β-Amyloid Protein is Higher in Alzheimer's Disease Brains: Description of a Quantitative Biochemical Assay

STEVEN J. FRUCHT, M.D. AND EDWARD H. KOO, M.D.

Abstract. Deposition of β-amyloid protein (Aβ) in senile plaques and in the walls of cerebral vessels is a pathologic hallmark of Alzheimer's disease (AD). The current diagnostic criteria for AD requires the presence of neurofibrillary tangles and a minimum number of senile plaques in cortex. Senile plaques are readily visualized by silver staining or immunocytochemistry using antibodies raised to Aβ. Available histochemical and immunocytochemical methods are sensitive but the results may occasionally be variable and sampling from many brain regions is difficult and impractical. This study describes a simple biochemical method for quantifying the Aβ load in unfixed brain homogenates. The immunoblot assay recognizes all forms of Aβ deposits (neuritic and diffuse plaques, and cerebrovascular amyloid) and has a sensitivity and specificity comparable to immunocytochemistry. In direct comparisons, results from the dot blot method correspond well with both Western blot analysis of partially purified Aβ and plaque counting by immunocytochemistry. In a retrospective series of 39 postmortem AD and control cases, the amount of Aβ in brain by dot blot immunoreactivity effectively separated the two groups. Therefore, this method provides a rapid, sensitive, and accurate quantitation of Aβ in postmortem brain tissue and represents an alternative approach for studying Aβ deposition in aging and AD.

Key Words: Alzheimer's disease; β-amyloid; Congophilic angiopathy; Immunocytochemistry; Senile plaques.

INTRODUCTION

Deposition of the β-amyloid protein (Aβ) in the central nervous system is a defining feature of Alzheimer's disease (AD), the leading cause of dementia in the elderly (1). In brains of individuals afflicted with AD, Aβ is found within senile plaques and in the walls of cerebral blood vessels. Similar Aβ deposits also occur in individuals with trisomy 21 (2) and, to a lesser extent, in some aged individuals (3, 4). Current neuropathologic criteria require, in addition to neurofibrillary tangles, a minimum number of cortical senile plaques for the diagnosis of AD (5). In addition, early studies suggested that the density of senile plaques in AD correlates with the severity of dementia (6), although more recent studies have shown that neurofibrillary tangles and synaptic density are better correlates of cognitive impairment (7–9). Recent evidence suggests that deposition of Aβ may be an early and possibly initiating event in the pathogenesis of AD (10). For these reasons, quantitation of senile plaques in brain has been an important focus in aging and AD research.

Senile plaques can be visualized by histochemical stains (silver, Congo red or thioflavin) or immunocytochemical staining using antibodies directed against Aβ. Several morphologic subtypes of senile plaques are currently recognized in brain: "classical" or "mature" plaques consisting of an amyloid core surrounded by dystrophic neurites, "primitive" plaques consisting primarily of neurites, "burnt-out" plaques with scant to no neurites, and diffuse plaques with no neurites or compacted fibrillar amyloid (11–13). The latter deposits have gained increasing attention because they may represent an early form of amyloid deposits (13–15) and may often constitute the majority of plaques present in brain (12).

Immunocytochemistry is currently the most sensitive and reliable method for detecting all forms of senile plaques. This is in contrast to conventional histochemical staining methods that often fail to stain diffuse Aβ deposits. Nevertheless, there are limitations to the ability of immunocytochemistry or histochemistry to quantify amyloid deposits. All staining methods occasionally produce variable results in different laboratories. This is especially problematic with tissue processed after prolonged fixation. Thus, accurate assessment and quantitation of Aβ is sometimes difficult to obtain. Even when automated, plaque counting from stained sections is time consuming and labor intensive. Finally, small punctate amyloid deposits which frequently accompany senile plaques and vascular deposits are difficult to quantitate.

In this study, we asked whether a biochemical assay could be designed to accurately quantitate amyloid load in postmortem brain. We also wanted to determine whether the amount of Aβ could reliably differentiate AD from control brains. Building upon the studies in scrapie prion protein and Aβ (Alz 50) protein (16, 17), this report describes a similar immunoblot technique for rapid quantitation of total amount of Aβ in unfixed brain homogenates. The dot blot technique for detecting Aβ has the sensitivity and specificity of immunocytochemistry. More importantly, the biochemical approach may...
be more sensitive and accurate than plaque counting in quantifying the total amount of Aβ in brain, i.e., amyloid in plaques and blood vessels. Therefore, this method may prove to be a useful tool in studying the pathogenesis of Aβ deposition and may contribute to an understanding of the role of Aβ in aging and AD.

MATERIALS AND METHODS

Brain Tissue

Brain tissue was obtained from autopsy in 46 cases of AD, Down's syndrome (DS), and control individuals. For 39 retrospective cases, one half of each brain was frozen at the time of autopsy, and the remaining half was fixed in formalin and examined by hematoxylin and eosin, modified Bielschowsky and Congo red staining. Cases were assigned to one of three groups using standard neuropathologic criteria (5): Aβ (n = 17), <Aβ (n = 4) (cases which contained greater than 5 plaques/20× field but did not meet the criteria), and control (n = 15). The amount of vascular amyloid was semiquantitatively assessed from sections as severe, moderate, mild, or absent. The average age of each group was 77 ± 2 (SEM), 83 ± 6, and 68 ± 3 years, respectively; the average postmortem interval (P.M.I.) in each group was 15 ± 2, 16 ± 1, and 15 ± 2 hours, respectively. Seven cases of neurodegenerative diseases without AD-type changes (three Pick's disease, three Parkinson's disease and one progressive supranuclear palsy [PSP]) were included in the control group. In addition, three individuals with DS (47, 55 and 58 years old) were part of the 39 cases and had sufficient changes to meet pathologic criteria for AD. Tissue from two AD (ages 83 and 88, P.M.I. 12 and 14 hours) and two control cases (ages 73 and 82, P.M.I. 15 and 19 hours) was prospectively sampled at autopsy (see below). Three additional AD cases were also used for control purposes: one for plaque core isolation using published methods (18) and two with exclusively diffuse plaques in the cortex.

Tissue Sampling

Five areas of cortex (0.2–0.5 g), consisting predominantly of gray matter, were sampled from the frozen hemisphere of each case: frontal pole, occipital pole near the striate cortex, mid-superior temporal gyrus, anterior cingulate gyrus and posterior cingulate gyrus at the level of the splenium. These areas were chosen to include: frontal, parietal, temporal and occipital cortices and for the ease in identifying these regions. The meninges were stripped as much as possible from the tissue before sampling. In four prospective cases (two control and two AD), immediately adjacent blocks were obtained from fresh tissue at the time of autopsy for histologic examination and immunohistochemistry in order to directly compare the different methods. Care was exercised on sampling so that full thickness of cortex was present in both tissue blocks.

Immunocytochemistry

Immunocytochemical detection of Aβ in tissue sections was carried out essentially as described using the rabbit polyclonal antibody R1280 directed against synthetic Aβ (1–40) (19, 20). Immunostaining was performed on 8 μm sections with pretreatment in 100% formic acid for 10 minutes. The avidin-biotin system with diaminobenzidine as the chromogen was used to visualize the primary antibody.

Quantitation of Senile Plaques

Nine cortical fields from each area, distributed evenly through the full thickness of gray matter, were counted at 200× magnification (approximately 1 mm²) from silver- or immunocytochemically stained sections in order to produce an average plaque count per region. All forms of plaques, including diffuse plaques and punctate deposits, were tabulated when larger than approximately 15–20 microns. In the retrospective series, silver-stained plaque counts from the five regions were then combined to produce an average value per case.

Dot Blot Quantitation of Amyloid Load

The immunoblotting method was adapted from a published procedure (16). Two hundred mg of frozen brain tissue (primarily gray matter) was homogenized with a hand-held glass homogenizer in 10× volume of buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4; 0.5% NP40, 0.5% sodium deoxycholate, 0.1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml aprotonin and 7 μg/ml pepstatin). Total protein was measured using the bicinchoninic acid reagent (Pierce, Rockford, IL). Two μl of each sample (0.2 mg of original wet weight of brain) was diluted with 98 μl of water and the entire mixture was spotted onto a 0.2 μm nitrocellulose membrane using a dot blot apparatus. The nitrocellulose filters were blocked in non-fat milk, reacted with R1280 at 1:1,000 dilution and then incubated with 125I-labeled goat anti-rabbit antibody (200 μCi/ml) at 0.1 μCi/ml. For preabsorption experiments, 5 μl of antibody was pre-incubated with 100 μg of synthetic Aβ peptide (1–40) in 1 ml of non-fat milk for 1 hour. Following exposure to X-ray film for 1–3 days, radioactivity minus background (membrane with no sample) was determined from each sample using a gamma counter. In the retrospective series, counts from the five brain regions were averaged to produce a mean Aβ load for each case. On all filters, a standard dilution series of HPLC purified synthetic Aβ peptide (1–40) dissolved in water at 1 mg/ml was included.

Isolation and Demonstration of Aβ from Brain by Western Blot

Frozen brain tissue (500 mg) from five cases in the retrospective series (three AD [average age 63], one DS [age 55] and one <AD [age 78]) was sampled from frontal cortex directly adjacent to tissue used for Aβ dot blot quantitation. Tissue was homogenized using a hand-held glass homogenizer in 10× volume of SDS buffer (10% SDS, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% sodium azide, 1 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 0.2 μM PMSF, and 1 μg/ml TLCK). Homogenates were centrifuged at 100,000 g for 30 minutes. The resulting pellets were rehomogenized in SDS buffer and centrifuged a second time as above. Pellets were then resuspended in 100% formic acid and left at room temperature for 1 hour, after which they were centrifuged for 40 minutes at 350,000 g (21). Formic acid supernatants were dried, resuspended in Laemmli sample buffer with 6 M urea, and fractionated by 14% tris-tricine SDS-PAGE (22). Following separation, proteins were trans-
Fig. 1. Dot blot of Aβ synthetic peptide. Different amounts of synthetic Aβ (1–40) peptide were spotted onto nitrocellulose membrane and reacted with the polyclonal antibody R1280. Radioactivity (cpm) was determined following incubation with an [3H]-goat anti-rabbit secondary antibody. The graph displays the level of radioactivity plotted against increasing amounts of Aβ peptide. Note that the curve is linear in the lower end but approaches an asymptotic limit as the amount of Aβ increases. Each point represents the average of triplicate samples ± SEM.

Fig. 2. Aβ dot blot immunoreactivity from brain. (A) Representative Aβ dot blot autoradiograms from several brain regions from a case of AD and a case of DS are shown. Tissue homogenates from frontal pole (FP), superior temporal gyrus (STG), occipital pole (OP), anterior cingulate gyrus (ACG), and posterior cingulate gyrus (PCG) were sampled. (B) Dot blots of purified plaque cores (CORE), diffuse amyloid plaque (DP) (obtained from two selected cases containing only diffuse plaques in cortex), and vascular amyloid (CAA) (from a control case with congophilic angiopathy but without senile plaques) are shown. The Aβ dot blot immunoassay recognizes all forms of β-amyloid deposits.

RESULTS

Dot Blot of Aβ Synthetic Peptide

In initial studies, the immunoreactivity of synthetic Aβ peptide (1–40) to the polyclonal antibody R1280 was determined in a dot blot assay (Fig. 1). The limit of detection by this technique was approximately two nanograms of protein. At low levels of Aβ, there was a linear relationship between the amount of peptide and radioactivity. Aβ immunoreactivity approached an asymptotic limit as the amount of synthetic peptide increased. On multiple repetitions of the standard peptide dilutions, the shape of the curve was highly reproducible although the absolute radioactivity varied. In addition, increasing amounts of synthetic Aβ peptide (1–40) were mixed with control brain tissue (containing no plaques or vascular amyloid) and the resultant immunoreactivity was unaffected by proteins present in brain tissue (data not shown).

Detection of Aβ from Brain by Dot Blot Immunoreactivity

In postmortem brain samples, preliminary studies showed that for cases with a wide spectrum of total Aβ, loading the equivalent of only 0.2 mg of original wet weight of brain produced autoradiograms with optimal signal-to-noise (Fig. 2A). Further, this amount of sample is within the linear range of an asymptotic standard curve (similar to Fig. 1) generated by loading increasing amounts of AD brain. Normalizing to an equivalent amount of starting total protein instead of wet weight of tissue did not significantly alter the pattern or intensity of immunoreactivity. The immunoblot results are reliable and consistent: when the same samples were analyzed on different days and radioactive counts normalized to an equivalent amount of synthetic Aβ peptide (within the linear range of the peptide dilution curve), relative Aβ immunoreactivity was highly reproducible. The dot blot immunoassay recognized Aβ deposits in multiple forms, including senile plaque cores, diffuse amyloid deposits and vascular amyloid (Fig. 2B). Immunoreactivity was abolished by preabsorption of antibody with Aβ peptide (data not shown). Finally, the method was not antibodiespecific; similar results were obtained on two of the prospective cases using the monoclonal antibody 10D5 raised against amino acid residues 1–28 of Aβ (20) (data not shown).
Correlation of Dot Blot Immunoreactivity with Partially Purified Aβ

Using adjacent blocks of tissue from several representative cases, dot blot immunoreactivity was compared to partially purified Aβ extracted by SDS precipitation and formic acid solubilization. There was a good correlation between dot blot immunoreactivity and the amount of \( \approx 4 \) kDa Aβ species identified after partial purification and Western blotting (Fig. 3). These results confirmed the specificity of the dot blot immunoassay for Aβ in brain.

Correlation of Dot Blot Immunoreactivity, Immunocytochemistry and Silver Staining

The sensitivity of the dot blot method was compared to conventional techniques (silver and immunostaining) using adjacent sections of fresh cortex from four prospective cases, two AD and two controls (Fig. 4A). In all cases, plaque counts were higher with immunocytochemistry than silver staining, consistent with the greater sensitivity of the former method. Examination of the individual cases revealed several trends. In general, the changes in plaque density by immunostaining and Aβ load by dot bloting were consistent with each other. However, in both AD 1 and AD 2 (Fig. 4A), plaque counts and immunoreactivity by dot blot were discordant in the occipital pole. The disparity may have resulted either from sampling differences or from the fact that plaques with compacted cores predominated in these areas (see below).

In case AD 2, the presence of mostly diffuse plaques in the superior temporal gyrus resulted in prominent Aβ dot blot values and immunostained plaque counts but very low plaque numbers by silver staining. Similarly, three regions in the two control cases (frontal pole and posterior cingulate gyrus of Cntr 1 and superior temporal gyrus of Cntr 2) contained low numbers of diffuse amyloid deposits that were recognized by immunocytochemistry and dot blot assay but not by silver staining. These results, therefore, demonstrate the superior sensitivity of immunocytochemistry and the dot blot assay. The findings also suggest that there is a close correlation between the two immunodetection methods. This correspondence is better demonstrated when the results from Aβ dot blotting and plaque counts by immunocytochemistry from these four cases are analyzed together (coefficient of determination \( r^2 = 0.93 \), Fig. 4B).

Dot Blot Immunoreactivity in 39 Postmortem Cases

In view of the correlation between Aβ dot blot and Aβ immunoreactivity in our prospectively studied samples, we asked whether the former method could differentiate AD cases from control cases in a large series. The four cases detailed above and illustrated in Figure 4 were not included in this series. Because this was a retrospective study, the tissue samples used for histologic staining and dot blot immunoassay were not obtained from precisely opposite regions in both hemispheres. In general, there was a good correlation between the average amount of Aβ by dot blot and the average number of senile plaques from silver-stained sections for each case (\( r^2 = 0.84 \), Fig. 5). In addition, two levels of radioactivity at approximately 850 and 500 cpm segregated the cases into three groups. All of the control and neurologic disease cases (Parkinson’s disease, Pick’s disease, and PSP) were below

Fig. 3. Correlation of Aβ dot blot with partially purified Aβ separated by Western blot. Aβ from three cases of AD (1–3), one case of DS (4), and one case of <AD (5) was partially purified by formic acid extraction and separated by SDS-PAGE. Equal volume of protein was fractionated and transferred to nitrocellulose membrane for incubation with R1280. One μg of synthetic Aβ (left lane) with a molecular weight of 4.3 kDa migrates anomalously with an apparent molecular weight of approximately 3 kDa on the 14% tris-tricine gel system (22). Molecular weights (kDa) of prestained markers are displayed on the left. The amount of Aβ in adjacent tissue blocks obtained by dot blotting, expressed as radioactivity (in cpm), is shown in the lower panel. Changes in Aβ immunoreactivity by dot blot correlate in general with Western blot detection of partially purified Aβ from the same brain regions in these cases.
Fig. 4. Correlation of dot blot, silver stain, and immunocytochemical detection of Aβ from four prospective cases. (A) Adjacent cortical tissue blocks from four cases (two AD [AD 1, AD 2] and two controls [Cntr 1, Cntr 2]) were analyzed by Aβ dot blotting (solid bar) and plaque counting from silver (open bar) and immunostained (hatched bar) sections. For plaque quantitation (right vertical axis), the average of nine cortical fields (±SEM) was calculated for each block at 200× magnification from five cortical regions (FP, STG, OP, ACG, and PCG). Samples were taken from only four areas (FP, STG, OP and TP) in AD 1. Aβ immunoreactivity from dot blotting (left vertical axis) expressed as radioactivity (in cpm) was obtained from the average of triplicate samples (±SEM). Plaque density was generally higher in immunostained than Bielschowsky-stained sections. When Aβ immunoreactivity by dot blotting is plotted against plaque counts by immunocytochemistry for these four cases (B), there is a close correlation between the two immunologic methods (coefficient of determination $r^2 = 0.93$).

the lower cutoff, and no AD brain had values in this lowest region. Four of the 17 AD cases and two of four <AD cases had Aβ values between 500 and 850 cpm. Only one of four <AD cases had Aβ amounts greater than the upper level and this case had a significant amount of coexistent congophilic angiopathy that may have accounted for the anomalously high value (Fig. 5). In the AD group, one case that showed an expectedly high dot blot value compared to plaque count also had severe cerebrovascular amyloid (Fig. 5). Although other AD cases had moderate to severe focal congophilic angiopathy, the resultant Aβ load did not share this trend. This may be due to the fact
that each case was represented by an average value of five cortical areas, thereby diluting out the contribution of focal deposits of vascular amyloid.

DISCUSSION

The presence of senile plaques in sufficient density in neocortex is one of the morphologic hallmarks of A.D. Indeed, numerous studies have attempted to correlate the number of plaques in various brain regions to aging, clinical signs and symptoms, and severity of dementia. Traditionally, silver staining is used to visualize plaques, although immunocytochemistry has been gaining popularity in recent years. The staining quality of these methods can be variable and is particularly sensitive to the preservation state of the tissue. Moreover, quantitation of plaque density from histologic sections is laborious and time consuming.

In this study, we have adapted a dot blot method, used previously to qualitatively assay prion protein in Creutzfeldt-Jakob disease, to quantitate the Aβ load in unfixed postmortem brain. Our results indicate that the dot blot assay is specific for Aβ, as demonstrated by the correlation in Aβ levels obtained by dot blotting of crude brain homogenates and Western blotting of partially purified Aβ. This method detects small amounts of Aβ and has a sensitivity and specificity comparable to immunocytochemistry. From four prospectively studied cases, there was a close correlation between the amount of Aβ measured by dot blot and the number of senile plaques visualized by immunocytochemistry in multiple brain regions \( r^2 = 0.93 \). Finally, in a large postmortem series, the amount of Aβ measured by this technique was able to differentiate most A.D. cases from non-A.D. cases. In this retrospective survey, the amount of Aβ by dot blotting and average plaque counts from silver-stained sections showed a surprisingly close correlation \( r^2 = 0.84 \), demonstrating the utility of this approach for measuring the amyloid load from tissue homogenates.

The immunoblotting technique described in this study offers several advantages over existing methods for measuring Aβ. The method is extremely practical, requiring only 0.2 mg of wet weight of brain tissue per analysis. The method is easy to perform so that many samples can be assessed simultaneously. The results are highly reproducible and quantitative, and comparison can be made from a number of experiments by standardizing the data to a relative amount of synthetic Aβ peptide (within the linear range). The method is particularly valuable for measuring small amounts of Aβ. In two aged control cases, dot blot assay and immunocytochemistry detected low numbers of diffuse amyloid deposits that were not seen by silver staining.

There are a number of limitations to the dot blot technique. Analysis of archival tissue embedded in paraffin or fixed in formalin is not possible. Because the method requires only 0.2 mg of tissue and the density of amyloid plaques varied considerably within brain regions, sampling errors are unavoidable. It is therefore important to sample multiple regions using larger blocks of tissue in order to obtain a representative estimate of the amyloid load. Further, the method is a variant of immunocytochemistry and is subject to similar limitations, such as antibody affinity and antibody access to the antigen. Thus, Aβ deposits bound to the nitrocellulose membrane but not accessible to the antibody will not be detected. Moreover, homogenization of the tissue probably leaves many Aβ deposits intact, especially compact cores, such that the antibody may not have access to the majority of the protein. As a result, the assay may be somewhat more sensitive to diffuse than compacted amyloid deposits. This could be one factor contributing to the asymptotic im-
munoreactivity at higher amounts of Aβ and to the discrepancy between dot blot and plaque counts at higher plaque densities.

One additional drawback is that the assay does not differentiate Aβ from senile plaque cores, diffuse plaques, and vascular amyloid. However, it is possible that by taking advantage of the biochemical differences between these three types of deposits, one may be able to separate and quantitate their relative amounts. For example, plaque cores are denser than diffuse plaques and vascular amyloid, and can be selectively pelleted by low speed centrifugation (23). Vascular amyloid can be separated from senile plaques by the former species’ solubility in guanidine hydrochloride. While such an approach requires more effort, it could provide a biochemical measure of the contribution of each type of Aβ deposit to the total amyloid load.

To establish a diagnosis of AD, current criteria rely on the quantitation of senile plaques and neurofibrillary tangles. There is still controversy regarding the accuracy of the diagnostic criteria and some neuropathologists question our ability to separate AD from changes in normal or “pathologic” aging (24, 25). There are many instances where the criteria appear to be inadequate. For example, a number of cases have been reported that fulfill pathologic criteria of AD for which no clinical correlate of dementing illness exists (26, 27). One of the questions asked in this study is whether the amount of Aβ is different between AD and non-AD brains. The analysis showed that total Aβ as determined by the dot blot immunoassay effectively segregated the majority of AD from control cases, although there is a gray zone where an overlap exists between the two groups. Indeed, the latter result is anticipated because this assay correlates well with immunocytochemistry and, as described above, the presence of senile plaques is not quantitatively or qualitatively able to separate some cases of AD from aged non-demented individuals (24, 26, 28). Nonetheless, the sensitivity, specificity, and simplicity of this method illustrates its potential as an adjunct in neuropathologic evaluations. It is our hope that biochemical measurements of Aβ and other cytoskeletal markers, such as tau protein, may provide important insights in establishing a more refined diagnostic criteria for AD.

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