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β-Amyloid-(1–42) is a major component of cerebrovascular amyloid deposits: Implications for the pathology of Alzheimer disease

(cerebrovasculature/blood brain barrier)

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ABSTRACT **Reinvestigation of the chemical structure of β-amyloid peptide (Aβ) deposits in the vascular tissue of Alzheimer disease brains revealed that the 42-residue form Aβ-(1–42), rather than the more soluble Aβ-(1–40) form, is the predominant peptide. Following removal of the surrounding tissue with SDS and collagenase, Aβ was solubilized in formic acid and purified by Superose 12 chromatography. Peptides generated by enzymatic and chemical digestion of the Aβ were purified by HPLC and characterized by amino acid analysis, sequence analysis, and mass spectrometry. In the leptomeningeal vessels, the average ratio of Aβ-(1–42)/Aβ-(1–40) was 58:42, whereas in the parenchymal vessels this ratio was 75:25. Interestingly, vascular Aβ contains considerably less isomerized and racemized aspartyl residues than does neuritic plaque Aβ, suggesting that the vascular amyloid is "younger." The discrete nature of the bands and spherical deposits of Aβ associated with arterioles and capillaries, respectively, suggests that this amyloid arises from the vascular tissue itself. Increasing Aβ deposition appears to lead to the distortion and occlusion of capillaries, which may contribute significantly to the pathology of Alzheimer disease.**

β-Amyloid peptide (Aβ) is an important component of the lesions found in the brains of individuals with Alzheimer disease (AD). Three types of extracellular Aβ aggregates have been observed that differ in morphology and localization (1). In the gray matter, Aβ can occur in diffuse deposits that appear to have little effect on adjacent tissue and in dense neuritic plaques, which are surrounded by dystrophic neurites and glial processes. The third type of Aβ deposit accumulates in the blood vessels of the brain. Although the association between dense cores of Aβ and neuronal pathology indicates a role for these deposits in the progression of AD (2), the consequences of vascular Aβ deposition have been less well established.

Characterization of the Aβ aggregates described above has been difficult because of their insolubility and heterogeneity; their chemical structures are still under investigation. We have recently reported that a 42-residue form of Aβ [Aβ-(1–42)] containing significant amounts of isomerized and racemized aspartyl residues predominates in dense neuritic plaques (6). Previous findings that a form shortened by two residues at the C terminus [Aβ-(1–40)] is more abundant than Aβ-(1–42) (7) probably resulted from the use of purification procedures that selectively removed the less soluble Aβ-(1–42) peptide. In fact, because Aβ-(1–40) and a variety of shorter species are present in the cerebrospinal fluid of normal as well as AD-afflicted individuals (8, 9), it may be a nonpathological by-product of cellular metabolism, whereas Aβ-(1–42) may have the more important role in the formation of neuritic plaques.

With this in mind, we reexamined amyloid from the cerebrovasculature of AD brains and found that Aβ-(1–42) is also the major form in these deposits. Interestingly, the amount of racemization and isomerization at aspartyl residues is much less than in neuritic plaque Aβ-(1–42). The localization of these undegradable aggregates suggests that their deposition might be linked to a compromised blood–brain barrier.

MATERIALS AND METHODS

Human brains obtained at autopsy met the diagnostic criteria for AD established by the National Institutes of Health Neuropathology Panel (10) and by the Consortium to Establish a Registry for AD (CERAD) (11). Morphometric analyses identified brains that contained large amounts of Aβ in compact cores and in the blood vessels. Left hemispheres were analyzed histopathologically, while right hemispheres were stored at −70°C for subsequent Aβ isolation.

Purification of Aβ from Leptomeningeal Blood Vessels. The leptomeninges were gently pulled from the surface of 1-cm-thick coronal sections with the aid of a dissecting microscope and immersed in 0.1 M Tris·HCl (pH 8.0; TB) at 4°C. Blood vessels larger than 1 mm in diameter were discarded, and the remaining tissue was cut with scissors into 1- to 2-mm pieces. The tissue was washed eight times with 1 liter of TB at 4°C with continuous stirring for 10 min and was collected by filtration (45-μm mesh). After resuspension of this material in 20 vol of 2 mM $CaCl_2$ in TB, 0.3 mg of collagenase CLS-3 (Worthington) and 10 μg of DNase I (Worthington) were added per ml, and the suspension was shaken for 18 hr at 37°C. Large debris was removed by filtration (350-μm mesh), and the smaller insoluble material was recovered by centrifugation at 6000 × *g* for 15 min. The resulting pellet was resuspended in 100 vol of 2% SDS in TB and incubated for 2 hr at room temperature, after which the insoluble material was again recovered by centrifugation (see above) and washed twice with distilled water. The pellet was then dissolved with 8 vol of 98% (vol/vol) glass-distilled formic acid (15 min at room temperature) and centrifuged at 430,000 × *g* for 15 min in Polyallomer tubes in a TLA 100.2 rotor (Beckman). Pure Aβ was isolated from the clear supernatant by size-exclusion chromatography with a Superose 12 column (10 × 300 mm) on a Pharmacia-LKB fast protein liquid chromatography (FPLC) system with a running buffer of 75%

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Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer disease; Aβ-(1–42) and Aβ-(1–40), 42- and 40-residue Aβ peptides; RP-HPLC, reverse-phase HPLC.

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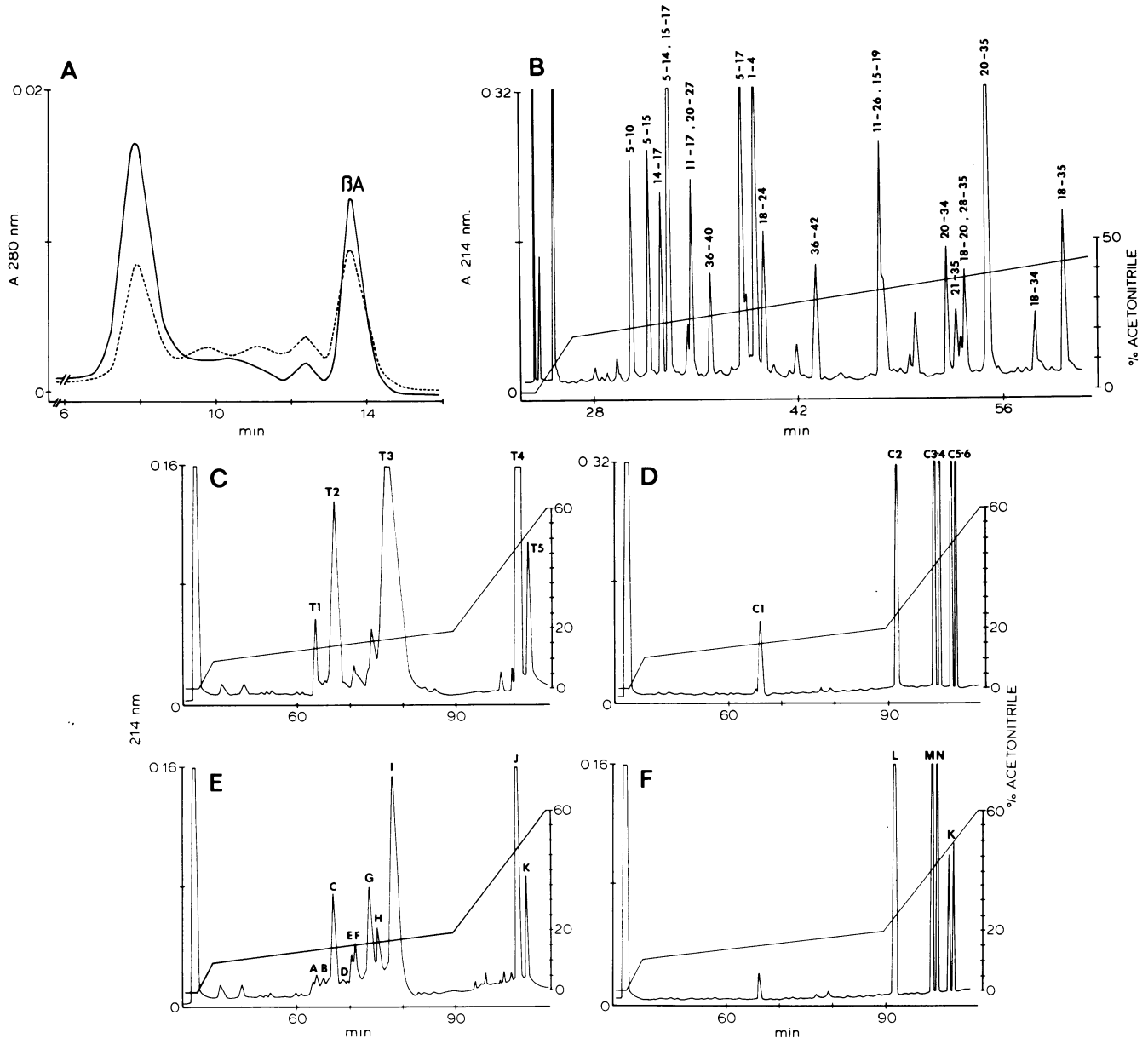

FIG. 1. Chemical characterization of Aβ peptides from leptomeningeal and parenchymal blood vessels. (*A*) Superose 12 gel filtration of leptomeningeal (solid line) and parenchymal (dotted line) vascular Aβ. Monomeric Aβ is eluted in the large peak with 13.5 ml of buffer (4500 Da), while the dimeric form is eluted with about 12.5 ml (compare ref. 6). (*B–F*) Elution on RP-HPLC of peptides obtained by enzymatic and chemical digestion of vascular Aβ. The identities of these peptides are given in Table 1. (*B*) Leptomeningeal Aβ digested by chymotrypsin, showing two peptides (residues 36–40 and 36–42) that correspond to the C terminus. (*C*) Leptomeningeal Aβ digested by trypsin. The N-terminal peptides T1 (residues 2–5) and T2 (residues 1–5) are present in a molar ratio of 20%:80%. The single peak representing the C terminus (T5; residues 29–40) is relatively small. (*D*) CNBr digest of the insoluble core produced by tryptic digestion of leptomeningeal Aβ. The majority of the C-terminal peptide in the insoluble core is present in peak C2 (residues 36–42) rather than in peak C1 (residues 36–40). (*E*) Parenchymal vascular Aβ digested by trypsin. The profile corresponds closely to that previously reported for Aβ purified from the neuritic plaque cores (6). (*F*) CNBr digest of the insoluble core produced by tryptic digestion of parenchymal vascular Aβ. As in *D*, the majority of the C-terminal peptide is present as residues 36–42 (peak L).

(vol/vol) glass-distilled formic acid at a flow rate of 0.2 ml/min. The eluent was monitored at 280 nm.

Purification of Aβ from Parenchymal Blood Vessels. After removal of the underlying white matter, the gray matter was cut into 1- to 1.5-cm cubes, and the parenchyma was dissolved in 30 vol of 15% SDS in TB with continuous stirring for 48 hr at room temperature. After elimination of soluble material by filtration through stainless steel meshes (350, 150, and 75 μm), the SDS-insoluble blood vessels were washed with 20 vol of 1% SDS in TB and again filtered (75 μm mesh). This wash was repeated six times to remove all material not firmly associated with the vessels. The vessels were then washed with TB, digested by collagenase CLS-3, filtered (75-μm mesh), and collected by centrifugation as described for the leptomeningeal vessels. The pellet containing Aβ was washed with 0.1% SDS in TB, and the Aβ was purified by using Superose 12 chromatography as described above.

Characterization of Leptomeningeal and Parenchymal Vascular Aβ. Superose 12 fractions containing the Aβ monomer were pooled. Betaine [0.1% (wt/vol) final concentration] was added to the column eluent to prevent adsorption of the peptides to the glass tubes. After reduction of the volume by vacuum centrifugation, the formic acid was removed by dialysis (1000-Da cutoff) against first 8% betaine and then 2%

Table 1. Plasma desorption mass spectrometry of peptides derived from vascular Aβ

Peak	M_r	MH^+	Sequence	Residues
T1	521.6	521.8	AEFR	2–5
T2	636.7	637.3	DAEFR	1–5
T3	1336.5	1337.1	HDSGYEVHHQK	6–16
T4	1325.7	1326.1	LVFFAEDVGSNK	17–28
T5	1085.5	1086.1	GAIIGLMVGGVV	29–40
C1	429.6	431.1	VGGVV	36–40
C2	613.8	614.2	VGGVVIA	36–42
C3	625.8	626.0	GAIIGLM	29–35
C4*	625.8		GAIIGLM	29–35
C5	1325.7	1325.9	LVFFAEDVGSNK	17–28
C6	1325.6	1327.1	LVFFAEDVGSNK	17–28
P1	560.8	561.1	MVGGVV	35–40
P2	613.8	613.1	VGGVVIA	36–42
P3	745.1	746.1	MVGGVVIA	35–42
P4	1999.4	1999.2	FRHDSGYEVHHQKLVF	4–19
	2199.4	2199.9	AEFRHDSGYEVHHQKLVF	2–19
	2314.8	2315.2	DAEFRHDSGYEVHHQKLVF	1–19
P5	1490.7	1492.2	FAEDVGSNKGAIIGL	20–34
A	636.7	637.3	DAEFR	1–5
	521.6	522.4	AEFR	2–5
B	636.7	637.8	DAEFR	1–5
	521.6	522.4	AEFR	2–5
C	636.7	637.6	DAEFR	1–5
D	636.7	637.6	DAEFR	1–5
E	997.2	997.5	GYEVHHQK	9–16
F	1084.3	1084.6	SGYEVHHQK	8–16
G	1336.5	1336.4	HDSGYEVHHQK	6–16
H	1336.5	1336.5	HDSGYEVHHQK	6–16
I	1336.5	1336.5	HDSGYEVHHQK	6–16
J	1325.6	1326.7	LVFFAEDVGSNK	17–28
K	1085.5	1085.0	GAIIGLMVGGVV	29–40
L	613.8	613.7	VGGVVIA	36–42
M	625.8	627.5	GAIIGLM	29–35
N*	625.8	627.5	GAIIGLM	29–35

Peptides T1–T5, C1–C6, and P1–P5 result from trypsin, CNBr, and pepsin digests of leptomeningeal vascular Aβ, respectively. Peptides A–N result from trypsin and CNBr digests of parenchymal vascular Aβ as shown in Fig. 1.

*Homoserine lactone is converted to homoserine during preparation of the peptide.

betaine in 0.1 M ammonium bicarbonate (pH 7.8). Both leptomeningeal and parenchymal vascular Aβ were digested by trypsin and chymotrypsin (Worthington) for 16 hr at 37°C at an enzyme-to-substrate ratio of ≈1:50 (wt/wt). After lyophilization, the digests were resuspended in 0.1% trifluoroacetic acid, and insoluble material was separated from the soluble peptides by centrifugation. The insoluble material from the tryptic digest was resuspended in 1 ml of 80% (vol/vol) formic acid, cleaved by treatment with cyanogen bromide (CNBr) for 16 hr at room temperature, and then analyzed as described below. Another aliquot of Superose 12-purified Aβ was concentrated, dialyzed against 2% (wt/vol) betaine in 3% (vol/vol) formic acid, and digested with pepsin (Worthington) under the same conditions as those used for the serine proteases. Aβ peptides in the enzymatic and chemical digests were separated by reverse-phase HPLC (RP-HPLC) on C_{18} columns (Pharmacia-LKB or Bio-Rad) and were characterized by mass spectrometry, amino acid analysis, and sequence analysis as described (6, 12).

RESULTS AND DISCUSSION

Chemical Structure of the Vascular Aβ. The protocol we have devised for the purification of vascular Aβ involves fewer steps than do previously published techniques (13, 14).

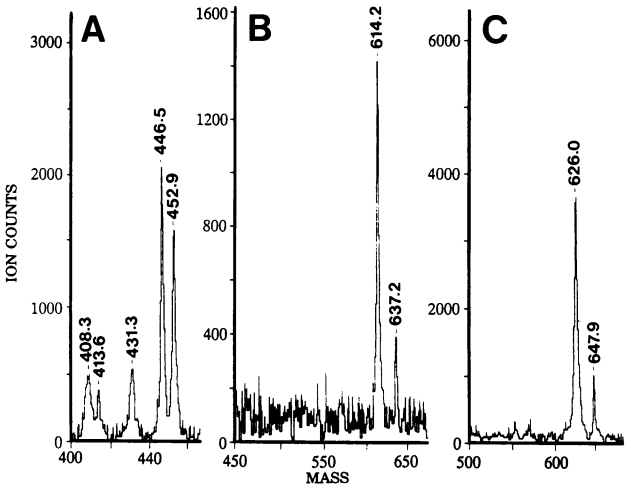

FIG. 2. Plasma desorption mass spectra showing the MH^+ and MNa^+ ions of peak C1 (peptide residues 36–40) (*A*), peak C2 (residues 36–42) (*B*), and peak C3 (residues 29–35) (*C*) from the CNBr digest of the insoluble material resulting from tryptic digestion of vascular Aβ. Positive ion mass spectra were obtained on an Applied Biosystems/BIO-ION Nordic AB model 20 plasma desorption mass spectrometer (Uppsala, Sweden) at an accelerative voltage of 16 kV, with the ion signal integrated for 1.6–2.0 million counts. In general, peptides were deposited on nitrocellulose sample foil as 20-μl aqueous solutions in 0.1% CCl_3COOH and washed with 10 μl of 0.1% CCl_3COOH. In *A*, the peak at m/a = 446 is from the nitrocellulose in the foil.

Recovery of pure Aβ from a single Superose 12 size-exclusion chromatography step (Fig. 1*A*) makes the C_4 RP-HPLC step previously used unnecessary. This is important because Aβ-(1–42), but not Aβ-(1–40), is insoluble in the acetonitrile buffers used with RP-HPLC (6, 7, 13, 14) and thus is not observed in these preparations. In contrast, Superose 12 gel filtration in formic acid yields both of these Aβ peptides.

Because we had recently found that modified Aβ-(1–42) is the major form of Aβ in the cores of the neuritic plaques (6), we wanted to determine whether this form is also present in the vascular tissue of the brain. Aβ was extracted from leptomeningeal blood vessels and from the parenchymal microvasculature and purified by gel filtration on Superose 12 as shown in Fig. 1*A*. Digestion of the leptomeningeal Aβ by chymotrypsin revealed two peptides corresponding to the C terminus (residues 36–40 and 36–42) (Fig. 1*B*), indicating the presence of both Aβ-(1–40) and Aβ-(1–42). Similar results were observed with pepsin, which generated peptides containing residues 35–42, 36–42, and 35–40 (Table 1). To better quantitate Aβ-(1–40) and Aβ-(1–42), the leptomeningeal Aβ was also digested with trypsin. After removal of insoluble material generated during the digestion, the soluble tryptic peptides were isolated by RP-HPLC (Fig. 1*C*) and characterized by amino acid analysis and plasma desorption mass spectrometry (Table 1). Interestingly, the C terminus was represented only by peptide T5 (residues 29–40); however, the recovery of this peptide was just 15% of that of the peptides T1/T2 (residues 2–5 and 1–5) and T3 (residues 6–16), suggesting that peptide 29–42 was insoluble. Therefore, we resuspended the insoluble pellet in 80% formic acid and cleaved it with CNBr. Analysis of this CNBr digest (Fig. 1*D*, Fig. 2, and Table 1) revealed that peptide C2 (residues 36–42) was more abundant than peptide C1 (residues 36–40). After quantitation of the tryptic and CNBr peptides by amino acid analysis, the yields of Aβ-(1–42) and Aβ-(1–40) were calculated by dividing the sum of peptides T5 (29–40) and C1 (36–40) (or peptides 29–42 and 36–42) by the averaged amounts of peptides 6–16 and 17–28. In four independent

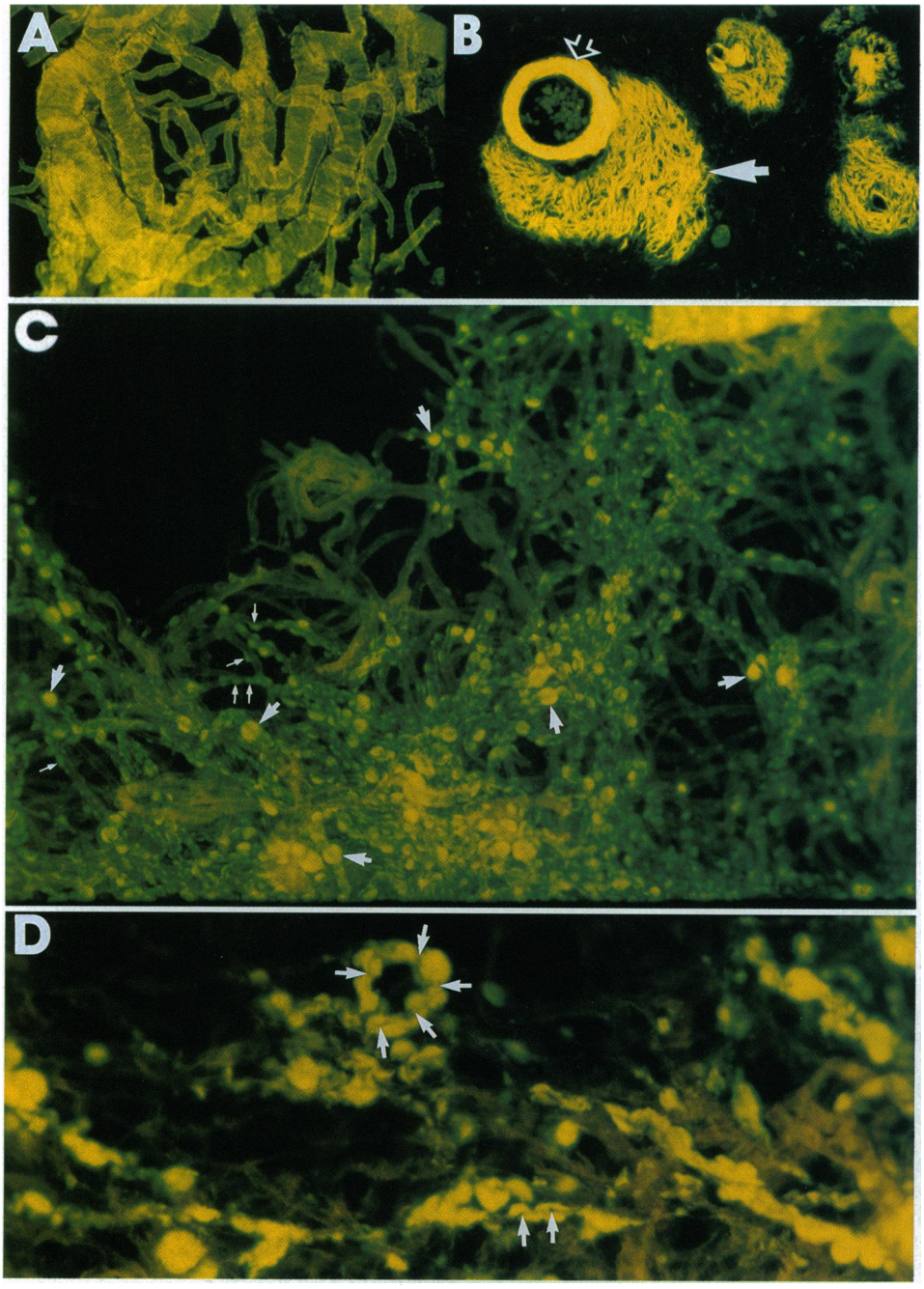

FIG. 3. Accumulation of Aβ deposits in leptomeningeal and parenchymal blood vessels in AD. Vessels were prepared and stained with thioflavin S to visualize Aβ by fluorescence microscopy as described (12). (*A*) Subarachnoidal arterioles of the leptomeninges. Washed only with buffer, these vessels retain cellular and stromal components. The Aβ is deposited in vessels localized in the subarachnoidal space as well as on pial vessels at the surface of the cerebral gyri and sulci. These arterioles show irregular diameters with numerous dilations and constrictions. The Aβ deposits are arranged perpendicularly to the main axis of the vessel. (×85.) (*B*) Cross-section of parenchymal arterioles laden with Aβ. The greater density of the Aβ in the vessel (open arrow) than in the surrounding tissue (solid arrow) suggests that the Aβ may first saturate the vessel wall and then diffuse into the surrounding parenchyma. (×85.) (*C*) Parenchymal capillaries. All of the cellular elements of the neuropil have been removed by treatment with SDS. This whole mount tuft exhibits a wide range of core sizes intimately associated with the insoluble basal lamina. The early small Aβ deposits (small arrows) are flat and ellipsoidal. Their main axis is parallel to that of the endothelial cells and thus that of the vessel as well. More condensed cores (large arrows) are spherical. (×180.) (*D*) Parenchymal capillaries at higher magnification. The larger deposits of Aβ appear to distort and in some cases obliterate the capillary lumens (arrows). This is most apparent when the focal point is varied during observation of these vessels. Obstruction of the lumens has also been seen in electron microscopy studies of capillary cross-sections (A.E.R., unpublished results). (×355.)

preparations of AD brains, Aβ-(1–42) made up 48–68% of the total Aβ.

Similar analyses were performed on Aβ purified from the parenchymal microvasculature. Peptides generated by tryptic and CNBr digestion yielded the same pattern of peaks on RP-HPLC (Fig. 1 *E* and *F*; Table 1) as has been recently published for Aβ purified from the cores of the neuritic plaques of the parenchyma (6). As was seen with leptomeningeal Aβ, the tryptic digest of parenchymal vascular Aβ produced a soluble peptide containing residues 29–40 (peptide K in Fig. 1*E*), but most of the C-terminal sequence released from precipitated material by CNBr digestion was in the peptide containing residues 36–42 (peptide L in Fig. 1*F*). Quantitation of these results as described above indicated that 75% of the Aβ in the parenchymal microvasculature is Aβ-(1–42).

Neuritic plaque Aβ has recently been shown to contain high levels of posttranslational modifications, including N-terminal degradation and the isomerization and racemization of aspartyl residues 1 and 7 (6). Like leptomeningeal Aβ, however, parenchymal vascular Aβ contains much fewer of these modifications. For example, 73% of the neuritic plaque Aβ molecules contain an isoaspartyl residue at position 7, while only 25% of the parenchymal vascular Aβ molecules contain this residue (compare peaks G, H, and I in Fig. 1*E* to the same peaks in Figures 2 *A* and *B* in ref. 6). Because isomerized and racemized aspartyl residues arise spontaneously in proteins, the abundance of these residues might be an indication of the relative age of the protein (15). Together with the greater N-terminal degradation observed for plaque Aβ compared with vascular Aβ (6, 13, 14, 16, 17), these results suggest that the Aβ found in the cores of the neuritic plaques is older than the Aβ associated with vascular tissues.

We conclude that Aβ-(1–42) and Aβ-(1–40) are the major forms of Aβ in the vascular deposits. The more soluble Aβ-(1–40), which appears to arise from normal metabolism in brain tissues, might be trapped at the sites of Aβ-(1–42) deposition by its association with this more insoluble form

(18–20). We do not observe, however, the Aβ-(1–39) form that had been reported (17).

Distribution of Vascular Aβ. Although the cell type(s) responsible for the processing of the Aβ precursor protein is not known, the presence of Aβ-(1–42) in both vascular tissues and neuritic plaques suggests that these deposits might arise from a common source. Fluorescence microscopy of thioflavin S-stained cerebrovascular tissues was used to investigate this possibility. In purified leptomeningeal arterioles, the deposition of Aβ forms discrete bands, with the axes of the Aβ deposits oriented perpendicularly to the main axis of the vessel (Fig. 3*A*). Interestingly, this distribution appears to follow the pattern of the extracellular matrix associated with the smooth muscle cells of the arteriole, suggesting that the source of this Aβ is the vascular tissue itself. If this Aβ came instead from the cerebrospinal fluid (CSF), it might be expected to be evenly distributed on the adventitia, which surrounds the arteriole and lacks the banded pattern of the smooth muscle cells. Smaller vessels more likely to participate in the blood–brain barrier exhibit more extensive Aβ deposition than do thicker-walled arteries (not shown), and the Aβ observed in the CSF might be fact originate from these Aβ-saturated arterioles.

In parenchymal arterioles, the morphological distribution of the Aβ resembled that seen in the leptomeninges, leading to similar irregularities in the vessel walls (not shown). However, the walls of the capillary network exhibited abundant discrete cores of Aβ at different stages of condensation (Fig. 3 *C* and *D*). During the isolation of these capillaries, all cellular components from the vascular wall and surrounding parenchyma were removed except for the capillary basal lamina, which remains insoluble in 15% SDS. The Aβ deposits appeared to be tightly anchored to the basal lamina, because repeated washings with SDS fail to detach them. Interestingly, other tufts of parenchymal capillaries in the same preparation were found to be completely free of Aβ cores (not shown). This indicates that the tight interaction between Aβ and the basal lamina did not arise artifactually during the preparation of the sample. At higher magnification, the lumen of some capillaries appeared to be occluded, particularly at sites surrounded by larger rounded cores (Fig. 3*D*). If the pressure exerted by the parenchymal tissue on the capillary wall is equal along the length of the capillary, then the continuous addition of Aβ originating from a single point on the capillary wall should generate spherical deposits like those observed in Fig. 3 *C* and *D*, and this pressure on the growing sphere should eventually squeeze the capillary shut. It is possible that obliteration of the lumen is then followed by degeneration and disappearance of the capillary, leaving the cores of amyloid free in the parenchyma; this, however, remains to be established. While the stronger walls of the larger arterioles may limit their occlusion, Aβ generated by these vessels would be expected to diffuse into the matrix of the media and the adventitia and then spill over into the surrounding parenchyma, as appears to be occurring in the tissue section shown in Fig. 3*B*.

Although it is possible that neuritic plaque and vascular Aβ deposits arise independently, with the former deposits forming earlier in the progression of AD than the latter, the results presented here suggest that Aβ-(1–42) in at least some cases of AD might originate primarily in the vascular tissue. The Aβ-(1–42) that escapes into the parenchyma from the Aβ-laden blood vessel or simply remains there after degeneration of this vessel may end up as the cores of the neuritic plaques. Supporting this model are the chemical similarity between vascular and neuritic plaque Aβ and the finding that the apparent origin of vascular Aβ is the vascular tissue itself. Furthermore, if neuritic core Aβ-(1–42) arises from vascular Aβ-(1–42), it would be inherently older than the vascular Aβ-(1–42), as is suggested by the levels of posttranslational modifications.

Given the observations described above, it is possible that Aβ functions physiologically as a hydrophobic patch to seal defects in the blood–brain barrier. This hypothesis is supported by the observance of vascular Aβ deposits in cases of cerebral vasculitis of different origins and cerebrovascular malformations (3), as well as the tight linkage between Aβ deposits and the basal lamina or extracellular matrix (4, 5). If this is true, the initial deposition of Aβ may be advantageous to the brain. Growth of these deposits with time, however, may lead to widespread obliteration of the capillaries. As a result, the protracted compromise of oxygen and nutrient delivery may contribute to the demise of vulnerable neurons and result in the pathology observed in some cases of AD.

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