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Short Communication

Full-Length Amyloid-β(1–42(43)) and Amino-Terminally Modified and Truncated Amyloid-β42(43) Deposit in Diffuse Plaques

Takeshi Iwatsubo,* Takaomi C. Saido,† David M. A. Mann,‡ Virginia M.-Y. Lee,§ and John Q. Trojanowski§

From the Department of Neuropathology and Neuroscience, Faculty of Pharmaceutical Sciences, University of Tokyo, and the Department of Molecular Biology,† Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; the Department of Pathological Sciences,‡ University of Manchester, Manchester, United Kingdom; and the Department of Pathology and Laboratory Medicine,§ University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania*

The amino- and carboxyl-terminal properties of the amyloid-β (Aβ) peptides deposited in diffuse plaques, one of the earliest forms of Aβ deposition, were examined in the brains of patients with Down's syndrome and Alzheimer's disease and in aged individuals without dementia by immunocytochemistry. This was done using a panel of antibodies that specifically discriminate the terminal structures and modifications at the amino and carboxyl termini of Aβ. Diffuse plaques found in the cerebral and cerebellar cortex, neostriatum, and hypothalamus of Down's syndrome, Alzheimer's disease, and nondemented brains were strongly immunoreactive for AβN1(L-Asp), AβN1(L-isoAsp), AβN1(D-Asp), and AβN3(pyroGlu) and weakly positive for AβN11(pyroGlu) and AβN17(Leu). Diffuse plaques also were positive for Aβ42(43) but negative for Aβ40, using carboxyl-terminal-specific anti-Aβ antibodies. These results suggest that the amino termini of the Aβ species that initially deposit in diffuse plaques begin with AβN1(Asp) with or without structural modifications (isomerization and racemization), as well as with AβN3(pyroGlu), and terminate preferentially at Aβ42(43) rather than Aβ40. (Am J Pathol 1996, 149:1823–1830)

The deposition of amyloid-β (Aβ) peptides as senile plaques (SPs) and in the walls of blood vessels is one of the most prominent pathological features of the Alzheimer's disease (AD), the Down's syndrome (DS), and the aging brain, and these deposits are considered to be closely related to the pathogenesis of the dementia in AD.[1–3] Aβ peptides are 39- to 43-amino-acid-long peptides that are proteolytically cleaved from β-amyloid protein precursors (βAPPs). This results from the activity of as yet unidentified proteases (designated as β-secretase and γ-secretase) that cleave the amino (N) terminus and the carboxyl (C) terminus of Aβ, respectively.[4] Two major C-terminal variants of Aβ, ie, Aβ40 ending at Val40 and Aβ42(43) ending at Ala42 or Thr43, are found in the amyloid deposits of AD brains,[5,6] and soluble forms of these Aβ peptides are secreted from cells.[7] Whereas the major species of secreted Aβ is Aβ40,[8,9] Aβ42(43) is the initially and predominantly deposited species in the amyloid plaques of AD[10] and DS brains[11] as well as in the brains of nondemented aged individuals.[12] One of the most significant biochemical differences in the C-terminal properties of these two forms of Aβ is considered to be their susceptibility to form aggregates. For example, as Aβ42(43) aggregates more readily than

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Address reprint requests to Dr. Takeshi Iwatsubo, Department of Neuropathology and Neuroscience, Faculty of Pharmaceutical Sciences, University of Tokyo, 7–3-1 Hongo, Bunkyoku, Tokyo, 113, Japan.

Aβ40,[13] this suggests a critical role of the γ-cleavage in amyloid formation. However, β-cleavage, which produces the N terminus of Aβ, also is thought to be a crucial rate-limiting step in the production of Aβ, because it is likely to precede γ-cleavage of Aβ to form the potentially amyloidogenic C-terminal fragment of βAPP starting from the N terminus of Aβ.[4] Indeed, the increase in the production of Aβ that results from the double mutations at βAPP670/671 is accompanied by an increase in the amount of C-terminal fragments of βAPP.[14,15] It has been shown that the N terminus of the Aβ peptides that are deposited as amyloid in AD brains harbors several different modifications including ragged N termini,[6,16] pyroglutamation,[17,18] isomerization, and racemization.[5] Moreover, it has been suggested that the major Aβ species deposited in diffuse plaques (DPs) is Aβ17-42,[19] which is produced by an entirely different mode of βAPP processing (ie, α-cleavage) and is thought to preclude amyloid formation.[4] However, little is known about the N-terminal properties of Aβ, including their chronological and morphological contributions to the formation of brain amyloid *in situ*. Considering the importance of β-cleavage in the production of Aβ, it is extremely critical to determine the N-terminal properties of the Aβ peptides that initially and predominantly accumulate as amyloid to develop therapeutic methods to reduce the production of Aβ and the formation of amyloid deposits in AD. For this reason, we have systematically investigated the N-terminal as well as the C-terminal properties of Aβ deposited as different types of the Aβ peptides that form different types of Aβ deposits in the brains of patients with DS and AD as well as in nondemented aged individuals. This was done using a panel of well characterized antibodies to Aβ that discriminate the terminal sequence or structure of various truncated forms of Aβ as well as different modified forms of Aβ. Furthermore, our studies focused on the molecular nature of Aβ in DPs, as DPs are considered to be one of the earliest types of Aβ deposits in the human brain.[20]

Materials and Methods

Cases

Samples from seven patients with DS (cases 1 to 7) and five with AD (cases 8 to 12) as well as samples from four nondemented individuals with some neocortical SPs (cases 13 to 16) were used in this study. All of the AD and DS patients (except case 6) had pathologically confirmed AD based on the consensus criteria of the National Institute of Aging,[21] and the pathological features of all seven DS brains have been previously described.[11,22] At autopsy, small blocks from middle frontal and superior temporal neocortices, cerebellar hemisphere, corpus striatum (caudate nucleus), and hypothalamus were collected and immersed in 70% ethanol (EtOH) containing 150 mmol/L sodium chloride for 24 hours and then embedded in paraffin as previously described.[22–24] Serial sections were cut at 6 μm thickness and immunostained with the antibodies described below. Additionally, sections from the middle frontal cortices of two patients with DS (cases 6 and 7) fixed in 10% formalin for more than 2 weeks and then embedded in paraffin[11] were similarly immunostained for comparison with the tissues fixed in EtOH. Clinicopathological information on the cases included in this study is summarized in Table 1.

Antibodies and Immunocytochemistry

Six different antibodies that specifically recognize distinct N-terminal structures of Aβ were used. These included antibodies to AβN1(L-Asp)(9204), AβN1(L-isoAsp), AβN1(D-Asp), AβN3(pyroGlu), AβN11(pyroGlu), and AβN17(Leu). Production and characterization of these antibodies are described elsewhere.[18,25] Briefly, each antibody was raised by immunizing rabbits with synthetic 5- or 6-amino-acid-long peptides with modified or unmodified N-terminal residues and conjugated to keyhole limpet hemocyanin at the C terminus and then affinity purified using columns conjugated with each peptide immunogen.[18,25] Anti-AβN17(Leu) antibody was raised to a Aβ17-40 peptide and affinity purified with several synthetic Aβ peptides so as to collect antibodies that specifically recognize Aβ beginning with AβN17(Leu) and eliminate those cross-reacting with full-length Aβ.[25] The specific reactions of each antibody exclusively to full-length Aβ peptides with a given Aβ N terminus (Aβn–40 or –42) have been rigorously characterized by dot- or immunoblotting as described elsewhere.[18,25] Two sets of monoclonal and polyclonal antibodies, ie, BA27 and BC05 (monoclonal antibodies specific for the C termini of Aβ40 and Aβ42(43), respectively[7,10–12]) and anti-AβC40 and anti-AβC42 (affinity-purified rabbit polyclonal antibodies raised against Aβ36-40 and Aβ38-42, respectively[18]) were used as specific immunoprobes for the C terminus of Aβ. Epitope locations of the Aβ N- and C-terminal-specific antibodies used in this study are schematically shown in Figure 1. After deparaffinization, serial sections

Table 1. *Summary of the Clinicopathological Features of the Cases Included in This Study*

Case	Diagnosis	Age (years)	Sex	PMI (hours)	Duration of dementia (years)	SPs					
						Cerebral cortex*		Cerebellum		Striatum, DPs	Hypothalamus, DPs
						DPs	MPs	DPs	MPs		
1	DS	62	M	6	1	1+	4+	3+	1+	4+	3+
2	DS	54	M	14	2	2+	3+	3+	1+	4+	3+
3	DS	58	M	13	10	2+	3+	3+	1+	4+	3+
4	DS	57	F	6	2	1+	4+	3+	1+	4+	3+
5	DS	53	F	10	0	3+	2+	1+	1+	3+	2+
6	DS†	36	M	13.5	0	3+	–	–	–	–	–
7	DS†	57	M	42	?‡	2+	3+	3+	1+	4+	3+
8	AD	79	F	11	2	3+	3+	1+	–	4+	NA
9	AD	80	M	23	11	3+	2+	1+	–	4+	3+
10	AD	74	M	11	7	2+	3+	–	–	4+	2+
11	AD	82	M	16.5	10	3+	2+	2+	–	4+	3+
12	AD	80	F	19	8	4+	1+	–	1+	4+	3+
13	ND	91	F	13	0	2+	1+	–	–	–	–
14	ND	60	M	14	0	3+	–	–	–	–	–
15	ND	67	F	5.5	0	3+	1+	–	–	–	–
16	ND	71	M	13	0	3+	3+	–	–	–	–

The number of SPs was counted in a 1.5-mm^2 area with highest SP density and evaluated semiquantitatively. PMI, postmortem interval; MPs, mature plaques; M, male; F, female; ND, nondemented individuals with neocortical SP; NA, not available. Scoring was as follows: –, absent; 1+, few SPs in some microscopic fields; 2+, 1 to 10 SPs/mm^2 in every microscopic field; 3+, 10 to 50 SPs/mm^2; 4+, >50 SPs/mm^2.

*Middle frontal cortex.

†Formalin-fixed, paraffin-embedded materials.

‡Demented, but duration is unknown.

were immunostained with each primary antibody in the following sequence: 9204, AβN1(L-isoAsp), AβN1(D-Asp), AβN3(pyroGlu), AβN11(pyroGlu), AβN17(Leu), BA27 (or anti-AβC40), and BC05 (or anti-AβC42). This was followed by a standard avidin-biotin complex procedure using Tris-buffered saline (50 mmol/L Tris/HCl, 150 mmol/L NaCl, pH 7.6) for dilution of the antibodies and wash of the specimens and then visualization using 3,3′-diaminobenzidine as chromogen. As antibody 9204 could show weak cross-reaction with isomerized and racemized AβN1(Asp) under 150 mmol/L NaCl conditions on dot-blots,[25] sections were also immunostained with AβN1(Asp) antibodies under 500 mmol/L NaCl conditions, which completely abolished these cross-reactions. Sections were stained with or without formic acid pretreatment.

Results

In the cerebral neocortices of AD and DS brains fixed in EtOH, numerous DPs as well as mature SPs were observed, and virtually all of these plaques were strongly positive for 9204 (Figure 2A, row 1), AβN1(L-isoAsp) (Figure 2A, row 2), AβN1(D-Asp) (Figure 2A, row 3), AβN3(pyroGlu) (Figure 2A, row 4), and Aβ42(43) (Figure 2A, row 8). In contrast, AβN11(pyroGlu) (Figure 2A, row 5) and AβN17(Leu) (Figure 2A, row 6) were only weakly or hardly positive in DPs but were positive, especially in the core portions, of mature SPs (upper part of the figures). Aβ40 was also preferentially detected in mature SPs in the cerebral neocortices (Figure 2A, row 7). Cerebral cortices from nondemented individuals showed scattered ma-

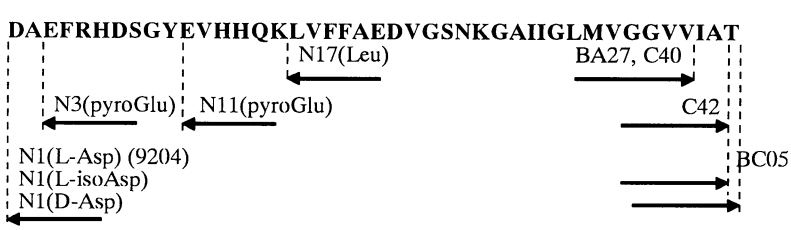

Figure 1. *Schematic representation of the epitope location and specificities of the Aβ N- and C-terminal antibodies. The N or C termini of variously truncated Aβ with or without modifications that are specifically recognized by each antibody are indicated by* arrows *below the sequence of Aβ.*

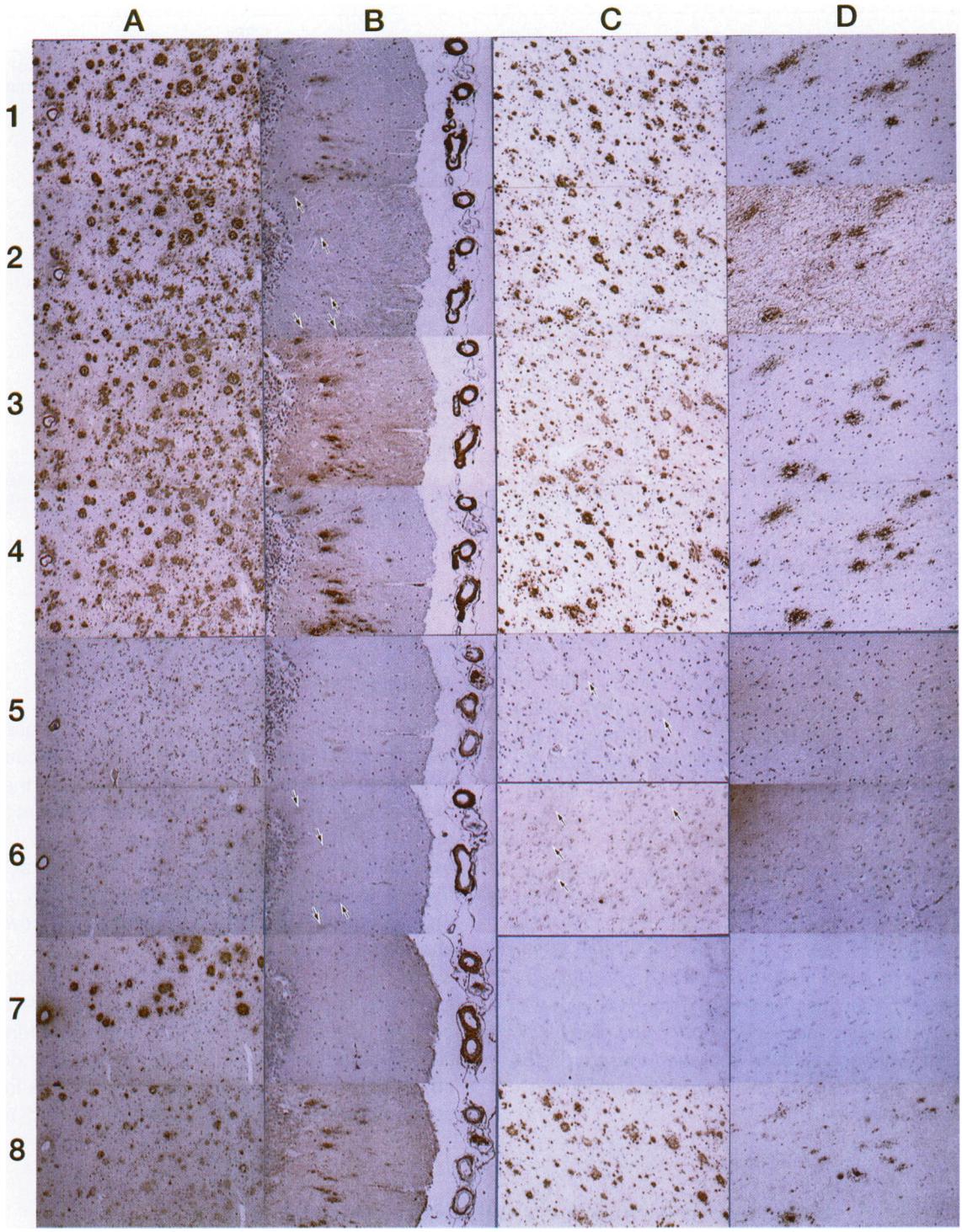

Figure 2. *Immunostaining patterns of Aβ deposits in Down's syndrome brains with AD pathology. Patterns of immunostaining of various types of Aβ deposits including DPs, MPs, and vascular amyloid deposits in DS brains were examined with Aβ N- and C-terminal antibodies. In the middle frontal neocortex* (**A, case 1**), *all SPs were strongly positive for 9204 (anti-AβN1(L-Asp);* **row 1**), *AβN1(L-isoAsp)* (**row 2**), *AβN1(D-Asp)* (**row 3**), *and AβN3(pyroGlu)* (**row 4**) *as well as for BC05 (Aβ42(43);* **row 8**), *whereas immunoreactivities for AβN11(pyroGlu)* (**row 5**) *and AβN17(Leu)* (**row 6**) *were exclusively detectable in the core portions of MPs in the upper half of the field, which were more intensely immunostained with BA27* (**row 7**). *DPs in the cerebellum* (**B, case 1**), *striatum* (**C, case 4**), *and hypothalamus* (**D, case 4**; *paraventricular nucleus*) *showed similar staining properties to those in neocortex* (**A**), *ie, relatively strong immunostaining for 9204* (**row 1**), *AβN1(L-isoAsp)* (**row 2**), *AβN1(D-Asp)* (**row 3**), *and AβN3(pyroGlu)* (**row 4**) *as well as for BC05 (Aβ42(43);* **row 8**), *except for modest staining in* **B2** (**arrows**). *However, DPs in these regions were negative for AβN11(pyroGlu)* (**row 5**) *and AβN17(Leu)* (**row 6**) *or only faintly positive for these epitopes* (**B** *and* **C, rows 5** *and* **6, arrows**). *In contrast, vascular amyloid deposits were strongly positive for all Aβ N- and C-terminal antibodies examined* (**A, left end**, *and* **B**). *Magnifications, ×41* (**A**), *×52* (**B**), *and ×66* (**C** *and* **D**).

ture SPs and many DPs that exhibited cotton-wool-like homogeneous staining patterns with ill defined boundaries, which were similar to those seen in comparable areas of young DS patients.[10,11] These DPs were also intensely immunostained with the three types of AβN1(Asp) and AβN3(pyroGlu) antibodies (Figure 3, A–D) as well as with anti-AβC42 (Figure 3H), but they were weakly positive for AβN11(pyroGlu) (Figure 3E), variably or equivocally positive for AβN17(Leu) (Figure 3F), and entirely negative for Aβ40 (Figure 3G). In most of the AD and DS cases, DPs were found in the molecular layer of the cerebellar cortex (Figure 2B), corpus striatum (Figure 2C), and hypothalamus (Figure 2D). The DPs in each of these regions also showed immunostaining patterns that were very similar to DPs in the neocortex of the AD, DS, and nondemented individuals. Specifically, they were positive for 9204 (Figure 2, row 1), AβN1(L-isoAsp) (Figure 2, row 2), AβN1(D-Asp) (Figure 2, row 3), AβN3(pyroGlu) (Figure 2, row 4), and Aβ42(43) (Figure 2, row 8) but weakly or equivocally positive for AβN11(pyroGlu) (Figure 2, row 5) and AβN17(Leu) (Figure 2, row 6), and they were negative for Aβ40 (Figure 2, row 7). Vascular amyloid deposits were strongly positive for all antibodies examined, ie, those for AβN1(Asp) (Figure 2, A and B, rows 1 to 3), AβN3(pyroGlu) (Figure 2, row 4), AβN11(pyroGlu) (Figure 2, row 5), AβN17(Leu) (Figure 2, row 6), Aβ40 (Figure 2, row 7), and Aβ42(43) (Figure 2, row 8). Furthermore, the antibodies to AβN1(L-isoAsp), AβN1(D-Asp), AβN3(pyroGlu), and AβN11(pyroGlu) as well as 9204 all immunostained these EtOH-fixed sections equally well regardless of the use of formic acid pretreatment. In contrast, immunostaining for AβN17(Leu), Aβ40, and Aβ42(43) was weaker without this pretreatment. Immunostaining with 9204 and other AβN1(Asp) antibodies under 500 mmol/L NaCl conditions gave essentially the same results as with 150 mmol/L NaCl, except that anti-AβN1(L-isoAsp) labeled slightly fewer numbers of SPs under 500 mmol/L NaCl conditions (data not shown).

On routinely prepared, formalin-fixed, paraffin-embedded sections, immunostaining of SPs for 9204, AβN1(L-isoAsp), and AβN1(D-Asp) was markedly diminished (Figure 3K). Furthermore, core portions of mature SPs and vascular amyloid deposits and a small number of DPs were positively stained with 9204 (Figure 3K), whereas anti-AβN1(L-isoAsp) and AβN1(D-Asp) stained occasional amyloid cores and vascular amyloid but no DPs. Anti-AβN3(pyroGlu) stained some DPs (Figure 3J), but the number and extent of the immunostained DPs were less compared with the Aβ42(43)-positive DPs (Figure 3I) and compared with the DPs in EtOH-fixed sections stained with anti-AβN3(pyroGlu) (Figure 3D). Notably, the immunoreactivity of these antibodies could not be restored by formic acid pretreatment or with hydrated autoclaving[26] (data not shown). In contrast, the immunostaining patterns for Aβ40 and Aβ42(43) on formic-acid-treated sections were almost the same as in EtOH-fixed specimens (Figure 3I).

Discussion

In the present study, we have clearly shown by immunocytochemistry 1) that Aβ peptides deposited in DPs in a wide variety of locations in the brains of AD and DS patients as well as nondemented individuals are largely composed of full-length Aβ species beginning with the N1(Asp) residue at the N terminus with or without structural modifications, including isomerization and racemization, as well as of those beginning with pyroglutamate-modified N3(Glu) (N3(pyroGlu)), 2) that Aβ species beginning with N11(pyroGlu) or N17(Leu) are scant in DPs but present in mature SPs and vascular amyloid deposits together with other Aβ42(43) species, and 3) that the C terminus of Aβ in these DPs is Aβ42(43) and not Aβ40.

The question of the N-terminal extent of Aβ that is deposited initially in DPs is an important one because it may provide important information to facilitate the identification of the β-secretase(s) involved in this rate-limiting step of Aβ production. Previous protein chemical studies have shown that β-amyloid deposits in human brains are composed of Aβ peptides with different N termini,[5,6] although it has been difficult to localize the origin of each molecular species of Aβ to specific types of amyloid lesions. It has been shown by immunocytochemistry using routine formalin-fixed and paraffin-embedded specimens that AβN3(pyroGlu) sometimes predominates over AβN1(L-Asp) in SPs of AD and nondemented individuals.[18,27] Alternatively, in young DS brains, a considerable number of DPs have been shown to be negative for both AβN1(L-Asp) and AβN3(pyroGlu), suggesting that the β-cleavage site for the initially deposited Aβ species takes place at residues other than these positions.[27] Although we confirmed these findings for many Aβ deposits in routinely processed, formalin-fixed specimens, virtually all SPs and DPs showed strong positive immunostaining for AβN1(L-Asp), AβN1(L-isoAsp), AβN1(D-Asp), and AβN3(pyroGlu) in EtOH-fixed sections. Hence, it is

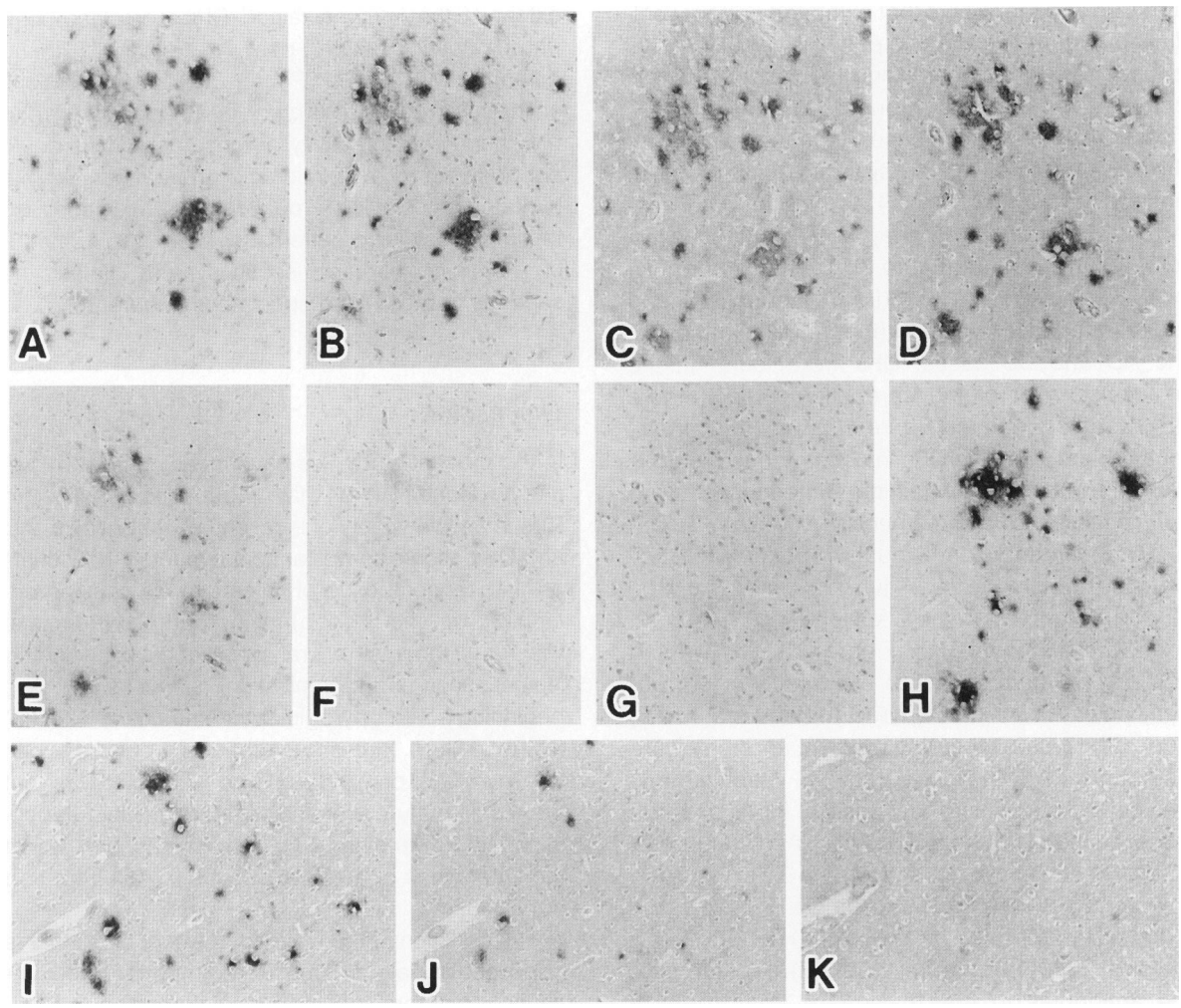

Figure 3. *Immunostaining patterns of diffuse plaques in the neocortex of nondemented individuals and a young DS patient. The immunostaining patterns of DPs in the middle frontal cortex of a nondemented individual (case 15, EtOH fixed) with Aβ N- and C-terminal-specific antibodies were compared with those in a young DS patient (case 6) routinely fixed in 10% formalin. In the EtOH-fixed specimens, all DPs were strongly positive for 9204 (anti-AβN1(L-Asp);* **A**), *AβN1(L-isoAsp)* (**B**), *AβN1(D-Asp)* (**C**), *AβN3(pyroGlu)* (**D**), *and AβC42* (**H**), *whereas they were less intensely positive for AβN11(pyroGlu)* (**E**), *faintly positive for AβN17 Leu)* (**F**), *and entirely negative for AβC40* (**G**). *In the formalin-fixed specimens, DPs are strongly positive for AβC42* (**I**), *of which a limited number and area are positive for AβN3(pyroGlu)* (**J**), *in sharp contrast to* **D** *and* **H**, *which showed a comparable extent of DP staining on EtOH-fixed sections. AβN1(L-Asp) was only faintly positive in a few DPs* (**K**). *Magnification, ×63.*

quite possible that these Aβ N-terminal epitopes are especially sensitive to prolonged aldehyde fixation and that aldehyde fixation results in epitope masking for these N-terminal Aβ antibodies.

In sharp contrast to the previous biochemical data suggesting that Aβ17-42 (a longer form of the p3 fragment of βAPP that is produced by α-cleavage at the N terminus) is the major molecular species of Aβ in DPs,[19] our findings imply that Aβ fragments beginning with N17(Leu) are detectable in mature SPs and vascular amyloid. Thus, p3 may not be the predominant Aβ species initially deposited in SPs, but it may accumulate substantially only at later stages of plaque formation. However, we cannot completely exclude the possibility that the DPs in neocortex, cerebellum,[22] striatum,[28] and hypothalamus[29] of AD as well as of elderly DS patients are not identical in their biochemical characteristics to the DPs in young DS brains, which are believed to be the most representative examples of early Aβ deposits. Nonetheless, in specific areas outside cerebral cortex (ie, cerebellum, striatum, and hypothalamus), DPs are the sole or nearly exclusive form of Aβ deposit even in advanced stages of AD.[22,28,29] Moreover, DPs in the cerebral cortex of nondemented aged individuals are morphologically indistinguishable from DPs in young DS patients, and they also show very similar Aβ N-terminal properties. Thus, we conclude that our present findings can be generalized to apply

to all situations in which DPs occur and that DPs probably represent the initial stages of Aβ deposition.

Furthermore, our data indicate that Aβ peptides beginning with AβN1(Asp) and AβN3(Glu) (including modifications) may be the major molecular species in DPs. This may suggest that β-secretase activated at the early stages of Aβ deposition cleaves the βAPP between N − 1(Met)/N1(Asp) and N2(Ala)/N3(Glu). Considering the biochemical data that Aβ beginning with AβN3(pyroGlu) predominates in Aβ deposits,[18,25] it is possible that AβN3(pyroGlu)-42 is the major molecular species of early Aβ deposits. Thus, it will be important to determine whether AβN3(pyroGlu)-42 or AβN3(Glu)-42 is present in a soluble state and whether it is readily incorporated into insoluble deposits, or alternatively, whether Aβ1-42 is initially deposited and then cleaved by two residues. Moreover, it is important to determine whether pyroglutamation at the Glu residues as well as the isomerization and racemization at the N1(Asp) occur before or after the deposition of Aβ. Regardless, it is plausible to consider that these modifications at the N terminus of Aβ may hamper proteolytic attack on these peptides by aminopeptidases, thereby contributing to the stabilization of Aβ deposits in stable plaques.

Although the present findings add substantially to our understanding of the molecular species of Aβ found in DPs, it is still difficult to determine the precise chronology for the deposition of various Aβ species using data obtained from postmortem human brains, as each brain represents only a single point in the course of AD/DS, which progresses at a variable rate in each patient. Thus, studies of the time course of Aβ deposition in the transgenic mice that develop Aβ plaques[30] using the same panel of Aβ antibodies could provide a better understanding of the chronological sequence in which various species of Aβ become incorporated in amyloid plaques in the AD brain, although the N-terminal properties of Aβ deposits in mouse brain could be somewhat different from those in human brains because of the differences in proteases that produce Aβ.

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