

Truncated beta-amyloid peptide species in pre-clinical Alzheimer's disease as new targets for the vaccination approach

Nicolas Sergeant,* Stepanie Bombois,* Antoine Ghestem,* Hervé Drobecq,† Vesna Kostanjevecki,‡ Carla Missiaen,‡ Annick Wattez,* Jean-Phillipe David,* Eugene Vanmechelen,‡ Christian Sergheraert† and André Delacourte*

*INSERM U422, Groupe VCDN, Equipe Protéomique, Lille, France

†UMR 8525, CNRS – Institut Pasteur de Lille – Université de Lille 2 – Institut de Biologie de Lille – Département Synthèse Structure et Fonction des Biomolécules, Lille, France

‡Innogenetics NV, Zwijnaarde, Belgium

Abstract

Vaccination against human beta-amyloid peptide (A β) has been shown to remove the amyloid burden produced in transgenic mice overexpressing the mutated human amyloid precursor protein (APP) gene. For human beings, the efficiency of this therapeutic strategy has to take into account the specificities of human amyloid, especially at the early stages of 'sporadic' Alzheimer's disease (AD). A β 40/42 were previously quantified in tissues from our well-established brain bank, including non-demented individuals with both mild amyloid and tau pathologies, hence corresponding to the earliest stages of Alzheimer pathology. Herein, we have adapted a proteomic method combined with western blotting and mass spectrometry for the characterization of insoluble A β extracted in pure-formic acid. We demonstrated that

amino-truncated A β species represented more than 60% of all A β species, not only in full blown AD, but also, and more interestingly, at the earliest stage of Alzheimer pathology. At this stage, A β oligomers were exclusively made of A β -42 species, most of them being amino-truncated. Thus, our results strongly suggest that amino-truncated A β -42 species are instrumental in the amyloidosis process. In conclusion, a vaccine specifically targeting these pathological amino-truncated species of A β -42 are likely to be doubly beneficial, by inducing the production of specific antibodies against pathological A β products that are, in addition, involved in the early and basic mechanisms of amyloidosis in humans.

Keywords: Alzheimer's disease, beta-amyloid peptide, proteomic, diagnostic, vaccination, physiopathology.
J. Neurochem. (2003) **85**, 1581–1591.

Alzheimer's disease (AD) is a progressive dementing disorder characterized by the conjunction of two degenerative processes: tau pathology and amyloid pathology. Both degenerative processes are found in familial autosomal dominant AD and in 'sporadic' AD (non-mendelian). However, familial autosomal dominant AD due to mutations on amyloid precursor protein (APP) or presenilin genes are extremely rare (Campion *et al.* 1999) and furthermore, less is known about the physiopathology of 'sporadic' AD.

The cognitive decline that characterizes AD is well correlated to the cortical spreading of tau pathology (Delacourte *et al.* 1999). However, the amyloid pathology is in close relationship with the aetiology of the disease. We have recently shown a strong correlation between the amyloid pathology and the dynamic of progression of tau pathology in cortical brain areas, hence demonstrating a synergy

between tau and amyloid pathologies in 'sporadic' AD (Delacourte *et al.* 2002; Sergeant *et al.* 2002). It suggests overall that the amyloid pathology could trigger the spreading of tau pathology, thus leading to AD. This hypothesis is also illustrated in transgenic mouse models, in which the tau

Received February 18, 2003; 2003; accepted March 14, 2003.

Address correspondence and reprints requests to André Delacourte, INSERM U422, Groupe VCDN, Equipe Protéomique 1, place de Verdun 59045 Lille cedex, France.

E-mail: delacourte@lille.inserm.fr

Abbreviations used: A β , beta-amyloid peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; MALDI-TOF, Matrix-Assisted Desorption and Ionization–Time-of-Flight; MS, mass spectrometry; nano-LC, nanoliquid chromatography; pI, isoelectric point; Q-TOF, Quadrupole–Time-of-Flight.

pathology is enhanced by the induction or coexistence of an amyloid pathology (Gotz *et al.* 2001; Lewis *et al.* 2001).

A possible treatment of AD would be to reduce the progression of tau pathology by targeting directly the amyloid pathology. Such treatment is already tested and opens new perspectives in AD therapy. The vaccination of transgenic mice producing amyloid plaques by targeting actively or passively the human beta-amyloid peptide (A β), the major component of amyloid deposits, leads to a reduced burden of amyloid deposition (Schenk *et al.* 1999; 2001; DeMattos *et al.* 2001) and restores cognitive function (Dodart *et al.* 2002; Janus *et al.* 2000). In human, vaccination leads to antibodies against amyloid deposits (Hock *et al.* 2002). Physiologically, A β is a 37–42 amino acid peptide that derives from the catabolism of a type I transmembrane glycoprotein, named amyloid precursor protein (APP) (Wiltfang *et al.* 2002). APP is ubiquitously expressed in all tissues, and in large amounts in the central nervous system. In transgenic mice, vaccination aimed to target the human A β sequence that is different from the A β sequence of mouse (R to G at position 5, Y to F at position 10 and H to R at position 13 of A β sequence, respectively). Therefore, the risk of autoimmunity response for transgenic mice is reduced.

Moreover, identified A β peptides extracted from the amyloid deposits of Alzheimer brain are composed of heterogeneous species that include the full-length A β 40/42 peptides and additional shorter carboxy-terminal A β peptides as well as amino-terminal truncated species (Kalback *et al.* 2002). However, little is known about which species of A β peptides are present at the very first steps of amyloid deposition, even before the appearance of clinical symptoms of dementia. We have investigated and characterized the A β peptides involved in the first steps of amyloid deposition. Those early deposits were found in non-demented individuals that had developed evidences of Alzheimer pathogenesis. They had both an amyloid and tau pathology demonstrated at the neuropathological and biochemical level (Delacourte *et al.* 2002) but were clinically asymptomatic. They were thus defined to belong to the infra-clinical or pre-clinical stages of Alzheimer pathology (Delacourte *et al.* 1999, 2002). The aggregated species of A β were directly extracted from the brain tissue using formic acid and two-dimensional gel electrophoresis analysis was adapted to formic acid-treated samples. A β species were identified using mass spectrometry (MS) and confirmed using multiple immunological tools against A β peptides. Our data demonstrated for the first time that the major A β species found to aggregate at the earliest stages of Alzheimer pathology were amino-truncated and, as previously established (Delacourte *et al.* 2002), they all corresponded to A β -42 peptides. Moreover, by using a western blotting approach to determine the ratio of full-length A β -42 upon all A β x-42 species, these amino-truncated species of A β were shown to represent more

than 60% of all A β species in non-demented as well as in confirmed AD individuals.

In conclusion, the present work is important at a physiological point of view, as well as essential in developing new diagnostic and therapeutic strategies, including vaccination. Among the already existing vaccination approach, a unique opportunity to develop further vaccination strategy would be to target non-physiological species of A β peptides that are, in addition, specifically found in the most prevalent 'sporadic' form of Alzheimer's pathology, and involved in the very first steps of amyloid deposition.

Materials and methods

Patients

All of the brain autopsy materials used in the present study were from our brain bank. Ten non-familial (sporadic) AD cases and eight non-demented cases have been extensively described (Delacourte *et al.* 1999, 2002). The five AD cases fulfilled the neuropathological diagnostic criteria of AD as established by the National Institute on Aging and the Reagan Institute Working Group on diagnostic criteria for the neuropathological assessment of Alzheimer disease (Hyman and Trojanowski 1997). The five non-demented cases correspond to neurofibrillary stages I and II [similar to the tau pathology stages 1–6, according to Delacourte *et al.* (1999, 2002)] and stage B and C for amyloid deposition, according to neuropathological staging of Braak and Braak (1991). Postmortem intervals ranged from 5 to 61 h (mean of 30 ± 10 h). At autopsy, one brain hemisphere was deep-frozen for biochemical analysis and the other hemisphere was formalin-fixed for both neuropathological examination and histochemistry.

Antibodies

The amino-terminal region of A β peptides was analysed with WO2 (Abeta GmbH, Heidelberg, Germany) and 6E10 (Senetek PLC, Napa, CA, USA) antibodies. These recognize the amino-acid sequences 5–8 [N-ter (5–8)] and 4–13 [N-ter (4–13)], respectively (Cherny *et al.* 1999; Terai *et al.* 2001). A β x-42 species were studied using 21F12 antibody (Athena Neurosciences, Worcester, MA, USA) and ADA42 antiserum. A β x-40 species were analysed with our antiserum ADA40 (Delacourte *et al.* 2002). Monoclonal antibody 3D6 (Athena Neurosciences) was used to detect the full-length A β 1-x species. It is specific to the free amino-terminal region (sequence 1–5) of A β , necessarily including the first aspartate residue (Johnson-Wood *et al.* 1997; Walsh *et al.* 2000, 2002; Bard *et al.* 2003).

Formic acid isolation of A β aggregates and two-dimensional gel electrophoresis

The brain tissue samples from the temporal, frontal, parietal and occipital cortex were processed as already described (Delacourte *et al.* 2002). Formic acid (Prolabo, Fontenay s/Bois, France) extracted brain tissue homogenate (100 μ L) was evaporated under nitrogen and dissolved in 400 μ L of two-dimensional electrophoresis lysis buffer [7 M urea, 2 M thiourea, 4% (v/v) Triton X-100, 20 mM dithiothreitol and 0.6% (v/v) Pharyaltes^a pH 3–10]. The sample was sonicated. Immobilized pH gradient strip pH 4–7

(Bio-Rad, Marnes la Coquette, France) was equilibrated with the sample for 15 h (Sergeant *et al.* 2002). Isoelectrofocusing was performed using the Protean IEF cell following the manufacturer's (Bio-Rad) instructions. Polypeptides were resolved on Tris-Tricine gels as described previously (Sergeant *et al.* 2002). The gels were transferred for immunodetection using the Multiphor transfer unit (Amersham-Pharmacia Biotech, Saclay, France), according to the manufacturer's instructions, or they were stained with Coomassie Brilliant Blue G250 (Sigma, Saint Quentin Fallavier, France) for MS analyses. Isoelectric points, molecular weights and the percentage of volume (mean of spot intensities over the area of the spot) of each A β peptide variant were determined using Melanie III two-dimensional gel analysis software (Genebio, Geneva, Switzerland). Calibration of the location of full-length A β peptide 40 or 42 was performed by adding 10 ng of synthetic A β -40 and A β -42 (Bachem AG, Bubendorf, Switzerland) to a formic acid brain tissue homogenate (not shown).

Mass spectrometry characterization

Coomassie Blue stained polypeptides spots were cut into 1 mm² gel pieces and washed twice with 50% (v/v) CH₃CN in 25 mM Tris-HCl pH 9. Gel pieces were dehydrated in a Speed-Vac and then in-gel digested overnight with 10 ng of Endoproteinase Lys-C (EC 3.4.21.19, Roche Molecular Biochemicals, Meylan, France) in 3 μ L of Tris-HCl pH 9. The resulting digested peptides were recovered in 10 μ L of 50% (v/v) CH₃CN and 1% trifluoroacetic acid (TFA). Samples were then prepared by the dry-droplet method. One mL of the peptide mixture was mixed with freshly dissolved β -cyano-4-hydroxycinnamic acid 0.5 mL [5 mg/mL in 50% (v/v) CH₃CN and 0.1% trifluoroacetic acid], and spotted on the sample plate. The dry spot was then washed with 5 μ L of 0.1% trifluoroacetic acid. Mass spectrometry was performed with a Matrix-Assisted Desorption and Ionization-Time-of-Flight (MALDI-TOF) Voyager-DE-STR (Applied Biosystems, Palo Alto, CA, USA) set to the following parameters: positive mode, reflector, voltage 20 kV, grid 61%, delayed extraction 90 ns, low mass gate 500 amu. The laser energy required to desorb/ionize samples was kept to a low value, compatible with a good signal/noise ratio. Spectra were calibrated externally using the [M + H]⁺ monoisotopic ions from trypsinized lysozyme. The characterization of two two-dimensional spots, that were digested with trypsin (EC 3.4.21.4, Promega) (spot 1 and spot 2, Table 2), was performed by nanoliquid chromatography (nano-LC/MS) coupled with a Quadrupole-Time-of-Flight (Q-TOF) mass spectrometer (Micromass Limited, Manchester, UK). Digested peptides were sequenced in tandem MS mode.

Western blot quantification of the proportion of full-length A β

Tris-Tricine gels and electrophoresis of formic acid-treated brain tissue homogenates was performed as already described (Delacourte *et al.* 2002). In addition to brain tissue homogenates, 10 and 20 ng of synthetic A β 1–42 (Bachem) were loaded. Two Tris-Tricine gels were processed at the same time. Following semi-dry protein transfer, one membrane was incubated with 3D6 antibody. The second membrane was incubated with 21F12 antibody. Multiple exposures were performed, ECL films were digitized using Labscan and ImageMaster 1D Elite Software (Pharmacia), the amount of A β detected was obtained in the linear range between the two quantities

of synthetic A β loaded. Thus, corresponding amounts of A β 1-x and A β x-42 were determined following 3D6 and 21F12 labelling, respectively. The quantification was only performed for monomeric A β species. The proportion of full-length A β was established by dividing the amount of A β 1-x by that of A β x-42.

Results

Two-dimensional characterization of aggregates species of A β of Alzheimer's disease brain tissue

Aggregates consisting of A β in Alzheimer brains were soluble only in pure formic acid, as previously described (Delacourte *et al.* 2002; Kalback *et al.* 2002). Such formic acid-soluble A β species were resolved by two-dimensional electrophoresis and detected using a panel of specific A β antibodies, including A β x-40 and A β x-42 antibodies (Fig. 1, A β -40 and A β -42 panels), as well as amino-terminal specific A β antibodies [Fig. 1, N-ter (5–8) and N-ter (4–13) panels]. Further characterizations were performed by MS analysis of the two-dimensional spots with identical locations to A β peptides detected with A β antibodies (Fig. 1, Coomassie Blue panel). This characterization of A β species was performed on brain tissues of AD cases in which the total amount of formic acid-soluble A β (above 500 μ g/g of brain tissue; as established in Delacourte *et al.* 2002) enabled subsequent MS analysis. Ten detected A β spots were stained also by Coomassie Blue (Fig. 1, Coomassie Blue panel). An in-gel digestion with Endoprotease-Lys C was performed and the resulting peptides were analysed by MS. The peptide masses of nine spots positively matched with A β sequence following MALDI-TOF/MS analysis (Fig. 2 and Table 1). In all spots analysed, the peptide mass 1325.6 was observed, corresponding to the A β sequence 17–28. The peptides corresponding to the sequence 29–40/42 were not detected using MALDI-TOF. Using nano-LC/MS, the carboxy-terminal A β 29–40/42 peptides were detected in two-dimensional spots 1 and 2 following in-gel digestion with trypsin (Table 2). No additional shorter carboxy-terminal A β 1-x were observed following Q-TOF MS analysis (Table 2). Altogether, two-dimensional gel electrophoresis followed either by western blotting with specific A β antibodies or MS analysis enabled the characterization of the human brain tissue A β species solubilized with formic acid. Only A β monomers were characterized using MS. The immunodetected dimeric species at 8 kDa (Fig. 1) were not stained with Coomassie, hence not analysed by MS.

According to the isoelectric points (pI) of the spots analysed, the labelling with N-terminal and C-terminal antibodies and MS analyses, the following spots were characterized. The results are summarized on Table 1. Spot 1 and 2 corresponded to full-length A β -40/42 peptides (Table 1 and Table 2). Spots 3–7 and 9–10 corresponded to amino-terminal truncated and post-translationally modified

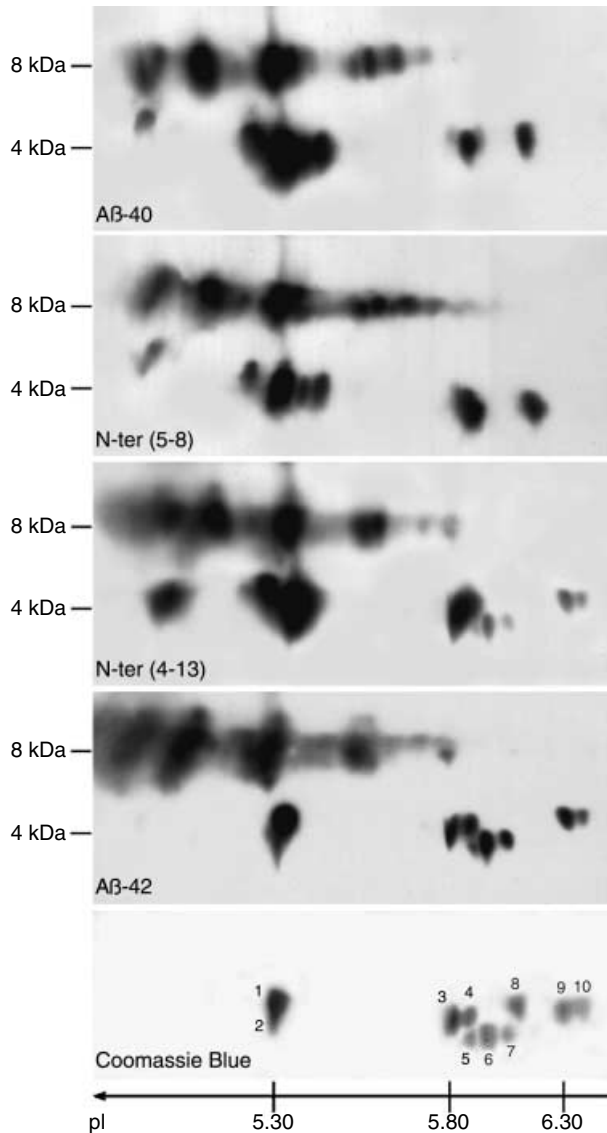


Fig. 1 Two-dimensional electrophoretic analysis of A β species in Alzheimer's disease. A β aggregates solubilized with formic acid were resolved by two-dimensional gel electrophoresis. A β monomers (4 kDa) and dimers (8 kDa) were labelled with antibodies WO2 and 6E10 against the amino-terminal region of A β [N-ter(5–8) and N-ter(4–13) panels, respectively]. The carboxy-terminal tails of Ab-42 and Ab-40 were detected with 21F12 and ADA40, respectively (Ab-42 and Ab-40 panels). The major Ab species recovered with our extraction method were stained with Coomassie Blue and 10 spots were subsequently analysed by MS (Table 1). The results presented were obtained from the AD patient showing the largest quantity of amyloid deposits. The pI and the Ab spots used for MS analysis are indicated. Note Coomassie Blue does not stained dimeric species of A β .

variants of A β . The major truncated variants consisted of A β starting at amino-acid positions 2–5 and 8–10. The post-translational modifications characterized were pyroglutamylation at position 3 and methylation (Table 1). The mass

spectra are presented for spots 4, 5, 7, 9 and 10 (Fig. 2). Interestingly, spots 6, 7, 9 and 10 contained similar A β variants but were separated as two spots, suggesting that an as yet unidentified modification was present.

The proportion of each A β species, following the immunolabelling with 21F12 (A β -42) antibody, was determined using Melanie III software and expressed as the percentage of volume (Table 1). The full-length A β peptides represented $37 \pm 7\%$ of all A β species. Taken together, the truncated variants thus accounted for more than 60%, among which $17 \pm 7\%$ and $20 \pm 4\%$ corresponded to truncated species starting at residues 4, 5 and 8, 9 and 10, respectively (Table 1). Surprisingly, the two-dimensional pattern of A β -40 as revealed by the ADA40 antiserum completely overlapped the pattern obtained with WO2, which detects the amino-terminal region of A β . These results further suggest that the identified truncated A β derived from the A β -42 and not from A β -40 species.

Characterization of A β species in the first amyloid deposits in non-demented individuals

The A β species that aggregate in the first steps of amyloidosis were investigated in the brain tissue of non-demented patients. These patients were previously shown to have amyloid deposits and tau pathology at the neuropathological level and the biochemical level, but they had no cognitive deficits. The three cases analysed by two-dimensional western blotting had traces or low amounts of aggregated A β (below 50 $\mu\text{g/g}$ of brain tissue) (Delacourte *et al.* 2002). A β aggregates were exclusively comprised of A β -42 species (Fig. 3, A β -40 and A β -42 panels). Antibodies against the amino-terminal region of A β only detected a single spot corresponding to the full-length A β peptide [Fig. 3, N-ter (5–8) and N-ter (4–13) panels]. The A β -42 specific antibody 21F12 labelled in addition spots 4, 5, 6 and 10 (Fig. 3, A β -42 panel) as well as dimers. The A β -42 species in the brain of non-demented patients, as in Alzheimer brain, corresponded to amino-terminal truncated variants starting at position 3-pyroglutamyl, 4, 5, 8 and 9. The lack of staining of A β dimers with amino-terminal A β antibodies WO2 and 6E10 on two-dimensional western blots [Fig. 3, N-ter (5–8) and N-ter (4–13) panels] and WO2 antibody on one-dimensional western blot [Fig. 4, N-ter (5–8) panel] suggested that A β dimers were exclusively composed of amino-terminally truncated A β -42 species (Figs 3 and 4, A β -42 panels).

Proportion of A β full-length in formic acid-treated brain tissue of non-demented and AD individuals

The quantification of the proportion of full-length A β peptide was investigated further using 3D6 antibody. This monoclonal antibody is specific for the free amino-terminal region of A β including the first aspartic residue (Johnson-Wood *et al.* 1997; Walsh *et al.* 2000). This specificity was verified using our two-dimensional gel electrophoresis method (Fig. 5).

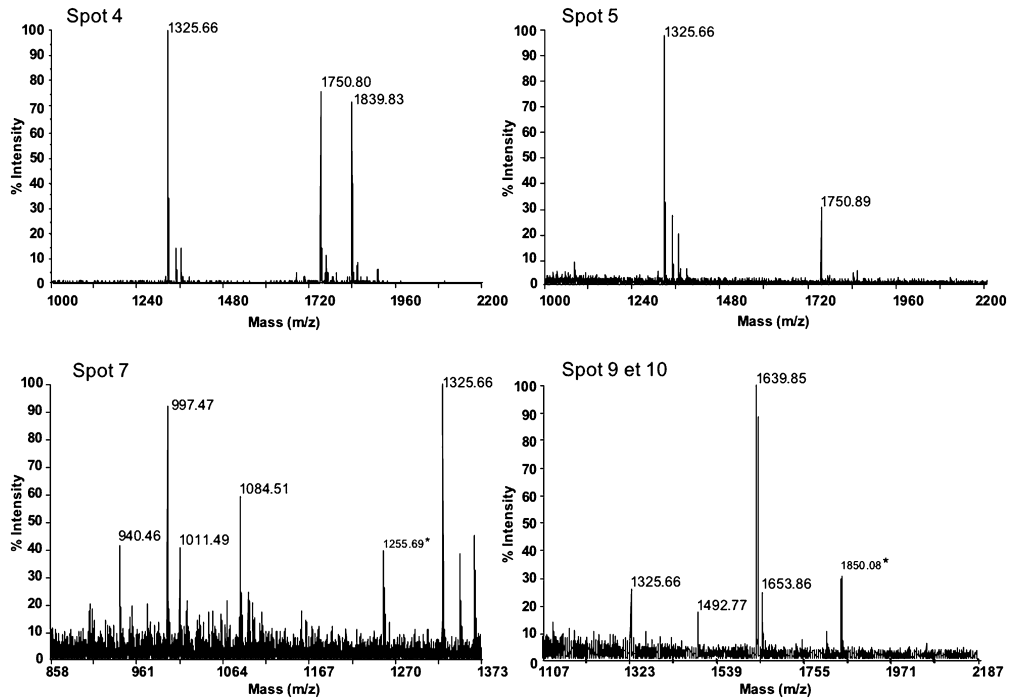


Fig. 2 Mass spectrometric analysis of two-dimensional gels A β spots. Formic acid brain tissue homogenate was resolved by two-dimensional gel electrophoresis and stained with Coomassie blue G250. Spots with identical pI and apparent molecular weights than A β x-42 peptides detected by 21F12 antibody were excised and in-gel digested with Endo-Lys C. The resulting peptides were analysed by MALDI-

TOF MS. The mass spectra are presented for spots 4, 5, 7 and 9 and 10 and masses are indicated at the top of the peaks. Results are summarized in Table 1. Masses not related to A β are indicated by an asterisk (*). Note that the mass of 1325.66 is observed on each spectra and corresponds to the A β sequence 17–28.

Among all A β species detected in an AD individual (Fig. 5a, A β x-42 panel), 3D6 immunolabelling revealed only the spot corresponding to full-length A β and little dimeric species at 8 kDa [Fig. 5a, N-ter (1–5)].

To establish the ratio of full-length A β peptides, formic acid-treated brain tissue homogenates were loaded on two Tris-Tricine gels, known quantities of synthetic A β 1–42 peptide was loaded in parallel. One membrane was incubated with 3D6 antibody, the second membrane with 21F12 antibody (Fig. 5b, A β 1-x and A β x-42 panel, respectively). The signal for the A β monomers (at 4 kDa) was quantified according to the immunoreactivity of each antibody with the none amount of synthetic A β -42 peptide. Thus, the amount of A β quantified following 3D6 or 21F12 labelling corresponded only to the full-length A β 1-x or A β x-42, respectively. By dividing the quantified amounts the ratio of the full-length A β was established.

Thus, additional AD and non-demented cases were analysed. The global amounts of full-length A β was ranging from 75% (Fig. 5b, lane 8) to 5% (Fig. 5b, lane 10), with an average amount of $29\% \pm 16.4\%$ and $32\% \pm 27.1\%$ for AD ($n = 12$) and non-demented individuals ($n = 8$), respectively. Autolysis of A β peptides could occur during the postmortem interval and be responsible for underestimating the proportion of full-length A β -42 peptides. The

postmortem delays of all individuals analysed (AD $n = 12$, non-demented $n = 8$) were compared to the A β ratio calculated, using the non-parametric correlation test of Spearman's range. No correlation was found between postmortem intervals and A β ratio ($Z = -0.127$, $p \leq 0.9$). Interestingly, 3D6 faintly detected A β dimers at 8 kDa dimers of A β when compared to that detected with 21F12 (Fig. 5b, panel A β 1-x and A β x-42). Overall, both two-dimensional gel analyses and one-dimensional western-blotting strongly suggest that amino-truncated A β x-42 altogether are the major species found in amyloid deposits, even in non-demented individuals with both tau and amyloid pathology.

Discussion

We describe here the aggregated A β species that seed the first amyloid deposits in non-demented individuals using a precise proteomic approach adapted to the analysis of A β solubilized in formic acid-treated human brain tissue.

A proteomic approach adapted to the qualitative study of aggregate A β peptides of human brain tissue

Two-dimensional gel electrophoresis is well adapted for the analysis of soluble proteins. This technique has never been

Spot	Relative proportion	Theoretical pI	Observed pI	A β identity proposed	Theoretical mass [M + H ⁺]	Observed mass [M + H ⁺]
1	25 \pm 5%	5.31	5.3	1–16	1954.879	1954.875
				1–16 + CH ₃	1968.905	1968.863
2	12 \pm 2%	5.31	5.3	1–16	1954.879	1954.875
				1–16 + CH ₃	1968.905	1968.863
3	10 \pm 3%	5.78	5.8	2–16	1839.852	1839.851
				2–16 + CH ₃	1853.878	1853.854
				(3–16)	1768.815	1768.804
4	9 \pm 3%	6.27	5.9	PyrE 3–16	1751.784	1750.790
				(2–16)	1839.852	1839.833
5	7 \pm 3%	6.27	6.3	PyrE 3–16	1751.784	1750.881
6	12 \pm 4%	5.96	6.0	8–16	1084.517	1084.557
				9–16	997.485	998.525
7	8 \pm 3%	5.96	6.1	8–16	1084.517	1084.518
				9–16	997.485	997.477
				9–16 + CH ₃	1011.500	1011.496
				10–16	940.463	940.460
9 and 10	17 \pm 7%	6.27	6.3	4–16	1639.772	1639.848
				4–16 + CH ₃	1653.798	1653.859
				5–16	1492.704	1492.770

Methylated fragments are indicated with a CH₃. PyrE corresponds to a pyroglutamyl residue at the amino-terminus of the identified fragment. The peptide corresponding to amino acid sequence 17–28 of A β was found in all spots (not shown). The relative proportion corresponds to the quantification of spots following western blotting with 21F12, using Melanie III software (mean \pm SD %, $n = 8$).

Peak	A β proposed identity	Theoretical mass [M + H ⁺]	Observed mass [M + H ⁺]	Peptide sequence confirmed
1	1–5	637.29	–	
2	6–16	1336.60	–	
3	17–28	1325.67	1325.53	LVFFAEDVGSNK
	29–42 + ox	1285.76	1285.64	
4	29–40	1085.63	1085.48	GAIIGLMVGGVV + ox
	29–40 + ox	1101.62	1101.49	

The sequences indicated were obtained by switching from MS to tandem MS mode. The oxidized Met₃₅ is indicated by ox.

described for the analysis of aggregated A β peptides found in amyloid deposits that are extremely insoluble. Herein, we have adapted this method to the analysis of A β peptides that are recovered following formic acid treatment of human brain tissue. These A β peptides were recently demonstrated to correspond to aggregated A β peptides found in amyloid deposits (Delacourte *et al.* 2002). However, the usefulness of this proteomic approach necessitates to compare our results to that already published using different approaches (Kumar-Singh *et al.* 2000; Kalback *et al.* 2002; Pype *et al.* 2003). The A β species thus characterized was performed only in fully developed AD individuals (Kalback *et al.* 2002). Therefore, we first characterized the A β peptides found in large amounts in fully developed Alzheimer individuals. A β species were characterized according to three parameters:

(i) two-dimensional gel characteristics (pI and molecular weights), (ii) immunostaining with amino-terminal specific (WO2, 6E10, 3D6) and carboxy-terminal specific A β antibodies (ADA40, 21F12), (iii) MS analysis of the two-dimensional spots corresponding to that stained with A β antibodies. Altogether, these parameters enable to unambiguously identify the A β peptides from formic acid brain tissue homogenates.

Our proteomic analysis gives very similar results to that recently described in AD using a combined LC/MS analysis (Kalback *et al.* 2002). Nine major A β species that consist mainly of A β -42 peptides, including full-length, amino-truncated and post-translationally modified peptides, were characterized. The carboxy-terminal identity of A β peptides was essentially established using A β -40 and A β -42

Table 1 Digested endo-Lys-C peptides of A β species in Alzheimer's disease

Table 2 Analysis of tryptic peptides spot 1 and 2 peptides using nano LC-MS and nano-LC-MS/MS

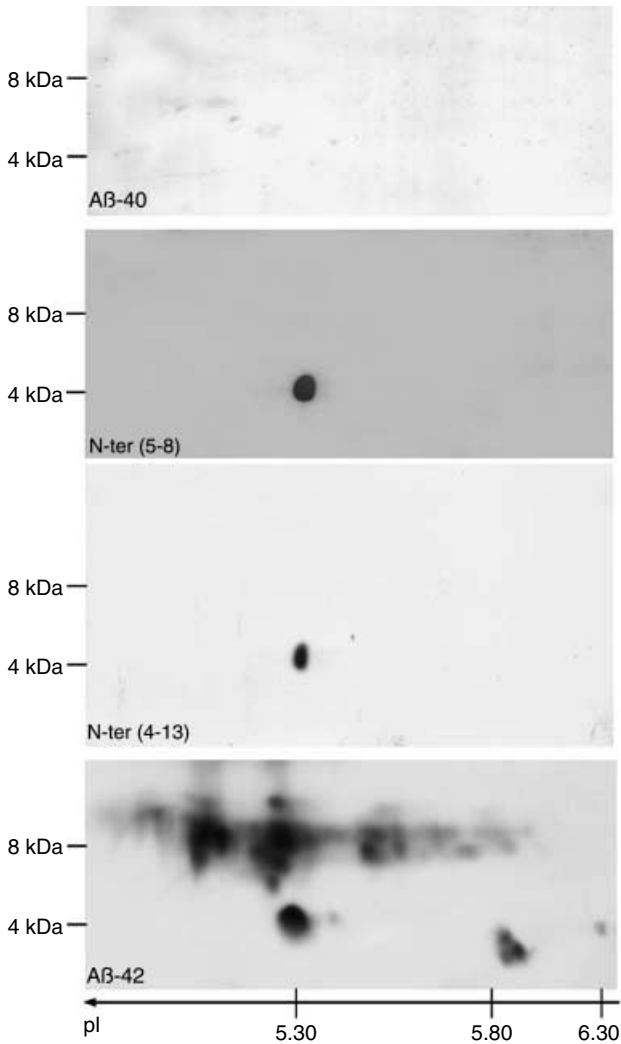


Fig. 3 Two-dimensional analysis of aggregated Aβ species in a non-demented case. Formic acid-solubilized Aβ species derived from the brain tissue of a non-demented patient were resolved by two-dimensional gel electrophoresis. Aβ-40 species are not detected with our ADA40 antiserum. 21F12 detects both Aβ-42 monomers (4 kDa staining) and dimers (8 kDa staining). Both WO2 [panel N-ter (5–8)] and 6E10 [panel N-ter (4–13)] antibodies stained a single Aβ peptide spot with pI of 5.30.

antibodies. Using MALDI-TOF/MS, the carboxy-terminal fragments of Aβ (29–40/42 or shorter) were not detected and hence suggested not to be ionized or desorbed. These Aβ 1-x have the same pI as Aβ-40/42 and would therefore be located at the same position as Aβ 1–40/42 on two-dimensional gels. Aβ 1–40 and Aβ 1–42 were fully characterized using nano-LC/MS in a tandem mass mode, but shorter Aβ 1-x were not evidenced, hence suggesting that these shorter Aβ 1-x are under the limit of detection, and thus, probably not the major Aβ peptides recovered in formic acid brain tissue homogenates of AD individuals. Shorter Aβ 1-x have been

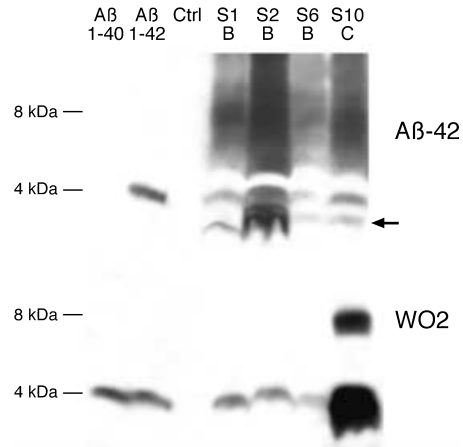


Fig. 4 Dimers of Aβ are essentially composed of amino-truncated Aβ peptides. The brain tissue of a control individual (Ctrl, S0), three non-demented cases (S1, S2 and S6) and one AD case (S10) were homogenized in formic acid. According to the nomenclatures defined by Delacourte *et al.* (2002) and Braak and Braak (1991), the stages of tau pathology (S0 to S10) and the amyloid staging classification (B or C) are indicated at the top of the lanes, respectively. Ten ng of Aβ peptides 1–40 and 1–42 solubilized in formic acid were loaded in parallel (first and second lane). Aβ x-42 was identified with 21F12 (panel Aβ-42) and the amino-terminal region with WO2 (WO2 panel). Molecular weights are indicated on the left and an arrow indicates the amino-truncated variants labelled by 21F12. Note that the same AD case was used as for two-dimensional electrophoretic analysis and MS.

characterized in human or transgenic mouse brain tissue (Kalback *et al.* 2002; Pype *et al.* 2003). In transgenic mice the most insoluble brain material was shown to comprise predominantly Aβ x-40, Aβ x-42 and, to a lesser extent, Aβ 1–38 (Pype *et al.* 2003). Shorter Aβ 1-x were detected in the soluble mouse brain extracts. By similarity to our data, we can speculate that the absence of detection of shorter Aβ 1-x peptides confirms the hypothesis that the Aβ species recovered following formic acid treatment of the brain correspond to aggregated Aβ species, as already described (Delacourte *et al.* 2002).

Aβ-40 peptides were observed in lower amounts than Aβ-42 and they were found late, rather than systematically, in the course of AD (Delacourte *et al.* 2002). We observed that in AD patients, there are fewer species of amino-truncated Aβ-40 than amino-truncated species of Aβ-42, suggesting that the former arise from a distinct mechanism (e.g. inflammation, vascular deposits, etc.). Also, full-length Aβ-40 peptide is physiologically produced and it can therefore be hypothesized that Aβ-40 coaggregates with Aβ-42 deposits. Taken together, our results suggest that aggregation of Aβ-40 species is a late event of Alzheimer physiopathology. This hypothesis is also supported by observation of the amyloidosis process in the brain of individuals affected by Down's

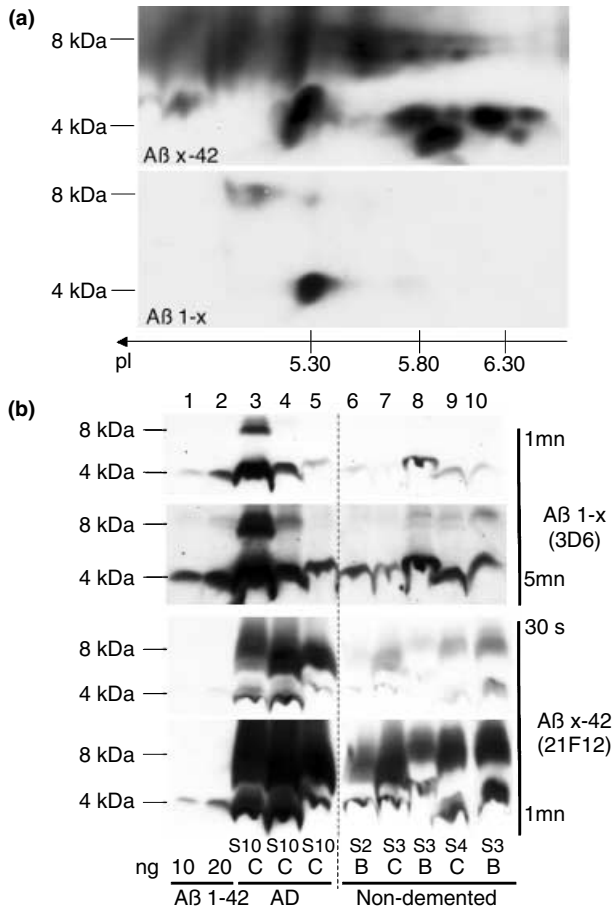


Fig. 5 Two-dimensional analysis of the specificity of 3D6 antibody and comparison of A β 1-x and A β x-42 in formic acid brain tissue homogenates of non-demented and AD individuals. (a) Brain tissue of an AD individual (S9 of tau pathology, stage C of amyloidosis, according to Delacourte *et al.* 2002 and Braak and Braak 1991, respectively) was homogenized in pure formic acid and resolved twice by two-dimensional gel electrophoresis. A β x-42 (upper panel) or A β 1-x (lower panel) peptides were detected with 21F12 or 3D6 monoclonal antibodies, respectively. Molecular weights are indicated on the left and pI are indicated on the x-axis. (b) Formic acid-treated brain tissue of three AD individuals and five non-demented individuals were loaded on the same Tris-Tricine gel. The experiment was performed twice, A β 1-x was detected with 3D6 and A β x-42 was detected with 21F12. In parallel, 10 ng and 20 ng of synthetic A β 1-42 were loaded (lanes 1 and 2). Two times of exposure are presented (times of exposure are indicated). The tau pathology and amyloidosis stage is indicated under each lane. Note that the same AD individual was analysed by two-dimensional electrophoresis (a) and one-dimensional western-blotting (b, lane 4). The non-demented individual in lane 6 corresponds to that analysed as well as on Fig. 4, lane 5. Note that A β dimers at 8 kDa are strongly detected with 21F12 (A β x-42 panels) whereas faintly (lanes 8-10) or not (lanes 6 and 7) stained with 3D6 antibody in non-demented individuals.

syndrome. A β -40 deposits are mainly observed in the oldest Down's syndrome individuals (Lemere *et al.* 1996) whereas intraneuronal A β -42 are the earliest species to accumulate and, interestingly, they are not immunoreactive with amino-terminal A β antibody (Mori *et al.* 2002).

A β species comprised in the first amyloid deposits of non-demented individuals

Previous study on our brain bank evidenced the existence of individuals that had no cognitive impairment but showed tau pathology in the hippocampal formation, extending to the anterior and mid-temporal cortex (Delacourte *et al.* 1999). Diffuse amyloid deposits in different neocortical areas were also observed at neuropathological examination and confirmed at the biochemical level (Delacourte *et al.* 2002). Thus, in accordance with the progression of AD lesions in the human brain cortical areas, as defined by Braak and Braak (1991) and Delacourte *et al.* (1999, 2002), the non-demented individuals certainly corresponded to early stage of 'sporadic' form of AD. The knowledge of the dynamic of progression of tau pathology and amyloid pathology (Delacourte *et al.* 2002) strongly supports that the lesions observed are likely to be the first lesions to develop.

Here, we demonstrate for the first time that the backbone of A β aggregates at the first stage of amyloid deposition in these non-demented individuals comprise amino-terminal truncated variants of A β -42, including A β starting at positions 4-, 5-, 8- and 9-42, or with a pyroglutamyl residue at position 3. A β peptides starting at residue 3-pyroglutamyl have been suggested to be early aggregating species in AD (Saïdo *et al.* 1995). Moreover, we show that the additional truncated species identified here, which all correspond to A β -42, are also early aggregating species. These A β -42 species do not result from treatment of brain tissue with formic acid (Delacourte *et al.* 2002; Kalback *et al.* 2002), as the treatment of synthetic A β peptides 1-40 and 1-42 with formic acid did not generate the truncated species observed in the human brain tissue (Delacourte *et al.* 2002).

One can ask if truncated A β species at the earliest stages of AD are not generated before aggregation, as normal or pathological products. These truncated species could play a decisive role as seeds for fibrillogenesis and amyloid deposition. Our data support indeed that specific amino-terminal cleavage occurs very early in the aggregation process. Firstly, these amino-truncated species are observed in non-demented individuals with little amyloid deposits and tau pathology. Secondly, biochemical analyses show that dimers of A β are lightly reactive with amino-specific A β antibodies, thus suggesting that they are mainly comprised of amino-truncated A β -42 peptides. *In vitro* experiments have shown that truncated variants of A β -42 are more fibrillogenic at physiological pH than the full-length A β and certainly more toxic (Pike *et al.* 1995; Russo *et al.* 2002). Furthermore, amino-truncated A β peptides were observed in cell

models, demonstrating that they can be generated directly by cells, and thus do not result from truncation following extracellular aggregation within tissue (Cescato *et al.* 2000; Shirovani *et al.* 2002). The truncated species of A β -42 are also found in large amount in cell models transfected with the human APP gene mutated at position close to the gamma-secretase cleavage (Ancolio *et al.* 1999; De Jonghe *et al.* 2001). These observations in addition to ours further support the early dysfunction of gamma-secretase complex in 'sporadic' AD individuals. Altogether, our results demonstrate that specific amino-truncated species of A β -42 are implicated in the very first steps of amyloidosis in Alzheimer pathology and give new insight toward the understanding of the pathophysiological process of the usual 'sporadic' form of AD.

Amino-truncated A β species are major species of early amyloid deposits

Two-dimensional gel electrophoresis followed by western blotting enables a semi-quantitative analysis of the proportion of each A β species. This proteomic method was previously described to be highly resolutive and sensitive for the quantification of A β in cerebrospinal fluid (Wiltfang *et al.* 2002). Using this approach, we showed that more than 60% of all A β species correspond to amino-truncated A β species. This result was further confirmed by the quantification of full-length A β peptides using 3D6 antibody. Moreover, we showed that the proportion of amino-truncated A β peptides was not correlated to postmortem intervals, thus suggesting that autolysis is not responsible for the generation of amino-truncated species of A β . The isoelectric focusing necessitates a long active rehydration step, during which reaggregation of A β peptides could occur. If such a reaggregation process did occur then it would alter the focusing of A β species and a long horizontal smear would be visualized. Indeed, all A β peptides were well-focused and even dimeric A β species. Moreover, the results of quantification either obtained with two-dimensional gel analysis or with one-dimensional western blotting are very similar. Russo *et al.* (2000) reported that full-length A β represented 30% of all A β species in sporadic AD and that in familial AD this ratio was even lowered. Our results are similar to that obtained by Russo *et al.* (2000) and can be extended to non-demented individuals. Therefore, we show that our proteomic method is also a very useful method for determining the proportion of each A β species in formic acid-treated human brain tissue. Moreover, we propose that the implication of amino-truncated A β peptides in the amyloidosis process of AD is of major importance and is likely to occur very early during amyloid deposition.

Implication for the human vaccination strategy

Aggregates of A β are removed following vaccination of transgenic mice against the amino-terminal region of A β or

full-length A β (Schenk *et al.* 1999, 2001). This amino-terminal region, as the 3-EFRH-6 A β sequence or the 4–10 A β sequence, generates the greatest immunogenic response in mice, reducing amyloid burden, A β fibril assembly and toxicity (Schenk *et al.* 1999; Frenkel *et al.* 2001; Lemere *et al.* 2001; Monsonego *et al.* 2001; McLaurin *et al.* 2002). Immunization demonstrated as well that antibodies against amino-terminal region of A β are more efficient to reduce the amyloid burden in transgenic mice (Bard *et al.* 2003). However, this higher immunogenic property could be explained by the fact that the human A β sequence differs specifically from that of mouse in this amino-terminal region of A β . Passive immunization using an antibody against the central core of A β is also an alternate and efficient therapeutic approach to reduce the amyloid burden or restore the cognitive function in transgenic mouse models of AD (DeMattos *et al.* 2001; Dodart *et al.* 2002). Yet, all vaccination strategies do not discriminate between the full-length or truncated A β species. Full-length A β is a physiological product with an unknown function that could generate an undesirable immune reaction. The ideal vaccination strategy in humans should have two aims: (i) to use antigens that correspond to the initial amyloid species formed at the earliest stage of AD, because they are at the heart of Alzheimer physiopathological process; (ii) the antigen should be a pathological epitope rather than a physiological one, thus specifically targeting the amino-truncated species rather than the full-length A β peptide. The fact that truncated A β species are early, pathological and abundant antigens of AD indicates that they could act as an ideal target for vaccination. Indeed, it is possible to design synthetic peptides or develop immunological tools that will target specifically the pathological amino-terminal truncated A β species and not physiological A β peptides, as the full-length A β .

In conclusion, our data show that the amino-truncated A β -42 peptides are the first species to seed amyloid deposition in infraclinal AD. Consequently, we propose a small but significant modification to the AD vaccination strategy that is likely to completely change the response in the early events of the amyloid cascade in 'sporadic' AD.

Acknowledgements

This work was supported by INSERM, CNRS and IMPRT of Lille. We are very grateful to Malcom Lyon (Manchester, UK), and Maryse Delehedde for their critical advice.

References

- Ancolio K., Dumanchin C., Barelli H., Warter J. M., Brice A., Campion D., Frébourg T. and Checler F. (1999) Unusual phenotypic alteration of β amyloid precursor protein (β APP) maturation by a new Val-715 \rightarrow Met β APP-770 mutation responsible for probable early-onset Alzheimer's disease. *Proc. Natl Acad. Sci. USA* **96**, 4119–4124.

- Bard F. *et al.* (2003) Epitope and isotype specificities of antibodies to {beta}-amyloid peptide for protection against Alzheimer's disease-like neuropathology. *Proc. Natl Acad. Sci. USA* **100**, 2023–2028.
- Braak H. and Braak E. (1991) Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol.* **82**, 239–259.
- Campion D. *et al.* (1999) Early-onset autosomal dominant Alzheimer disease: prevalence, genetic heterogeneity, and mutation spectrum. *Am. J. Hum. Genet.* **65**, 664–670.
- Cescato R., Dumermuth E., Spiess M. and Paganetti P. A. (2000) Increased generation of alternatively cleaved beta-amyloid peptides in cells expressing mutants of the amyloid precursor protein defective in endocytosis. *J. Neurochem.* **74**, 1131–1139.
- Cherny R. A., Legg J. T., McLean C. A., Fairlie D. P., Huang X., Atwood C. S., Beyreuther K., Tanzi R. E., Masters C.L. and Bush A. I. (1999) Aqueous dissolution of Alzheimer's disease A β amyloid deposits by biometal depletion. *J. Biol. Chem.* **274** (33), 23223–23228.
- De Jonghe C., Esselens C., Kumar-Singh S., Craessaerts K., Serneels S., Checler F., Annaert W., Van Broeckhoven C. and De Strooper B. (2001) Pathogenic APP mutations near the γ -secretase cleavage site differentially affect A β secretion and APP C-terminal fragment stability. *Hum. Mol. Genet.* **10**, 1665–1671.
- Delacourte A. *et al.* (1999) The biochemical pathway of neurofibrillary degeneration in aging and Alzheimer's disease. *Neurology* **52**, 1158–1165.
- Delacourte A., Sergeant N., Champain D., Watzet A., Maurage C. A., Lebert F., Pasquier F. and David J. P. (2002) Nonoverlapping but synergistic tau and APP pathologies in sporadic Alzheimer's disease. *Neurology* **59**, 398–407.
- DeMattos R. B., Bales K. R., Cummins D. J., Dodart J. C., Paul S. M. and Holtzman D. M. (2001) Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. *Proc. Natl Acad. Sci. USA* **98**, 8850–8855.
- Dodart J. C. *et al.* (2002) Immunization reverses memory deficits without reducing brain A beta burden in Alzheimer's disease model. *Nat. Neurosci.* **5**, 452–457.
- Frenkel D., Kariv N. and Solomon B. (2001) Generation of auto-antibodies towards Alzheimer's disease vaccination. *Vaccine* **19**, 2615–2619.
- Gotz J., Chen F., Van Dorpe J. and Nitsch R. M. (2001) Formation of neurofibrillary tangles in P301L tau transgenic mice induced by A beta 42 fibrils. *Science* **293**, 1491–1495.
- Hock C., Konietzko U., Papassotiropoulos A., Wollmer A., Streffer J., Von Rotz R. C., Davey G., Moritz E. and Nitsch R. M. (2002) Generation of antibodies specific for beta-amyloid by vaccination of patients with Alzheimer disease. *Nat. Med.* **8**, 1270–1275.
- Hyman B. T. and Trojanowski J. Q. (1997) Consensus recommendations for the postmortem diagnosis of Alzheimer disease from the National Institute on Aging and the Reagan Institute Working Group on diagnostic criteria for the neuropathological assessment of Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **56**, 1095–1097.
- Janus C. *et al.* (2000) A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature* **408**, 979–982.
- Johnson-Wood K. *et al.* (1997) Amyloid precursor protein processing and A beta42 deposition in a transgenic mouse model of Alzheimer disease. *Proc. Natl Acad. Sci. USA* **94**, 1550–1555.
- Kalback W. *et al.* (2002) APP transgenic mice Tg2576 accumulate A beta peptides that are distinct from the chemically modified and insoluble peptides deposited in Alzheimer's disease senile plaques. *Biochemistry* **41**, 922–928.
- Kumar-Singh S. *et al.* (2000) Nonfibrillar diffuse amyloid deposition due to a gamma (42)-secretase site mutation points to an essential role for N-truncated A beta (42) in Alzheimer's disease. *Hum. Mol. Genet.* **9**, 2589–2598.
- Lemere C. A., Blusztajn J. K., Yamaguchi H., Wisniewski T., Saido T. C. and Selkoe D. J. (1996) Sequence of deposition of heterogeneous amyloid beta-peptides and APO E in Down syndrome: implications for initial events in amyloid plaque formation. *Neurobiol. Dis.* **3**, 16–32.
- Lemere C. A., Maron R., Selkoe D. J. and Weiner H. L. (2001) Nasal vaccination with beta-amyloid peptide for the treatment of Alzheimer's disease. *DNA Cell Biol.* **20**, 705–711.
- Lewis J. *et al.* (2001) Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science* **293**, 1487–1491.
- McLaurin J. *et al.* (2002) Therapeutically effective antibodies against amyloid-beta peptide target amyloid-beta residues 4–10 and inhibit cytotoxicity and fibrillogenesis. *Nat. Med.* **8**, 1263–1269.
- Monsonogo A., Maron R., Zota V., Selkoe D. J. and Weiner H. L. (2001) Immune hyporesponsiveness to amyloid beta-peptide in amyloid precursor protein transgenic mice: implications for the pathogenesis and treatment of Alzheimer's disease. *Proc. Natl Acad. Sci. USA* **98**, 10273–10278.
- Mori C., Spooner E. T., Wisniewski K. E., Wisniewski T. M., Yamaguchi H., Saido T. C., Tolan D. R., Selkoe D. J. and Lemere C. A. (2002) Intraneuronal Abeta42 accumulation in Down syndrome brain. *Amyloid* **9**, 88–102.
- Pike C. J., Overman M. J. and Cotman C. W. (1995) Amino-terminal deletions enhance aggregation of beta amyloid peptides in vitro. *J. Biol. Chem.* **270**, 23895–23898.
- Pype S., Moechars D., Dillen I. and Mercken M. (2003) Characterization of amyloid beta peptides from brain extracts of transgenic mice overexpressing the London mutant of human amyloid precursor protein. *J. Neurochem.* **84**, 602–609.
- Russo C., Schettini G., Saido T. C., Hulette C., Lippa C., Lannfelt L., Ghetti B., Gambetti P., Tabaton M. and Teller J. K. (2000) Presenilin-1 mutations in Alzheimer's disease. *Nature* **405**, 531–532.
- Russo C. *et al.* (2002) Pyroglutamate-modified amyloid beta-peptides – A beta N3 (pE) – strongly affect cultured neuron and astrocyte survival. *J. Neurochem.* **82**, 1480–1489.
- Saido T. C., Iwatsubo T., Mann D. M., Shimada H., Ihara Y. and Kawashima S. (1995) Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3 (pE), in senile plaques. *Neuron* **14**, 457–466.
- Schenk D. *et al.* (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* **400**, 173–177.
- Schenk D., Seubert P. and Ciccarelli R. B. (2001) Immunotherapy with beta-amyloid for Alzheimer's disease: a new frontier. *DNA Cell Biol.* **20**, 679–681.
- Sergeant N., David J. P., Champain D., Ghestem A., Watzet A., Delacourte A. (2002) Progressive decrease of amyloid precursor protein carboxy terminal fragments (APP-CTFs), associated with tau pathology stages, in Alzheimer's disease. *J. Neurochem.* **81**, 663–672.
- Shirovani K., Tsubuki S., Lee H. J., Maruyama K. and Saido T. C. (2002) Generation of amyloid beta peptide with pyroglutamate at position 3 in primary cortical neurons. *Neurosci. Lett.* **327**, 25–28.
- Terai K., Iwai A., Kawabata S., Tasaki T., Watanabe K., Miyata K. and Yamaguchi T. (2001) β -Amyloid deposits in transgenic mice expressing human β -amyloid precursor protein have the same characteristics as those in Alzheimer's disease. *Neuroscience* **104**, 299–310.

- Walsh D. M., Tseng B. P., Rydel R. E., Podlisny M. B. and Selkoe D. J. (2000) The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain. *Biochemistry* **39**, 10831–10839.
- Walsh D. M., Klyubin I., Fadeeva J. V., Cullen W. K., Anwyl R., Wolfe M. S., Rowan M. J. and Selkoe D. J. (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* **416**, 535–539.
- Wiltfang J. *et al.* (2002) Highly conserved and disease-specific patterns of carboxyterminally truncated Abeta peptides 1–37/38/39 in addition to 1–40/42 in Alzheimer's disease and in patients with chronic neuroinflammation. *J. Neurochem.* **81**, 481–496.