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Short Communication

A Method for the Detection of Amyloid-β_{1-40}, Amyloid-β_{1-42} and Amyloid-β Oligomers in Blood Using Magnetic Beads in Combination with Flow Cytometry and its Application in the Diagnostics of Alzheimer's Disease

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Abstract. A method for simultaneous quantification of amyloid-β_{1-40}, amyloid-β_{1-42} and amyloid-β oligomers in human plasma is described. The method consists of a combination of immunoprecipitation using specific antibodies against the different forms of amyloid-β, and immobilization of the immunocomplexes to magnetic beads. Addition of fluorescence-labelled antibodies which recognize the specific antibodies to the amyloid-β subsets allows the peptide/associates detection in the sample by flow cytometry. The clinical assay performance was tested using blood samples from Alzheimer disease's patients and control donors. A sensitivity of 70% and a specificity of 81% was achieved.

Keywords: Alzheimer's disease, amyloid-β, flow cytometry, human plasma, immunoprecipitation, magnetic beads

INTRODUCTION

Recent studies have shown that oligomers of the amyloid-β (Aβ) protein play an important role in the early stage of Alzheimer's disease (AD) [5,6, 9,14]. However, we could show the presence of Aβ oligomers in cerebrospinal fluid (CSF) from non-demented donors [16] demonstrating that oligomerization of Aβ occurs in patients suffering from neurological disorders different from AD. Up to now, a reliable method for the detection and quantification of Aβ oligomers in blood does not exist. Here we describe a

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blood-based method for the detection of Aβ oligomers. For the evaluation of the assay, human plasma samples from AD patients and healthy donors (control group) were obtained. The study was approved by the ethics committee of the University of Dresden. All patients gave their informed consent. The AD group ($n = 17$) consisted of patients diagnosed according to the ICD-10:FOO.1. The degree of mental impairment was assessed in all patients with the minimental state examination (MMSE). The control group ($n = 16$) was comprised of healthy subjects without memory complaints. Blood was collected from all subjects by venipuncture into a standard monovette containing lithium heparin (Sarsted). After collection the blood was centrifuged and the plasma was divided into aliquots and immediately frozen at $-80\,^{\circ}$C.

The plasma levels of the different forms of Aβ were determined using a combination of immunoprecipitation and flow cytometry: I) purification of total Aβ molecules from plasma using the antibodies 6E10 and 4G8 and goat-anti mouse coated beads; II) specific precipitation of the different forms of Aβ with rabbit anti Aβ_{1-40}, Aβ_{1-42} and Aβ oligomers; and III) labelling of the immunoprecipitated molecules with a goat anti-rabbit antibody fluorescence-labelled with Alexa-fluor 488 followed by flow cytometry detection. First, 2.5 ml of lithium heparin plasma were mixed with 250 μl of 10x immunoprecipitation buffer (250 mM Tris-HCl, pH 8.0, 5% Triton X-100) 5% Nonidet P-40 alternative (Calbiochem) and 1 tablet of protease inhibitor mixture Complete$^{\mathrm{TM}}$ Mini (Roche Applied Science) per 2 ml of 10x buffer) and incubated for 10 min at room temperature with continuous rocking of the tubes. After that, 4 μg of the antibody 6E10 and 4 μg of the antibody 4G8 were added and incubated at 23 °C for 16 h with continuous rocking of the tubes. The addition of 200 μl of goat anti mouse magnetic beads (Polysciences) and an incubation for 1 h at 23 °C followed. The beads coupled with the immunocomplex were then recovered using a Dynal magnetic separator and washed 3 times with TBS-T buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% Tween 20). For the differentiation between Aβ_{1-40}, Aβ_{1-42} and Aβ oligomers and expecting that the amount of Aβ_{1-40} > Aβ_{1-42} > Aβ oligomers, the beads coupled with total Aβ were dissolved in 200 μl TBS-T and divided in four samples: I) 20 μl of beads; II) 30 μl beads; and III-IV) 75 μl beads. In the next step, 200 μl of TBS-T buffer containing 0.1% bovine serum albumin were added to the samples I-IV and incubated with a rabbit specific Aβ antibody as follows: no antibody was added to the sample I (negative control), 40 ng of a rabbit anti-Aβ_{1-40} antibody were added to the sample II, 40 ng of a rabbit anti-Aβ_{1-42} antibody (Abcam) were added to the sample III, and 40 ng of a rabbit anti-Aβ oligomers antibody (Chemicon) were added to the sample IV. The samples were incubated at 23°C for 45 min. After washing off the free anti rabbit antibodies, the immunocomplex tagged to the beads was fluorescence-labelled using 40 ng of a goat anti-rabbit Alexa-fluor 488 labelled antibody at 23 °C for 45 min. After washing off the free fluorescence-labelled antibody, the concentration of the beads was equalized by adding the following amounts of TBS-T buffer to the beads: to the sample I) 400 μl, to the sample II) 600 μl, and to the samples III-IV) 1,500 μl. The detection of the different Aβ forms tagged to the magnetic beads followed by flow cytometry analysis using a FACS Calibur flow cytometer (BD Biosciences). The fluorescence-labelled beads were gated in linear forward/sideward scatter dot plots (FSC vs SSC). The green fluorescence of the dye Alexa-fluor 488 was detected by the corresponding FL1 (logarithmic scale) photomultiplier through 530/30 band pass filters. In order to avoid differences in the measurement due to variation in the plasma samples, all samples were measured in TruCount Tubes (BD Biosciences). Due to the differences in volume of the samples, the amount of measured sample was regulated by the measurement of different amounts of TruCount beads: for sample I) 5,000, for sample II) 7,500, and for sample III-IV) 18,750 beads. The sample I aimed to determine the unspecific binding of the goat anti-rabbit fluorescence-labelled antibody to the immunocomplex-tagged beads. The final calculation of the Aβ molecules detected was determined as followed:

Step 1: calculation of the background (C_0), $C_0 = C_0/(C_0 + B_0)$, where C_0 was the percentage of fluorescence beads in the region C (positive signal for the samples II-IV or background for sample I) and B_0 was the percentage of fluorescence beads from sample I in the region B (threshold fluorescence) of the flow cytometry data analysis.

Step 2: correction of the measured beads in region C for Aβ_{1-40}, Aβ_{1-42} and Aβ oligomers (C_{cx}), $C_{cx} = C_{x*}(1\text{-}C_0)$, whereas C_x was the percentage of fluorescence beads in the region C from sample II-IV.

Step 3: final calculation of the Aβ molecules (C_f), $C_f = C_{cx}/(C_{cx} + B_x)$, where B_x was the percentage of fluorescence beads in the region B from sample II-IV.

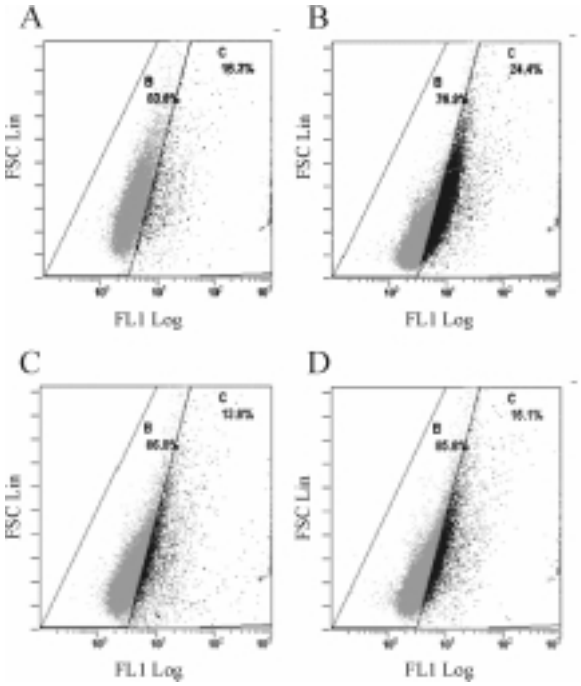

Fig. 1. Typical measurement of a plasma sample by flow cytometry. A) Sample I: sample without the rabbit antibodies but with the fluorescence labelled Alexa-fluor 488 goat anti-rabbit antibody which allows determining the background of the sample. B) Sample II containing a rabbit anti-Aβ40 antibody and the fluorescence labelled Alexa-fluor 488 goat anti-rabbit antibody. C) Sample III containing a rabbit anti-Aβ42 antibody and the fluorescence labelled Alexa-fluor 488 goat anti-rabbit antibody. D) Sample IV containing a rabbit anti-Aβ oligomers antibody and the fluorescence labelled Alexa-fluor 488 goat anti-rabbit antibody.

The Example 1 demonstrates how to calculate the amounts of Aβ_{1-40}, Aβ_{1-42} and Aβ oligomers in a plasma sample.

For the Sample I (background, Fig. 1A) the value in the region C is 16.3% and in the region B is 83.8%. Therefore, $C_0 = C/(C + B) = 16.3\,\%/(16.3\% + 83.8\%) = \textit{0.162}$.

For Aβ_{1-40} $C_{c40} = C_{40}[\%]*(1 - C_0) = 24.4\%*(1 - 0.162) = 20.44\%$ (Fig. 1B) and

$Cf_{40} = C_{c40}/(C_{c40} + B_{40}) = 20.44\%/(20.44\% + 76.9\%) = \textit{0.209}$

For Aβ_{1-42} $C_{c42} = C_{42}*[\%]\,(1 - C_0) = 13.8\%*(1-0.162) = 11.56\%$ (Fig. 1C) and

$Cf_{42} = C_{c42}/(C_{c42} + B_{42}) = 11.56\%/(11.56\% + 86.8\%) = \textit{0.117}$

For Aβ Oligos $C_{cOligos} = C_{Oligos}[\%]*(1 - C_0) = 15.1\%(1 - 0.162) = 12.65\%$ (Fig. 1D) and

$Cf_{Oligos} = C_{cOligos}/(C_{cOligos} + B_{Oligos}) = 12.65\%/(12.65\% + 85.8\%) = \textit{0.128}$

Table 1
Estimated amounts of Aβ_{1-40}, Aβ_{1-42} and Aβ oligomers in plasma of AD patients (relative units)

AD	Aβ_{1-40}	Aβ_{1-42}	Aβ oligomers
1	0.0341	0.0280	0.0226
2	0.0380	0.0247	0.0339
3	0.0176	0.0572	0.0245
4	0.0299	0.0219	0.0164
5	0.0250	0.0311	0.0250
6	0.0397	0.0393	0.0463
7	0.0329	0.0244	0.0222
8	0.0401	0.0598	0.0508
9	0.0235	0.0183	0.0178
10	0.0168	0.0272	0.0242
11	0.0222	0.0168	0.0294
12	0.0264	0.0266	0.0174
13	0.0242	0.0181	0.0106
14	0.0239	0.0115	0.0208
15	0.0351	0.0429	0.0260
16	0.0238	0.0263	0.0246
17	0.0283	0.0359	0.0252

Table 2
Estimated amounts of Aβ_{1-40}, Aβ_{1-42} and Aβ oligomers in plasma of healthy donors (relative units)

Control	Aβ_{1-40}	Aβ_{1-42}	Aβ oligomers
1	0.0242	0.0246	0.0178
2	0.0346	0.0197	0.0189
3	0.0251	0.0179	0.0183
4	0.0277	0.0174	0.0158
5	0.0393	0.0391	0.0363
6	0.0213	0.0338	0.0219
7	0.0374	0.0391	0.0340
8	0.0269	0.0189	0.0158
9	0.0222	0.0243	0.0164
10	0.0229	0.0199	0.0139
11	0.0404	0.0289	0.0175
12	0.0280	0.0280	0.0184
13	0.0267	0.0407	0.0188
14	0.0136	0.0276	0.0283
15	0.0153	0.0190	0.0137
16	0.0200	0.0310	0.0170

In order to test the clinical assay performance, 17 plasma AD samples and 16 control plasma samples were analyzed. The values for Aβ_{1-40}, Aβ_{1-42} and Aβ oligomers are summarized in Table 1 for AD and Table 2 for the controls.

The sensitivity and specificity of the assay was calculated using the MedCalc software. The amount of Aβ oligomers allowed a differentiation between the AD and the control group. The ROC curve analysis in this case showed a specificity of 81.2% and a sensitivity of 70.6%. The area under the curve was 0.707. The 95% confidence intervals were at 0.52 and 0.853, with the significance level $P < 0.025$ (Fig. 2A, B). A discrimination of both groups using the Aβ_{1-40} and Aβ_{1-42} values did not improve the sensitivity and specificity.

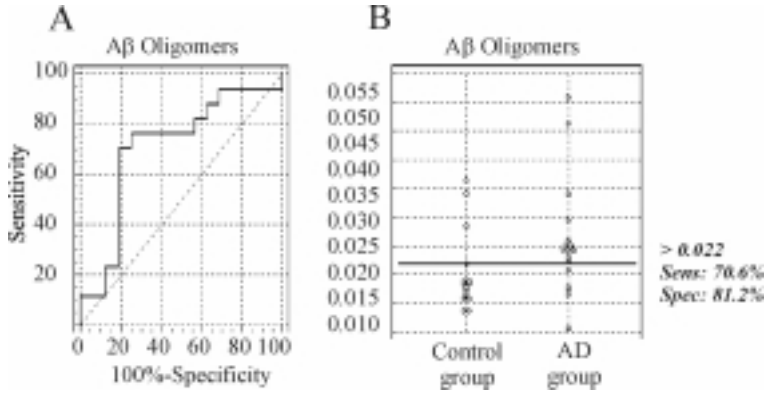

Fig. 2. The Aβ oligomer concentration in plasma allows the differentiation between AD and control donors. Aβ oligomers were detected by a combination of immunoprecipitation using magnetic beads and monoclonal antibodies against total Aβ forms. The differentiation of the different Aβ forms was achieved using polyclonal antibodies against Aβ_{1-40}, Aβ_{1-42} and Aβ oligomers. Finally, the detection of the different Aβ forms was completed by flow cytometry using a fluorescent labelled (Alexa-fluor 488) goat anti rabbit antibody. The ROC curve for Aβ oligomers (A) and discrimination between the AD and the Control group using the concentration of Aβ oligomers (B) is shown. For the ROC curve the area under the curve was 0.707. The 95% confidence intervals were 0.52 to 0.853 with the significance level $P < 0.025$. A sensitivity of 71% with a specificity of 81% was achieved.

Although the sensitivity and specificity of the assay were acceptable, the assay has to be further improved before it should be tested for differentiation between AD samples and sub clinical forms of AD such as Mild Cognitive Impairment.

Many laboratories have been undertaking great efforts for the identification of biological markers for AD in body fluids. In CSF, a reduced Aβ_{1-42} concentration in AD patients in comparison to age-matched controls allows discrimination between both groups by 86% with specificity of 89% [3]. However, no correlation between Aβ levels in plasma and CSF was found [13]. The measurement of Aβ in plasma is difficult. The peptide is very sticky and binds to plasma proteins such as albumin and lipoproteins [1,11]. On the other hand, the oligomerization of Aβ could mask Aβ epitopes, resulting in measurement of only a fraction of the peptide. This may be the reason why the results on Aβ concentration in plasma are very contradictory. For example, van Oijen et al. showed increased concentration of plasma Aβ_{1-40} but not Aβ_{1-42} associated with increased risk of dementia [17]. In contrast, other groups found high concentrations in plasma for Aβ_{1-40} and Aβ_{1-42} in AD, although with a broad overlap between patients and control, whereas most groups find no significant changes [reviewed in 10]. High plasma Aβ_{1-42} but not Aβ_{1-40} concentrations were reported in non-demented elderly people who later developed either progressive cognitive decline or AD [12,15]. In a recent study, it was shown that medial temporal lobe atrophy but not plasma Aβ_{1-42} measured at baseline predicted which persons remained cognitively healthy and who developed AD 2.5 years later [2]. It was proposed that plasma Aβ_{1-42} alone is not suitable as a biomarker for AD. In our study, the concentration of Aβ_{1-40} and Aβ_{1-42} measured in plasma from AD patients and control donors did not allow discrimination between both groups.

Increasing evidence indicates that oligomeric assemblies of Aβ may represent the molecular species responsible for cytotoxicity in AD [4,7,8,18,19]. Oligomers of Aβ were already detected in CSF [5,6,9,14,16]. A method allowing the detection of aggregated Aβ in blood was not available so far. The data presented here represent the first study showing Aβ oligomers in blood and their use as a diagnostic tool for AD. However, more measurements are necessary for the confirmation of Aβ oligomers as a marker of AD in blood. Nevertheless, the availability of a novel method is the first step in this direction.

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