The “Nonamyloidogenic” p3 Fragment (Amyloid β17–42) Is a Major Constituent of Down’s Syndrome Cerebellar Preamyloid*

(Received for publication, July 8, 1996, and in revised form, September 16, 1996)

Maciej Lalowski‡‡‡, Adam Golabek‡, Cynthia A. Lemere§, Dennis J. Selkoe§, Henryk M. Wisniewski‡, Ronald C. Beavis, Blas Frangione‡, and Thomas Wisniewski***‡‡‡

From the Departments of *Pathology, **Pharmacology, and ***Neurology, New York University Medical Center, New York, New York 10016, §Center for Neurologic Diseases, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115, and ¶New York State Institute for Basic Research in Development Disabilities, Staten Island, New York 10314

Down’s syndrome (DS) patients show accelerated Alzheimer’s disease (AD) neuropathology, which consists of preamyloid lesions followed by the development of neurofibrillary tangles. The major constituents of preamyloid and neuritic plaques are amyloid β (Aβ) peptides. Preamyloid lesions are defined as being Aβ immunoreactive lesions, which unlike neuritic plaque amyloid are Congo red-negative and largely nonfibrillar ultrastructurally. DS patients can develop extensive preamyloid deposits in the cerebellum, without neuritic plaques; hence, DS cerebellums are a source of relatively pure preamyloid. We biochemically characterized the composition of DS preamyloid and compared it to amyloid in the neuritic plaques and leptomeninges in the same patients. We found that Aβ17–42 or p3 is a major Aβ peptide of DS cerebellar preamyloid. This 26-residue peptide is also present in low quantities in neuritic plaques. We suggest that preamyloid can now be defined biochemically as lesions in which a major Aβ peptide is p3.

When the temporal profile of Aβ lesions has been evaluated in DS with current methods, it is evident that the earliest deposits are diffuse plaques or “preamyloid” (4–6). These are amorphous, roughly spherical areas immunoreactive with anti-Aβ antibodies, having irregular borders, and are associated with few or no dystrophic neurites. Preamyloid deposits, unlike amyloid, are not stained by Congo red or thioflavine S (7–11). Ultrastructurally, these deposits are mainly nonfibrillar. Extensive numbers of preamyloid lesions can be found in aged individuals, with no reported clinical symptoms (12–14). In DS, preamyloid lesions can appear as early as the age of 12 years (3, 15, 16). According to some investigators, these lesions are thought to become compacted over a period of many years, at which point they acquire the characteristics of amyloid and are associated with neuronal damage and neurofibrillary tangle formation in the form of neuritic plaques (1). Congo red-positive, neuritic plaques begin to appear in DS typically by the end of the third decade of life (3, 15, 16). It has also been suggested that preamyloid plaques never fibrillize and that neuritic, primitive, classical, and preamyloid plaques have separate cellular origins (17). The apparent lack of associated cerebral dysfunction from preamyloid lesions can be correlated with in vitro studies using Aβ synthetic peptides, where it has been suggested that toxicity is dependent on the presence of a fibrillar, predominantly β-sheet conformation (18–22). Hence, important questions for elucidating the pathogenesis of AD include what are the differences in the Aβ peptide content in preamyloid versus neuritic plaques and does preamyloid represent a precursor to neuritic plaques or is it a separate pathway of Aβ peptide deposition?*

Neuritic plaque amyloid, first sequenced in 1985, was found to extend mainly to residues 42 or 43 (23). Extensive amino-terminal heterogeneity was also documented in this and later studies with starts at residues 1, 2, 4, 8, and 11, as well as blocked peptides starting with the glutamate at residue 3 converted to pyroglutamate or isomerization of the aspartate at residue 1 (24–27). Vascular amyloid was initially reported to extend to Aβ residue 28 and later to Aβ39 or Aβ40 (28–31). More recent reports, using different techniques, have also found Aβ42 in vascular amyloid (32).

The biochemical composition of DS preamyloid has not been previously studied. In AD tissue it has been suggested earlier that Aβ17–42 is a major component of preamyloid (33). However, this peptide was extracted from AD tissue that contained a mixture of preamyloid deposits and neuritic plaques; hence it was uncertain that this extraction represented “pure” preamyloid. In addition, we have recently extracted preamyloid from the aged canine model of AD (34). The sequence of canine Aβ is the same as human. Extensive preamyloid deposition can occur in the aged dog in the absence of neuritic plaques (35, 36). The major Aβ
peptide of canine preamyloid was found biochemically to be Aβ17–42; although, other Aβ peptides were also present (34). In the current study, we chose to evaluate DS cerebellar preamyloid, because extensive cerebellar preamyloid deposits occur in DS patients that, throughout the life span of the patient, fail to convert into fibrillar deposits or do so very rarely (3, 37). Hence, this brain tissue serves as a source of relatively pure human preamyloid. In addition, we were able to compare and contrast the biochemical composition of these preamyloid deposits to that of cerebral, cortical neuritic amyloid, as well as leptomeningeal vascular amyloid in the same patients. Our biochemical studies were also correlated with immunohistochemical assays using epitope-specific anti-Aβ antibodies. In order to elucidate further the possible role of Aβ17–42 in neuritic plaque formation, we conducted in vitro studies using synthetic peptides.

MATERIALS AND METHODS

Down’s Syndrome Brain Tissue—Brain tissue was used from six patients with a clinical diagnosis of DS, which was confirmed by chromosomal analysis. Three cases aged 46, 57, and 65 years at the time of death were obtained from the brain bank at the Center for Neurological Diseases at the Brigham and Women’s Hospital (Boston, MA). Three other cases, aged 57, 62, and 61 were obtained from the Institute for Basic Research (IBR) in Mental Retardation (Staten Island, NY). From all three frozen embedded and adjacent cerebellar fresh frozen tissue were available, while from the IBR cases fresh frozen cortical tissue was also available. Extensive preamyloid deposits, which were immunoreactive with an anti-Aβ antibody (4G8) (38), were present in the cerebellums of each patient. These deposits were all Congo red and thioflavine S-negative. Numerous neuritic plaques were present in the frontal cortex in all cases by silver stains and 4G8 immunohistochemistry. In addition, in three of the DS cerebellums, extensive amyloid deposition was noted in the leptomeningeal vessels. These vessel deposits were Congo red-positive. As a control for the biochemical studies, one frozen cerebellum from an AD patient, aged 65 years, with no Aβ immunoreactive lesions in the cerebellum was used.

Preamyloid Extraction—All six cerebellums were subject to the following preamyloid extraction procedure, which is a modification of our and other published protocols (33, 34). Approximately 20 g of cerebellar cortex was dissected free of any large vessel contamination with the use of a dissecting microscope. To avoid contamination of our preamyloid fraction with soluble Aβ (sAβ) peptides, which are known to be present in DS and AD brains, the tissue was first homogenized in a Dounce homogenizer in a buffer containing no detergents (20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.02% sodium azide, 200 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin) at 4 °C and centrifuged at 100,000 × g for 1 h (39, 40). The supernatant contained the pool of free sAβ, as described previously (39, 40). The pellet was used for the preamyloid extraction. The pellets were rehomogenized in a Dounce homogenizer and a blender in 10 volumes of TB buffer (0.1 M Tris-HCl, pH 7.4, 1% SDS, 5 mM EDTA, with 0.02% sodium azide, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). To this buffer a 30% SDS solution was added to obtain a final concentration of 15% SDS. This solution was stirred for 36 h, followed by filtration through a series of nylon mesh of 350, 150, 70, and finally 30 μm, in order to remove small vessel contamination. The filtrate was centrifuged at 135,000 × g for 2 h at 20 °C. The resultant pellet was suspended in TB and centrifuged at 1000 × g for 15 min. The pellet from this 1000 × g spin was subjected to purification according to a protocol for neuritic plaques described below.

The supernatant from the 1000 × g centrifugation was recentrifuged for 1 h at 20 °C at 135,000 × g. The resultant pellet (P-135) was dissolved in 80% formic acid and was obtained from each of the six DS patients. To remove lipids, 1.5 ml of chloroform were added. After vigorous mixing the material was centrifuged at 1500 × g for 15 min. The top layer was redissolved to liquid chromatography Superose 12 column (30 × 1 cm) prior to reverse-phase HPLC. The chromatography was performed in 80% formic acid at 0.4 ml/min and recorded at 280 nm. The material from the Superose 12 column eluting at a volume corresponding to less than 10 kDa was then applied to the HPLC C4-reverse. The material from the 135,000 centrifugation was treated as described previously (33). The resulting P-275 and P-500 pellets were applied either to a Superose 12 column or the C4-reverse column as described for the P-135.

Each of the eluted fractions from the gel filtration and HPLC were assessed for the presence of Aβ peptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. A portion of the fractions were biotinylated and solubilized in 4% saponin sample buffer under reducing conditions. Samples were boiled and run on 16% Tris Tricine SDS-PAGE system. The separated proteins were transferred to a nitrocellulose membrane by electroblotting. The membrane was blocked by 5% nonfat milk in 150 mM NaCl, 20 mM Tris, 0.05% Tween 20, pH 7.4 (TBST) and probed with a monoclonal antibody 6E10 (against the N-terminus of Aβ1–16 of Aβ) and antibody 4G8 (directed against residues 17–24 of Aβ) (38). After incubation with horseradish peroxidase-labeled sheep anti-mouse immunoglobulin, antibody binding was visualized with an enhanced chemiluminescence detection system (ECL, Amersham Corp.). All steps of the purification procedure were also followed by Congo red staining. Aβ-containing fractions were subjected to NH4-terminal sequencing on a 477A protein sequencer (Applied Biosystems). Aβ peptides eluting from HPLC were quantitated by amino acid analysis. Samples were hydrolyzed with 0.2 ml of 6 N HCl containing 0.02% phenol in vacuo at 112 °C. The samples were dried, resuspended in 20 μl of methanol-water-triethylamine (40:40:20, v/v) solution and reduced. HPLC fractions were derivatized with phenylisothiocyanate. Samples were then incubated for 3 min at room temperature with 20 μl of phenylisothiocyanate reagent, consisting of water-triethylamine-phenylisothiocyanate-ethanol (10:10:10:70, v/v). Following evaporation of reagents, the residues were analyzed by reverse-phase HPLC on a Fico-Tag amino acid analyzer (Waters). Aβ-containing fragments were also subjected to mass spectroscopy. Laser desorption mass spectroscopy (LDMS) was carried out on a custom-built machine at the Skirball Institute at NYU Medical Center (41). Protein and peptide samples were prepared for LDMS using the dried droplet method (42). The matrix used was cyano-4-hydroxycinnamic acid (Sigma), which was purified by recrystallization. To produce the dried droplets, a saturated solution of matrix was prepared using either 2:1 aqueous 0.1% trifluoroacetic acid, acetonitrile or 1:2:3 formic acid:2-propanol:water at room temperature (43). The sample was added to this solution so the final sample concentration was 1–10 μM. One half of the solution was placed on the mass spectrometer’s probe and allowed to dry. The laser beam was aimed at several different spots within each position. Over 30 shots were averaged for each spectrum, with over five spectra being acquired for each sample. The LDMS was calibrated with 30 known standards, including Aβ 1–42 (m/z = 4514.1), Aβ 17–42 (m/z = 2577.2), gramicidin S (m/z = 1142.5), and insulin (m/z = 5734.5). The accuracy of the LDMS spectra was determined to be ±0.5 mass unit.

Cerebellar Leptomeningeal Amyloid Extraction—The cerebellar leptomeningeal vessels from three DS patients with amyloid deposition were subjected to a vessel amyloid extraction. This was done using our previously published protocol, which was a modification of other established techniques (26, 34).

Frontal Cortex Neuritic Plaque Amyloid Extraction—From the three DS patients where frozen frontal cortex tissue was available, a neuritic plaque extraction was performed, which was a modification of our and other previously published protocols (26, 34). The frontal cortex tissue contains a mixture of fresh sAβ, preamyloid lesions, and neuritic plaques. To separate out the brain sAβ and preamyloid from the extractions, the same protocol was followed as for the preamyloid extraction described above. The pellet from the 1000 × g spin was washed in 0.1 M Tris-HCl, pH 8.0, four times and digested by collagenase (CLS-3) ( Worthington, 0.2 mg/ml) and DNase I (Worthington, 10 μg/ml) in the presence of 2 mM CaCl2, for 16 h at 37 °C. Following digestion, the material was pelleted at 5000 × g and washed in 0.1 M Tris-HCl, pH 8.0, four times. The pellet was then suspended in 1 M sucrose and layered over a discontinuous density gradient of 1.8, 1.2, 1.15, 1.1, and 1.05 M sucrose. The material was centrifuged at 5000 × g for 1 h. The pellet and the 1.21.8 interface was enriched in amyloid by Congo red staining. The amyloid-enriched neuritic plaques were digested with 0.01% trypsin. A material was washed in distilled water with protease inhibitors and centrifuged at 5000 × g twice. The resultant pellet was resuspended in 0.32 M sucrose with protease inhibitors and 2% SDS. This was mixed at room temperature for 2 h. The sucrose concentration was brought up to 1 M, and the material was layered over a discontinuous gradient of 2.2 and 1.1 M sucrose, followed by centrifugation at 5000 × g for 1 h. The amyloid was enriched in the pellet and...
2.2/1.1 interface. This material was solubilized in formic acid and first subjected to gel filtration, followed by reverse-phase HPLC as described above for the preamyloid extraction. All fractions during the purification were followed by Congo red staining and Western blotting. The Aβ-containing fractions were subjected to NH₂-terminal sequencing and LDMS as described above.

Immunohistochemical Studies—5-μm paraffin sections of the DS cerebellums were subjected to immunohistochemical staining by first deparaffinization followed by treatment with 100% formic acid for 20 min. The slides were blocked in phosphate-buffered saline with 0.1% Tween 20, 10% fetal calf serum (PBST-FCS). Following washing sequential sections had the following primary antibodies applied: BC40 (1:500), BC42 (1:500), 4G8 (1:2000), and 6E10 (1:15000) in PBST-FCS (38, 44). A sequential section was also subjected to Congo red staining. 4G8 recognizes residues 1–17 of Aβ, while 6E10 recognizes residues 1–17 of Aβ (38). BC40 specifically immunoreacts with the carboxyl end of Aβ40, and BC42 specifically labels the carboxyl end of Aβ42 (44). The secondary antibodies used were goat anti-rabbit IgG, biotin-conjugated (1:800) (Sigma), and goat anti-mouse IgG, biotin-conjugated (1:2000) (Sigma), followed by streptavidin-horseradish peroxidase conjugate (1:300) (Amersham) in PBST-FCS. The slides were developed in diaminobenzidine and H₂O₂, with cobalt chloride hexahydrate (0.005%) enhancement. Aβ immunoreactive parenchymal lesions and Aβ-positive vessels were counted in 20 1-mm² areas, on sequential slides for each of the four primary antibodies. The number of immunoreactive cerebellar vessels or preamyloid lesions for each of 6E10, BC40, and BC42 antibody sections was matched to the adjacent 4G8 sections.

Fluorescence Studies—Aβ peptides corresponding to residues 17–42 and 1–42 were obtained by custom order from Quality Controlled Biochemicals Inc. (Hopkin, MA). Aβ1–40 peptide was obtained by custom order at the W. M. Keck Facility at Yale University. The purity of all peptides was assessed by reverse-phase HPLC and LDMS. Amyloid fibril formation by these three synthetic peptides was compared by using the thioflavin T fluorescence method. Thioflavin T binds specifically to amyloid, and this binding produces a shift in its emission spectrum and a fluorescent signal proportional to the mass of amyloid formed (45–47). Aliquots of Aβ peptides were incubated for 5 days at room temperature in 0.1 M Tris/HCl, pH 7.4, at 0.1 mg/ml. Peptides after zero time and 5-day incubations were added to 50 μM thioflavin T in a final volume of 2 μl. Fluorescence was then measured at excitation 435 nm and emission 485 nm in a Hitachi F-2000 fluorescence spectrometer. A time scan of fluorescence was performed, and three values after the decay reached a plateau (280, 290, and 300 s) were averaged after subtracting the background fluorescence of 2 μM thioflavin T.

For electron microscopic studies, synthetic peptide Aβ17–42 was incubated at 1 mg/ml in 100 mM Tris, pH 7.4, for 3 days at room temperature in a 20-μl volume. 5 μl of the sample were applied onto Formvar/silicone oxide-coated 200 mesh copper grids (Pella Inc., Redding, CA) followed by negative staining with 2% uranyl acetate under a vapor of 2% glutaraldehyde. The grids were visualized on a Hitachi H-7000 electron microscope operating at 75 kV. For Congo red staining, samples were applied onto poly-L-lysine-coated or air-dried, anes, sprayed with saturated NaCl, and dried at 75 kV for 20 min. The slides were then stained for 1 h in saturated NaCl in 80% ethanol with 0.1% Congo red. The slides were viewed under polarized light.

RESULTS

Cerebellar Preamyloid Extraction—Preamyloid was extracted individually from the cerebellums of all six DS patients. The purification procedure was followed by SDS-PAGE and Western blotting for Aβ peptides (see Fig. 1, inset). The purified preamyloid material was also subject to Congo red staining, to make sure that there was no amyloid contamination from the cerebellar vessel amyloid, which was present in three of the DS cases. None of the purified preamyloid fractions showed the tinctorial properties of amyloid after Congo red staining. Aβ peptides were found in pellets P-135 and P-275 from all the DS cerebellums, with most of the preamyloid being in P-135. This was found to be the case in each extraction from the six DS patients. The pellet from the 1000 × g centrifugation, which was subject to the purification protocol for neuritic plaques, did not show any Aβ peptides. From the RP-HPLC of P-135 and P-275, Aβ peptides eluted at 37–44% acetonitrile, with an additional major peak at 72% acetonitrile. The HPLC profile was similar from each of the six DS patients. A representative RP-HPLC is shown in Fig. 1. Synthetic peptides Aβ1–42, Aβ1–40, and Aβ17–42 were also run in this HPLC system. It was found that Aβ1–40 eluted at 37–40% acetonitrile, Aβ1–42 at 39–43%, and Aβ17–42 at 72% acetonitrile. Application of the partially purified preamyloid directly onto the C4-RP column gave a higher yield but with slightly more contaminants than when the material was first applied to the Superose 12 column. All the Aβ-containing fractions were subjected to NH₂-terminal amino acid sequencing and mass spectrometry.

NH₂-terminal Edman degradation sequencing of the preamyloid fraction eluting at 72% acetonitrile yielded the following sequence: LVFFAEDVGS, corresponding to residues 17–26 of Aβ. The LDMS of this fraction showed a major peak of 2577.2 (Fig. 2A, the peak is labeled 1). The accuracy of obtained mass is estimated to be ±0.5 mass unit. Over 30 shots were averaged for each spectrum, with over five spectra being acquired for each sample. The expected mass of Aβ17–42 is 2577.1 (see Table I). When synthetic Aβ17–42 was subjected to LDMS for comparison a peak was obtained at 2577.1 ± 0.1 mass units. In addition two minor peaks were seen and are labeled 2 and 3 in Fig. 2A. Peak 2 has a mass of 2463.8. This corresponds to Aβ18–42 which has an expected mass of 2463.9. It is not surprising, given the difference in sensitivity between NH₂-terminal sequencing and LDMS, that we were unable to obtain the sequence of this minor peptide. This peak labeled 2 was obtained from each of the six DS cerebellar P-135 pellets on LDMS. The third minor peak, labeled 3 in Fig. 2A, has a mass of 2336.8. This very small peak was seen in three out of the six DS cerebellar extractions. Peak 3 may correspond to Aβ15–36, which has an expected mass of 2336.8. NH₂-terminal sequencing of the Aβ peptides eluting at 37–43% acetonitrile yielded the following sequences: DAEFRHDSGYEVH, SGYEVH, FRHDSG, and AEFR, corresponding to residues 1–13, 8–13, 4–9, and 2–5 of Aβ. The lag corrected yields in picomoles of each amino acid during Edman degradation sequencing of this pool of Aβ peptides are shown in Table II. This fraction was also subject to LDMS, which is shown in Fig. 2B.
FIG. 2. A, the mass obtained by LDMS of the DS preamyloid extraction of the P-135 pellet, eluting at 72% acetonitrile on RP-HPLC. Each of the six DS cerebellar extractions were subject to LDMS with similar results. The accuracy of the mass spectrum is ±0.5 mass unit. The major peak, labeled 1, shows a mass of 2577.2, corresponding to Aβ17–42 that has an expected mass of 2577.1. In addition, two minor peaks were identified. Peak 2 has a mass of 2463.8. This corresponds to Aβ18–42 that has an expected mass of 2463.9. This peak was obtained from each of the six DS cerebellar P-135 pellets on LDMS. A third minor peak, labeled 3, has a mass of 2336.8. This very small peak was seen in three out of the six DS cerebellar extractions. Peak 3 may correspond to Aβ15–36 that has an expected mass of 2336.8. B, the mass spectrum obtained from the Aβ peptides eluting at 37–43% acetonitrile on RP-HPLC (see Fig. 1). The major peak is labeled 1 and has an observed mass of 4541.7, corresponding to Aβ1–42 that has a calculated mass of 4541.1. The observed and calculated masses of the other peaks are given in Table I.

The Table I

| Aβ peptides | Obs. mass | Calc. mass | Yield |
|-------------|-----------|------------|-------|
| 17–42 (peak 1, Fig. 2A) | 2577.2 | 2577.1 | 70 |
| 1–42 (peak 1, Fig. 2B) | 4541.7 | 4541.1 | 12 |
| 8–42 (peak 5, Fig. 2B) | 3643.4 | 3643.2 | 5 |
| 4–42 (peak 4, Fig. 2B) | 4199.4 | 4198.8 | 5 |
| 2–42 (peak 2, Fig. 2B) | 4398.1 | 4399 | 4 |
| 1–40 (peak 3, Fig. 2B) | 4329 | 4329.9 | 4 |

The peak labeled 1 in this figure had an observed mass of 4541.7, which corresponds to Aβ1–42 that has an expected mass of 4541.1. Peak 2 had an observed mass of 4398.1, corresponding to Aβ2–42 that has a calculated mass of 4399. Peak 3 had an observed mass of 4329, corresponding to Aβ1–40 that has a calculated mass of 4329.9. Peak 4 had an observed mass of 4199.4, corresponding to Aβ4–42 that has a calculated mass of 4198.8. Peak 5 had an observed mass of 3643.4, corresponding to Aβ8–42 that has a calculated mass of 3643.2. These observed and calculated masses are also shown in Table I. The approximate yield for each of the Aβ peptides from the P-135 pellets is also given in Table I. This yield was calculated in the following manner. The ratio of Aβ17–42, eluting at 72% acetonitrile on RP-HPLC, to the other Aβ peptides eluting at 37–44% acetonitrile was determined by integration of the area defined by the chromatographic peaks. This gave a ratio of 7.3 (Aβ17–42/other Aβ peptides). The percentage yield of the other Aβ peptides was estimated by comparing the size of their peaks obtained on LDMS, which is shown in Fig. 2B. These calculations assume that the losses of each of the different Aβ peptides during our purification procedure are approximately the same. From each 20 g of DS cerebellar tissue about 100 μg of Aβ17–42 was obtained. As expected, Aβ peptides were not detected in the preamyloid extraction of the control cerebellum, which did not have Aβ immunoreactive lesions.

Cerebellar Vessel Amyloid Extraction—After the partially purified cerebellar vessel amyloid was applied to the RP-HPLC column, Aβ peptides eluted at 37–41% acetonitrile. No peak was present at 72% acetonitrile. Amino-terminal sequencing gave a major sequence of DAEFRHDSG, corresponding to residues 1 through 9 of Aβ and a minor sequence of AEFR, corresponding to residues 2 through 5 of Aβ. LDMS of these fractions showed two major peaks of 4330.2, corresponding to Aβ1–40 (expected mass of 4329.9) and 4398.3 corresponding to Aβ2–42 (expected mass of 4399.0). In addition, two minor LDMS peaks of 4345.3, corresponding to oxidized Aβ1–40 (expected mass of 4344.9) and 4510.0, corresponding to Aβ1–42 (expected mass of 4514.1) were obtained. No mass was obtained corresponding to Aβ17–42 in the leptomeningeal amyloid preparation.

Immunohistochemistry of DS Cerebellums—The DS cerebellum of all six cases was immunoreacted with 4G8, 6E10, BC40, and BC42 antibodies (Fig. 3). 4G8 recognizes the epitope Aβ17–24; 6E10 recognizes Aβ1–17; BC40 recognizes the carboxyl end of Aβ1–40, and BC42 recognizes the carboxyl end of Aβ1–42 (38, 44). None of the DS cerebellar parenchymal Aβ immunoreactive deposits was Congo red-positive; however, three of the cerebellums contained leptomeningeal vessel deposits which were Congo red-positive. 4G8 and BC42 immunoreacted with all the diffuse deposits of Aβ (Fig. 3, A and B). 6E10 and BC40 immunolabeled the parenchymal diffuse deposits much less or not at all (Fig. 3, C and D). This immunolabeling is consistent with our biochemical findings that the major peptide of the diffuse deposits is Aβ17–42. In three of the patients’ cerebellar sections, Aβ deposits were also present in the leptomeningeal vessels. These were immunodetected equally by 6E10 and 4G8, while BC40 labeled the vessels more intensely than BC42 (data not shown), consistent with prior reports and our biochemical studies that the major Aβ peptides in vessel amyloid deposits extend to residue 40 but also contain some peptides extending to residue 42 (32, 34, 44, 48).

Neuritic Plaque Amyloid Extraction—Gel filtration of partially purified amyloid from the frontal cortex of three of the six cases yielded a major peak corresponding to a molecular mass of less than 10 kDa. When this was run on the reverse-phase HPLC C4 column, a major peak eluted at 39–44% acetonitrile; in addition, a very small peak was noted at 72% acetonitrile. Western blotting of the Aβ fractions eluting from 39–44% acetonitrile with 4G8 showed 3–4 kDa bands. A 2.5-kDa band was present in the fraction eluting at 72% acetonitrile. Western blotting of the Aβ fractions eluting from 39–44% acetonitrile with 4G8 showed 3–4 kDa bands. A 2.5-kDa band was present in the fraction eluting at 72% acetonitrile, as well as higher molecular mass aggregates of Aβ (Fig. 4A, lane 1). 6E10 revealed a 4-kDa band in the fractions eluting from 39 to 44% acetonitrile (not shown), while no Aβ 6E10 immunoreactive band was noted in the fraction eluting at 72% acetonitrile (Fig. 4A, lane 3). This is consistent with the amino-terminal
epitope of Aβ1–17 being absent in the Aβ peptides eluting at 72% acetonitrile. NH₂-terminal Edman degradation of the Aβ eluting from 39–44% acetonitrile yielded Aβ sequences starting at residues 1, 4, and 8. The sequences obtained were DAEFRHDSGYE, FRHDSG, and SGYEV. Similar amino-terminal heterogeneity is well characterized in neuritic plaque amyloid (23, 27, 49). No sequence was obtained for the small peak eluting at 72% acetonitrile; we were unable to sequence the Aβ17–42 peptide due to its small quantity in this extraction.

LDMS of the Aβ peptide immunoreactive with 4G8, eluting at 72% acetonitrile, showed a peak of 2577.2 corresponding to Aβ17–42 (expected mass of 2577.1) (Fig. 4B). In addition, a second peak was present of 2593.1, suggesting a substantial portion of the Aβ17–42 was oxidized (oxidation adds 16 mass units) (Fig. 4B). The accuracy of the LDMS measurement is approximately 0.5 mass unit. Extensive amino-terminal heterogeneity was identified. Additional smaller mass spectrometry

### TABLE II

| Amino acid sequence data of DS cerebellar preamyloid eluting on RP-HPLC at 37–43% acetonitrile from cycle 1 to 13 |
|---|
| Cycle | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Aβ1–42/40 | Asp | Ala | Glu | Phe | Arg | His | Asp | Ser | Gly | Tyr | Glu | Val | His |
| | 11.9 | 24.2 | 15.7 | 39.0 | 21.0 | 10.8 | 20.8 | 9.9 | 24.0 | 16.5 | 15 | 18 | 4.3 |
| Aβ4–42 | Phe | Arg | His | Asp | Ser | Gly | | | | | | | |
| | 12.0 | 2.9 | 1.5 | 4 | 12 | 5.8 |
| Aβ8–42 | Ser | Gly | Tyr | Gly | Val | His |
| | 5.8 | 7.5 | 4.6 | 6.1 | 11 | 10.8 |
| Aβ2–42 | Ala | Glu | Phe | Arg |
| | 12.0 | 2.0 | 3.5 | 3.7 |

**Fig. 3. Immunohistochemical studies of DS cerebellar preamyloid deposits.** A, B, C, and D are sequential sections from a 62-year-old DS patient. In panel A the section was immunoreacted with 4G8 (recognizes Aβ17–24) (38). Panel B was immunoreacted with BC42 (recognizes the carboxyl end of Aβ42) (44). Panel C was immunoreacted with 6E10 (recognizes Aβ1–17) (38). Panel D was immunoreacted with BC40 (recognizes the carboxyl end of Aβ40) (44). 4G8 and BC42 immunoreact equally with the extensive preamyloid deposits in the molecular layer of the cerebellum (see arrow), although BC42 has a higher background staining since it is a polyclonal antibody. Both 6E10 and BC40 give little or no immunoreactivity, consistent with Aβ17–42 being the major constituent of these lesions.
peaks were also obtained corresponding to formylated derivatives of these Aβ peptides. The peak labeled 4 in Fig. 4C has a mass of 4309, which corresponds to 3–42 where the glutamate at the amino terminus is dehydrated to form pyroglutamate (Aβ3–42p). The calculated mass of Aβ3–42p is 4309.9, and the presence of this peptide is well documented in neuritic plaques (25, 50). Each of the other identified Aβ peptides in Fig. 4C has also been previously reported as a component of neuritic plaque amyloid (23, 26, 27).
biochemical studies show that the presence of A residues 16–17 and tase at the A amyloid-like fibrils that are Congo red-positive; however, it were present on the edge of A chemical studies in DS patients (16, 37, 52). In addition, our posits. The latter is consistent with previous immunohisto-

vitro

genic,” because it involved cleavage within the A dipexin II (2, 53). This pathway was initially call “nonamyloido-

ger. Alterationsof p3 in Down Syndrome Preamyloid

composition of AD preamyloid in the same location will be similar. Our studies have confirmed and extended previous biochemical work from AD brains and the aged canine model of AD, showing that a major Aβ species in preamyloid is Aβ17–42 (33, 34). This finding was corroborated by our immunohistochemical studies which showed little immunoreaction with antibodies specific to the Aβ40 COOH terminus or antibodies to amino-terminal epitopes of Aβ, whereas antibodies to Aβ17–24 and Aβ42 were strongly positive on cerebellar preamyloid de-

posit. The latter is consistent with previous immunohisto-

chemical studies in DS patients (16, 37, 52). In addition, our in vitro studies have clearly demonstrated that Aβ17–42 can form amyloid-like fibrils that are Congo red-positive; however, it does so much less well than either Aβ1–40 or Aβ1–42. Under the conditions we used, this highly hydrophobic peptide tended to form amorphous aggregates. This correlates well with the known, largely nonfibrillar nature of preamyloid (10, 11) and may, in part, explain the lack of clear clinical signs of cerebral dysfunction associated with preamyloid lesions. Aβ17–42 or p3 arises form βPP by the combined actions of α-secretase at residues 16–17 and γ-secretase at the carboxyl end of Aβ, whereas Aβ1–42 is derived by the combined actions of β-secretase at the Aβ amino terminus and γ-secretase (2, 53). The α-secretase pathway of βPP processing is known to give rise to a larger, 90–100-kDa amino fragment of βPP known as protease nexin II (2, 53). This pathway was initially call “nonamyloidogenic,” because it involved cleavage within the Aβ domain, precluding the formation of Aβ1–40/42. Our studies clearly document that this pathway is not necessarily nonamyloidogenic. Alterations of βPP processing which favor the production of p3 versus other Aβ peptides may not necessarily benefit AD patients because this peptide appears to be the major constituent of at least some initial Aβ preamyloid deposits.

Several in vitro peptide and immunohistochemical studies have suggested that the deposition of Aβ peptides extending to residue 42 is a critical factor in plaque formation (48, 54–56). The normal sAβ peptides which are found in most biological fluids extends mainly to residue 40, with Aβ42 being a minor component (57–61). The extra two residues of isoleucine and alanine render the peptide more hydrophobic and, at least in vitro, Aβ1–42 is more fibrillogenic than Aβ1–40, leading to the suggestion that Aβ42 “seeds” fibril growth (55). However, our biochemical studies show that the presence of Aβ42 peptides can not be the only factor in vivo which determines the devel-

Fig. 5. In vitro studies on Aβ17–42 fibrillogenesis. A, thioflavin 

T assay comparing synthetic Aβ1–42, 1–40, and 17–42. The fluorescence of these peptides in solution in the presence of thioflavin T is proportional to amyloid-like fibril formation (45–47). The black bars show the fluorescence at time 0. The hatched bars show the fluorescence after 5 days of incubation. B, negatively stained electron micrograph of synthetic Aβ17–42. The scale bar in the top corner corresponds to 100 nm. Aβ17–42 mainly formed amorphous aggregates, similar to preamyloid in vivo; however, on the edge of some of these aggregates amyloid-like fibrils were noted, as shown. C, the characteristic apple-green birifringence of amyloid produced by synthetic Aβ17–42 after 5 days of incubation, as seen in some foci (magnification × 200).
amenti of neuritic plaques. Other factors, as well as impaired clearance and the local tissue concentration of Aβ42 peptides are likely to play a role. We have previously shown that in the aged canine model of AD, where extensive preamyloid deposits occur but neuritic plaques are rare, Aβ17–42 and other Aβ42 peptides are the major components of the preamyloid (34). Similarly, in DS cerebellums where parenchymal amyloid deposits rarely occur, Aβ42 is also the major Aβ component. DS cerebellar preamyloid deposits have been documented to persist for many years without fibrillization, with DS cerebellar preamyloid being observed in patients as young as 21 years (15). There is also substantial in vitro evidence to suggest that other regions of Aβ are important determinants of fibrillization. Aβ residues 16–20 have been shown to serve as a binding sequence during Aβ polymerization and fibril formation (62). Amino acid substitutions at residue 18 as well as at positions 17–20 in vitro have been shown to dramatically alter fibril formation (63, 64). Furthermore, it has been suggested that in hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D; a familial variant of AD), the substitution of glutamine for glutamic acid at residue 22 increases the fibrillogenic potential and hence amyloid formation within cerebral vessels (65, 66). HCHWA-D is characterized by extensive cerebrovascular amyloid deposition, as well as parenchymal diffuse plaques (67). Current studies suggest that these preamyloid deposits do not progress onto neuritic plaques. Interestingly, recent immunohistochemical studies using Aβ42 carboxy epitope specific antibodies have documented that these preamyloid deposits are also composed mainly of Aβ42 peptides (68, 69). Each of these studies indicate that other factors, in addition to the deposition of Aβ42, are involved in plaque formation.

There are at least three possible pathways: preamyloid may be a required initial stage of neuritic plaque formation, neuritic plaques and preamyloid may have separate origins, or preamyloid may develop into neuritic plaques only in certain brain regions and under the influence of other factors. We favor the last possibility. We found a substantial difference in the amount of Aβ17–42 contained in preamyloid versus neuritic plaques, suggesting separate origins for these two lesions. However, this difference in Aβ17–42 content may in part be related to a very low yield of this peptide from the neuritic plaque extraction; it is possible that, during our neuritic plaque purification procedure, Aβ17–42 is subject to greater losses. Using different biochemical techniques, the yield of Aβ17–42 from neuritic plaques may be higher. Furthermore recent immunohistochemical studies of cortical preamyloid deposits with antibodies that are specific for p3 have suggested that the Aβ17–42 content in preamyloid lesions may not be as high as we have found biochemically in the DS cerebellum (70). An alternative possibility is that the degradation and/or clearance of Aβ17–42 from the brain may be greater than that of other Aβ peptides, which subsequentially are deposited during the maturation of preamyloid lesions. That preamyloid is the precursor to neuritic plaques is suggested by the earlier appearance of preamyloid during normal aging and in DS patients. Furthermore, in transgenic mice in which βPP is overexpressed (71), diffuse deposits of Aβ immunoreactivity presage the development of Congo red-positive, fibrillar amyloid deposits. Additional evidence linking preamyloid to neuritic plaques comes from recent studies in aged individuals with extensive diffuse plaques, who have shown evidence of presymptomatic or very mild cognitive dysfunction, suggesting that preamyloid deposition is not necessarily benign, but represents unrecognized early symptomatic AD (72). Biochemically we found that approximately 30% of Aβ peptides in preamyloid deposits are similar to the major peptides in neuritic plaques in the same patients, suggesting a relationship between these lesions. Interestingly, a substantial portion of the Aβ17–42 which we identified in neuritic plaques was oxidized, while the Aβ17–42 in the cerebellar preamyloid deposits of the same patient and all the other patients showed little evidence of this post-translational modification. Oxidation is a well recognized alteration of Aβ peptides that was first recognized in vascular AD amyloid, and it has been shown in vitro to increase Aβ aggregation (31, 73). It is likely that this post-translational modification will alter the fibrillogenicity of Aβ17–42, and our preliminary studies using oxidized synthetic Aβ17–42 suggest that fibrillogenicity is promoted, based on a thioflavin T assay. Compatible with this, the Aβ17–42 from our DS cerebellar extraction ran on SDS-PAGE mainly as a monomer (see Fig. 1, inset, lane 1), while the Aβ17–42 form the neuritic plaque ran mainly as higher molecular weight aggregates (Fig. 4A, lane 1). In addition to the oxidation of Aβ17–42 in neuritic plaques, the other Aβ peptides showed greater amino-terminal heterogeneity and evidence for modifications such as pyroglutamate formation, as seen in the LDMS shown in Fig. 4C. Such amino-terminal heterogeneity has been shown in vitro to increase fibrillogenicity (51). Hence, it is possible that under some conditions and in particular brain locations, Aβ17–42 and the other Aβ peptides found in preamyloid are modified, promoting the development of neuritic plaques.

It has also been suggested that preamyloid is a separate pathway of cerebral Aβ deposition which is largely unrelated to neuritic plaque formation (17). This viewpoint suggests that diffuse plaque formation is a nonpathological age-related phenomenon that is unassociated with dementia; hence the production of Aβ17–42 would be viewed as a benign event. Our preamyloid extraction could be used to support this view since the major Aβ peptide constituent was found to be distinct from what has previously been reported in neuritic plaques. If preamyloid is the precursor to neuritic plaques, then plaque maturation would require the selective removal of Aβ17–42. However, our in vitro studies of Aβ17–42 fibrillogenesis have shown that this peptide can form amyloid-like fibrils, albeit at a reduced rate compared to Aβ1–42. Therefore, the deposition of p3 may be associated with the development of subsequent pathological changes.

A number of different factors play a role in neuritic plaque formation besides the presence or absence of Aβ17–42. Further identification of the different biochemical composition of preamyloid and neuritic plaques, as well as study of the evolution of these lesions in animal models of AD are needed before it will be clear whether preamyloid is the precursor to neuritic plaques. Post-translational modifications such as oxidation, isomerization, or racemization of Aβ17–42 and Aβ1–42, as well as the presence of other truncated, altered Aβ peptides are likely to augment fibrillogenesis in vitro (27, 51, 73, 74). Furthermore AD is recognized to be a polygenic disorder, with linkage to the presenilin-1, presenilin-2, apolipoprotein E, and βPP genes on chromosomes 14, 1, 19, and 21 respectively, as well as other uncharacterized loci (75, 76). The neuropathological features of each of these subtypes of AD are similar or identical. The presence of the apoE4 allele is recognized to be a major risk factor for late-onset AD (77). We and others have shown by biochemical methods that apoE is present in senile plaques complexed to Aβ and that apoE in vitro modulates Aβ fibrillogenesis (47, 78–81). The role of the homologous presenilin-1 and -2 gene products in AD is unknown, but a carboxyl epitope of presenilin has also been found to be present in some amyloid plaques (82). Other factors, such as proteoglycans, heparin binding-growth associated molecule (HB-GAM), and α1-antichymotrypsin, and the presence of heavy metals may also play a role (83–86). Multiple lines of evidence suggest that
the presence of Aβ42 is not by itself sufficient for neuritic plaque formation. Hence, neuritic plaque formation is a multipletime step process occurring over many years, involving the participation of multiple factors and different proteins.

Acknowledgment—We thank Dr. H. Yamaguchi for providing antibodies BC40 and BC42.

REFERENCES

1. Wisniewski, T., Ghio, J., and Frangione, B. (1994) Neurobiol. Aging 15, 143–152
2. Selkoe, D. J. (1993) Annu. Rev. Cell Biol. 10, 371–403
3. Wisniewski, H. M., Wegiel, J., and Popovich, E. R. (1994) Dev. Brain Dysfunct. 7, 330–339
4. Gaigl, B., Tagliavini, F., Linolli, G., Bouras, C., Frigerio, L., Frangione, B., and Bugiani, O. (1998) Neurosci. Lett. 272, 233–238
5. Mann, D. M. (1999) Neurobiol. Aging 20, 397–399
6. Motte, J. and Williams, R. S. (1989) Acta Neuroangiologia 19, 31–113
7. Yamaguchi, H., Nakazato, Y., Shoji, M., Takatama, M., and Hirai, S. (1991) J. Neurosci. 11, 330–339
8. Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M., and Harigaya, Y. (1988) Acta Neuruphysiology 77, 113–119
9. Tagliavini, F., Gaigl, B., Frangione, B., and Bugiani, O. (1998) Neurosci. Lett. 255, 53–54
10. Yamazaki, T., Yamaguchi, H., Okamoto, K., and Hirai, S. (1991) Acta Neuroangiologia 19, 845–854
11. Yamaguchi, H., Nakazato, Y., Shoji, M., Takatama, M., and Hirai, S. (1991) Acta Neuruphysiology 82, 13–18
12. Crystal, H. A., Dickson, D. W., Sliwinski, M. J., Lipton, R. B., Grober, E., Marks-Nelsen, H., and Antis, P. (1993) Ann. Neurol. 34, 566–573
13. Davies, P., Duyskaerts, C., Beyreuther, K., Masters, C. L., Peitte, F., and Selkoe, D. J. (1993) Neurobiology 18, 1685–1700
14. Delaere, P., Duyskaerts, C., Beyreuther, K., Masters, C. L., Peitte, F., and Hauw, J. J. (1990) Neurosci. Lett. 116, 87–93
15. Kida, E., Choi-Miura, N. H., and Wisniewski, K. E. (1995) Brain Res. 685, 211–216
16. Lemere, C. A., Blustnajst, K. Y., Yamaguchi, H., Scattoni, M., and Selkoe, D. J. (1996) Neurobiol. Dis. 3, 16–32
17. Wisniewski, H. M., Wegiel, J., and Kotula, L. (1996) Neuruphysiology Appl. Neuruphysiology 22, 3–11
18. Kosik, K. S., and Coleman, P. (1992) Neurobiol. Aging 13, 535–627
19. Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. (1994) J. Neurosci. 14, 1598–1603
20. Leib, G., Takeda, T., and Shoji, M. (1988) Cell 57, 31–37
21. Ueda, K., Fukui, Y., and Kageyama, H. (1994) Brain Res. 639, 240–244
22. Lorenzo, A. and Yankner, B. A. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 12643–12647
23. Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 4245–4249
24. Selkoe, D. J., Abraham, C. R., Podlisny, M. B., and Duffy, L. K. (1986) J. Neurochem. 46, 1820–1834
25. Mori, H., Takio, K., Ogawara, M., and Selkoe, D. J. (1992) J. Biol. Chem. 267, 5092–5096
26. Miller, D. L., Papayannopoulos, I. A., Styles, J., Bobin, S. A., Lin, Y. Y., and Selkoe, D. J. (1993) J. Neurochem. 61, 4745–4749
27. Roher, A. E., Lownard, J. D., Clarke, S., Selkoe, D. J., and Selkoe, D. J. (1996) J. Biol. Chem. 271, 100–111
28. Glenner, G. G., and Wong, C. W. (1984) Arch. Biochem. Biophys. 238, 647–664
29. Gorry, P. D., Man, S. M., and Li, S. (1993) J. Biol. Chem. 268, 2403–2409
30. Takeda, T., Tani, M., Shoji, M., and Shoji, M. (1988) Cell 57, 31–37
31. Tsai, S. H., and Reis, E. M. (1993) J. Biol. Chem. 268, 2403–2409
32. Selkoe, D. J., Abraham, C. R., Podlisny, M. B., and Duffy, L. K. (1986) Brain Res. 370, 240–244
33. Selkoe, D. J., Abraham, C. R., Podlisny, M. B., and Duffy, L. K. (1986) Brain Res. 370, 240–244
34. Wisniewski, T., Lalowski, M., Bobik, M., Russell, M., Strosznajder, J., and Frangione, B. (1991) Neuroscience 336, 1–11
35. Kim, K. S., Miller, D. L., Sapienza, V. J., Chen, C. M. J., Bai, C., Grundke-Iqbal, I., Currie, J., and Wisniewski, H. M. (1990) Neurobiol. Dis. 2, 127–137
36. Tabaton, M., Nunzi, M. G., Xue, R., Usiak, M., Autilio-Gambetti, L., and Frangione, B. (1994) Biochem. Biophys. Res. Commun. 200, 1508–1603
37. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neuron 14, 921–932
38. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neuron 14, 921–932
39. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neuron 14, 921–932
40. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neuron 14, 921–932
41. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neurobiol. Dis. 2, 127–137
42. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neurobiol. Dis. 2, 127–137
43. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neurobiol. Dis. 2, 127–137
44. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neurobiol. Dis. 2, 127–137
45. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neurobiol. Dis. 2, 127–137
46. Kida, E., Wisniewski, K. E., and Wisniewski, H. M. (1995) Neurobiol. Dis. 2, 127–137