Correlation Between Aβx-40–, Aβx-42–, and Aβx-43–Containing Amyloid Plaques and Cognitive Decline

S. Parvathy, PhD; Peter Davies, PhD; Vahram Haroutunian, PhD; Dushyant P. Purohit, MD; Kenneth L. Davis, MD; Richard C. Mohs, PhD; Helen Park; Thomas M. Moran, PhD; Joseph Y. Chan, MS; Joseph D. Buxbaum, PhD

Context: Accumulation of senile plaques containing amyloid β (Aβ)-protein is a pathologic hallmark of Alzheimer disease. Amyloid β-peptide is heterogeneous, with carboxyterminal variants ending at residues Val40 (Aβx-40), Ala42 (Aβx-42), or Thr43 (Aβx-43). The relative importance of each of these variants in dementia or cognitive decline remains unclear.

Objective: To study whether Aβ deposition correlates with dementia and occurs at the earliest signs of cognitive decline.

Design, Setting, and Patients: Postmortem cross-sectional study comparing the deposition of Aβ variants in the prefrontal cortex of 79 nursing home residents having no, questionable, mild, moderate, or severe dementia.

Main Outcome Measures: Levels of staining of Aβ-peptides ending at amino acid 40, 42, or 43 in the frontal cortex, as a function of Clinical Dementia Rating score.

Results: There were significant deposits of all 3 Aβ species that strongly correlated with cognitive decline. Furthermore, deposition of Aβx-42 and Aβx-43 occurred very early in the disease process before there could be a diagnosis of Alzheimer disease. Levels of deposited Aβx-43 appeared surprisingly high given the low amounts synthesized.

Conclusions: These data indicate that Aβx-42 and Aβx-43 are important species associated with early disease progression and suggest that the physicochemical properties of the Aβ species may be a major determinant in amyloid deposition. The results support an important role for Aβ in mediating initial pathogenic events in Alzheimer disease dementia and reinforce that treatment strategies targeting the formation, accumulation, or cytotoxic effects of Aβ should be pursued.

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ALZHEIMER disease (AD) is characterized histopathologically by the intraneuronal accumulation of paired helical filaments composed of abnormal τ proteins and extracellular deposits of an amyloid β (Aβ)-peptide in plaques.1,2 Amyloid β is proteolytically derived from a large integral membrane protein, the amyloid precursor protein (APP). Amyloid precursor protein can be processed by at least 3 secretases, namely, α-, β-, and γ-secretases. β-Secretase initiates Aβ generation by cleaving APP after methionine 671 (using APP770 numbering), creating an approximate 12-kd membrane retained carboxy terminal fragment.3 The 12-kd fragment may then undergo γ-secretase cleavage within the hydrophobic transmembrane domain at valine 710, alanine 712, or threonine 713 to release the 40, 42, or 43 residue Aβ-peptides, respectively.4 Four groups have recently identified a candidate for β-secretase, known as BACE (for β-site APP-cleaving enzyme).5-8 There has been increasing evidence that presenilin might be a functional γ-secretase or it might act as a cofactor for γ-secretase.9,10 Both β-secretase and γ-secretase are targets for drug discovery.

Immunocytochemical studies using antibodies against Aβ have established that Aβ is deposited in cored plaques, which include well-defined amyloid cores, and in diffuse plaques, which are poorly circumscribed immunoreactive lesions showing minimal neuritic change.11,12 Different Aβ species have been identified in the AD-affected brain. Mass spectrometric studies on Aβ derived from AD-affected brains identified that the carboxy terminal of Aβ typically ends at residues 40, 42, or 43.13,14 In vitro experiments have clearly demonstrated that Aβx-42 and Aβx-43 polymerize faster than Aβx-40, suggesting that the carboxy terminal of Aβ determines the aggregation potential, and there-
SUBJECTS, MATERIALS, AND METHODS

SUBJECTS

Seventy-nine subjects who had been residents of the Jewish Home and Hospital in Manhattan and the Bronx, NY, were included in the study. These subjects were selected from a larger group of 278 consecutive autopsies performed between November 11, 1986, and April 27, 1997, after excluding patients with neuropathologic lesions other than those of AD (eg, lesions of Pick disease, diffuse Lewy body disease, Parkinson disease, stroke, multi-infarct dementia, and severe cerebrovascular disease). A multistep approach was used to assign Clinical Dementia Rating (CDR) scores based on cognitive and functional status during the last 6 months of life. A consensus CDR score was obtained following a careful review of all information contained within each patient’s medical record, including admitting diagnosis, nurses’ notes, social work records, results of psychiatric and neuropsychologic consultations, medication histories, results of mental status testing, and results of laboratory studies. The methods used for subject selection and cognitive and neuropathologic assessment have been described previously in detail.26,28,29

Because one of our aims was to identify whether there is a relationship between amyloid plaque deposition and clinical dementia, 5 groups of subjects were formed, consisting of cases falling into CDR score categories of 0.0 (no dementia), 0.5 (questionable dementia), 1.0 (mild dementia), 2.0 (moderate dementia), and 4.0 or 5.0 (severe or terminal dementia). The demographic data for the final selection of the subjects is given in Table 1. Twenty of the subjects had 1 apolipoprotein E allele of the ε4 type and 4 subjects had 2. The cases with 1 allele were evenly distributed within the CDR groups. Of those 4 cases with 2 alleles, 1 case was classified as CDR 0.0; 1, CDR 1.0; and 2, CDR 5.0.

PREPARATION OF MONOCLONAL ANTIBODIES

The carboxy terminal–specific antibodies were prepared by injecting mice with the peptides corresponding to the last 6 amino acids of Aβx-40, Aβx-42, or Aβx-43, which had been coupled to keyhole limpet hemocyanin through the addition of an amino-terminal cysteine. Hybridomas that tested positive by enzyme-linked immunosorbent assay (ELISA) were then further characterized as detailed in the “Results” section. The Aβx-40, Aβx-42, and Aβx-43–specific antibodies were identified as AβC40, AβC42, and AβC43, respectively.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed in sections of frontal cortex (5 µm) that were cut from paraffin-embedded tissue blocks, deparaffinized with xylene, and rehydrated using 100%, 95%, and 80% ethanol. Following rehydration, the endogenous horseradish-peroxidase activity was quenched by incubating in hydrogen peroxide, and formic acid was used to enhance immunoreactivity. Sections were blocked for 1 hour at room temperature in blocking buffer (5% nonfat milk in 10mM Tris, 140mM sodium chloride [NaCl], pH 7.4), followed by an overnight incubation with primary antibody (anti–Aβx40 [AβC40] at a dilution of 1:250, anti–Aβx42 [AβC42] at a dilution of 1:2000, and anti–Aβx43 [AβC43] at a dilution of 1:500) at 4°C in blocking solution. Sections were washed 5 times in Tris-buffered saline (10mM Tris, 140mM NaCl, pH 7.4), incubated in secondary antibody (biotin-conjugated, isotype-specific goat anti–mouse IgG1 or IgG2B at 1:500 dilution; Southern Biotechnology Associates, Birmingham, Ala) for 2 hours, washed again, and incubated with streptavidine conjugated–horseradish-peroxidase for 1 hour at room temperature. After reaction with substrate (diaminobenzidine) (0.3 mg/mL in 100mM Tris, pH 7.4 with 0.01% hydrogen peroxide), slides were washed for 5 minutes in Tris-buffered saline, dehydrated in 80%, 95%, 100% ethanol and xylene, and counterstained with toluidine blue and cover slipped using synthetic mounting medium (Permount; Fisher Scientific, Morris Plains, NJ).

For double labeling, AβC42 was used with alkaline phosphatase conjugated goat anti–mouse IgG2B (Southern Biotechnology Associates) and the substrate nitroblue tetrazolium chloride (Pierce Chemical Co, Rockford, Ill).

RESULTS

CHARACTERIZATION OF CARBOXY TERMINAL–SPECIFIC ANTI-Aβ ANTIBODIES

Three monoclonal antibodies that specifically detected the different carboxy terminal ends of Aβ–peptide were developed and characterized. The carboxy terminal–specific antibodies were named AβC40, AβC42, and AβC43, recognizing the carboxy terminal of Aβx-40, Aβx-42, and Aβx-43, respectively. The specificity and cross reactivity of the 3 antibodies were examined using dot blots, immunoblotting, and ELISA (Figure 1). Each antibody was specific for the corresponding peptide to which it was prepared.
for 1 hour at room temperature for blue color development. In addition, the dehydroxy step did not include xylene, and counterstaining with toluidine blue was excluded.

IMMUNOELECTROPHORETIC BLOT

Synthetic Aβ1-40, Aβ1-42, and Aβ1-43 (1 µg) were resolved on a 10% to 20% Tris-tricine gel in running buffer (100mM Tris, 100mM tricine, 0.1% sodium dodecyl sulfate, pH 7.4) and transferred to a polyvinylidene difluoride membrane using transfer buffer (20mM Tris, 120mM glycine, 20% methanol) at 30 mA for 1 hour. The polyvinylidene difluoride membrane was blocked in blocking solution (50mM Tris, 150mM NaCl, 0.05% polysorbate [Tween] 20, pH 8.0 containing 5% milk) for 3 hours at room temperature and probed overnight at 4°C with AβC40, AβC42, or AβC43 at a 1:1000 dilution, followed by 1-hour incubation with peroxidase-conjugated anti-mouse antibody at a 1:2000 dilution. Bound antibody was detected using enhanced chemiluminescent system (Amersham Pharmacia Biotech, Piscataway, NJ).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Microtiter plates were coated with the respective synthetic peptides in carbonate-bicarbonate (50mM) buffer, pH 9.6, at amounts ranging from 0 to 1000 ng per well and incubated for 18 hours at 37°C. After blocking with ELISA coupling and wash buffer (phosphate-buffered saline, 0.1% bovine serum albumin, 0.05% polysorbate 20, 0.2% 3-(cyclohexylamino)-1-propanesulphonic acid, 5mM EDTA, 2mM betaine, 0.05% sodium azide) containing 1% casein for 4 hours at room temperature, wells were incubated with antibody at 1 µg/mL for 15 hours. Following washing with ELISA coupling and wash buffer, wells were incubated for 5 hours at room temperature with goat anti-mouse antibody conjugated with biotin, followed by an incubation with streptavidine conjugated–alkaline phosphatase. The substrate AttoPhos (Promega, San Luis Obispo, Calif) was used to generate the fluorescent product.

QUANTIFICATION OF AMYLOID LOAD

Two methods for quantifying amyloid load were used. For cored plaques, the area of cortex occupied by amyloid was determined in a blinded fashion using semiautomated, quantitative computer analysis as previously documented. For each case image from 5 different fields were captured and analyzed. For diffuse plaques, this method could not be reliably used. Instead, the extent of diffuse plaque staining was categorized into 1 of 4 levels (0-3, with 0 reflecting no plaques). To determine the accuracy of this semiquantitative approach, 25 cases (5 cases from each CDR group) were evaluated for cored plaques using both the semiautomated quantitative computer analysis and the semiquantitative approach, and the results compared. In these analyses, the quantitative values correlated well with the semiquantitative rating (r values were 0.81 for AβC40, 0.89 for AβC42, and 0.75 for AβC43). In 12 cases (3 with a CDR 0.0 score, 2 with a CDR 0.5 score, 3 with a CDR 1.0 score, 1 with a CDR 2.0 score, and 3 with CDR score 4.0 or 5.0) that were double labeled for AβX–40 and AβX–42 or AβX–42 and AβX–43, 4 areas were quantified for the number of AβX–40, AβX–42, and AβX–43-containing diffuse and cored plaques.

DATA ANALYSIS

Diffuse and cored amyloid plaque load detected by each of the carboxy terminal–specific antibodies was used as the independent variables for subsequent analyses. The dependent variable consisted of 5 CDR categories. For further exploratory analyses, quantitative plaque counts measured by silver staining, soluble Aβ levels, and phosphorylated τ levels determined by ELISA were used as dependent variables. Spearman rank order correlation was used to calculate the P values. Statistical significance was set at P<.05.

IMMUNOCYTOCHEMISTRY OF FRONTAL CORTEX

Frontal cortical sections (Brodman area 9) of controls and cases with AD were immunostained with the antibodies; the bound antibody was detected using diaminobenzidine substrate. Typical staining patterns of subjects with a CDR 0.0 and 5.0 scores are shown in Figure 2. We distinguished between 2 types of plaques—cored, which have an intensely stained central core with a weakly stained peripheral (halo) region, and diffuse, which do not have a core and the immunoreactivity is uniform over the plaque. AβC40 and AβC42 labeled both cores and the plaque periphery of cored plaques as well as diffuse plaques. AβC43 also labeled both diffuse and cored plaques. However, in cored plaques, we noted that AβC43 essentially only labeled what appeared to be the plaque core, with little or no AβX–43 reactivity in the halos of the cored plaques.

DOUBLE LABELING OF PLAQUE TYPES

To determine whether the plaques identified by each of these antibodies were the same or different, double labeling with AβC42 and AβC40 (Figure 3A-C) or with AβC42 and AβC43 (Figure 3D-F) was carried out. Because the isotypes of AβC40 and AβC43 were identical, double labeling could not be performed with this pair of antibodies. Conditions were developed so that reactivity with AβC42 antibody generated a blue product while reactivity with AβC40 or AβC43 generated a brown product. When double labeling for AβX–40 and AβX–42, most deposits were blue, representing AβX–42. Some deposits were brown, representing AβX–40, while others were purple, indicating colocalization of both AβX–40 and AβX–42.

When double labeling for AβX–42 and AβX–43, most deposits were blue, i.e., were composed of AβX–42. Some cored plaques were very intensely stained purple in the core of the plaque, representing colocalization of AβX–42 and...
Aβx-43. Diffuse plaques were either exclusively blue (Aβx-42) or purple (Aβx-42 and Aβx-43). No plaques were stained only brown, indicating all Aβx-43 plaques contain Aβx-42. In addition, no cored plaques demonstrated significant Aβx-43 reactivity outside of the core (Figure 3).

**CORRELATION OF AMYLOID PLAQUE DEPOSITION WITH DEMENTIA**

Immunostaining was carried out in 79 patients with varying degrees of dementia. The subjects chosen for this study were classified according to their CDR scores; the demographic characteristics of the subjects are given in Table 1. The cohort included subjects without dementia (CDR score 0.0 [n = 16]), those with questionable dementia (CDR score 0.5 [n = 11]), those with mild dementia (CDR score 1.0 [n = 22]), those with moderate dementia (CDR score 2.0 [n = 15]), and those with severe dementia (CDR score 4.0 or 5.0 [n = 15]). Amyloid load was determined in the sections of frontal cortex of these cases for both cored and diffuse plaques. Amyloid load for each antibody as a function of CDR scores is shown in Figure 4. With all 3 antibodies there was a strong correlation between CDR scores and cored plaques (Table 2). In the
case of diffuse plaques, levels of Aβx-42 and Aβx-43 plaques correlated with dementia while levels of Aβx-40 did not. The lack of correlation of diffuse Aβx-40 plaques is not surprising given that levels of these plaques remain relatively unchanged across CDR scores.

In further analysis, correlations were determined for a subset of CDR scores (ie, 0.0-2.0 and 0.5-5.0) (Table 2). The purpose of these analyses was to determine to what degree the correlations observed for amyloid load with CDR score were driven by changes in the extreme CDR groups. In these analyses it was clear that for Aβx-42 and Aβx-43 the correlations observed for CDR 0.0-5.0 were not driven exclusively by changes between controls and cases with dementia or between cases with severe dementia and the rest of the cohort.

RELATIVE LEVELS OF PLAQUE TYPES DURING DISEASE PROGRESSION

The proportion of cored and diffuse plaques containing Aβx-40, Aβx-42, or Aβx-43 was determined from double-labeled sections. Twelve cases were stained for Aβx-40 and Aβx-42 or Aβx-42 and Aβx-43, and the number of cored and diffuse plaques stained for 1 or both antibodies counted in 4 regions. Levels of Aβx-42–reactive plaques were highest (Figure 5), representing more than 50% of the total plaques in both labeling conditions. However, significant levels of Aβx-40– and particularly Aβx-43–reactive plaques were observed as well. All Aβx-43–containing and most of the Aβx-40–containing plaques also contained Aβx-42.

CORRELATION OF AMYLOID PLAQUE DEPOSITION WITH OTHER MARKERS OF DISEASE

In further exploratory analyses, Spearman rank order correlation was performed to find the correlation between amyloid plaque load and silver-stained plaques, soluble Aβ levels, or phosphorylated τ levels. Amyloid load in the frontal cortex correlated with the level of plaques determined by silver staining (r ranging from 0.3889-0.6254; P<.001 in all cases). Soluble Aβ levels measured in the same region by ELISA correlated with the levels of both diffuse and cored plaques (Table 3), with Aβx-40–containing diffuse plaques correlating apparently less well. There was also a correlation between the diffuse and cored plaques and the amount of phosphorylated τ in the frontal cortex measured by the phospho-state–specific antibody MC1 using ELISA, but again correlation with diffuse Aβx-40 plaques was apparently lower (Table 4).

COMMENT

In the current study we have developed specific antibodies for Aβx-40, Aβx-42, and Aβx-43. Staining with these
antibodies identified diffuse and cored plaques reacting with each of these antibodies. The level of diffuse Aβx-40– and Aβx-43–containing plaques increased with increasing disease severity while the levels of diffuse Aβx-40–containing plaques did not. Levels of cored Aβx-40–, Aβx-42–, and Aβx-43–containing plaques increased with disease severity, but there were no cored Aβx-40–containing plaques in early dementia (the areas shown in Figure 4 represent background). Plaque levels correlated with dementia, plaque load determined by silver staining, and the levels of total Aβ and phosphorylated τ as measured by ELISA. Note that the number of subjects with 1 or 2 apolipoprotein E alleles of the type ε4 were too small for any reliable analysis by apolipoprotein E allele genotype.

With double labeling it appears that there are Aβx-40–containing plaques that do not contain Aβx-42. In addition, there appear to be Aβx-42–containing plaques that contain neither Aβx-40 nor Aβx-43. There are plaques containing both Aβx-40 and Aβx-42 as well as plaques containing both Aβx-42 and Aβx-43. We did not detect any Aβx-43–containing plaques that were not reactive for Aβx-42. By the careful examination of double-labeled sections, we were able to estimate the relative amount of each of the plaque species. Approximately 20% of the plaques were reactive for Aβx-40 and 20% of the plaques were reactive for Aβx-43. From a great number of studies with cells in culture, it appears that Aβx-40 is the most common species synthesized, with low levels of Aβx-42 and even lower levels of Aβx-43. These ratios appear to be true in vivo as well. The accumulation of large amounts of Aβx-42–containing plaques and significant levels of Aβx-43–containing plaques appear to be disproportionate to the levels of synthesis of these variants. This suggests that factors aside from the level synthesized are critical for accumulation. One such factor could be the physiochemical properties of the peptides and the increased propensity for the larger variants to aggregate.

**CONCLUSIONS**

In the current study we show that there is significant deposition of all Aβ species that strongly correlate with cognitive decline. In addition, our studies show the correlation of Aβx-43 deposition with dementia in a very large cohort. Furthermore, deposition, particularly of Aβx-42 and Aβx-43, occurs very early in the disease process even
Figure 4. Amyloid load as a function of Clinical Dementia Rating (CDR) score where 0.0 indicates no dementia; 0.5, questionable dementia; 1.0, mild dementia; 2.0, moderate dementia; and 4.0 and 5.0, severe or terminal dementia. Sections were incubated with each of the carboxy terminal-specific antibodies and the bound antibodies were detected using horseradish peroxidase-conjugates and diaminobenzidine substrate. Sections were counterstained with toluidine blue and the amyloid load was determined. Average (±SEM) scores were plotted against the CDR scores. Aβ/H9252C40, Aβ/H9252C42, and Aβ/H9252C43 are antibodies reactive to amyloid βx-40.

Table 2. Correlation Between Clinical Dementia Rating (CDR) Score and Amyloid Plaque Load

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<tr>
<th>Type of Amyloid Plaque</th>
<th>CDR Rating Score*</th>
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<tr>
<td></td>
<td>0.0-5.0 (n = 79)</td>
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<tr>
<td></td>
<td>r</td>
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<tr>
<td>Diffuse 40</td>
<td>0.24</td>
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<tr>
<td>Cored 40</td>
<td>0.31</td>
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<tr>
<td>Diffuse 42</td>
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<tr>
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<td>Cored 43</td>
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*See “Subjects” subsection of “Subjects, Materials, and Methods” section for explanation of rating.

Figure 5. Relative proportion of amyloid β (Aβ) plaque types. The number of cored and diffuse plaques in double-labeled sections stained for either Aβ/H9252C40 and Aβ/H9252C42 (A) or Aβ/H9252C42 and Aβ/H9252C43 (B) antibodies was determined and is given as a percentage of total plaques counted in each condition. In each condition, the number of plaques containing either 1 or 2 Aβ variants was determined. Aβ/C40, Aβ/C42, and Aβ/C43 are antibodies reactive to Aβ x-40.
before there is a diagnosis of AD. These data also suggest that the physiochemical properties of the Aβ species are a major determinant in amyloid deposition. Altogether, these data support Aβ accumulation as an important component of disease progression and suggest that reducing Aβ levels in the brain may be beneficial in AD.

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Corresponding author: Joseph D. Buxbaum, PhD, Laboratory of Molecular Neurosurgery, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1230, New York, NY 10029 (e-mail: buxbaj01@doc.mssm.edu).

REFERENCES


Errors in Figures 1 and 5 and Accompanying Legends, for a Sentence in Legends to Figures 2 Through 4, and in Lead Author’s Name in Affiliation Footnote. In the Original Contribution by Parvathy et al titled “Correlation Between Aβx-40, Aβx-42, and Aβx-43–Containing Amyloid Plaques and Cognitive Decline,” published in the December issue of ARCHIVES (2001;58:2025-2032), **Figure 1** and **Figure 5** had errors in the body of the figure and its accompanying legend. We are reproducing the correct figures and legends below. In the legends to Figures 1 through 5, the sentence that reads “AβC40, AβC42, and AβC43 are antibodies reactive to amyloid βx-40.” should have read: “AβC40, AβC42, and AβC43 are antibodies reactive to amyloid βx-40, amyloid βx-42, and amyloid βx-43, respectively. In addition, the second mention of the lead author’s name was misspelled in the affiliation footnote at the bottom left on page 2025. It should have read Dr Parvathy. The ARCHIVES regrets these errors.

**Figure 1.** Characterization of antibodies. AβC40, AβC42, and AβC43 are antibodies reactive to amyloid βx-40, amyloid βx-42, and amyloid βx-43, respectively. Specificity of antibodies AβC40, AβC42, and AβC43 was determined by dot blotting (A), immunoblotting (B), and enzyme-linked immunosorbent assay (C). Synthetic Aβ1-40, Aβ1-42, or Aβ1-43 was either spotted on a nitrocellulose membrane (A) or electrophoresed on a Tris-tricine gel and transferred to a nitrocellulose membrane (B). The membranes were probed with the indicated antibodies and detected using horseradish peroxidase–conjugated secondary antibody with enhanced chemiluminescent reagent. In part C indicated amounts of synthetic Aβ1-40, Aβ1-42, and Aβ1-43 peptides were coated onto enzyme-linked immunosorbent assay plates and incubated with antibody. Bound antibody was detected using a calorimetric assay.

**Figure 5.** Relative proportion of amyloid β (Aβ) plaque types. The number of cored and diffuse plaques in double-labeled sections stained with either AβC40 and AβC42 (A) or AβC42 and AβC43 (B) antibodies was determined and is given as a percentage of total plaques counted in each condition. In each condition, the number of plaques containing either 1 or 2 Aβ variants was determined. AβC40, AβC42, and AβC43 are antibodies reactive to amyloid βx-40, amyloid βx-42, and amyloid βx-43, respectively.