Increased Number of Plasma B Cells Producing Autoantibodies Against Aβ<sub>42</sub> Protofibrils in Alzheimer’s Disease

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Handling Associate Editor: Fabrizio Piazza

Accepted 26 May 2015

Abstract. The Alzheimer’s disease (AD)-related peptide amyloid-β (Aβ) has a propensity to aggregate into various assemblies including toxic soluble Aβ protofibrils. Several studies have reported the existence of anti-Aβ antibodies in humans. However, it is still debated whether levels of anti-Aβ antibodies are altered in AD patients compared to healthy individuals. Formation of immune complexes with plasma Aβ makes it difficult to reliably measure the concentration of circulating anti-Aβ antibodies with certain immunoassays, potentially leading to an underestimation. Here we have investigated anti-Aβ antibody production on a cellular level by measuring the amount of anti-Aβ antibody producing cells instead of the plasma level of anti-Aβ antibodies. To our knowledge, this is the first time the anti-Aβ antibody response in plasma has been compared in AD patients and age-matched healthy individuals using the enzyme-linked immunospot (ELISpot) technique. Both AD patients and healthy individuals had low levels of B cells producing antibodies binding Aβ<sub>40</sub> monomers, whereas the number of cells producing antibodies toward Aβ<sub>42</sub> protofibrils was higher overall and significantly higher in AD compared to healthy controls. This study shows, by an alternative and reliable method, that there is a specific immune response to the toxic Aβ protofibrils, which is significantly increased in AD patients.

Keywords: Amyloid-β, amyloid-β protofibrils, anti-amyloid-β antibodies, enzyme-linked immunospot assay

INTRODUCTION

In Alzheimer’s disease (AD) brains, extracellular amyloid deposits are found in the parenchyma as plaques and in vessel walls as congophilic amyloid angiopathy. These hallmarks of AD are composed of insoluble fibrils of amyloid-β (Aβ), a hydrophobic and self-aggregating peptide. Prior to plaque deposition, Aβ gradually polymerizes from monomers into larger soluble molecular forms (oligomers/protofibrils) and eventually into insoluble fibrils. Several of the intermediate Aβ species have been shown to elicit adverse biological effects both in vitro and in vivo [1–5], suggesting that they play a central role in the pathogenesis. We have previously identified a pathogenic mutation in the amyloid-β protein precursor (AβPP) gene, the Arctic mutation (E693G). This mutation, causing early onset AD, is located within the Aβ domain and enhances the formation of Aβ protofibrils, suggesting that this Aβ species may be causative of the disease [6]. Moreover, it has been shown that Aβ protofibrils are present in cerebrospinal fluid (CSF) and that the levels
of large soluble Aβ aggregates in CSF correlate with cognitive impairment, implying that the progression of the disease is dependent on Aβ protofibrils [7–10].

Autoantibodies, reactive to self-antigens, are often associated with pathological functions leading to autoimmune diseases. However, some autoantibodies play a physiological role in the body without leading to pathological processes and disease progression. These naturally occurring autoantibodies are believed to have a role in maintaining homeostasis and preventing inflammation [11]. Natural autoantibodies against Aβ (anti-Aβ antibodies) have been found in both AD and non-demented individuals [12, 13]. A vast part of anti-Aβ antibodies are believed to be produced in response to Aβ in the periphery and central nervous system. It has been proposed that anti-Aβ antibodies predominantly show affinity toward the toxic aggregated forms of Aβ, including oligomers and protofibrils, which may be considered nonself-antigens, and not to monomers [14–17]. The function of anti-Aβ antibodies is not understood but they have been implied to interfere with Aβ toxicity and the oligomerization and fibrillization of Aβ [1, 14, 16, 18, 19]. As a therapeutic approach, intravenous infusion of polyvalent IgG antibodies from healthy individuals (IVIG), proposed to contain anti-Aβ antibodies, has been studied in AD with positive effects on mouse behavior [14, 20] and AD cognition [21–23]. Nevertheless, to this date, clinical trials have failed to show any positive treatment effects of IVIG in AD [24, 25]. Aducanumab, originally derived from healthy aged individuals, makes up yet another therapeutic strategy for the use of anti-Aβ antibodies in AD and it has recently entered phase 3 clinical trials. Aducanumab recognizes aggregated forms of Aβ and appears to reduce Aβ deposits in patients with early and mild forms of the disease and to slow cognitive decline [26].

Different studies report discrepancies regarding plasma levels of anti-Aβ antibodies in AD. Numerous reports have demonstrated decreased [12, 13, 15, 27–31], elevated [16, 18, 32, 33], or equal [1, 34, 35] levels in AD patients compared to control subjects. Anti-Aβ42 antibodies levels in Down syndrome patients have been found to be higher in plasma compared to non-Down syndrome mentally retarded subjects with perinatal brain injury and to age-matched healthy controls [36]. Moreover, an autoimmune reaction against Aβ, mediated by anti-Aβ antibodies, has been implied to be directly involved in the pathogenesis of cerebral amyloid-related inflammation with increased levels of anti-Aβ antibodies in CSF [37, 38]. In the current study, instead of studying antibodies in plasma, we utilized the enzyme-linked immunospot (ELISpot) technique to investigate the presence of anti-Aβ antibody secreting cells in the blood. With this technique, first described by Czerkinsky et al. [39], secretion of anti-Aβ antibodies can be analyzed on the cellular level (Fig. 1) avoiding enzyme-linked immunosorbent assay (ELISA) associated problems, e.g. formation of immune complexes between circulating Aβ antibodies and native Aβ, when analyzing plasma samples. In addition, the ELISpot method enabled us to compare the antibody response toward monomeric and protofibrillar forms of Aβ. Here we have found that the number of B cells producing IgG antibodies towards Aβ42 protofibrils was significantly higher in AD patients compared to healthy individuals.

**MATERIALS AND METHODS**

**Study material**

Blood sampling from AD patients (n = 50) and age-matched healthy individuals (n = 55) was performed according to standardized procedures at the Memory Disorder Unit, Uppsala University Hospital, Uppsala, Sweden as approved by the Regional ethical committee in Uppsala (decision number 2009/097). Clinical AD
diagnosis was determined by the NINCDS-ADRDA criteria, after a comprehensive clinical diagnostic procedure including neuroimaging. Mini-Mental State Examination (MMSE, Folstein –75) was administered and scores were used as a rough measurement of cognitive function. Age-matched healthy individuals were recruited by local advertisement at the Memory Disorder Unit, Uppsala University. Inclusion of healthy individuals required absence of cognitive impairment or other dementia disorders. Both AD patients and age-matched healthy individuals volunteered to be part of the study and written informed consent was obtained from all subjects or their closest relative.

Preparation of biotinylated Aβ40 monomers and Aβ42 protofibrils

Synthetic Aβ40 monomers and Aβ42 protofibrils were prepared as previously described [18, 40, 41]. Synthetic Aβ40 (Polypeptide Laboratories AB, Limhamn, Sweden) dissolved in 10 mM NaOH, was diluted in 2 × phosphate buffered saline (PBS) to 100 μM. Synthetic Aβ42 (American Peptide Company Inc., Sunnyvale, CA, USA) dissolved in 10 mM NaOH, diluted in 10 PBS to 443 μM (2 mg/ml), was incubated for 30 min at 37°C and centrifuged for 5 min at 17 900 × g to remove any insoluble aggregates and then diluted with PBS to a final concentration of 100 μM. Both Aβ40 monomers and Aβ42 protofibrils were biotinylated with Sulfo-NHS-LC-Biotin (Thermo Fischer Scientific Inc., Waltham, MA, USA) according to manufacturer’s guidelines with an Aβ/biotin molar ratio of 1/20. The Aβ42 protofibril concentration is expressed in monomer concentration units.

Aβ protofibril ELISA

Aβ protofibril ELISA using the monoclonal antibody 158 (mAb158) was performed according to the protocol described by Englund et al. [42]. In short, 96-well ELISA plates (Corning Inc., Corning, NY, USA) were coated with 200 ng/well of mAb158 antibody in 100 μl PBS at 4°C overnight. Plates were blocked with 1% bovine serum albumin (BSA) in PBS with 0.15% Kathon. Standard series of synthetic Aβ42 protofibrils, either biotinylated or not, were diluted in ELISA incubation buffer (0.05% Tween 0.1% BSA and 0.15% Kathon in PBS at pH 7.4) and added to the plates for incubation 2h at room temperature (RT). After washing, the plates were incubated for 1 h at RT with 0.5 μg/ml mAb158 conjugated to horseradish peroxidase. K-blue aqueous TMB substrate (Neogen Corporation, Lansing, MI, USA) was used as horseradish peroxidase substrate, and the reaction was stopped with 1 M H2SO4. Absorbance was measured by Tecan Infinite M200 PRO (Tecan Group Ltd., Männedorf, Switzerland) spectrophotometer at 450 nm and analyzed with Magellan v7.0 software (Tecan Group Ltd.). Washing was performed by adding 250 μl washing buffer (PBS with 0.1% Tween 20 and 0.15% Kathon) repeated three times between each step of the ELISA.

PBMC preparation

Whole blood, sampled in BD Vacutainer CPT™ Cell Preparation Tubes with sodium heparin (BD bioscience, Franklin Lakes, NJ, USA), was diluted 1:2 in PBS and gently layered over cold Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK) and kept on ice before centrifuged for 20 min at 900 × g without brake at 18–20°C. The cell layer on top of the Ficoll-Paque PLUS consisting of peripheral blood mononuclear cells (PBMCs) was collected and diluted in PBS. PBMCs were centrifuged again for 7 min at 450 × g at 18–20°C. The supernatant was removed and cells were resuspended in 5 ml PBS and counted before they were centrifuged at 350 × g for 7 min at 18–20°C. Finally, cells were resuspended in freezing medium (10% DMSO in heat inactivated fetal bovine serum (FBS) (Nordic Bioslabs AB, Täby, Sweden) and placed at −70°C in a Mr. Frosty™ Freezing Container (Thermo Fisher) for at least 3 h before being transferred to liquid nitrogen for extended storage.

Flow cytometry

For each individual cell sample, 5 × 10⁶ PBMCs were quickly thawed at 37°C and diluted in 40 ml cold wash buffer (PBS supplemented with 2.5% FBS (Life Technologies Ltd. Paisley, UK) and 0.1% sodium azide). The cell suspension was centrifuged at 250 × g for 5 min and after discarding the supernatant, the pellet was resuspended in 400 μl wash buffer. Each sample was stained for 1 h in a light-protected environment at 4°C with titrated amounts of anti CD10 labelled with Alexa Fluor 700 (BD Biosciences). Samples were analyzed using a BD LSR II Special Order System, controlled by the BD FACSDiva 6.0 software (BD Biosciences). A preliminary forward scatter (FSC) versus side scatter (SSC) gate was used to identify lymphocyte and, depending on sample size, a total of up to 100,000 in-gate events were recorded. All datasets were migrated to FlowJo 7.6.5 (Treestar Inc. Ashland, OR, USA) for further gating and analysis.
Activation of B cell antibody production

Five million PBMCs were thawed and diluted in complete medium (RPMI 1640 (Nordic Bioslabs), supplemented with a mix of penicillin (100 U/ml) and streptomycin (100 μg/ml) (Thermo Fisher), L-glutamine (2 mM) (Life Technologies), Heps (10mM) (Sigma-Aldrich, Saint Louis, MO, USA), and FBS (10%) (Nordic Bioslabs). The PBMCs were washed twice in complete medium by centrifugation for 10 min at 200 × g before incubation in complete medium at 37 C, 5% CO2 for 1 h. After resuspension, any debris or cell aggregates were allowed to sediment and the cells were transferred to a new centrifuge vial.

To activate the IgG production, cells were stimulated for 72 h at 37 C, 5% CO2 in complete medium supplemented with the polyclonal activator R848 (1 μg/ml) (Mabtech AB, Nacka Strand, Sweden) and recombinant human Interleukin-2 (10 ng/ml) (Mabtech AB). 

ELISpot

EliSpotPLUS kit for human IgG (Mabtech AB) was used to study the IgG producing B cells. First, ELISpot plates were activated with 70% Ethanol (50 μl/well) during 1 min and washed with sterile H2O and coated with mouse anti-human IgG mAbs MT01/145 (15 μg/ml) overnight at 4 C. Plates were then washed five times with sterile PBS followed by 1 h incubation in complete medium. PBMCs were added in triplicates to the plates at a concentration of 500,000 cells/well for the analysis of anti-Ab antibodies and 50,000 cells/well for measuring the total number of IgG secreting cells. After incubation for 24 h in 37 C, 5% CO2, cells were removed by washing four times in PBS in a Tecan Hydrospeed plate washer (Tecan Group Ltd.) followed by incubation with biotinylated Aβ42 protofibrils (500 mM) or biotinylated Aβ40 monomers (500 nM) for 2 h at RT to detect anti-Aβ antibodies and with biotinylated α-human IgG mAbs MT78/145 (1 μg/ml) for determination of the total number of IgG producing cells. After another round of washing, the plates were incubated for 1 h at RT with the conjugate Streptavidin-Alkaline Phosphatase (Streptavidin-ALP) (1:1000 in PBS supplemented with 0.5% FBS). Finally, plates were washed and developed with filtered 5-bromo-4-chloro-3-indolyl phosphate in conjunction with nitro blue tetrazolium (BCIP/NBT) substrate until spots were evident. After stopping the substrate reaction by washing extensively in tap water, plates were dried and analyzed with an ELISpot reader (AID, Strassberg, Germany).

Statistical analysis

GraphPad Prism 5 was used for all statistical analyses (GraphPad Software, Inc., La Jolla, USA). Unpaired t test with Welch’s correction was used throughout the study and the results are presented in scattergrams or box plots with mean ± standard deviation. For correlation, Spearman rank correlation was used with the 95% confidence interval, spearman r and p values included in the graph. Level of significance was set at “p<0.05, “p<0.01, and “p<0.001. The study was blinded until time of statistical analysis.

RESULTS

Blood from age-matched AD patients (n = 50, mean age 77.8 years) and healthy individuals (n = 55, mean age 74.3 years) were used for the preparation of PBMCs. For the analysis of cells secreting anti-Aβ antibodies, 500,000 cells stimulated for 72 h with the polyclonal activator R848 and recombinant IL-2 were added to each well of ELISpot plates coated with anti-IgG. As a positive control, cells (50,000 cells/well) were added in parallel wells but analyzed for the total number of IgG secreting cells. Samples with no or very few IgG producing cells in the positive control wells (AD patients (n = 7), healthy individuals (n =10)) were excluded from the study. Similarly, samples (AD patients (n = 4), healthy individuals (n = 11)) with too low numbers of PBMCs already after extraction or after thawing were excluded. The remaining samples that were included in the study are displayed in Table 1. In general, more IgG-producing B cells were found in blood from individuals with AD compared to age matched controls (Fig 2A). However, no differences in B cell numbers were detected when comparing AD patients to healthy individuals by flow cytometry (Fig 2B).

To investigate the presence of B cells secreting anti-Aβ antibodies, two different biotinylated Aβ preparations were used; synthetic Aβ40 monomers and synthetic Aβ42 protofibrils. Synthetic Aβ42 protofibrils were here defined as soluble Aβ aggregates larger than 100 kDa, i.e., eluting in the void volume on a size exclusion Sephadex 75 column. The Aβ42 protofibrils were first run in an Aβ protofibril specific ELISA to control that the protofibril structure was intact after the biotinylation step. The biotinylated Aβ42 protofibrils were readily detected by a protofibril specific ELISA and there was no significant difference between Aβ42 protofibrils with or without biotin confirming conformational maintenance (Fig 2C). In general, a...
Table 1
AD patients and age-matched healthy individuals included in the study. The PBMCs used in the study were derived from AD patients and age-matched healthy controls. The table presents gender, age and ApoE allele frequencies for AD patients and healthy controls, respectively.

|                        | AD patients (n=46) | Healthy individuals (n=44) |
|------------------------|-------------------|---------------------------|
| **Age (years)**        |                   |                           |
| Mean ± SD              | 78.0 ± 6.5        | 74.8 ± 7.0                |
| Range                  | 63–90             | 51–87                     |
| **Gender**             |                   |                           |
| Female/Male            | 20/26             | 24/20                     |
| **MMSE score**         |                   |                           |
| Mean ± SD              | 21.0 ± 3.8        |                            |
| Range                  | 10–27             |                            |
| **ApolE allele**       |                   |                           |
| E2                     | 11.1%             | 15.9%                     |
| E3                     | 91.1%             | 90.9%                     |
| E4                     | 62.2%             | 38.6%                     |

*MMSE score from 1 patient was not available and was therefore not included in the calculations. *ApoE genotype from 1 AD patient was not available and was therefore not included in the calculations.

higher number of cells produced antibodies recognizing synthetic $\alpha$42 protofibrils than synthetic $\alpha$40 monomers ($p<0.0001$) (Fig. 3A). While there was no significant difference between AD patients and healthy individuals with regard to the number of plasma B cells producing antibodies against monomeric $\alpha$40 (Fig. 3B), the number of cells secreting antibodies to $\alpha$42 protofibrils was significantly higher in AD individuals compared to healthy individuals ($p=0.043$) (Fig. 3C). This difference was even more evident for ApoE4 allele homo- or heterozygous AD individuals ($p=0.031$). Moreover, in AD patients the number of B cells producing antibodies to $\alpha$42 protofibrils correlated weakly with the total number of IgG producing B cells (Spearman $r=0.59$, $p<0.001$) (Fig. 3D).

No statistically significant correlation was found between the frequencies of anti-$\alpha$ antibodies secreting cells in the AD group and Mini Mental Status (based on Mini Mental Test) (Fig. 4). Nor was there a correlation between the number of $\alpha$-specific cells and age or gender except for in the control group, where females had significantly higher frequencies of B cells producing antibodies towards $\alpha$42 protofibrils than males ($p=0.025$).

**DISCUSSION**

Use of different methods, study setups and various forms of $\alpha$ may explain the conflicting results regarding anti-$\alpha$ antibodies in AD and healthy individuals. The vast majority of earlier publications reporting $\alpha$ antibody levels in human serum/plasma have used ELISA [12, 13, 28, 31–35, 43–48]. With ELISA, the reliability of the measurements can be questioned as there is a risk of immune complex formation between circulating $\alpha$ antibodies and native $\alpha$ in the blood. Thus, antibodies may be prevented from binding $\alpha$ in ELISA plates, leading to an underestimation of antibody levels. This problem can be overcome by measuring anti-$\alpha$ antibodies after dissolving the complexes, typically through a brief treatment with acid. Gustaw et al. measured the levels using ELISA before and after dissociation and found that anti-$\alpha$ antibody levels were increased in both AD patients and normal control subjects and that AD patients displayed higher levels of anti-$\alpha$ antibodies compared to normal controls after treatment [29, 30]. Another

Fig. 2. Characterization of PBMCs and validation of the antigen. A) Total IgG production from activated B cells. Number of spots representing IgG producing cells detected in the ELISpot. AD individuals (n=39) (■) had higher number of spots than healthy controls (n=30) (●) ($p \leq 0.05$). PBMCs from individuals failing activation (spots <20) (AD patients (n=7), healthy controls (n=10)) were excluded from the study (below horizontal line). B) B cell levels in AD patients and healthy individuals. The proportion of B cells in PBMCs was determined with flow cytometry by determining the number of CD19+ cells. There was no significant difference in the percentage of B cell in AD patients compared to healthy controls ($p=0.085$). C) $\alpha$42 protofibril evaluation with protofibril specific ELISA. No considerable difference was noticed comparing biotinylated $\alpha$42 protofibrils with non-biotinylated $\alpha$42 protofibrils.

S. Sollvander et al. / B Cell ELISpot Analysis of $\alpha$ Antibodies in AD
problem when using ELISA is that Aβ monomers may artificially adopt amyloidogenic epitopes due to surface adsorption [15], potentially causing misinterpretations regarding antibody recognition of specific Aβ forms. Therefore, to distinguish different forms of Aβ in immunoassays, Aβ should preferably be kept in solution. We used the ELISpot assay since this not only eliminates the problem of antibodies occurring in complexes but also allows the testing of Aβ in a soluble form. The ELISpot assay is also very sensitive and enables the detection of the very small populations typically seen in antigen-specific responses. This way ELISpot enabled us to accurately measure the presence of B cells producing anti-Aβ antibodies in AD patients and age-matched healthy individuals and to compare the anti-Aβ antibody selectivity toward Aβ40 monomers and Aβ42 protofibrils. The choice of different Aβ peptides for generation of monomers and protofibrils was motivated by their different propensity to aggregate and thus maintain their conformation throughout the experiment. Since the C-terminus of aggregated Aβ is less accessible for antibody binding [49, 50], we do not expect the two extra C-terminal amino acids in the Aβ42 protofibril preparation to affect the results.

Before measuring the Aβ specific IgG production, flow cytometry was performed to estimate the total B cell numbers in the blood of both AD patients and healthy individuals. Earlier reports have found lower levels of B cells in AD compared to healthy individuals.

Fig. 3. B cell production of anti-Aβ40 monomer and anti-Aβ42 protofibril antibodies. A) Number of spots representing cells producing antibodies to Aβ40 monomers (■) and Aβ42 protofibrils (●). Looking at the whole study material (n = 63), antibodies detected Aβ42 protofibrils to a higher degree than Aβ40 monomers (p < 0.0001). B) B cell production of anti-Aβ40 monomer antibodies. Number of spots representing cells producing antibodies to Aβ40 monomers in AD patients (n = 37) (■) and healthy individuals (n = 26) (●). No significant difference was seen when comparing AD patients with healthy controls (p < 0.23). C) Number of spots representing cells producing antibodies detecting Aβ42 protofibrils in AD individuals (n = 37) (■) and healthy individuals (n = 26) (●). There was a significant difference in number of spots comparing AD patients with healthy individuals (p < 0.05). D) In AD patients, the number of spots representing B cells producing antibodies binding to Aβ42 protofibrils correlated weakly with the number of spots representing the total number of IgG producing B cells (Spearman r = 0.59, p < 0.001).
cognition in AD patients (cells producing antibodies specific for Aβ and has also been shown before [55]. In contrast, B cells of the peptide in the human body, this was expected of the anti-Aβ antibodies, thereby abating the role of such antibodies as a potential biomarker for AD. Nevertheless, it would be interesting to follow AD patients over time to see if and how the number of B cells producing anti-Aβ antibodies correlates with the cognitive decline on an individual level. Conti et al. have previously described that AD patients receiving acetylcholinesterase inhibitors (AChEI) increased the anti-Aβ antibody levels in plasma compared to untreated AD patients [57]. AD patients show an increased production of interleukin-4 and monocyte chemotactic protein-1, two positive regulators of Th2 differentiation, following AChEI treatment. Possibly, AChEI treatment can induce a Th2-mediated immune response leading to an increase of immunoglobulin production by B lymphocytes, including anti-Aβ antibodies [58]. However, as anti-Aβ antibodies levels were evaluated by ELISA, the differences between AChEI treated and untreated AD patients could reflect variations in Aβ/anti-Aβ antibody complexes rather than number of anti-Aβ antibodies. As all patients in the AD group except for four had been or were still on treatment with AChEI in our study, evaluation regarding AChEI influence on anti-Aβ antibody production from B cells among AD patients was not possible.

In conclusion, we report for the first time that ELISpot can be used to measure anti-Aβ antibodies from B cells in the blood. Low levels of B cells producing antibodies to Aβ40 monomers were seen in both AD and healthy individuals whereas the number of cells producing antibodies to Aβ42 protofibrils was significantly higher in AD compared to healthy individuals. Both IVIG and therapeutic anti-Aβ antibodies originally derived from healthy individuals are evaluated as AD therapies. Thus, the role of anti-Aβ antibodies in AD, as well as their physiological importance need to be further evaluated to learn if they could contribute to new therapeutic approaches for AD.

ACKNOWLEDGMENTS

We are grateful to Anne-Marie Ljungberg for her assistance during PBMC preparation and Käthe Ström for blood sampling. This work was supported by the Swedish Research Council (#2012-2172, LL), Alzheimerfonden (FEP, SS) and Uppsala Berzelii
Technology Centre for Neurodiagnostics (DS, SS, LL, FEP). Authors’ disclosures available online (http://j-alz.com manuscript-disclosures/15-0236r1).

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