

Relative abundance of Alzheimer A β amyloid peptide variants in Alzheimer disease and normal aging

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ABSTRACT The Alzheimer A β amyloid peptide (A β) is the principal proteinaceous component of amyloid associated with Alzheimer disease (AD). We have determined the relative abundance of A β structural variants present in amyloid from brains of 10 individuals with sporadic AD, 2 individuals with familial AD carrying specific mutations in the Alzheimer amyloid precursor protein gene, and 5 nondemented elderly controls. A procedure of isolation based on the extreme insolubility of A β amyloid was used. The purified, nondigested A β was analyzed by N-terminal sequencing and electrospray-ionization mass spectrometry. Three principal A β variants were detected—A β (1–40), A β (1–42), and A β (11–42)—in all brains analyzed. The predominant variant in sporadic AD was A β (1–40), whereas the principal A β variant in nondemented elderly controls was A β (1–42). The ratio A β (1–40)/A β (1–42) differed by 10-fold between brains from nondemented controls and those with sporadic AD.

Alzheimer disease (AD) is associated with deposition of amyloid in the brain parenchyma and within the cerebrovascular vasculature (for review, see ref. 1). Amyloid displaying properties similar to those of AD amyloid can also be detected in normal aging (2). Whether this amyloid accompanies normal aging or is an early histopathological sign of presymptomatic AD is not known. The AD-associated amyloid deposits are mainly composed of the 4-kDa Alzheimer A β amyloid peptide (A β) (3, 4). A β is a proteolytic fragment of a transmembrane glycoprotein, the Alzheimer A β amyloid precursor protein (APP) (5).

Since the initial isolation of A β from amyloid deposits (3), a variety of methods for purification and analysis of the peptide have been used (4, 6–8). Various forms of the native peptide have been reported. For instance, it has been stated that the N terminus of A β is blocked (6), that A β is deposited as a mixture of N-terminally truncated (“ragged”) variants (4), and that the C terminus is different in vascular and parenchymal A β (7). More recently, it was proposed that A β (1–40) is the major variant in brain (9) and that cerebrovascular amyloid is composed primarily of A β (1–40) and A β (1–42) (10) (Fig. 1). Some of the discrepancies may be technical but they could also be due to interindividual differences in A β composition in the small number of AD cases investigated. The primary structure of A β is an important issue, since A β variants of different lengths display distinct characteristics regarding solubility and amyloid fibril formation (11). For instance, A β (1–40) is relatively soluble in aqueous media, whereas the C-terminally extended variant

A β (1–42) rapidly forms fibril-like, insoluble structures (12). Since slight differences in structure may affect amyloidogenesis, a thorough knowledge of the actual A β composition of amyloid is therefore important. Moreover, knowledge of the exact structure is important for characterization of the proteolytic enzymes involved in A β formation. In addition, amyloid associated with normal aging has, to our knowledge, not been characterized biochemically and its peptide composition is unknown.

In the present work, A β was purified from the cerebral cortex of a number of sporadic AD cases and nondemented elderly controls, as well as two familial AD (FAD) cases. One of the two FAD cases had the APP K670N/M671L mutation (13), and the other had the APP V717I mutation (14). Primary structures and relative abundances of the purified A β variants were determined by N-terminal microsequencing and electrospray-ionization mass spectrometry (ESI-MS).

MATERIALS AND METHODS

Brain tissue from sporadic AD cases, two control cases, and the K670N/M671L case was obtained from the Alzheimer's Disease Research Centre, Huddinge University Hospital, Stockholm. The APP V717I FAD brain tissue was obtained from the Joseph and Kathleen Bryan Alzheimer's Disease Research Center, Duke University. AD diagnosis was confirmed as described (15). The sporadic AD and FAD cases were of either sex with an average age of 81 years (range, 63–101 years). Brain tissue from other control cases was obtained from the Department of Forensic Pathology, Karolinska Institute, Stockholm. The controls were of either sex with an average age of 75 years (range, 57–81 years). Two of the controls were subjected to the same neuropathological examination as the AD cases. In the other three controls (including the high-A β control; see *Results*), neither gross pathological examination nor clinical records showed any signs of AD or other neurological disorders. In both AD and control cases, occipital cortex was removed at autopsy, immediately frozen, and stored at -70°C until further processing. Occipital cortex was used for reasons of availability. All reagents used in extraction and purification of A β were from Sigma unless indicated otherwise.

Purification of A β from Human Brain Tissue. Frozen cortical tissue (10–40 g) was thawed and homogenized in 5 vol of a buffer containing 1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, in a

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Abbreviations: AD, Alzheimer disease; FAD, familial AD; A β , Alzheimer A β amyloid peptide; APP, Alzheimer A β amyloid precursor protein; ESI-MS, electrospray-ionization mass spectrometry; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol.

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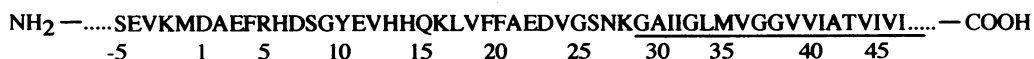


FIG. 1. Amino acid sequence of the A β region of APP. Transmembrane region is underlined. Single-letter code is used.

Potter-Elvehjem homogenizer (loose-fitting pestle). The SDS insoluble material was collected by centrifugation for 20 min at 100,000 $\times g$ (22°C). The pellet was homogenized and centrifuged again under the same conditions. This was repeated five times. The final pellet was washed with water, transferred to a glass tube, and extracted with 5 vol of 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) using mild stirring for 30 min (22°C). The A β -enriched HFIP extract was aspirated and coarse debris was removed by passing the extract through a column of glass beads (diameter, 500 μ m). The crude extract was collected in Eppendorf tubes and dried by vacuum centrifugation. After drying, the extract was delipidated by a modification of an established protocol (16). To the lyophilized material 1 ml of chloroform/methanol (1:2) was added, followed by centrifugation for 10 s at 10,000 $\times g$. After addition of 0.5 ml of water to the supernatant, the tube contents were thoroughly mixed before centrifugation for 1 min at 10,000 $\times g$. The aqueous upper phase was carefully removed by aspiration. Then, 0.6 ml of methanol was added, followed by centrifugation for 2 min at 10,000 $\times g$. The protein-containing pellets were dried under a stream of nitrogen and resuspended in 70% formic acid. After removal of insoluble material by centrifugation at 2500 $\times g$ for 3 min, A β was purified from the supernatant by size-exclusion chromatography on a Superose 12 column (10 \times 300 mm) (Pharmacia) developed with 70% formic acid at a flow rate of 200 μ l/min (17). UV absorbance was monitored at 280 nm and an aliquot of each fraction collected was subjected to immunoblotting.

SDS/PAGE and Immunoblotting of Superose 12 Fractions. SDS (final concentration, 1%) was added to an aliquot of each Superose 12 fraction, and formic acid was removed by vacuum centrifugation. The dried material was solubilized in Laemmli sample buffer containing 6 M urea. Samples were boiled and resolved by SDS/PAGE using a Tris Tricine buffer system (18). The separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) by electroblotting. The membrane was blocked in TBS-T (0.05% Tween 20/150 mM NaCl/50 mM Tris-HCl, pH 7.4) containing 5% defatted dry milk and probed with monoclonal antibody 6E10 directed against residues 1–16 of A β (19). After incubation with horseradish peroxidase-labeled sheep anti-mouse immunoglobulin (Amersham), antibody binding was visualized with the enhanced chemiluminescence detection system (ECL; Amersham). Fractions containing monomeric A β were pooled, lyophilized by vacuum centrifugation, and analyzed by ESI-MS. In some cases, A β was immobilized on poly(vinylidene difluoride) membranes and subjected to Edman degradation using an Applied Biosystems protein sequenator (model 477A) according to the manufacturer's recommendations.

ESI-MS. The samples were analyzed on a VG BIO-Q (Fisons, Manchester, U.K.), an instrument that essentially consists of an atmospheric pressure electrospray ion source followed by a triple quadrupole mass analyzer with a maximum mass range of 4000. The lyophilized material was dissolved in 99% formic acid and diluted 10 times in a mixture of methanol/isopropanol/water (2:2:1; vol/vol). Methanol/water (1:1; vol/vol) containing 0.1% formic acid was pumped through the capillary at a flow rate of 4 μ l/min, and the samples were introduced into the instrument via loop injection. Mass/charge (m/z) values of the multiple charged peptides were acquired by scanning from m/z 600 to m/z 1300 for 10 s. The spectra (positive-ion mode) were transformed to give the molecular size of the different peptides on a mass

scale. Mass scale calibration was performed with horse heart myoglobin. The calculated masses of the A β variants refer to the average mass of the uncharged molecules.

RESULTS

Purification of A β Peptide. A β amyloid, in contrast to most other brain peptide/protein constituents, is insoluble in 1% SDS (4). After SDS extraction of brain tissue, a pellet remained that contained partially purified amyloid. The amyloid peptides were extracted with HFIP, which is a superior solvent for A β variants of >40 residues (11). Moreover, by using HFIP, exposure of the proteins to formic acid could be reduced and, hence, artifactual formylation of A β could be minimized. As shown in Fig. 2, A β could be purified to near homogeneity from a delipidated HFIP extract in a single size-exclusion chromatography run. The indicated 4-kDa peptide (Fig. 2B) was identified as A β peptide by N-terminal microsequencing (data not shown). The double band appearance of A β was also observed with synthetic A β (1–40) and therefore is attributable to the SDS/PAGE procedure.

MS Analysis of A β . ESI-MS of nondigested, purified A β peptides yielded high-quality spectra (Fig. 3). The signal intensities of the peptides in the mass spectrum are directly proportional to their relative abundance in the sample. The primary structure of each peptide was deduced by comparing the observed mass to the calculated mass for peptides derived from the A β sequence (Fig. 3 *Insert*). In the spectrum shown in Fig. 3, which represents A β purified from a sporadic AD patient, the most intense signal (peak C) corresponded to A β (1–40) while the signal corresponding to A β (1–42) (peak E) was less intense. Minor components in the spectrum included pyroglutamate A β (11–42) (peak A), the methionine sulfoxide derivative of A β (4–42) (peak B), and the formyl derivative of A β (1–40) (peak D). There were also a number of peptides present whose observed masses were not assignable to any A β peptide variants.

All mass spectra analyzed contained one or more distinct peaks in the mass region from 3318 to 3408. These peaks corresponded to A β (11–42) and the formyl and methionine sulfoxide derivatives of A β (11–42). Although A β (11–42) species with an intact N terminus could be detected, the majority of these peptides apparently existed in the pyroglutamate form—i.e., the N-terminal glutamate residue had cyclized to form a pyroglutamate residue. Levels of the native A β (11–42) and the pyroglutamate derivative were combined and are referred to as A β (11–42) in Table 1. In some spectra, extensive formylation and methionine oxidation of the peptides was evident. In control experiments, we could show that synthetic A β (1–40) was also oxidized and formylated when subjected to the same Superose 12 purification and ESI-MS analysis as the samples. As pointed out by Roher *et al.* (17), commercial grade formic acid might contain impurities catalyzing formylation of peptides.

Relative Abundance of A β Variants in Human Brain. Each of the brains of the individuals with sporadic AD had sufficiently high levels of A β to analyze the relative abundance of the principal variant forms (e.g., see Fig. 3). Of the 12 brains of the nondemented elderly controls, the levels in seven were too low to allow ESI-MS. The brains from four of the nondemented individuals had levels of A β substantially lower than those found in sporadic AD brains but were sufficiently high for ESI-MS and therefore have been used as the control group. One brain from a nondemented elderly individual had levels of A β much higher than those found in any of the

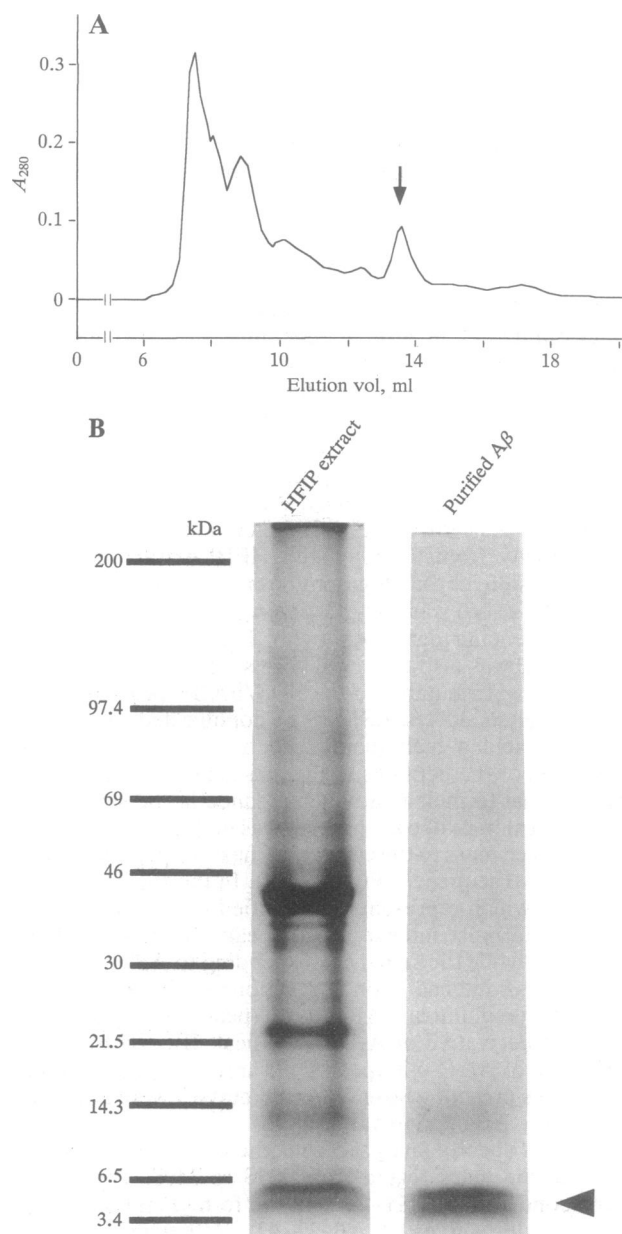


FIG. 2. Purification of $A\beta$ from the brain of an individual with sporadic AD. (A) Size-exclusion chromatography of the delipidated HFIP extract on a Superose 12 column. (B) SDS/PAGE analysis of a HFIP extract before size-exclusion chromatography (left lane) and the material indicated by the arrow in A, showing highly purified $A\beta$ (right lane). Proteins were visualized with Coomassie brilliant blue stain. Positions of molecular size markers are shown on the left and monomeric $A\beta$ is indicated by an arrowhead. Faint band migrating with an apparent molecular mass of 12 kDa in the right lane represents $A\beta$ dimer formed in the gel.

sporadic AD brains and therefore is treated separately from the control group.

The $A\beta$ variants detected in the amyloid are listed in Table 1. All brains studied contained $A\beta(1-40)$, $A\beta(1-42)$, and $A\beta(11-42)$. In 15 of 17 samples, either $A\beta(1-40)$ or $A\beta(1-42)$ was the principal component. In two cases, one sporadic AD brain (case 3) and one FAD brain (APP V717I), the $A\beta(11-42)$ variant was the predominant form. N-terminally truncated variants other than $A\beta(11-42)$ were detectable in all but one sample. The $A\beta(4-42)$ and $A\beta(8-42)$ variants appeared to be the most prevalent minor $A\beta$ variants in AD samples, a finding in part supporting the finding of Miller *et*

al. (20). Interestingly, all detected $A\beta$ variants terminated with either Val-40 or Ala-42.

A prominent difference in the ratio $A\beta(1-40)/A\beta(1-42)$ was observed in the sporadic AD group in comparison to the control group. Thus, in 9 of 10 sporadic AD brains the ratio was >1.0 and the mean ratio for all sporadic AD brains was 3.65 (Table 1; Fig. 4). In contrast, in all four of the control brains the ratio was <1.0 , with a mean ratio of 0.37—i.e., 10-fold less than that found in the sporadic AD brains. Interestingly, the nondemented elderly individual with a high level of $A\beta$ had an $A\beta(1-40)/A\beta(1-42)$ ratio of 1.90, consistent with the possibility that the high ratio in sporadic AD versus control might be a reflection of the amyloid burden *per se*.

DISCUSSION

We have determined the relative abundance of the $A\beta$ variants deposited in amyloid. The study included material from individuals with sporadic AD, individuals with FAD-associated APP mutations, and nondemented elderly. The general conclusions from the data presented here are as follows: (i) the predominant $A\beta$ variants in amyloid are $A\beta(1-40)$, $A\beta(1-42)$, and $A\beta(11-42)$; (ii) amyloid contains some N-terminally truncated $A\beta$ variants besides $A\beta(11-42)$, which is the shortest and also most abundant; (iii) no $A\beta$ variants attributable to the mutant APP alleles could be detected; (iv) all $A\beta$ variants present in amyloid terminate in either Val-40 or Ala-42; (v) $A\beta$ amyloid in normally aged brains contains the same $A\beta$ variants, although in different proportions, as AD-associated amyloid. Since the yields of the extraction procedure are not known, absolute levels have not been calculated. However, the sporadic AD and FAD cases certainly contained severalfold more $A\beta$ than the controls (with the one exception noted), as determined by immunoblotting. This difference in brain $A\beta$ content between AD cases and controls has been reported previously (21).

In all brains, both the soluble $A\beta(1-40)$ and the insoluble $A\beta(1-42)$ were detected. This suggests that they may act in concert during amyloidogenesis. A possible mechanism for interaction between $A\beta(1-40)$ and $A\beta(1-42)$ was outlined by Jarret *et al.* (12). They showed that aggregated $A\beta(1-42)$ can serve as a nucleus for the polymerization of soluble $A\beta(1-40)$ into amyloid fibrils. This process resembles protein crystallization and has been termed nucleation-dependent polymerization (for review, see ref. 22). Hence, it is possible that insoluble $A\beta(1-42)$ is generated and deposited locally in the brain. These aggregates may serve as nuclei for the conversion of soluble $A\beta(1-40)$, present in blood and cerebrospinal fluid (23), into amyloid fibrils. However, it is also possible that the different ratios merely reflect the occurrence of differing amounts of deposited $A\beta$. Deposited $A\beta(1-42)$ may also be converted by exopeptidases to $A\beta(1-40)$ during the progression of the disease.

The $A\beta$ peptide used here was purified from total brain cortex. It is conceivable that the difference in the $A\beta(1-40)/A\beta(1-42)$ ratio between AD and nondemented controls reflects a different distribution between parenchymal amyloid and cerebrovascular amyloid in the individuals. However, in order to conclusively demonstrate differences in the amyloid composition between these two compartments and to determine their relative contribution to the total brain amyloid requires methodology that is not yet available.

In accordance with previous studies (4, 20), we found that amyloid contains N-terminally truncated variants. The most prominent truncated variant, $A\beta(11-42)$, could be detected in all brains to varying degrees. In one sporadic AD and one FAD brain (APP V717I), pyroglutamate $A\beta(11-42)$ was the predominant $A\beta$ variant. After conversion of the N-terminal glutamate to pyroglutamate, the peptide appears blocked when subjected to N-terminal sequencing. Formation of the

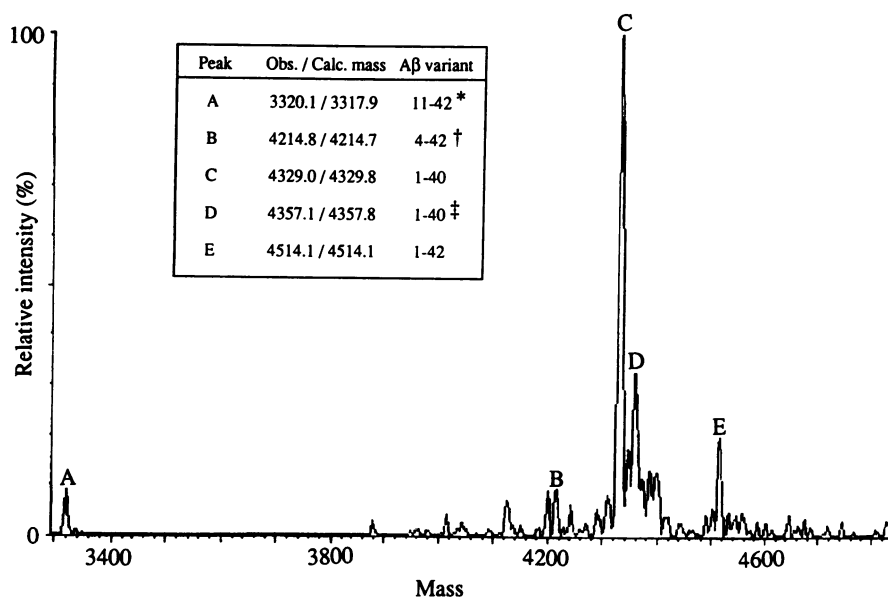


FIG. 3. Mass spectrum of A β purified by size-exclusion chromatography from an individual with sporadic AD. The spectrum was acquired in the mass range m/z 600–1300 and transformed to give the molecular mass of the uncharged molecules. (Inset) Molecular masses and predicted sequences of A β peaks A–E. *, Pyroglutamate, –18 mass units; †, methionine oxidized, +16 mass units; ‡, formylated, +28 mass units.

pyroglutamate A β -(11–42) variant may explain reports stating that the N terminus of A β is blocked (6, 24). Whether N-terminally truncated variants are genuine products from the cellular metabolic pathway secreting A β or arise as a result of partial proteolytic digestion after secretion is presently unknown. Seubert *et al.* (23) reported that the medium from human mixed-brain cell cultures contained an A β variant with N terminus at Glu-11, suggesting that A β -(11–42) is a secretory product from cells. The relatively low abundance and irregular appearance of N-terminally truncated variants other than A β -(11–42) suggests that they have a minor, or no, specific role in amyloidogenesis.

ESI-MS analysis of low molecular weight Superose 12 fractions failed to detect p3, an A β variant devoid of the N-terminal 16 amino acids [i.e., A β -(17–40) or A β -(17–42)] that is produced in relatively large quantities by, e.g., transfected human kidney 293 cells (25) and CHO cells (26). The

fact that A β -(11–42) was found in amyloid deposits whereas p3 was not suggests either that the region between residue Glu-11 and Lys-16 of A β is obligate for deposition of the peptide in amyloid or that p3 is not produced *in vivo*.

The APP V717I mutation occurs at a residue C-terminal to the A β region and, as reported here and previously by Liepnieks *et al.* (27), this mutation does not lead to detectable A β variants longer than A β -(1–42). Neither does the APP K670N/M671L double mutation, which changes the wild-type Lys-Met sequence adjacent to the N terminus of A β for Asn-Leu, lead to incorporation of N-terminally elongated forms in the amyloid.

A β formation is a consequence of a limited number of proteolytic events, none of which have, however, been identified. APP is also proteolytically cleaved by an as yet unidentified α -secretase at a site within the A β sequence (28), precluding the formation of intact A β . Consequently, the risk

Table 1. Distribution of A β variants in amyloid from human brain

Group	Case	% of total A β									
		1–40	1–42	3–40	3–42	4–42	6–42	7–42	8–42	9–42	11–42
Sporadic AD	1	59.9	7.8	—	—	5.5	—	—	—	—	26.8
	2	62.3	9.3	11.4	—	5.7	—	—	—	—	11.2
	3	29.7	27.5	—	—	—	—	—	—	—	42.8
	4	59.0	16.5	—	—	11.9	—	—	—	—	12.6
	5	52.9	13.2	—	—	—	—	—	8.0	9.5	16.3
	6	34.4	23.9	—	—	—	9.1	—	10.4	—	22.1
	7	40.1	16.4	—	5.7	9.9	—	3.3	8.0	6.2	10.3
	8	38.8	16.8	—	6.0	8.5	2.8	3.4	5.9	—	17.6
	9	15.9	39.3	—	10.2	—	—	6.5	9.0	—	19.0
	10	65.4	9.9	—	—	17.9	—	—	—	—	6.8
	Mean	45.8	18.1	1.1	2.2	5.9	1.2	1.3	4.1	1.6	18.6
Nondemented elderly	1	10.7	60.7	—	—	8.9	—	—	—	—	19.6
	2	11.7	58.9	—	—	6.7	—	—	—	—	22.6
	3	23.7	51.1	—	—	10.9	—	—	—	—	14.3
	4	30.0	41.1	—	—	11.5	—	6.1	—	—	11.4
		Mean	19.0	53.0	0	0	9.5	0	1.5	0	0
High A β control		41.8	22.4	11.0	—	11.2	—	—	—	—	13.6
	FAD										
FAD	K670N/M671L	31.9	11.9	—	—	8.2	—	5.5	7.8	9.5	25.4
	V717I	16.4	21.4	—	—	9.2	—	7.3	6.7	7.8	31.2

A β variants not detected are indicated by a dash and entered as zero in calculation of means.

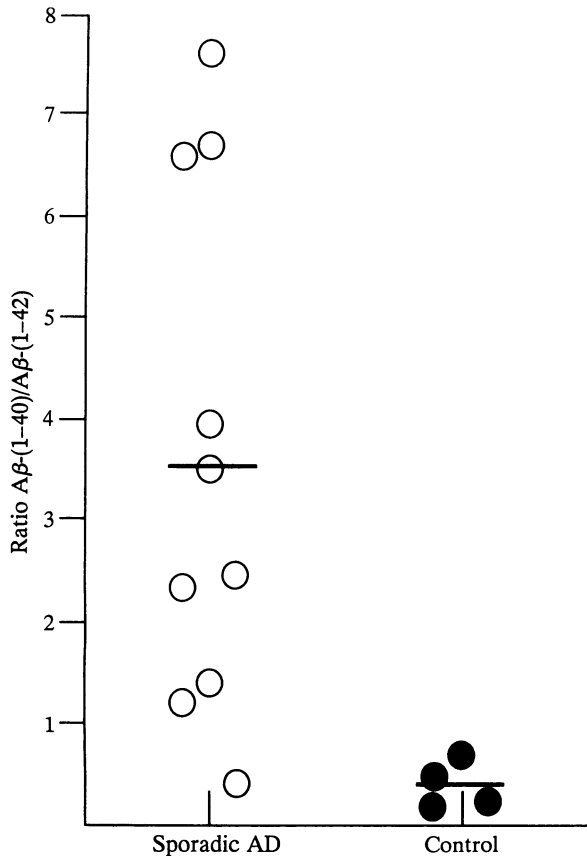


FIG. 4. Ratio between A β (1-40) and A β (1-42) in amyloid from individuals with sporadic AD (○) and elderly nondemented controls (●). Ratio was significantly higher in the sporadic AD group compared to the control group ($P < 0.01$; Wilcoxon rank sum test). Average ratio in each group is indicated by a bar.

for excessive A β formation is related to the relative activities of several proteolytic enzymes. The determination of the exact composition of A β should help in identifying these enzymes.

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