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Astrocytes Containing Amyloid β-Protein (Aβ)-Positive Granules Are Associated with Aβ40-Positive Diffuse Plaques in the Aged Human Brain

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Amyloid β-protein (Aβ) is the major component of senile plaques that emerge in the cortex during aging and appear most abundantly in Alzheimer's disease. In the course of our immunocytochemical study on a large number of autopsy cases, we noticed, in many aged nondemented cases, the presence of unique diffuse plaques in the cortex distinct from ordinary diffuse plaques by immunocytochemistry. The former were amorphous, very faintly Aβ-immunoreactive plaques resembling diffuse plaques, but they stained for Aβ40 and were associated with small cells containing Aβ-positive granules. A panel of amino- and carboxyl-terminal-specific Aβ antibodies showed that such Aβ40-positive diffuse plaques and cell-associated granules were composed exclusively of amino-terminally deleted Aβ terminating at Aβ40, -42, and -43. Double immunostaining also showed that those Aβ-immunoreactive granules are located in astrocytes and not in microglia or neurons. Immunoelectron microscopy revealed that nonfibrillar Aβ immunoreactivity was located within lipofuscin-like granules in somewhat swollen astrocytes. These findings raise the possibility that astrocytes take up Aβ and attempt to degrade it in lysosomes in the aged brain. ***(Am J Pathol 1998, 152:983–992)***

Amyloid β-protein (Aβ) is the major component of senile plaques that occur in normal aged brains and to a much greater extent in brains affected by Alzheimer's disease (AD). There are two major Aβ species terminating at Val-40 (Aβ40) and at Ala-42 (Aβ42), of which Aβ42 is now thought to be the major and earliest species to be deposited in the cortex.[1,2] We currently do not know why full-length Aβ1–42, a very minor species of secreted Aβ, accumulates in the cortex to form Aβ deposits and/or amyloid fibrils that can be visualized by immunocytochemistry. However, its deposition must take a long time, because Aβ accumulation that can be detected only by sensitive enzyme immunoassay appears to precede the formation of senile plaques or vascular amyloid deposition by more than several years (H. Funato, M. Yoshimura, and Y. Ihara, unpublished observations).[3] Amyloid fibrils thus formed gradually are naturally recognized as foreign bodies *in vivo*, and relevant cells in the brain attempt to remove these extracellular aggregates. Microglia that abundantly express scavenger receptors through which Aβ aggregates are internalized are well known (see also Ref. 4).[5,6] However, attempts by microglia to degrade Aβ would be relatively unsuccessful because aggregated Aβ is known to be highly resistant to many proteases.[7–9]

In the general population, Aβ42 levels in the various regions of the brain rise steeply between the ages of 50 and 70 years (H. Funato, M. Yoshimura, and Y. Ihara, unpublished observations).[3] This marked change in Aβ42 levels in the brain, in particular in the cortex, likely results from failure to effectively clear Aβ42 from the brain, although we cannot exclude the possibility that overproduction of Aβ42 occurs simultaneously. This, in turn, suggests that, under normal conditions, soluble Aβ42 monomer and/or oligomer should be quickly removed to maintain low Aβ42 levels in the extracellular space. Thus, we hypothesize that the clearance rate of Aβ42 in the brain decreases sharply at the critical period for unknown reasons, which leads to Aβ42 accumulation and finally to senile plaque formation. In this context, it is of note that cultured microglia and astrocytes can take up and degrade soluble Aβ.[10] Both types of cells are con-

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sidered to carry receptors for soluble Aβ. One candidate receptor is the serpin-enzyme complex receptor through which Aβ is endocytosed.[11] Another is the receptor for advanced glycation end product (RAGE), which is expressed in neurons and microglia. This receptor must also serve as one of the receptors for soluble Aβ.[12] It is also possible that unknown proteases secreted from brain cells effectively degrade Aβ42 in the extracellular space.[13,14]

In the course of our immunocytochemical examinations of aged brains, we noted unique Aβ immunoreactivities in the cortical sections of many nondemented aged subjects. Here we show that a subset of astrocytes containing Aβ-positive granules are associated with unusual diffuse plaques that are composed of both Aβ40, and Aβ42. This raises the possibility that astrocytes are involved in the removal of accumulating Aβ in the brain.

Materials and Methods

Materials

Samples examined here were obtained from fresh autopsied brains at the Tokyo Medical Examiner's Office (Otsuka, Tokyo, Japan) and the Tokyo Metropolitan Tama Geriatric Hospital (Tama, Tokyo, Japan). The ages of the 143 subjects ranged from 22 to 94 years (5 subjects in the 20- to 29-year range, 4 in the 30- to 39-year range, 13 in the 40- to 49-year range, 19 in the 50- to 59-year range, 29 in the 60- to 69-year range, 38 in the 70- to 79-year range, 26 in the 80- to 89-year range, and 9 in the 90- to 99-year range), and among these subjects there were 11 cases of AD (2 subjects in the 50- to 59-year range, 3 in the 70- to 79-year range, 5 in the 80- to 89-year range, and 1 who was 92 years old). Postmortem delay ranged from 2 to 12 hours. The diagnosis of AD was made based on clinical and neuropathological criteria.[15,16] Tissue blocks from the occipital lobes at the occipital pole (Brodmann areas 17/18) and the inferior temporal lobes, including the hippocampus and medial occipitotemporal cortex, were fixed in 10% buffered formalin and processed for histological and immunocytochemical examinations.

Antibodies

The locations of epitope for the used Aβ antibodies are illustrated in Figure 1. BA27, BC05, and BC65 were monoclonal antibodies specific for Aβ40, Aβ42(43), and Aβ43, respectively. Other Aβ monoclonal antibodies used included BAN50 (raised against residues 1 to 16 of Aβ), BAN052 (raised against residues 1 to 16 of Aβ), BNT77 (the epitope is located between residues 11 and 16 of Aβ), 4G8 (Senetek PLC, Maryland Heights, MO; the epitope is located between residues 17 and 24 of Aβ).[1,17–19] Also used were a polyclonal antibody to Aβ1–28 and the following polyclonal antibodies, which specifically recognize various, amino-terminally ragged Aβ species and their structurally modified forms: AβN1(L-Asp), AβN1(L-isoAsp),

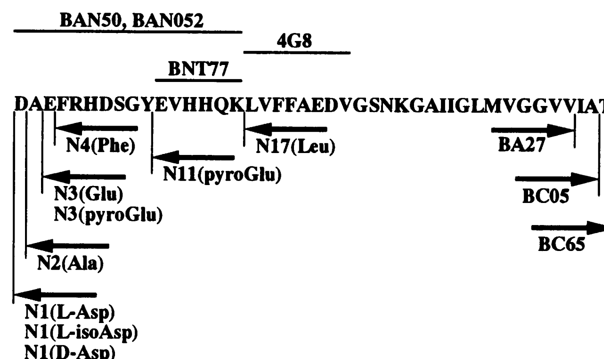

Figure 1. Schematic representation of the locations of epitope for the Aβ antibodies used in the present study.

AβN1(D-Asp), AβN2(Ala), AβN3(Glu), AβN3(pyroGlu), AβN4(Phe), AβN11(pyroGlu), and AβN17(Leu).[20,21] For β-amyloid precursor protein (APP) immunostaining, monoclonal antibody 22C11 (Boehringer Mannheim, Mannheim, Germany) and polyclonal antibody C9 (raised against APP676–695 according to the numbering of the shortest form of APP) were used. Other antibodies used were anti-human tau antibody, a monoclonal antibody to nonphosphorylated neurofilaments (SMI33, Sternberger, Baltimore, MD), two kinds of anti-glial fibrillary acidic protein (GFAP) polyclonal antibodies (Sigma Chemical Co., St. Louis, MO and a gift of H. Yamaguchi[22]), a monoclonal antibody to GFAP (IBL, Gunma, Japan), a monoclonal antibody to S100β (Sigma), and an affinity-purified antibody to growth inhibitory factor (GIF).[23,24] A polyclonal antibody to ferritin (Dako, Glostrup, Denmark) or a biotinylated lectin, *Ricinus communis* agglutinin 120 (RCA120, Sigma), was used for identifying microglia.[25,26]

Immunocytochemistry

Tissue sections were cut at 6 μm, mounted on albumin-coated slides, air dried on a hot plate at 55°C, and kept at 65°C for several hours. After deparaffinization, the sections were treated with 0.3% hydrogen peroxide in methanol for 15 minutes to destroy endogenous peroxidase activity. For retrieving Aβ, the sections were pretreated with 95% formic acid for 5 minutes. Thus, treated sections were incubated with Tris-buffered saline (TBS) containing 10% calf serum and then with an appropriately diluted primary antibody or a biotinylated lectin, RCA120, overnight at room temperature (RT). After being washed with TBS, the sections were incubated with secondary antibody (biotinylated anti-mouse IgG antibody or biotinylated anti-rabbit IgG antibody). The immunoreactive proteins were visualized by the avidin-biotin method (ABC kit, Vector Elite, Vector Laboratories, Burlingame, CA) using 0.02% 3,3′-diaminobenzidine.

Double Immunostaining

For double immunostaining, 4G8 or anti-Aβ1–28 was used for Aβ visualization, whereas GFAP, GIF, or S100β

was used as a marker for astrocytes. Neurons were labeled with anti-nonphosphorylated neurofilament antibody, and microglia were labeled with anti-ferritin antibody or lectin RCA120. To enhance immunoreactivity, deparaffinized sections were treated with 95% formic acid for 5 minutes for Aβ detection, preincubated with 0.1% trypsin for 15 minutes for S100β detection, autoclaved in distilled water for GFAP staining,[27] or heated in a microwave oven (500 W) in 0.01 mol/L citrate buffer (pH 6.0) three times, each for 5 minutes, for detection of GIF, neurofilament, or lectin RCA120.[28] After being treated with 0.3% hydrogen peroxide in methanol for 15 minutes, the first primary antibody was visualized by use of alkaline-phosphatase-conjugated avidin-biotin method (Vector) using Vector substrate kit III. The blue-developed sections were then treated with glycine/HCl buffer (pH 2.2) to remove the first primary antibody. The second primary antibody was applied to the sections and the bound antibody was visualized using horseradish-peroxidase-conjugated avidin-biotin method using Vector VIP kit chromogen (purple) or diaminobenzidine (brown). The blue- and brown- or purple-developed sections were counterstained briefly with hematoxylin.

Electron Microscopy

Tissue blocks of interest were fixed in 2.5% glutaraldehyde and then post-fixed with 1% OsO_4, followed by Epon embedding. The embedded block was serially cut into semi-thin (0.3-μm-thick) and ultra-thin (90-nm-thick) sections alternately, as described previously.[29,30] After removal of the Epon, semi-thin sections were treated with 0.3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity and then pretreated with 95% formic acid for 3 minutes to enhance Aβ immunoreactivity. The treated sections were incubated with TBS containing 10% calf serum and then with 4G8 or anti-Aβ1–28 overnight at RT. After being washed with TBS, the sections were incubated with biotinylated anti-mouse IgG antibody or biotinylated anti-rabbit IgG antibody. The bound antibodies were visualized by the avidin-biotin method using diaminobenzidine. Adjacent ultra-thin sections were stained with uranyl acetate and lead citrate and viewed under a JEOL 1200EX electron microscope.

Immunoelectron Microscopy

Formalin-fixed tissue blocks of interest were cut at a thickness of 50 μm on a vibratome. The vibratome sections were incubated in 2% Triton X-100 for 1 hour at RT, followed by trypsin (0.1%) treatment. These sections, after being treated with 95% formic acid for 3 minutes, were blocked with 10% calf serum and then incubated with one of the Aβ monoclonal antibodies for 24 hours at RT, followed by incubation with 10-nm gold-conjugated anti-mouse IgG for 24 hours at RT. These sections were fixed in 2.5% glutaraldehyde in phosphate buffer for 60 minutes and subsequently in 2% OsO_4 for 60 minutes, dehydrated, and embedded in Epon. Ultra-thin sections prepared from the area less than 2 μm deep from the cutting surface were viewed after uranyl acetate and lead citrate staining under a JEOL 1200EX electron microscope. As a control, vibratome sections were incubated with preimmune sera or Aβ antibodies preabsorbed with relevant synthetic Aβ peptides.

Results

Unusual Aβ40-Positive Diffuse Plaques Associated with Dot Aβ Immunoreactivities

The unique Aβ immunoreactivities we originally noted consisted of two distinct types of staining: very faint amorphous staining resembling that of diffuse plaques and scattered dot staining associated with small, round nuclei. Thus, we examined in a systematic manner the brain tissue sections from 143 subjects (age range, 22 to 94 years), including 11 with AD, for detection of the Aβ40-positive diffuse plaques. Among the 143 nondemented subjects, 60 subjects exhibited senile plaques, and 22 of the 60 subjects were found to exhibit these Aβ40-positive diffuse plaques: 3 subjects in the 50- to 59-year range, 5 in the 60- to 69-year range, 10 in the 70- to 79-year range, 3 in the 80- to 89-year range, and 1 who was 92 years old. Only two among the AD subjects exhibited the Aβ40-positive diffuse plaques. The nondemented subjects exhibiting Aβ40-positive diffuse plaques usually exhibited only small numbers of ordinary senile plaques and no neurofibrillary tangles. These Aβ40-positive diffuse plaques associated with scattered dot staining were located in layers II to VI, most commonly in layer III of the cortex.

Dot Aβ immunoreactivities, 5 to 10 μm in diameter, were scattered within or close to a diffuse plaque that stained for both Aβ42 and Aβ40 (Figures 2A-K). Each of the dots was associated with a small, round nucleus. The dots were composed of many fine granules (<1 μm in diameter) apparently located in the cytoplasm (see below).

The Aβ40-positive diffuse plaque stained for Aβ40, a unique characteristic that distinguishes it from an ordinary diffuse plaque (Figure 2, A and B). Conventional histological stains, including Bielschowsky's, Bodian's, thioflavin S, or Congo red stain, failed to detect the Aβ40-positive diffuse plaques associated with the dot Aβ immunoreactivities.

Aβ40-Positive Diffuse Plaques

The size of the Aβ40-positive diffuse plaques varied greatly just as did that of ordinary diffuse plaques. At high magnification, the Aβ40-positive diffuse plaques were found to consist of granules and somewhat reticular staining. This contrasts with the uniformly stained diffuse plaques typical of brains affected with Down's syndrome. The immunochemical profile of the Aβ40-positive diffuse plaque was similar to that of those cell-associated dots with small variations among cases. These Aβ40-positive diffuse plaques were la-

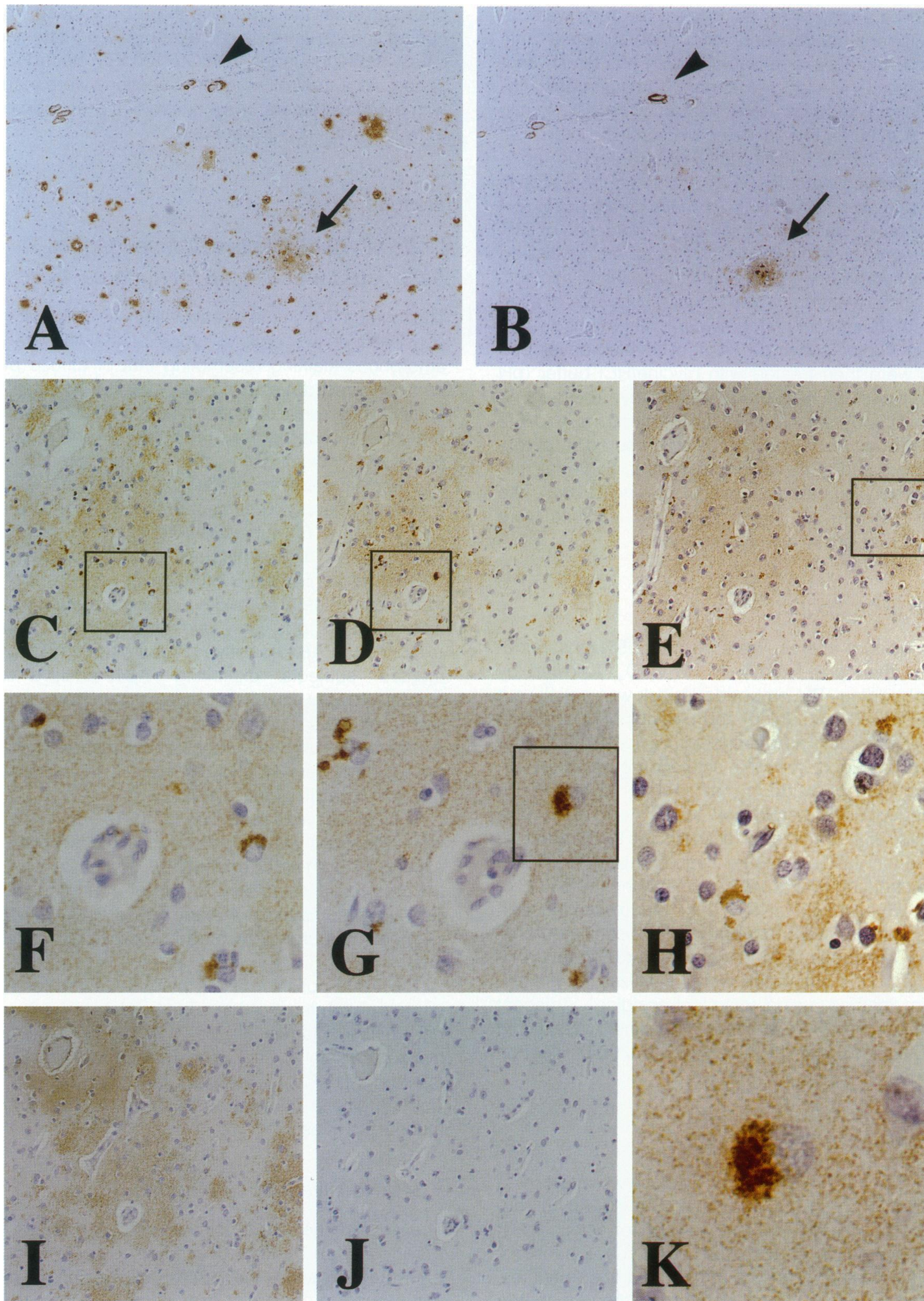

Figure 2. Dot Aβ immunoreactivities, scattered in Aβ40-positive diffuse plaques, visualized using a variety of Aβ antibodies. In one section (**A**) 4G8 labeled a variety of senile plaques, diffuse and mature plaques, and β-amyloid-bearing vessels (**arrowhead**), in addition to Aβ40-positive diffuse plaques associated with dot immunoreactivity (**arrow**). In contrast, in an adjacent section (**B**) BA27 labeled only the cell-associated dots and Aβ40-positive diffuse plaques (**arrow**) besides β-amyloid-bearing vessels (**arrowhead**). The five adjacent sections (**C**, **D**, **E**, **I**, and **J**) were immunostained with 4G8, BA27, BC05, BAN052, and BA27 preincubated with synthetic Aβ1–40, respectively. Each indicated area in **C**, **D**, or **E** is magnified in **F**, **G**, or **H**, respectively. 4G8 (**C** and **F**), BA27 (**D** and **G**), and BC05 (**E** and **H**) labeled the cell-associated dots and the diffuse plaques, whereas BAN052 (**I**) labeled only the diffuse plaques but not the cell-associated dots. At higher magnification (**K**), fine granular BA27 immunoreactivities are packed around small nuclei, presumably of astrocytes. Original magnification, ×40 (**A** and **B**), ×100 (**C** to **D**, **I**, and **J**), ×400 (**F** to **H**), and ×1000 (**K**).

beled with 4G8, BA27, BC05 (Figures 2, C–E), and BC65 (data not shown) but not with any of the following antibodies, which specifically recognized various, amino-terminally ragged Aβ species and their structurally modified forms: AβN1(L-Asp), AβN1(L-isoAsp), AβN1(D-Asp), AβN2(Ala), AβN3(Glu), AβN3(pyroGlu), AβN4(Phe), AβN11(pyroGlu), and AβN17(Leu). Sections treated without primary antibodies or with Aβ antibodies preabsorbed with corresponding synthetic Aβ peptides (Figure 2J) showed no immunoreactivity.

This may be because these amino-terminally ragged Aβ species are quite vulnerable to formalin fixation,[21] but under the same conditions, ordinary senile plaques in the same section and in the sections from AD brains stained very well with these antibodies (data not shown). BNT77 and anti-Aβ1–28 labeled only a certain area of the 4G8-stained domain. BAN50 labeled neither the Aβ40-positive diffuse plaques nor cell-associated dots, whereas BAN052 labeled the former (Figure 2I) but not the latter (see below). Overall, the Aβ species in the Aβ40-positive diffuse plaques consisted exclusively of Aβ40, Aβ42, and Aβ43 species that are amino-terminally deleted to extents that do not affect their 4G8 immunoreactivities. The lack of reactivities with the Aβ antibodies specific for various Aβ species with ragged amino termini are the second major characteristic of the Aβ40-positive diffuse plaques distinguishing them from the ordinary diffuse plaques. In particular, the Aβ40-positive diffuse plaques did not stain for AβN3 (pyroGlu), which is known to be a major component of ordinary diffuse plaques.[20,21,31]

Dot Aβ Immunoreactivities Localized to Astrocytes

To characterize the cell-associated dot Aβ immunoreactivities, we used the same panel of Aβ antibodies. BA27, BC05, and BC65 labeled the dots almost as intensely as did 4G8 (Figure 2, C–H, and data not shown). None of the antibodies to various Aβ species with ragged amino termini labeled the dots. BNT77 and anti-Aβ1–28 labeled less than half of the dots. BAN052 stained none of the dots (Figure 2I), whereas BAN50 faintly labeled only a few of them. Absorption with excess synthetic Aβ peptides resulted in no immunoreactivity on the sections (Figure 2J). None of the dots stained with the antibodies to the amino-terminal portion (22C11) or carboxy terminus of APP (C9; data not shown).

To determine whether the dot Aβ immunoreactivities are located in astrocytes, double immunostaining was performed using an Aβ monoclonal or polyclonal antibody and GFAP, GIF, or S100β as an astrocytic marker. Consistent with the previous results on human brains, anti-GIF intensely labeled astrocytes, especially their extended fine processes, both in the gray and white matter, but did not label neurons, oligodendrocytes, or microglia (data not shown). Almost all of the dots labeled with 4G8 or anti-Aβ1–28 co-localized with GFAP, GIF (Figure 3, A and B) and S100β immunoreactivity (data not shown). Thus, we concluded that almost all of the cells containing Aβ immunoreactivity are astrocytes. No immunoreactivities for APP were detected in the Aβ-positive astrocytes, suggesting that these astrocytes are of the protoplasmic type and not reactive ones (data not shown).

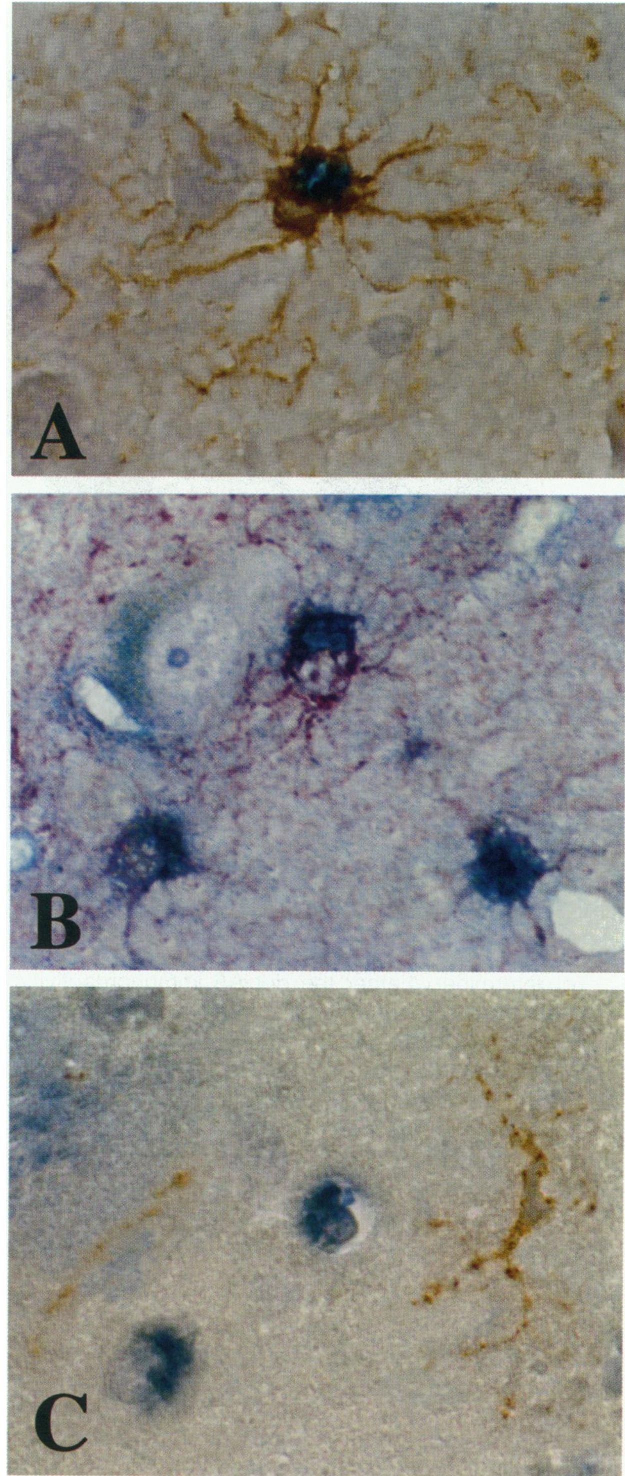

Figure 3. Dot Aβ immunoreactivities localized to astrocytes as shown by double immunostaining. **A:** GFAP-positive cortical astrocytes (brown) exhibited 4G8 immunoreactivity (blue). **B:** GIF-positive cortical astrocytes (purple) exhibited 4G8 immunoreactivity (blue). **C:** Lectin RCA120-positive microglia (brown) did not co-localize with Aβ immunoreactivity (blue). Original magnification, ×1000.

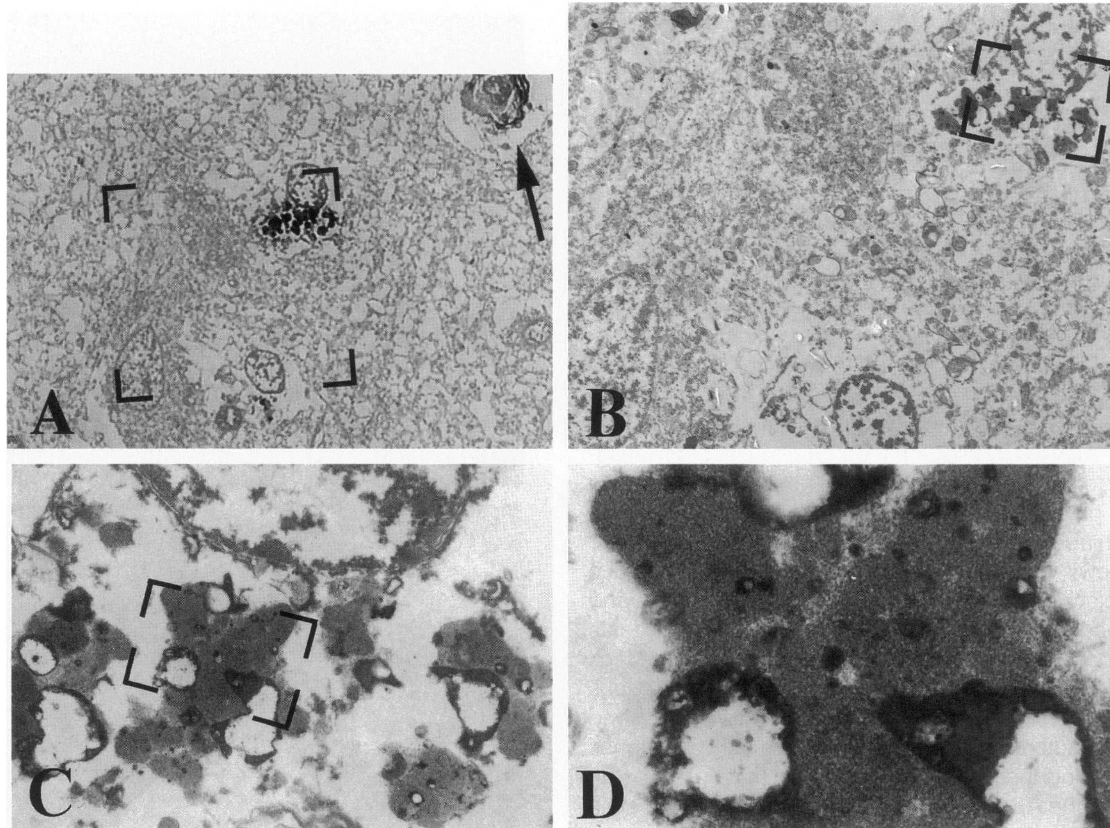

Figure 4. Electron microscopic identification of the dot immunoreactivities using the semi-thin/ultra-thin alternate sectioning protocol. Shown are light (**A**) and electron (**B** to **D**) micrographs of a dot Aβ immunoreactivity that is present in an edematous and organelle-poor cytoplasm associated with a medium-sized, round and clear nucleus. Each indicated area is magnified in the next photograph. **D:** Higher magnification of the lipofuscin-like granules containing no apparent fibrillar profiles. An **arrow** in **A** indicates a β-amyloid-bearing vessel. Original magnification, ×1000 (**A** and **B**), ×5000 (**C**), and ×20,000 (**D**).

To determine whether the dot Aβ immunoreactivities are also located in other types of cells, additional double immunostaining was performed using biotinylated lectin RCA120 or anti-ferritin antibody as a microglial marker and anti-nonphosphorylated neurofilament antibody as a neuronal marker. Microglia labeled with RCA120 or anti-ferritin exhibited no dot Aβ immunoreactivity (Figure 3C), except in extremely rare cases in which a few ferritin-positive microglia co-localized with the dot Aβ immunoreactivities. No neuron exhibited dot Aβ immunoreactivity.

Aβ-positive granules localized to lipofuscin-like granules

To further investigate the intracellular localization of the dot Aβ immunoreactivities, we selected four cases that exhibited abundant dot Aβ immunoreactivities but very few senile plaques for electron microscopic observations.

Using the semi-thin/ultra-thin alternate sectioning protocol, all dot Aβ immunoreactivities were found to be located in lipofuscin-like granules, in a rather edematous and organelle-poor cytoplasm, which were associated with a medium-sized, round and clear nucleus (Figure 4, A–D). These nuclear and cytoplasmic features are consistent with those of cortical protoplasmic astrocytes. The identified structures, 1 to 2 μm in diameter, had irregular shapes and varying electron densities and contained small lucent lipid droplets that are indistinguishable from lipofuscin in normal brain cells. No apparent fibrillar structures were discernible in these lipofuscin-like granules (Figure 4D). No individual amyloid fibrils were found in the vicinity of these astrocytes carrying Aβ-positive granules, a finding consistent with a previous observation on diffuse plaques.[29]

Vibratome sections containing many dot Aβ immunoreactivities were processed for immunoelectron microscopy using a colloidal-gold-conjugated secondary antibody. Almost all gold labeling for 4G8, BC05, or BA27 was confined in lipofuscin-like granules in somewhat edematous astrocytes (Figure 5, A–C). Neuronal lipofuscins were labeled very faintly with 4G8 but not with other monoclonal antibodies.[32] Occasionally, a few gold particles were found in secondary lysosomes. No gold labeling was found when the sections were incubated with preabsorbed Aβ antibodies (Figure 5D) or when primary antibodies were omitted.

Discussion

There are several previous reports on the presence of cytoplasmic Aβ immunoreactivities.[33–35] Kim et al[33]

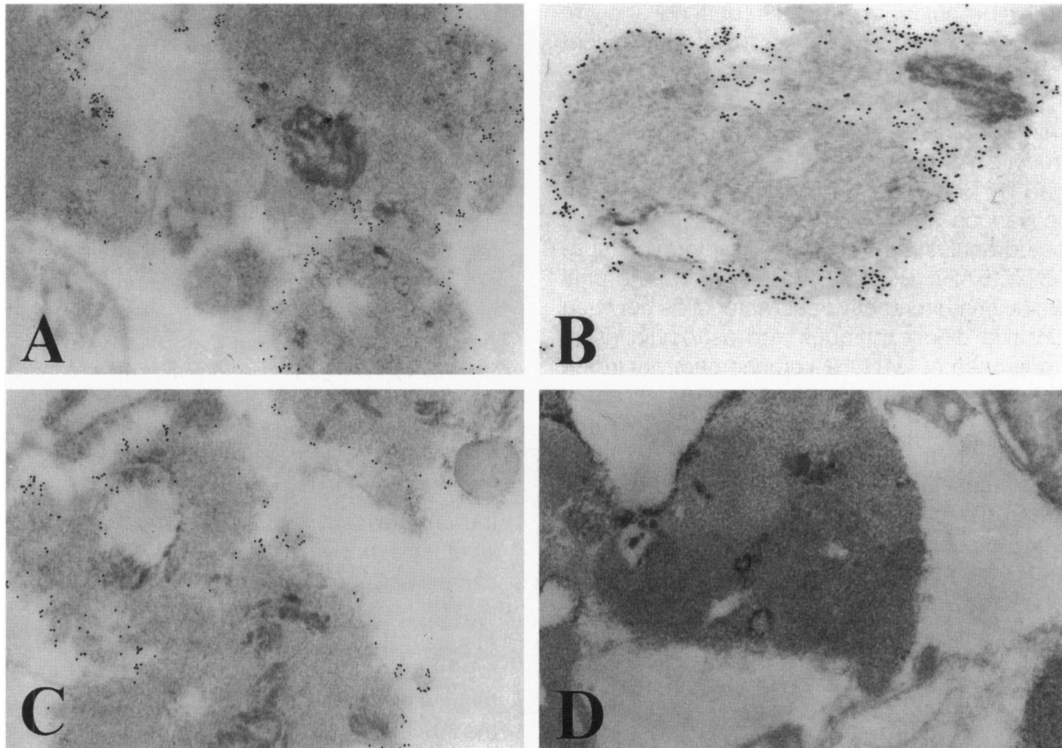

Figure 5. Immunogold labeling for Aβ. Numerous gold particles representing 4G8 (**A**), BA27 (**B**), or BC05 (**C**) are located mainly in the peripheral portion of lipofuscin-like granules in an edematous and organelle-poor cytoplasm associated with a medium-sized, relatively round and clear nucleus. At lower left in **A**, a swollen mitochondrion is not labeled. Lipofuscin labeling disappeared when BA27 was preabsorbed with synthetic Aβ 1–40 (**D**). Original magnification, ×20,000.

showed 4G8 immunoreactivity in neuronal lipofuscins. However, it is now shown that 4G8 recognized in lipofuscins is not Aβ but a 31-kd protein, possibly a fragment of APP.[32] It was also reported that two monoclonal antibodies against synthetic Aβ1–28 label not only senile plaques but also cytoplasm of neurons and other cells. Western blotting showed that those antibodies recognized a 36-kd protein,[34] which turned out to be glyceraldehyde-3-phosphate dehydrogenase.[36] Other monoclonal antibodies raised against synthetic Aβ1–28 were reported to immunostain neuronal and glial cytoplasm[35]; however, it was later shown that those Aβ antibodies cross-reacted with fibrinogen in human brain homogenate.[37] Thus, we should be cautious about the interpretation of the intracellular Aβ immunoreactivities in the present work. In particular, as lysosomes are the site for the degradation of the carboxyl-terminal portion of APP,[38,39] its presence in lysosomes may create false positive results with respect to Aβ immunoreactivity. However, the presence of Aβ in the granules is validated by the absence of C9 reactivity and by the reactivity with BA27, which shows no cross-reactivity with full-length APP or APP fragments containing the full-length Aβ sequence (M. Morishima-Kawashima and Y. Ihara, unpublished data). Despite some cross-reactivity, BC05 has affinity for Aβ42 ~300-fold more than for full-length APP (M. Morishima-Kawashima and Y. Ihara, unpublished data). Moreover, it is unlikely that all of the monoclonal antibodies exhibiting distinct specificities toward the Aβ molecule consistently labeled any molecule other than Aβ.

Double immunostaining for Aβ and GFAP or GIF clearly showed that Aβ-immunoreactive granules are located in astrocytes and not in microglia or neurons. Although definite intermediate filaments were not found at the electron microscopic levels in Aβ-positive cells (Figure 5), this is presumably because the materials used for the present study were fixed in formalin after a long postmortem interval. In our experience, intermediate filaments in protoplasmic astrocytes are recognizable only when the tissues are appropriately fixed with glutaraldehyde after a short postmortem interval (less than 7 hours). Thus, most of Aβ-positive cells should be astrocytes, although we cannot exclude completely the possibility that some neurons also contain similar Aβ-immunoreactive granules.

Immunoelectron microscopy showed that Aβ-positive immunoreactivity localized to lipofuscin-like granules in astrocytes. One may claim that immunogold particles are located mainly along the margin of the granules but not inside. This is presumably due to limited penetration of gold particles into the specimens, a problem caused by the pre-embedding protocol. To address this issue, the adjacent sections were similarly stained with anti-cathepsin D, again providing immunogold labeling along the margin of lysosomes where cathepsin D is located (data not shown). We attempted to employ the post-embedding method, but only few gold particles were observed

in lipofuscin-like granules in astrocytes (data not shown). This is presumably because of omission of formic acid treatment, an essential step to enhance the Aβ immunoreactivity. Taken together, we conclude that most of Aβ is located within lipofuscin-like granules in astrocytes.

Intra-astrocytic Aβ was labeled most intensely with 4G8, followed by BNT77, but not with BAN50. Whereas none of the various amino-terminal-specific antibodies labeled these granules, all of the carboxyl-terminal-specific antibodies, BA27, BC05, and BC65, did. The only difference in the immunoreactive characteristics between the Aβ40-positive diffuse plaques and astrocytic granules is the presence of BAN052 immunoreactivity in the former. This suggests that the Aβ in the astrocytic granules is processed to a greater extent than the Aβ in the Aβ40-positive diffuse plaques.

Faintly Aβ-stained diffuse plaques and scattered Aβ-positive dots characterize the Aβ40-positive diffuse plaque under the light microscope. Using these criteria, we noted that these plaques occur not infrequently in the sections of aged control subjects. The appearance of the Aβ40-positive diffuse plaques was not related to underlying diseases, or causes of death, and was not correlated with the abundance of ordinary senile plaques; only a very small proportion of the AD subjects (2/11) exhibited the Aβ40-positive diffuse plaques, and there were some control subjects exhibiting the Aβ40-positive diffuse plaques but not the ordinary ones. This suggests that the Aβ40-positive diffuse plaques with astrocytes carrying Aβ-positive granules emerge in the early stage of senile plaque formation and disappear or are replaced by ordinary plaques as plaque formation progresses.

Although morphologically it is difficult to distinguish between the Aβ40-positive diffuse plaques and ordinary diffuse plaques, the former stained more weakly and exhibited fine reticular staining with 4G8. Despite its morphological similarity to the ordinary diffuse plaque, the Aβ40-positive diffuse plaque has two characteristics distinct from those of the ordinary diffuse plaque. One is the presence of Aβ40, which apparently contradicts the immunocytochemical definition of a diffuse plaque.[1,2,21] The other is the negligible extent of their immunoreactivities with the Aβ antibodies specific for various, amino-terminally ragged species, with the exception of BAN052. The ordinary diffuse plaque stains to varying degrees with all of these antibodies, and particularly strongly with the specific antibodies to AβN1(L-Asp), AβN1(L-isoAsp), AβN1(D-Asp), and AβN3(pyroGlu).[20,21] The lack of staining with the above antibodies may be due to much smaller amounts of Aβ in the Aβ40-positive diffuse plaque than in the ordinary diffuse plaque, and thus each distinct species may be present at such a low level that even sensitive immunocytochemistry cannot detect them. Alternatively, Aβ in the Aβ40-positive diffuse plaques would have been rapidly cleaved up to the midportion of Aβ, resulting in neither sufficient amounts of various intermediary products nor sufficient time to induce structural modifications of Aβ including racemization, isomerization, and cyclization.

There are two possible sources of the Aβ in astrocytic granules. The first possibility is that the Aβ is generated in lysosomes from full-length APP and/or its carboxyl-terminal stump produced within astrocytes. Cathepsin D was reported to cleave Aβ at the Met-Asp bond and possibly at the carboxy terminus *in vitro*.[40,41] Once Aβ accumulation exceeds the capacity of lysosomes, some of the Aβ-containing lysosomes may be released from the cell and become the source of extracellular Aβ deposition, a situation postulated for some lysosomal enzymes detected in senile plaques in AD brain.[42] However, this seems to be unlikely because 1) the APP expression level is known to be low in resting astrocytes[43] in which Aβ-positive granules appear to accumulate and 2) cathepsin D deficiency results in no alterations in the levels of secreted Aβ.[44]

The second possibility is that the astrocytic Aβ may originate from extracellular fibrillar and/or aggregated Aβ, or nonfibrillar, soluble Aβ. Although astrocytes have the capability of phagocytosis *in vivo*,[45] it is not known whether astrocytes can incorporate Aβ aggregates like microglia. In particular, astrocytes lack the scavenger receptors that are abundantly expressed in microglia and monocytes/macrophages and play an important role in the uptake of Aβ aggregates.[5,6] It is thus unlikely that these astrocytes can take up Aβ aggregates deposited in the Aβ40-positive diffuse plaques. This raises the possibility that astrocytes take up soluble Aβ monomers or oligomers via a receptor-mediated or independent mechanism and digest them in lysosomes. In fact, cultured astrocytes and microglia were reported to remove soluble Aβ added to the culture medium.[10] This is also compatible with the present electron microscopic finding that nonfibrillar Aβ accumulates within lipofuscin-like granules.

If the above is the case, the Aβ in astrocytes should originate from soluble Aβ at the regionally high levels due to its inefficient clearance, leading to the formation of Aβ40-positive diffuse plaques. This may explain why astrocytic Aβ is amino-terminally processed to a greater extent than Aβ in the Aβ40-positive diffuse plaques (see also Ref. 8). In addition, this assumption is consistent with the observation that there are a few cases in which only particular astrocytes carrying Aβ-positive granules are present without associated Aβ40-positive diffuse plaques; there may be a stage during aging where Aβ-positive astrocytes only are present, followed by a stage where the Aβ40-positive plaques appear.

There may be several reasons why this type of Aβ40-positive diffuse plaques associated with Aβ-positive astrocytes has long been overlooked. One reason is that immunocytochemistry is only rarely used when studying autopsies. In our experience, only sensitive Aβ immunostaining can visualize these Aβ40-positive diffuse plaques. Even with immunostaining, the staining intensity of the Aβ40-positive diffuse plaques is so weak that they may be easily overlooked. In addition, dot Aβ immunoreactivities scattered in the Aβ40-positive plaques may be judged as artifacts. To detect such intracellular Aβ labeling, we first searched for Aβ40-positive diffuse plaques in the BA27-stained sections and then looked carefully for those cell-associated dot Aβ immunoreactivities.

We cannot offer a proper explanation why the unusual Aβ40-positive diffuse plaques accompanied by Aβ-bearing astrocytes are hardly seen in AD brains. Using double staining with GFAP and Aβ, their co-localization was hardly detected in AD cortex except for restricted areas in two cases (data not shown). In AD cortex, abundant amyloid fibrils may rapidly incorporate Aβ, before astrocytes take it up. In addition, the remaining soluble Aβ in AD brain may be more efficiently degraded by extracellular protease than that in control brain because AD cortex undergoes chronic inflammation and protease activity is enhanced there.[46]

It was reported that Aβ42 amino-terminally deleted up to residue 17 can be detected in activated microglia and a few reactive astrocytes near senile plaques in ischemic lesions of AD brains, although it was sometimes detected in AD brains without ischemic lesions.[47,48] These observations have been claimed to represent phagocytosis of insoluble Aβ derived from senile plaques by reactive microglia and some reactive astrocytes. In addition, there is one report showing that, in brains of aged macaque monkeys, activated microglia and reactive astrocytes surrounding senile plaques exhibited intracellular Aβ immunoreactivities.[49] In contrast, the present Aβ40-positive diffuse plaques are characterized by the presence of Aβ exhibiting loss of amino-terminal portions, a finding that may indicate that such processing occurs rapidly, possibly before Aβ deposition. The amino-terminally deleted Aβ species would then be deposited because of their higher aggregation potential.[50] Aβ40 specific to the Aβ40-positive diffuse plaque may be generated through the carboxyl-terminal cleavage of Aβ42 that is accumulating and being amino-terminally processed. Thus, Aβ in the Aβ40-positive diffuse plaque is characterized by higher susceptibility of its carboxy terminus to proteases than Aβ in the ordinary senile plaque.

Those Aβ40-positive plaque-associated astrocytes carrying Aβ-positive granules may be nonreactive ones because of the absence of APP immunoreactivities. This indicates that resting astrocytes can take up and digest Aβ under certain conditions. One straightforward interpretation of the above observations is that, distinct from the ordinary diffuse plaque, the Aβ40-positive diffuse plaque is transiently generated and disappears during the early stage of aging. Those Aβ40-positive plaque-associated astrocytes may be involved in the removal of transiently formed Aβ40-positive diffuse plaques in the cortex. This further suggests that astrocytes may have an important role in maintaining Aβ homeostasis in the extracellular space of the brain under normal conditions. The above assumption that the Aβ40-positive diffuse plaques are only transiently generated may also explain why these Aβ40-positive diffuse plaques have been overlooked to date.

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