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Dissociated Amyloid- β Antibody Levels as a Serum Biomarker for the Progression of Alzheimer's Disease: A Population-Based Study

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Abstract

With an ever growing population of aged individuals who are at risk of developing Alzheimer disease (AD), there is an urgent need for a sensitive, specific, and preferably non-invasive diagnostic standard of disease progression. Mainstream thinking suggests that early intervention is key to maximizing the opportunity for a successful treatment regimen in AD and, as such, an early and accurate means of diagnosis is essential. In this study, we applied a recently described antibody-antigen dissociation technique to samples obtained as part of a population-based analysis of the prevalence of AD. Stratified sampling and random selection strategies were combined to obtain a representative population for screening of individuals older than 55 years. Serum antibodies to amyloid- β (A β)₁₋₄₂ were measured before and after antigen dissociation. The difference between the two measurements was indicated as the dissociation delta (Δ). Our analyses showed that the levels of dissociated antibody in AD patients were always significantly different from controls and that levels of A β antibody after dissociation, but not non-dissociated levels, correlated negatively ($p < 0.05$) with both duration of the disease and age in the AD patients. Moreover, the change in concentration of A β antibody from pre- to post-dissociation (i.e., the dissociation Δ) directly reflected the progression of AD in terms of both time since diagnosis and age of the patients, with a lower dissociation Δ indicating a more advanced stage of AD. Ultimately, these data suggest that dissociated A β antibody levels are of significant diagnostic value at the onset of the neurodegenerative process and, thereafter, may be a useful biomarker for disease progression.

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Keywords

Alzheimer disease; amyloid- β ; antigen-antibody complexes; biomarker; diagnosis

Introduction

Amyloid- β (A β) is the major protein component of senile plaques that accumulate in specific brain regions of patients with Alzheimer disease (AD), and its presence is used for a definitive postmortem diagnosis of the disorder (Andreasen et al., 1999; Andreasen et al., 2001; Castellani et al., 2006; McKeel et al., 2004; Murayama and Saito, 2004). Unfortunately, current treatments provide only symptomatic relief that provides extremely limited impact on disease progression (Marlatt et al., 2008). Further, disease modifying clinical trials are often confounded by using patients at more advanced stages of disease. Therefore, it is currently thought that early treatment may provide greater opportunity for therapeutic efficacy. Unfortunately, such early diagnosis has been hampered by a lack of a diagnostic standard. Similarly, treatment efficacy in clinical trials relies on subjective changes since there are no current biomarkers of disease progression.

Given these voids, considerable efforts over the last decade have been expended on developing tools for the early diagnostic of disease and/or biomarkers of disease progression. Most efforts in this regard have focused on serum or cerebrospinal fluid (CSF) levels of amyloid- β (A β) or antibodies to A β (Kester et al., 2009; Pauwels et al., 2009; Verbeek et al., 2009; Verwey et al., 2009) and have been unequivocal with conflicting data with little diagnostic predictive value (Blennow, 2004). Unfortunately, ignored in most studies is the fact that, in biological fluids, antibodies and antigens are in a state of dynamic equilibrium between bound and unbound forms that is concentration dependent. Although the amount of antibody against a particular antigen in a given disease state may be strongly elevated, only a fraction of the total amount is likely detectable by ELISA (enzyme linked immunosorbent assay) due to interference by these antigen-antibody complexes. In this regard, previously we demonstrated the importance of measuring total amounts of antibody following dissociation of the antigen-antibody complexes and, by using both *in vitro* and *in vivo* approaches, we provided an explanation of the discrepancy in existing data and provided a novel diagnostic strategy (Gustaw et al., 2008a). Similar approaches were employed in a transgenic animal model of AD (Li et al., 2004).

In this study, we expand upon the use of this technique for the assessment of A β antibody levels in sera collected from a population-based series of newly diagnosed AD subjects and controls. Our findings show that dissociated A β antibody levels and the dissociation Δ (dissociated minus non-dissociated) may be useful tools for the reliable and early diagnosis of disease as well as for providing an assessment of disease progression. As such, this technique may be useful in monitoring therapeutic efficacy in clinical trials.

Patients and Methods

Population based sampling design

The current study is a part of a large population-based study named **BERCAL** (*Badanie Epidemiologiczne Rozpowszechnienia Choroby Alzheimer i innych form demencji w województwie Lubelskim*).

The participants of the BERCAL study were randomly selected from the population-based sample within the Lublin Region of Poland composed of 2,182,191 inhabitants in the year 2005 when the population was sampled. As a result of the project, the prevalence of AD in Lublin's

Region Poland was calculated as 1634.6/100,000 inhabitants (Gustaw et al., 2008b). The BERCAL study design is described in more detail elsewhere (Gustaw et al., 2008b), yet it is important to mention that dementia was diagnosed by the Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV) criteria (American Psychiatric Association, 2000) and AD was diagnosed in accordance to the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS - ADRDA) criteria (McKhann et al., 1984).

Normal control subjects were recruited from the same population. Control participants had normal cognition excluding mild cognitive impairment (MCI).

The study was carried out in accordance with the local IRB agreement. Written informed consent was obtained from the patient (if possible), the caregiver, and/or the patient's representative (if applicable) before beginning detailed screening activities.

In vivo dissociation of A β antibody in human sera

The technique for the *in vitro* dissociation of A β antibodies has been previously described in detail and shown to be reliable and reproducible (Gustaw et al., 2008a). Briefly, sera (AD, n=48 and healthy, age- and gender-matched controls, n=20) were diluted 1:100 with dissociation buffer (PBS buffer with 1.5% BSA and 0.2 M glycine-acetate pH 2.5) to a 500 μ l final volume and incubated for 20 min at room temperature (RT). The sera were then pipetted into the sample reservoir of Microcon centrifugal filter device, YM-10 (10,000 MW cut-off; Millipore) and centrifuged at 14,100 rpm for 20 min at RT. The sample reservoir was then separated from the flowthrough, which was inverted into a second tube and centrifuged at 5000 rpm for 3 min at RT. The collected solution containing the antibody dissociated from the A β peptide immediately was adjusted to pH 7.0 with 1 M Tris buffer, pH 9.0. The retentate volume was reconstituted to the initial volume (500 μ l) with ELISA dilution buffer (PBS with 1.5% BSA and 0.1% Tween-20). The collected sera were then added to an ELISA plate at several dilutions to determine the antibody titer. As a control, the same serum was treated in an identical process except that the sera were diluted into buffer at pH 7.0 instead of dissociation buffer, pH 2.5. The difference between the dissociated sera and the corresponding non-dissociated sera was analyzed as the dissociation Δ .

Measurements of antibody titers by ELISA

NUNC Maxisorp 96 well ELISA plates were coated with 50 μ l/well A β ₁₋₄₂ 5 μ g/ml in PBS, pH 7 and incubated overnight at 4°C. Plates were washed 5 times with washing buffer (0.45% BSA + 0.05% Tween-20), and then blocked (300 μ l/well) for one hour at 37°C with 1.5% BSA + 0.05% Tween 20 in PBS. Following blocking, the plates were washed 4 times with washing buffer and samples applied (50 μ l/well) in duplicate or triplicate and incubated at 37°C for one hour. The plates were then washed 10 times with washing buffer. Anti-human IgG (H+L) antibody (Southern Biotechnology Associates Inc.) was diluted 1:2500 and added at 50 μ l/well.

Samples in quadruplicate were incubated for 1 hour at 37°C. After incubation, the plates were washed 10 times, developed with TMB (Pierce 1-step Ultra, Sigma), the reaction stopped with 2M sulfuric acid (50 μ l) and the plates were analyzed spectrophotometrically at 450 nm.

Statistical Analysis

Quadruplicates of each sample were analyzed using the student's t-test to obtain mean values and standard deviation for further analysis. Mann-Whitney test and Pearson Product Moment Correlation were used to compare and correlate outcome parameters respectively.

Statistical significance was marked when Power of the test was > 0.8 for $\alpha=0.05$. Sigma Stat 3.0 for Windows was used for statistical analyses. All p values <0.05 were regarded as statistically significant.

Results

Sera collected from newly diagnosed AD patients and age-matched controls were analyzed for antibodies against A β both before and after dissociation of A β . Statistical analysis shows significantly greater antibody levels both before and after dissociation in AD groups when compared to control (Figure 1A,B). As expected, within the control group, differences in antibody level were statistically greater after dissociation, however, within the AD group, these differences were even larger (median O.D. 1.07 vs 0.7 respectively, $P<0.001$) (Figure 1B). In fact, the dissociation Δ , the difference between antibody levels after and before dissociation, was much greater in AD than in controls (median difference 0.4 vs 0.2, $P<0.001$) (Figure 1B) and also was related to disease progression with newly diagnosed cases.

The level of A β antibodies was correlated with other patient and disease parameters. Both the age of the patients examined (Figure 3B) and the time from when the first clinical symptoms were noticed (Figure 2), which approximately reflected the duration of the disease, were negatively correlated with levels of A β_{1-42} after dissociation in the AD patients (correlation coefficient: -0.7 and -0.5 , $p<0.05$). Moreover, the difference in antibody levels after dissociation (i.e., dissociation Δ) was the greatest in patients with less than one year following their clinical diagnosis (Figure 1B). The level of antibody detected after dissociation, however, did not correlate with age in the controls (Figure 3A), and the levels of antibody both before and after dissociation in both AD and control groups were not statistically correlated to Mini-Mental Status Examination (Figure 4A,B).

Discussion

To date, there has been considerable variation, with often contradictory data, in studies probing the potential for AD diagnosis using serum A β antibody levels. On the one hand, a number of reports describe that patients with AD have lower levels of A β serum antibodies as compared to age-matched controls (Du et al., 2001; Weksler et al., 2002), while other studies indicate the opposite: the level of anti-A β antibodies may be much higher in AD as compared to control subjects (Mruthinti et al., 2004). Similarly, although our study did not show, like many others, A β antibody titers have been previously reported to be negatively correlated to cognitive status, with more cognitively-impaired individuals exhibiting higher anti-A β titers (Mruthinti et al., 2004). Such discrepancies in data attest to the fact that the immunochemical measurement of plasma A β is complex and requires sufficient and particular analysis. Specifically, variables such as the binding of A β to other proteins (Biere et al., 1996; Sagare et al., 2007), its tendency to aggregate (Fullwood et al., 2006), and its strong affinity for A β antibodies (Gustaw et al., 2008a; Oh et al., 2008) must be taken into account, and in this study, our dissociation technique does just that.

In our previous *in vitro* experiments (Gustaw et al., 2008a), A β_{1-42} antigen effectively blocked antibody detection, yet following dissociation of the complex with low pH buffer, detection of the A β antibody was completely restored. This relatively simple method involved passing the A β peptide through a filter after its dissociation from the antibody, separating the antibody from its antigen, and preventing any rebinding, thus allowing it to be detectable at greater levels after dissociation *in vitro*. Ultimately, we developed the dissociation technique using human plasma samples from AD patients and age-matched controls (Gustaw et al., 2008a) and found the assay to be simple, fast, reliable, and reproducible.

It has been proposed that during the long preclinical, non-symptomatic phase of AD, the levels of A β ₄₂ are on average higher than normal (Ida et al., 1996). The formation of A β in the brain, as well as the progressive aggregation of A β ₄₂, then leads to the decline of plasma levels to within normal limits-not significantly different from the population with no AD risk. At the MCI stage, the levels of A β ₄₂ are high (Quesada et al., 1997), and later in the course of the disease, A β ₄₂ in plasma decreases, as shown by several studies that reported no difference of A β plasma levels between AD patients and controls (Ida et al., 1996). At the MCI stage, the levels of A β ₄₂ are still high presumably because the amyloid load is generally limited to the mesial temporal lobe (Petersen et al., 2001). A number of studies, however, reported a large variability of A β values in plasma that lead to a large overlap between normal controls and MCI, and longitudinal studies in amnesic MCI cases yielded conflicting results on the value of A β plasma levels as being predictive of AD (Blasko et al., 2008; Mayeux et al., 2003). According to these results, plasma A β levels reflect A β metabolism in the brain not only in the early phase of AD, but also in MCI. Bearing in mind the above mentioned data, the level of antibodies to A β , before and after disassociation, in MCI cases will be assessed in future studies.

The same process of disease progression, then, may in fact present a low, apparently basal level of A β in sera of the patient with advanced AD mainly because of the long process of antibody production and amyloid binding to antigen.

Measurement of A β in sera is complicated by the fact that peripheral tissues might contribute to both the circulating amyloid pool and AD pathology within the brain and its vasculature. The wide spread of plasma A β values is also due in part to the ability of A β to bind to a variety of plasma and membrane proteins. Sources outside the central nervous system (CNS) should therefore be taken into consideration, because any intervention to reduce cerebral A β is assessed by monitoring A β plasma levels.

A β , which accumulates in the cerebral microvessels in an age-dependent manner, plays a key role in the pathogenesis of cerebral amyloid angiopathy (Zhu et al., 2007). Platelets are an important cellular element in vasculopathy of various causes. A β may activate or potentiate platelet aggregation. Exogenous A β potentiates platelet aggregation caused by collagen and other agonists. At higher concentrations, A β induces platelet aggregation, which is accompanied by an increase in thromboxane A₂ formation. These actions of A β on platelets are causally related to A β activation of p38 mitogen-activated protein kinase (MAPK) (Cattabeni et al., 2004).

Roher et al. (2009) evaluated the amounts of A β peptides in the CNS and in reservoirs outside the CNS and their potential impact on A β plasma levels and AD pathology. A β levels were measured in the plasma of AD and non-demented controls in a longitudinal study, the plasma of a cohort of AD patients receiving a cholinesterase inhibitor, and the skeletal muscle, liver, aorta, platelets, leptomeningeal arteries, and in gray and white matter of AD and control subjects. Plasma A β levels fluctuated over time and among individuals, suggesting continuous contributions from brain and peripheral tissues and associations with reactive circulating proteins. Arteries with atherosclerosis had larger amounts of A β ₁₋₄₀ than disease-free vessels. Inactivated platelets contained more A β peptides than activated ones. All the measurements show, AD brain and skeletal muscle contained increased levels of A β (Roher et al., 2009).

In this current work, the dissociation assay was performed on newly diagnosed AD and control plasma samples obtained as a part of a BERCAL study protocol, where patients were recruited from the sample population. The experimental group was selected from those patients making initial visits following first signs of cognitive loss. The phenomenon of increasing antibody titers after dissociation was confirmed (Figure 1). Using this methodology, we previously reported that only following dissociation were the A β ₁₋₄₂ antibody levels consistently reliable

for distinguishing AD and control. In the present study, this is confirmed, and additional data is presented to show the validity of this assay to scrutinize even newly diagnosed cases, a major step in consideration of early diagnosis.

In accordance with the proposed hypothesis, it was indeed found that the longer the cognitive decline had been present, the lower the levels of A β in the human sera (Figure 2). Additionally, older people had lower levels of amyloid detected in sera (Figure 3). These correlations became significant after the process of amyloid dissociation (Figure 1). Thus, the dissociated A β _{1–42} antibody levels are of compelling diagnostic value in the beginning of the neurodegenerative process in AD patients. One conclusion is that early in the AD process, an increase in A β production elicits a strong immunological response that could be responsible for the negative correlation of A β _{1–42} antibodies and duration of symptoms.

Thus, evaluation of samples in our study demonstrated the basic premise of the aforementioned hypothesis: A β -antigen effectively masks the detection of A β auto-antibody, but its dissociation can increase the sensitivity of measurement, most notably when compared to a non-dissociated sample of the same sera. The longer the process lasts, the lower non-dissociated level of binding compounds. Significantly, as predicted by our model (Gustaw et al., 2008a), the increase in A β auto-antibody levels in AD cases following dissociation is much greater than that in controls (Figure 1A,B). This study provides an explanation for the variability reported in previous studies using non-dissociated samples as well as providing strong support for the dissociation method. The fact that the effect is dependent on A β level and more apparent in affected AD individuals may be of exceptional value in the search for a reliable biomarker.

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Figure 1.

Serum Aβ antibody levels were measured in control cases (n=18), cases having an AD diagnosis of less than 1 year (n=25), and AD cases with an AD diagnosis greater than 1 year (n=27) before and after antibody dissociation. A. Both groups of AD patients had significantly higher levels of serum Aβ antibodies compared to controls both before and after dissociation. Before dissociation, the levels were unchanged between the AD groups with a diagnosis of either <1 year or >1 year, yet, following dissociation, the levels in cases with a diagnosis of less than 1 year were significantly higher compared to the other AD cases. B. Closer examination of the antibody levels before and after dissociation within each case (the Dissociation Δ), revealed only the AD cases with a diagnosis of <1 year were significantly

different from control. All other AD patients divided according to years since diagnosis showed no differences from control based on the Dissociation Δ , yet were different from the AD cases diagnosed <1 year. (NS= not significant; *= $p<0.001$; **= $p<0.01$; *** $p<0.05$).

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Figure 2.

Serum A β antibodies before and after dissociation in AD patients with a diagnosis of less than 1 year to 6 years. Notably, only the dissociated antibody levels are significantly correlated with time since diagnosis ($p < 0.01$).

Figure 3. Serum A β antibodies in control patients were plotted versus age. Following dissociation, there is a significant positive correlation ($p < 0.001$) (A). Serum A β antibodies in AD patients both before and after dissociation reveal significant negative correlation with age (B).

Figure 4.

Serum A β antibodies measure both before and after dissociation are not correlated with Mini-Mental Status Examination scores in either control (A) or AD (B) patients.