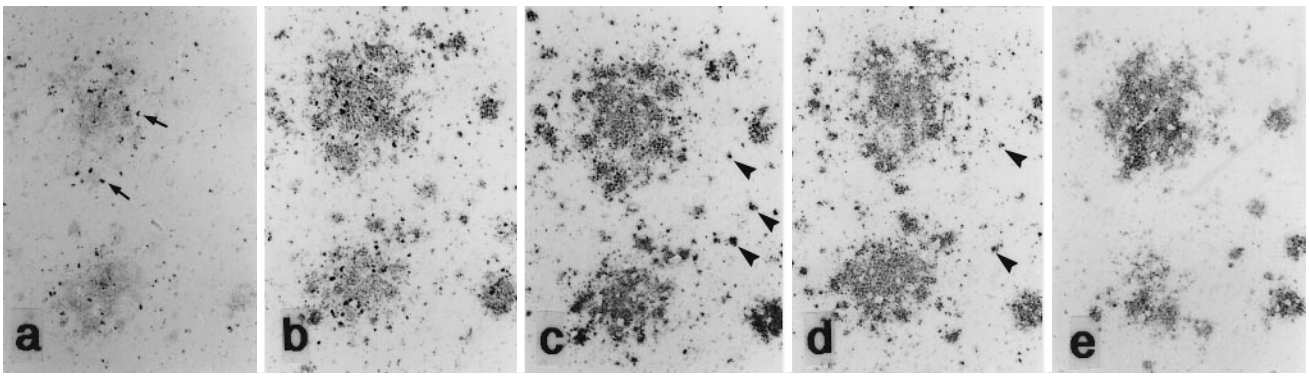

Fig. 2 a–e Comparison between different Aβ antibodies using serial sections in the occipital lobe of a 75-year-old male. Astroglial Aβ granules positive with MBC40 (**a**; *arrows*) and 4G8 (**b**) are negative with 6E10 (**c**), BN1 (**d**) and anti-N3(pE) (**e**), showing that they have a shorter N-terminal region than the Aβ of the plaque part which is positive with all antibodies. The N-termini of the Aβ in small stellate deposits (**c**, **d**; *arrowheads*) are preserved. **a–e** × 60

anti-N17(L). This indicated that the peptide of astroglial Aβ granules may have been somewhat longer than p3, Aβ17–42 (40).

Using frozen sections of AD brains, Akiyama et al. [1] reported similar Aβ-positive granules to a large extent in microglia and to some extent in astrocytes. In contrast, we found these granules markedly in astrocytes using paraffin sections of non-demented subjects, especially those in their forties and fifties. In the middle-aged subjects, glial reaction to β amyloid deposits was very weak. Moreover, immunoreactivity for microglial markers was not well preserved in the paraffin sections, possibly accounting for our finding Aβ granules predominantly in astrocytes.

Astrocytes, especially plaque-associated astrocytes, produce APP, a source of Aβ [19]. Therefore, astroglial Aβ granules could have been primarily produced within them. However, we considered that astroglial Aβ granules were a result of phagocytosis. First, DP-Aβ granules appeared in only some subjects, and were also a subset of DP, indicating that DP-Aβ granules were not a common form. Second, the plaque part of DP-Aβ granules was labeled only very weakly, suggesting small amounts of Aβ within this type of plaque. Third, the N-terminus of Aβ was more truncated in astroglial Aβ granules than in the plaque part, indicating degradation of Aβ in the astroglial cytoplasm. Fourth, most of the astroglial Aβ were lipofuscin granules, which were derived from lysosomes. Fifth, amyloid fibrils in DP were surrounded by astrocytic processes [18], and astrocytes had phagocytic activity [2]. We favor the phagocytosis theory, although our findings did not provide direct evidence.

Amyloid fibrils can be removed by phagocytic cells. In experimental amyloidosis with casein injection, splenic amyloid was gradually abolished after cessation of the injection [15]. In dialysis amyloidosis, hemoperfusion using a β_2-microglobulin-adsolvent column markedly reduced the serum β_2-microglobulin level and resulted in improved clinical symptoms [7]. In familial amyloid polyneuropathy of transthyretin type, liver transplantation improved the clinical manifestation [4]. Moreover, in the APP fragment gene-transgenic mouse, β amyloid was taken up by macrophages in the pancreas [9]. Direct injection of plaque cores into the rat cerebral cortex resulted in phagocytosis of the amyloid cores by macrophages [6]. These findings support the theory that β amyloid can be removed by scavenger cells.

We have detected a novel type of DP, DP-Aβ granules, and suggest that they indicate phagocytosis (the disappearing stage of senile plaques), rather than Aβ production within the astrocytes. Plaque density does not increase with age, although its prevalence increases with age [10]. Moreover, comparison between biopsy and autopsy samples from the same AD patient has shown that plaque density does not greatly increase during the course of the disease [11]. From these studies we suggested that plaque density reaches a plateau when plaque formation and destruction are equally balanced.

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