Nonoverlapping but synergetic tau and APP pathologies in sporadic Alzheimer’s disease

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Abstract—Objective: To determine the spatiotemporal mapping of tau pathologies and insoluble pools of Aβ in aging and sporadic AD, and their contribution to the physiopathologic, clinical, and neuropathologic features. Methods: The authors studied 130 patients of various ages and different cognitive status, from non-demented controls (n = 60) to patients with severe definite AD (n = 70) who were followed prospectively. Insoluble Aβ 42 and 40 species were fully solubilized and quantified in the main neocortical areas, with a new procedure adapted to human brain tissue. Tau pathology staging was determined in 10 different brain areas, using Western blots. Results: In AD, there is a constellation of amyloid phenotypes, extending from cases with exclusively aggregated Aβ 42 to cases with, in addition, large quantities of insoluble Aβ 40 species. Five other points were observed: 1) There was no spatial and temporal overlap in the distribution of these two insoluble Aβ species in cortical brain areas. 2) In contrast to solubilized Aβ 40 aggregates composed essentially of monomers and dimers, solubilized Aβ 42 was essentially observed as dimers and multimers. 3) Aβ 42 aggregates were observed at the early stages of tau pathology, whereas the insoluble Aβ 40 pool was found at the last stages. 4) During the progression of the disease, Aβ aggregates increase in quantity and heterogeneity, in close parallel to the extension of tau pathology. 5) There was no spatial overlap between Aβ aggregation that is widespread and heterogeneously distributed in cortical areas and tau pathology that is progressing sequentially, stereotypically, and hierarchically. Conclusions: These observations demonstrate that Aβ 42 aggregation, and not Aβ 40, is the marker that is close to Alzheimer etiology. It should be the main target for the early biological diagnosis of AD and modeling. Furthermore, the spatial mismatch between amyloid β-precursor protein (APP) and tau pathologies in cortical brain areas demonstrates that neurodegeneration is not a direct consequence of extracellular Aβ neurotoxicity. Hence, there is a synergetic effect of APP dysfunction, revealed by Aβ aggregation, on the neuron-to-neuron propagation of tau pathology.

AD is a neurodegenerative disorder characterized by the coexistence of two degenerating processes: amyloidosis and tau pathology. Amyloidosis corresponds to the extracellular aggregation of Aβ peptides into amyloid plaques.1 Tau pathology, also named taupathy, corresponds to the intraneuronal association of tau proteins into abnormal filaments.2,3 Amyloidosis is closely related to etiology1 and tau pathology is strongly correlated to the clinical expression of the disease.4–10 Little is known about the relationship between amyloid β-precursor protein (APP) and tau pathologies, which is the missing link in fully understanding AD. Indeed, although the pathway of tau pathology is very precise in the brain of AD patients,6,7 amyloidosis seems to be more heterogeneously and randomly distributed.8 Aβ peptides derive from the catabolism of a large transmembrane glycoprotein precursor (APP). Molecular heterogeneity of APP processing that generates Aβ peptides results from different types of mutations in familial autosomal dominant AD (FAD), located near the beta or the gamma cleavage sites.11 These different pathogenic mutations can be modeled in transgenic mice.12 However, AD is nonfamilial in more than 99% of patients, according to a large scale population study.13 The formation and turnover of amyloid deposits in the human brain are essentially known through immunohistochemical techniques,14 because a biochemical quantification is not easy, as these amyloid deposits are extremely insoluble, even in harsh detergents.15 ELISA immunoassays (EIA) and Western blot techniques were used to quantify soluble and insoluble Aβ species accessible to this technique.16–19 Together, all these studies tend to demonstrate that in the human brain, amyloidosis is observed first as diffuse aggregates of Aβ 42 peptides, which accumulate progressively as amyloid plaques, followed by the deposition of Aβ 40 pep-
tides. A microglial cell proteolysis of Aβ 42 into Aβ 40 species has also been suggested. A good correlation between soluble Aβ and the severity of the disease has been reported, although surprisingly this was not observed for the most insoluble fraction. Indeed, the insoluble pool of Aβ corresponds to one of the two brain lesions that characterize the disease. Furthermore, the definition of what is an insoluble pool of Aβ is different according to the studies, in that different extraction buffers were used (guanidinium, urea, urea + sodium dodecyl sulfate [SDS], different concentrations of formic acid) as well as different centrifugation forces to pellet the insoluble material.

We have developed a reproducible biochemical method to analyze at the quantitative and qualitative levels the amyloid deposits in aging and AD. This method is easy to perform, efficient in that it allows a complete solubilization of all Aβ aggregates, and informative. Quantification of Aβ 42 and 40 aggregates was performed in four brain regions of 65 cases from our prospective study, and compared to the quantification of tau pathology. This allows a better description of the natural and molecular history of AD and helps to set up strategies for diagnostic and therapeutic approaches. Here we show first that there is a good correlation between the amounts of insoluble Aβ 42 and the progression of the disease, and second that there is a synergetic interaction between APP and tau pathologies, despite their different spatiotemporal distribution.

Materials and methods. Patients. The 60 nonde-mented and 70 demented patients were from the geriatric department of E. Roux Hospital at Limeil-Brevannes and the Lille CH&U Hospital, France, as described in reference 7. They represent all patients who were hospitalized for various disorders and died at this hospital, excluding those whose family opposed autopsy, or for whom postmortem delay was more than 24 hours. Clinical data were detailed in reference 7. Cognitive status was evaluated using the Mini-Mental State Examination (MMSE) and the Clinical Dementing Rating (CDR) score. Clinical criteria for dementia were based on Diagnostic and Statistical Manual of Mental Disorders, 3rd ed, rev; for AD, National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer’s Disease and Related Disorders Association; for vascular dementia, National Institute of Neurological Disorders and Stroke–Association Internationale pour la Recherche et l’Enseignement en Neurosciences; and for mixed dementia, Hachinski score. Clinical diagnosis was summarized as AD (possible, probable), vascular dementia, mixed dementia (AD with a strong vascular involvement revealed by investigations), or dementia (for patients with an uncertain clinical diagnosis). Clinical data about the patients who were analyzed here in detail for their amyloid content are summarized in table e1 (available online at www.neurology.org). Similarly, neuropathologic data for each patient were reported in the previous study (amyloid plaques, amyloid angiopathy, Braak stages of neurofibrillary degeneration, vascular pathology, other lesions), and are summarized in table e1 (available online at www.neurology.org).

Mice. The APP Swedish mouse brain tissue was a generous gift of Dr. M. Staufenbiel. Twenty-four-month-old mice containing the highest amounts of amyloid deposits, as observed at the immunohistochemical level, were studied. Biochemical studies. Amyloid extraction. A total of 100 mg of brain tissue was homogenized in 1 mL of pure formic acid. Two microliters of the brain homogenate were used for the dot-blotting, or 50 µL were evaporated under nitrogen, solubilized in 50 µL of the SDS sample buffer (5% SDS, 20% glycerol, 2% β-mercaptoethanol, 150 mM Tris-HCl pH 6.8), and boiled 10 minutes before electrophoresis. A total of 10 µL (100 µg of protein) were loaded per well.

Immunologic probes. Tau pathology was revealed with AD2, a monoclonal antibody (mAb) against paired helical filaments that is directed against phosphorylated Tau proteins and quantified according to reference 7. Amyloid plaques and aggregated Aβ peptides were detected using rabbit polyclonal antisera, named ADA40 and ADA42, generated against synthetic peptides corresponding to the seven last carboxy-terminal amino acids of Aβ 40 and 42. The specificity of these antibodies was checked by absorption with the corresponding synthetic Aβ 1-40 and 1-42 (Bachem, Bubendorf, Switzerland), by the specific labeling of these commercial peptides at concentration up to 500 ng, both using dot-blot and electrophoresis. Their immunoreactivity, specificity, and sensitivity were similar to the well-characterized FC3542 and FC3340 antibodies. Aβ 42 species were also specifically detected using the monoclonal antibody 21F12 that recognized the 42, 43 carboxy-terminal end of Aβ. Aβ 40 and 42 species were also analyzed using the monoclonal antibody WO2 against the amino acid 4 to 10 of Aβ.

ImmunobLOTS. Tau pathology was investigated as already described. Amyloid pathology was analyzed using electrophoresis adapted to the separation of small peptides. After the proteins were blotted on nitrocellulose membranes, the upper part was reacted with AD2 for the estimation of tau pathology. The lower part of the membranes was reacted first with ADA40 for the detection of Aβ 40 and then, after stripping, with 21F12 or ADA42 for the detection of Aβ 42 species.

Dot-blOT analyses. Amyloid was detected and quantified by dot-blOT, using the procedure described in reference 29. The centrifugation step was removed and formic acid homogenates were directly loaded on the polyvinylidene fluoride (PVDF) membrane.

Image analysis of immunoblot and dot-blOT quantification. Immunoblots and dot-blot were analyzed using the ImageMaster 1D Elite software (Amersham-Pharmacia, Orsay, France). The quantification is expressed in micrograms per gram of tissue, using Aβ 1-40 or Aβ 1-42 synthetic peptides (Bachem) as standards.

Results. Quantification of Aβ aggregates. Human brain amyloid is extremely difficult to dissociate and solubi-lize. Aβ species from the human brain tissue were extracted with different lysis buffers: SDS, guanidinium chloride, urea, urea + SDS, and formic acid (FA), at differ-
The brain tissue was homogenized in a sodium dodecyl sulphate (SDS) buffer, either directly or after a formic acid (FA) treatment, and then solubilized Aβ peptides were resolved by SDS gel electrophoresis. Aβ 40 and 42 peptides were immunolabeled with specific polyclonal antibodies against Aβ 40 (ADA40, left part) and Aβ 42 (ADA42, right part). A total of 12.5 μg of synthetic Aβ 40 and 42 were loaded in the central wells. Note that for patient Fa.85, huge amounts of Aβ 40 monomers and dimers, solubilized in SDS (A) or after FA treatment (B) were detected, whereas Aβ 42 aggregates poorly solubilized in SDS (A’) but well extracted with FA, were detected as monomers, dimers, and oligomers (B’). The other patient, Ci.65, had exclusively Aβ 42 deposits, detected only after FA treatment (D’) as dimers and mainly multimers. Large amounts of Aβ 40 were detected in APPsw mice brain homogenized in SDS buffer (E) as well as after FA treatment (F), and low amounts of Aβ 42 species (F’) were detected after FA extraction.

Figure 1. Western blot detection of Aβ 40 and 42 species in brain homogenates from Alzheimer patients and APP Swedish mice. The brain tissue was homogenized in a sodium dodecyl sulphate (SDS) buffer, either directly or after a formic acid (FA) treatment, and then solubilized Aβ peptides were resolved by SDS gel electrophoresis. Aβ 40 and 42 peptides were immunolabeled with specific polyclonal antibodies against Aβ 40 (ADA40, left part) and Aβ 42 (ADA42, right part). A total of 12.5 μg of synthetic Aβ 40 and 42 were loaded in the central wells. Note that for patient Fa.85, huge amounts of Aβ 40 monomers and dimers, solubilized in SDS (A) or after FA treatment (B) were detected, whereas Aβ 42 aggregates poorly solubilized in SDS (A’) but well extracted with FA, were detected as monomers, dimers, and oligomers (B’). The other patient, Ci.65, had exclusively Aβ 42 deposits, detected only after FA treatment (D’) as dimers and mainly multimers. Large amounts of Aβ 40 were detected in APPsw mice brain homogenized in SDS buffer (E) as well as after FA treatment (F), and low amounts of Aβ 42 species (F’) were detected after FA extraction.

A comparison with other commercially available mAb was performed. In our hands, WO2 was the most sensitive antibody for the detection of both synthetic Aβ 40 and 42 species, in good agreement with the literature. However, it failed to detect the most insoluble human Aβ species, while it detected strongly Aβ products generated by transgenic mice with the Swedish mutation, as shown in figure 2. The absence of detection of a large pool of human insoluble Aβ material by WO2 is due to the fact that this antibody is directed against the N-terminal part of Aβ, which is truncated and chemically modified in the most insoluble aggregated Aβ species. This pool is essentially composed of N-truncated Aβ 42. The very insoluble Aβ material labeled by ADA42, but not by WO2, is strongly detected by 21F12, a mAb directed against the carboxy-terminal part of Aβ 42, 43.

Together, this comparison of immunologic probes led us to use mAb 21F12, a commercially available antibody against Aβ 42, following ADA40, a polyclonal antibody against Aβ 40, to detect with a good specificity and sensitivity all Aβ species on our Western blots (figure 3).

Different patterns of amyloidosis in Alzheimer cases. The different biochemical signatures of insoluble Aβ were investigated further. To understand better the physiopathologic significance of these different patterns, we quantified Aβ aggregates and tau pathology in different brain areas, randomly chosen among the 130 cases (60 nondemented, 70 demented patients) of our prospective study (see figure 3).

Alzheimer patients presented contrasted Aβ phenotypes. Some cases were characterized by their almost pure and large amounts of insoluble Aβ 42 variants (see figure 3A’, Case Ci.65). Other cases had moderate (see figure 3, Case Fa.85) to large amounts of insoluble Aβ 40 (see figure 3B’, Cases Ga.86, My.74, Pn.70). A second type of heterogeneity was found in the cerebral distribution of aggregated Aβ variants, which where sometimes in larger quantities...
either in the temporal or the parietal or the occipital cortex (see figure 3, A', B'). In general, the frontal pole contained less Aβ aggregates, while more insoluble Aβ 40 variants were found in the occipital region. A third type of heterogeneity was the absence of overlap between Aβ 40 and 42 aggregates in some cases. For a given brain, the two aggregated Aβ species mapped differently, as shown for Case Fa.85, where insoluble Aβ 42 was in larger quantities in the occipital cortex while insoluble Aβ 40 was found essentially in the frontal and parietal cortex (see figure 3, A', B'). A fourth type of heterogeneity was revealed by the detailed electrophoretic pattern of Aβ deposits. Interestingly, solubilized Aβ 42 variants mainly consisted of dimers and multimers (figure 3A') whereas solubilized Aβ 40 consisted mainly of monomers and dimers (figure 3B'). A fifth type of heterogeneity was observed in the relationship between the distribution of tau and Aβ pathologies. Insoluble Aβ was heterogeneous distributed in cortical areas, but frequently found in more important quantities in the occipital cortex. However, the occipital cortex was always the less affected brain area by tau pathology.

We also investigated the biochemical patterns of amyloidosis in nondemented patients. In good agreement with our previous studies, we found a few aged cases with no trace of Aβ aggregates, despite the presence of tau pathology (see figure 3A, Cases Bl.93 and Hn.95). Patients up to stage 7 of tau pathology had almost exclusively Aβ 42 aggregates (see figure 3A, Cases Pt.83, As.85, Ge.85).

Biochemical quantification of amyloid deposits. We quantified Aβ aggregates by dot-blot and Western blots, using Aβ 40 and 42 synthetic peptides as standards. Results obtained with both approaches were similar. The dot-blot technique was faster, whereas the Western blot approach gave additional qualitative values on the content of Aβ aggregates, such as the percentage of monomers, dimers, and multimers, as well as more specificity. Values obtained with these methods are reported in table e1 (at www.neurology.org) and figure 4. We analyzed at random at least three cases per Alzheimer stage of tau pathology, as defined in reference 7. With this approach, we found insoluble Aβ concentration ranging from 25 to 1250 µg per gram of human brain tissue from Alzheimer patients. Then, the quantification was performed in the four main brain areas of nondemented and Alzheimer patients. Our results demonstrate the strong heterogeneity of Aβ aggregates, in quantity and quality, with a Aβ 42/40 ratio that varied from infinite (absence of Aβ 40) to 0.4. For patients with a tau pathology restricted to the hippocampal area, such as Pt.83 and Ds.59, the amyloid burden was in the range of 2 to 145 µg of insoluble Aβ 42 per gram of wet tissue. For these patients, insoluble Aβ 40 species were not detected.

Using the same approach, we found Aβ insoluble material in APPsw mice at a concentration of 750 µg per gram of brain tissue, which were almost totally SDS soluble and essentially composed of Aβ 40 peptide aggregates (figure 1).
Spatiotemporal analysis of Aβ deposits in AD. In our prospective study, we demonstrated that the clinical features were well correlated with the extension of tau pathology in association polymodal cortical areas. For the present study, it was interesting to compare the clinical and neuropathologic features of these patients with tau pathology as well as with the different biochemical phenotypes of amyloid deposition and their concentrations.

For Alzheimer demented patients, we always found severe tau pathology (stage 7 to stage 10) well correlated with a huge and widespread Aβ burden. As shown in table e1 (available online at www.neurology.org), the presence of Aβ 40 aggregates is a landmark of the late stages of AD. Also, the most affected AD cases, with an early onset, a rapid cognitive degradation, and a huge neurofibrillary degeneration process, were cases with large amounts of Aβ 42, and rare or absent Aβ 40 aggregates (Cases To.54, Fu.63, Cl.65, Cu.70).

When all biochemical data were collected, it was striking to observe the excellent parallelism between the increase of the disease process, tau pathology, and the increase of Aβ 42 aggregates (table e1, figure 4). However, at the level of specific brain areas, there was no overlap between the mapping of tau and Aβ40 degenerating processes. It was interesting to observe brain areas such as the occipital cortex with enormous Aβ aggregates but with absolutely no trace of tau pathology in this region (see figure 3A, Case Gs.86, occipital). Conversely, brain areas with more tau pathology were not those that had larger amounts of Aβ 42 aggregates (see figure 3A, Fa.85, temporal cortex). Moreover, each time we were able to detect Aβ aggregates, we found a tau pathology, at least in the entorhinal region. The opposite was not true because we found cases with tau pathology up to stage 6 and no trace of Aβ aggregates (see figure 3, A through C).

Discussion. Our objective was to describe the natural and molecular history of AD as well as to develop a molecular basis for the definite diagnosis of AD. Indeed, at present, the definite diagnosis of AD, and the distinction from other neurologic disorders, is still difficult, even when working directly on the brain tissue: this is demonstrated by the fact that tau pathology has been recently reintroduced in the criteria for the definite diagnosis of AD, after a long absence, to fit now with Alois Alzheimer’s first and sound description. This is also demonstrated by the fact that the 1997 consensus report recommends a

Figure 3. Western blot analysis of Aβ deposition (A, A', B, B') and tau pathology (C, C') in the different brains areas of nondemented and Alzheimer patients. Top, aging and infraclinical AD; bottom, definite AD. The specific immunolabeling with Aβ 42 (A, A'), Aβ 40 (B, B'), and tau (C, C') antibodies is presented. The temporal (T), frontal (F), parietal (P), and occipital (O) cortices were studied. A, A', B, B': Aβ 42 (E), 40 (F), and tau (G) were quantified in different brain areas from nondemented and demented patients. We used successively the monoclonal 21F12 antibody, because of its very high sensitivity and specificity toward Aβ 42 species, and then the polyclonal ADA40 antibody. C, C': Tau pathology revealed with AD2 antibody. Tau detection was performed on the same membrane (upper part). Pathologic tau proteins correspond to the main triplet of immunodetected band (tau 60, 64 and 69 kDa). A–C: Some representative nondemented cases are presented here: two cases with tau pathology (C) and no Aβ aggregates (Bl.93 and Hn.95); Case Pt.83, Case As.85, and Case Ge.85 at stages 1, 3, and 6 of tau pathology, respectively, and with various amounts of aggregated Aβ 42 species, but no Aβ 40 aggregates. Note that some cases with tau pathology had no Aβ aggregates. Case Pt.83 had tau pathology in the entorhinal formation (not shown). A–C: Aβ 42 aggregates were found in large quantities in all brain areas of Alzheimer patients. Patient Ci.65 had exclusively Aβ 42 aggregates, as no trace or trace amounts of Aβ 40 were detected in the four brain regions. Patients Gs.86 and Fa.85 had huge aggregates of Aβ 40 peptides. Aβ 40 was in larger quantities in the occipital area of Gs.86 whereas it was essentially found in the frontal and parietal cortex of Patient Fa.85. Aβ 40 aggregates were essentially solubilized as Aβ monomers and dimers whereas Aβ 42 aggregates were dissociated and solubilized as Aβ dimers and multimers. They were abundant in all brain areas of Patients Ci.65 and Cu.70. For Patient Gs.86, at stage 9 and mildly affected, the temporal and parietal cortex were moderately affected by tau pathology, but not the frontal and occipital cortex. Note that the region with more Aβ 40 and 42 deposits, the occipital cortex, is not affected by tau pathology. In the same way, tau pathology and amyloid deposits do not overlap in the brain areas of Patient Fa.85.
probabilistic analysis. This is also obvious when we see that the molecular markers of neurodegenerative disorders, namely amyloid pathology, tau pathology, and synuclein, are overlapping markers of many different diseases. For example, tau pathology is observed in more than 24 neurodegenerative disorders. Also, amyloid pathology is frequently observed in Lewy body dementia, a disease with a synucleopathy. Aβ deposits are also basic components of amyloid angiopathy, a pathologic entity distinct from AD. Therefore, a reliable quantification of brain lesions is necessary, and only biochemical analyses can fulfill the work rapidly, precisely, and with a good interlaboratory reproducibility. The usefulness of such a procedure is that it could bring a quantitative and qualitative aspect to the diagnosis, as required by the consensus criteria, which is difficult to obtain with an immunohistochemical approach. Brain banks could use it to type precisely the extent of the degenerating processes in the frozen tissue that will be used for the search of markers or for the description of the pathologic process. A molecular basis for the diagnosis at the CNS level should also open the gates for a biological diagnosis in the blood or the CSF. A biochemical approach should also provide a possibility for a definite ante mortem diagnosis, based on the quantification of pathologic tau and Aβ in a stereotaxic biopsy. This controversial approach is not yet an issue, but could become one if an efficient anti-Alzheimer drug is discovered.

Aβ quantification was performed on the postmortem brain tissue of 70 patients from our prospective study. Western blot or dot-blot techniques gave similar values. The dot-blot technique is more efficient than EIA to quantify total Aβ because it enables one to quantify whole Aβ aggregated species (from the most soluble to the most insoluble). One important feature of this technique is that we were able to load directly the antigen dissolved in pure FA on the PVDF membrane. This methodology avoids the possible pitfalls due to Aβ reaggregation that would inevitably happen if the FA-treated tissue is then neutralized or treated in a less denaturant buffer. This strong tendency to reaggregation is demonstrated by the presence of smears of insoluble material on Western blots, when FA-treated material is removed and dissolved in a SDS buffer. These smears that are often in large quantities cannot be processed by ELISA. However, the dot-blot technique necessitates working with anti-Aβ antibodies that do not react with other higher molecular weight APP catabolic products, a property that is rarely found. The Western blot technique gives additional information such as the molecular weight of the detected Aβ species as well as their distribution into monomers, oligomers, and multimers, and guarantees the specificity of the analytical approach of insoluble Aβ pools.

Therefore, with our methodology, it has been possible to develop a large scale study for the quantitative and qualitative analyses of Aβ aggregation in the gradient of pathology of our series of 130 cases, from normal aging to severe AD, via infraclinical stages of AD. Furthermore, it has been possible to study the temporal distribution of Aβ aggregates in four brain areas (temporal, frontal, parietal, occipital, and temporal).
tal), which summarizes the precise and sequential degenerating process revealed by tau pathology. Also, all our biochemical data were compared to clinical and neuropathologic data.

Different biochemical phenotypes of amyloidosis were found in nonfamilial Alzheimer cases. The detection and quantification of amyloid deposits show the extensive heterogeneity and the different patterns of Aβ aggregation in the brain of Alzheimer patients. For some cases, the Aβ burden was almost exclusively composed of insoluble Aβ 42 species. These cases had generally an early onset and a rapid evolution, as indicated in the clinical data. Aβ 42 deposits were also exclusively found in cases that are at the infraclinical stage of AD. ADA42 and ADA40 had similar sensitivities, suggesting that Aβ 42 aggregation is the first event of amyloidosis. Conversely, some cases at the last stages of tau pathology were strongly affected by Aβ 40 aggregates. The profile of Aβ 40 consisted of well resolved monomers and dimers, partially soluble in SDS extracts, in contrast to highly SDS-insoluble Aβ 42 aggregates that consisted of dimers and multimers. Cases with Aβ 40 frequently had amyloid angiopathy, but this was not always the case, showing that the production of Aβ 40 can be partially independent of amyloid angiopathy. In general, we found that Aβ 42 burden was mainly found in temporal and parietal cortices, whereas Aβ 40 deposits were mostly in the occipital areas. There is no clear explanation for the heterogeneous Aβ 40 phenotypes. One explanation could be that Aβ 40 species derive from a carboxy-terminal proteolytic degradation of Aβ 42 species, which is lately and heterogeneously activated in brain areas and among individuals. For example, this activity could be triggered by microglial cells that are known to phagocyte amyloid debris, as already suggested.

Aβ 42 aggregates were exclusively found at the infraclinical stages of AD, showing that they are the exclusive biochemical phenotype associated with the early development of Alzheimer amyloidosis. Aβ 42 aggregates were also exclusively found in the most severe Alzheimer cases, showing again the close relationship with Alzheimer pathology.

The relationship between amyloidosis and tau pathology was studied. The correlation between amyloid deposits and tau pathology is weak at the level of their spatial distribution in brain areas, but strong if we compare the stages of tau pathology versus the average amount of Aβ 42 aggregates in the neocortical areas. Indeed, we demonstrated with our biochemical approach, and in excellent agreement with numerous neuropathologic studies, that the pathway of tau pathology is precise, sequential, predictable, stereotypical, and hierarchical. Ten stages of tau pathology were defined, according to the 10 brain areas that are successively affected. Here, our precise typing and quantification of amyloid deposits shows that the Aβ burden is diffuse, widespread, and extremely heterogeneous, also in good agreement with neuropathologic studies. It is interesting to note that each time we detected Aβ 42 cortical aggregates, we found a tau pathology, at least in the entorhinal region. But the opposite was not true, as a few rare cases had not a trace of amyloid but tau pathology up to stage 6. The absence of detection of Aβ aggregates in the brain of certain control patients should be considered as a function of two parameters: the definition of what is a soluble or insoluble Aβ pool and the sensitivity of the technique.

There are numerous pools of Aβ in the human brain of Alzheimer patients. First, Aβ is intracellular, resulting from a cleavage of APP that occurs in nerve cells and in glial cells. Then Aβ is extracellular and found in the parenchyma. The logical steps for a formation of Aβ fibrils suggest that there is a dimerisation of Aβ, then a formation of oligomers and multimers. In parallel, there is a transformation from soluble dimers to stable dimers, a loss of helical structures at the expense of beta sheet structures to form fibrils. Furthermore, in the human brain, there is also a chemical transformation of Aβ species, with a truncation at the N and C-carboxy terminal endings of Aβ, and also biochemical modifications: tyrosine cross-linking, pyroglutamate, oxidation. At the end of the cycle, it is also possible that the degradation of amyloid by glial and microglial cells releases soluble degradation products of Aβ. Therefore, the quantification of Aβ pools is complex. Soluble pools can be quantified using the sensitive ELISA methods. The quantification of soluble Aβ pools is more precisely related to the production of Aβ and its neurotoxicity as a soluble agent. It is well demonstrated that soluble Aβ pools are increased in FAD (demonstration in experimental models as well as in the peripheral fluids and tissues of FAD patients). Interestingly, several articles have reported a close relationship between the amounts of soluble Aβ pools and the severity of the disease in sporadic AD.

The analysis of insoluble Aβ pools is related to the physiopathologic process of aggregation, and its relationship to a possible neurotoxicity as an insoluble agent. In the current work, we demonstrate that the quantification of insoluble Aβ pools necessitates a complete solubilization with pure FA, a Western blot approach, and the use of precise immunologic probes. ELISA methods should be adapted to quantify stable Aβ complex, which represents over 40% of total Aβ species in the human brain. The major technical difficulty is the reaggregation of extracted Aβ when physiologic buffers are used for the immunologic detection. Other technical difficulties are reported in reference 19. Even using the Western blot approach, the amounts of insoluble Aβ pools can be underestimated using buffers that are not stringent enough or probes that do not detect chemically modified Aβ pools. Together, this explains why we found 10 to 50 times more insoluble Aβ than the amounts reported in the literature.

Another important point to address the physiopa-
thology of insoluble Aβ pools is the sensitivity of the technique. This is an essential point for nonde-
mented cases with tau pathology in the entorhinal formation and with no Aβ aggregates. Using mAb
21F12, which was, in our hands, the most sensitive and specific tool for the detection of insoluble Aβ
species, we were able to detect with a good reproduc-
ibility insoluble Aβ amounts at a concentration of 1
µg per gram of tissue. This concentration is 1000
times lower than the one detected in some Alzheimer
brains. However, we cannot exclude the presence of
an abnormal concentration of soluble Aβ pools for
cases with tau pathology and no Aβ aggregates, as
suggested by reports using the ELISA approach.

Together, our data presented here reinforce the
idea that nonfamilial autosomal AD is an amyloid
(neurototoxic) or APP (loss of trophic activities) burden
that will extend tau pathology from the temporal
cortex to the association brain areas.36 The data are
in favor of a synergy of tau and APP pathologies.
They show that tau pathology can be initiated before
and likely independently of amyloidosis or APP dys-
functions, up to stage 6 of tau pathology. Our data
also show that both neurodegenerative processes
evolve systematically in parallel after stage 6, and
that APP pathology amplifies tau pathology. Indeed,
the progression of tau pathology in the association
cortical areas is only observed when there is a simul-
taneous amyloid burden. Therefore, our study dem-
onstrates that tau and APP pathologies work
together to produce nonfamilial AD, and that tau
pathology cannot be considered as a late secondary
event of nonfamilial AD pathology, as frequently
suggested. In that respect, the recent mice models
proposed37,38 fit well with the human physiopatholo-
gy36 and as described here, because they demonstrate
a synergy between APP and tau pathology. Further-
more, it is interesting to note that in one model, the
amyloid burden is acting through neuronal connec-
tions and not by a direct contact to nerve cells, as
observed here in human brains.37

The quantification of Aβ and tau pathologies in
the different brain areas allowed us to refine the
criteria for a biochemical diagnosis of AD. We found
a good correlation among clinical data, amyloidosis,
and tau pathology, if a notion of threshold is added.
Indeed, brain lesions at low levels, observed at the
beginning of the pathology, do not impede cognitive
functions. Neuroplasticity is certainly a significant
factor that will modify the threshold of clinical man-
ifestations, almost independently of the physiopatho-
logic process.7,39

Our study shows that all clinically defined proba-
ble AD cases had huge amounts of amyloid deposits
and widespread tau pathology, in good agreement
with the recent neuropathologic criteria.32 As men-
tioned previously, the polymodal association areas
are the most informative brain areas for an accurate
definite diagnosis. The hippocampal area, early and
frequently affected by tau pathology and amyloidosis
in nondemented patients, is only informative to
stage the infraclinical stages of AD. In agreement
with our previous reports, this technique of amyloid
quantification is able to define the criteria to estab-
lish a biochemical diagnosis of AD (CEBDAD) and
shows that it is possible, with only biochemical
means, to distinguish between normal aging and in-
fraclinical stages of AD on one hand, and between
infraclinical and clinical AD on the other.

Clinical AD. All of our clinically and then neuro-
pathologically diagnosed cases with AD had signifi-
ant tau pathology in the frontal pole and the
parietal cortex (stage 7 to 10) and the presence of Aβ
42 aggregates above 50 µg/g of wet tissue in these
cortical areas. Therefore, these biochemical criteria
are necessary and sufficient to demonstrate that an
Alzheimer process is present that could explain the
cognitive deficit.

Infraclinical AD. All nondemented patients, or
those with a mild cognitive impairment, with insol-
uble Aβ 42 at a concentration of 10 µg/g of tissue in a
polymodal association area, such as the frontal pole
or the parietal cortex, and a tau pathology in the
hippocampal area can be reasonably considered as
patients at the infraclinical stage of AD. The choice
of 10 µg is arbitrary and corresponds to a sizable
detection of insoluble Aβ, 10 times higher than the
threshold of sensitivity of the technique. At this con-
centration, there is no doubt that the amyloidogenic
process of AD has started. Staging of tau pathology
is necessary to define the extent of the neurodegen-
erating process. Tau pathology can be asymptomatic
up to stage 6, and rarely but possibly up to stages 7
or even 8.

Normal aging. All nondemented patients can be
considered as pure ‘controls,’ as far as tau and APP
pathologies are concerned, if tau pathology is absent
in all cortical areas, including the entorhinal area,
and if there is no trace of Aβ 42 aggregates in neo-
cortical areas. If the patients are older that 75 years,
tau pathology, very discrete or moderate, is likely to
be found in the entorhinal and hippocampal areas
(stages 1 to 3), due to aging or a pathologic process
that remains to be determined. But these aged non-
demented patients can be considered as controls if
they have no detectable Aβ 42 aggregates (arbitrary
value of 10 µg/g of tissue).

Here we show that Aβ 42 aggregates is the main
and sufficient variable to demonstrate and quantify
amyloidosis. Our CEBDAD criteria can be used first
for the staging of AD, in complement to the neuro-
pathologic one. Our method gives a rapid and reli-
able quantification of amyloid deposition and tau
pathology. This method can be used to further char-
acterize Alzheimer pathology of the frozen tissue
from brain banks or from animal models. This ap-
proach is likely to be extremely valuable, as the
brain tissue is the source for the search of biochemi-
cal (proteomic) or molecular markers (differential
display, DNA chips). The CEBDAD should also be
useful for the search of markers for the biological
diagnosis, in the context of a prospective study.
Amyloid patterns led us to define their significance for a biological diagnosis of AD. The data presented here show the great variety of insoluble Aβ patterns in AD patients, from pure Aβ 42 to huge Aβ 40 peptide accumulations. The ratio Aβ 42/40 is varying from infinite to 0.4 according to the cases studied. This heterogeneity is likely to be mirrored by the Aβ peptides that are released in the CSF or the blood. It could explain the relative lack of specificity observed for the biological tests based on the ratio Aβ 40/42. Indeed, at the early stages of AD, we demonstrate here that all amylod deposits are in the Aβ 42 form. Biological parameters dealing with Aβ 40 could bring a lack of specificity, due to the extreme heterogeneity of Aβ 40 deposits as well as to the late appearance of insoluble Aβ 40 in the course of the disease. Therefore, we recommend testing exclusively Aβ 42 for the early diagnosis of AD.

Amyloid and tau patterns reveal some aspects of the etiology of AD. The quantification of Aβ in the different brain areas also demonstrates that the spreading pathway of tau pathology remains constant, whatever the cortical distribution of Aβ aggregates. We conclude that if there is a toxicity of amyloid, this one is acting through neuron to neuron connections, and not by a direct neurotoxicity of Aβ toward surrounding neuronal cell bodies. In that perspective, our results suggest more a loss of APP function as a neurotrophic factor than an amyloid toxicity as the cause of AD, Aβ 42 being clearly an early marker of Alzheimer pathology, but not necessarily the etiologic factor. A dysfunction of APP carboxy-terminal fragments, that generate Aβ, are likely to be upstream markers closer to Alzheimer etiology.

Aβ patterns described here can be used for setting up experimental models. Indeed, conversely to references 18 and 19, we also demonstrate herein that Aβ aggregates, and mainly Aβ 42 species, are well correlated to tau pathology and to the severity of the pathology. The amyloid substance from transgenic mice with mutated APP gene was quantified in comparison to human brains. Our biochemical approach shows that, unexpectedly, APPSw mice that are known to produce a fivefold increase of physiologic Aβ 40 and a 14-fold increase of Aβ 42 are essentially composed of Aβ 40 aggregates, as described here, that were easy to dissociate and solubilize in SDS buffers. Our results on these transgenic mice are similar to those obtained using HPLC means, showing that human Aβ aggregates are by far more strongly cohesive. However, our quantification of noncohesive amyloid in mouse brain fits well with previous studies. Together, we observe that Aβ aggregates from APPSw mice are similar in quantities from those of the human brain, but with different physical and biochemical properties. In that perspective, APPld mice could be more relevant to Alzheimer physiopathology, as they produce essentially Aβ 42 deposits, a feature of the first steps of AD. The values of Aβ 42 aggregates observed in the human brain could be used for cellular and animal models of Aβ 42 toxicity. At last, our quantitative approach was validated by comparison with the neuropathologic one, and led us to refine the criteria to establish a biochemical diagnosis of Alzheimer’s disease (CEBDAD).

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