Decreased Serum Amyloid β₁₋₄₂ Autoantibody Levels in Alzheimer’s Disease, Determined by a Newly Developed Immuno-Precipitation Assay with Radiolabeled Amyloid β₁₋₄₂ Peptide

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**Background:** Autoantibodies against amyloid β (Aβ) peptide found in patients with Alzheimer’s disease (AD) also occur naturally in the general population independently of the cognitive status.

**Methods:** We compared serum Aβ₁₋₄₂ autoantibody levels (Aβ₁₋₄₂-AL) of 96 AD patients and 30 healthy elderly control subjects (HC), assessing their diagnostic value for AD with a newly developed immunoprecipitation assay with radiolabeled Aβ₁₋₄₂ peptide.

**Results:** We found a highly significant decrease of Aβ₁₋₄₂-AL in AD patients (p = .001) independently of age, cognitive status, and apolipoprotein Eε4 carrier status. Amyloid β₁₋₄₂ autoantibody levels were correlated with gender in AD, with a higher level occurring in women. When Aβ₁₋₄₂ autoantibody sensitivity (specificity) was set >80%, specificity (sensitivity) was below 50% to correctly allocate patients and healthy control subjects.

**Conclusions:** Our data indicate a potentially pathophysiologic decrease of serum Aβ₁₋₄₂ antibodies in AD. Amyloid β₁₋₄₂ antibodies in the serum alone, however, seem not to be useful as a diagnostic marker of AD.

**Key Words:** Alzheimer’s disease, Aβ autoantibodies, biomarkers, cerebrospinal fluid (CSF), diagnosis, bioassay

The amyloid cascade hypothesis suggests a central role of the amyloid β (Aβ) peptide in the pathogenesis of Alzheimer’s disease (AD) (Hardy and Selkoe 2002). Amyloid β seems to induce autoantibodies because naturally occurring autoantibodies directed against Aβ were isolated from human B cells (Gaskin et al 1993; Xu and Gaskin 1997). They even were detected in intravenous immunoglobulin preparations (Dodel et al 2002). In an epidemiologic study, low Aβ₁₋₄₂ autoantibody levels (Aβ₁₋₄₂-AL) were detected in more than 50% of non-memory elderly individuals (Hyman et al 2001). Amyloid β autoantibodies might contribute to peripheral and central degradation of Aβ and to plaque clearance (for review see Dodel et al 2003). This potential pathophysiologic role of Aβ autoantibodies in AD is substantiated by study results of neuronal cell cultures (Du et al 2003), animal models of amyloid pathology (Bard et al 2000; DeMattos et al 2001, 2002; Janus et al 2000; Morgan et al 2000; Schenk et al 1999; Weiner et al 2000; Wilcock et al 2003), and the Aβ vaccination trial (Hock et al 2003; Nicoll et al 2003). In this study, we used a newly developed immunoassay to investigate differences in Aβ₁₋₄₂-AL in serum of mildly to moderately demented AD patients compared with healthy control subjects (HC). We investigated to what extent the variability of serum Aβ₁₋₄₂-AL across patients can be explained by age, gender, cognitive state, or apolipoprotein E (ApoE)-ε4 genotype. Finally, we assessed whether differences in Aβ₁₋₄₂-AL in serum can be used as a potential biomarker in AD.

**Methods and Materials**

All patients and control subjects were recruited at the Department of Psychiatry, Ludwig-Maximilian University, Munich, Germany. They are part of a thoroughly characterized cohort, as described earlier (Buerger et al 2002). All subjects gave written informed consent to participate in this study, which was approved by the institutional review board of the medical faculty of the Ludwig-Maximilian University Munich. For subjects’ characteristics, see Table 1. The clinical diagnosis of probable AD was made according to the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer’s Disease and Related Disorders Association criteria (McKhann et al 1984). Patients with AD differed from HC in age (t = 8.149, p < .001), gender (χ² = 6.222, p < .013) and Mini-Mental State Examination (MMSE) score (t = 13.317, p < .001). Serum samples were collected in vacutainers without further additives. After ½ hour of coagulation, samples were centrifuged for 10 min at 1000g and 4°C. The supernatant was aliquoted into Eppendorf cups (Eppendorf, Hamburg, Germany) and immediately frozen at −80°C.

For the labeling, Aβ peptide 1-42 (Aβ₁₋₄₂-P) (Sigma-Aldrich, Taufkirchen/Munich, Germany) was diluted in 200 mmol/L NaH₂PO₄ buffer pH 7.5 (Merck, Darmstadt, Germany) to a final concentration of 1 mg/mL and stored at −80°C. Amyloid β₁₋₄₂ peptide was subsequently labeled with Na¹²⁵I, applying the chloramine T method by incubating 5 µg of Aβ₁₋₄₂-P with 2 mCi Na¹²⁵I (15.1 mCi/µg in 20 µL volume, Amersham, Freiburg, Germany) and 10 µL chloramine T (.5 mg/mL, Merck) for 2 min at room temperature (RT). Reaction was terminated by the addition of 10 µL DTE (1,4-dithioerythritol, 1 mg/mL, Sigma-Aldrich) and 200 µL buffer A (950 mL H₂O, 50 mL acetonitrile, 1 mL trifluoro acidic acid). Labeled protein was purified by high performance liquid chromatography (HPLC) (Waters, Eschborn, Germany).
Germany) applying a C18-μBondapak-column (Waters). Column was equilibrated in buffer A and labeled peptide was eluted with a linear gradient of buffer B (450 mL acetoniitile, 50 mL H2O, 500 μL trifluoro acidic acid) at a flow rate of 1 mL/min, continuously monitored for radioactivity (Raytest, Straubenhardt, Germany). The eluted tracer was diluted with 1% protease-free bovine serum albumin (Sigma) and 50 mmol/L Tris-HCl (Merck), pH 7.5, aliquoted and stored at 0°C. The total yield was 2.2 × 106 cpm (1.0 mCi) of tracer. For each set of experiments, 400 μL of tracer concentration were thawed, immediately diluted in 25 mL of Tris dilution buffer containing protease inhibitors, and used for immunoprecipitation experiments. The final diluted amyloid peptide tracer contained 45,000 cpm per 50 μL.

Each experiment was carried out in conical polystyrene tubes (Greiner, Nürtingen, Germany) in duplicates, wherein 50 μL of final tracer were added to 10 μL of human serum, followed by overnight incubation at 4°C. Fifty μL of Protein A suspension (Sigma) were added, followed by further incubation for 1 hour at RT under agitation (300 rpm). Three mL of washing buffer (from Hemingtest anti-TPO, BRAHMS AG, Berlin, Germany) were added, followed by further incubation for 1 hour at RT under agitation (300 rpm). The suspension was centrifuged for 30 min at RT at 2000g, decanted, and the precipitated radioactivity was measured in a gamma counter (Berthold, Bad Wildbad, Germany).

Statistical analysis was performed with SPSS (version 11; SPSS, Chicago, Illinois) with nonparametric procedures (Mann-Whitney Test, Spearman rank correlation). Level of significance was set at p < .05. Sensitivity and specificity levels were calculated by receiver operating characteristic curve analysis. On the basis of criteria for a useful biomarker (Growdon et al. 1998), we derived levels of specificity (sensitivity), when sensitivity (specificity) for detecting AD was set at >80%.

Results

We developed an immunoprecipitation assay with radioiodine-labeled Aβ1-42-P. Labeled Aβ1-42-P was purified by HPLC under continuous monitoring of the radioactivity. Figure 1 shows an elution profile of the labeled peptide. The sharp peak indicating labeled Aβ1-42-P was collected and used as tracer. The specific radioactivity of the labeled peptide was 420 μCi/μg.

The specificity of the immunoprecipitation was examined by adding an excess of unlabeled Aβ1-42-P to the serum before the addition of Aβ1-42 tracer. Figure 2A shows the raw data as counts per minute. The binding of Aβ1-42 autoantibodies (gray bars) to the tracer could be inhibited up to 98% by the addition of unlabeled Aβ1-42-P (black bars), indicating a specific interaction in the immunoprecipitation assay.

We standardized the assay by a calibration curve to compare different sets of experiments. A serum strongly positive for Aβ1-42 autoantibodies was diluted serially in horse serum, resulting in dilutions of 1:2–1:16. The undiluted serum was arbitrarily defined as having 16 units, the horse serum as having 0 units. The final standard curve (Figure 2B) contained 5 standards between 0 and 16 units and was added to each set of experiments. The counts per minute data of individual sera were subsequently expressed in arbitrary units.

To assess the precision of the assay, we plotted the intra-assay coefficient of variation (CV) of 25 sera, covering the range of the standard curve (Figure 2C). We defined as technical sensitivity an intra-assay CV of 10%. The corresponding arbitrary unit was 2. Hence, all data below 2 units were considered as not detectable. In statistical calculations, this value was arbitrarily determined as 1 unit.

Amyloid β1-42 autoantibody levels were significantly reduced in patients with AD compared with HC (p = .001). Mean Aβ1-42-AL of both groups are presented in Table 2. Owing to significant age difference in both groups, we analyzed age-matched subgroups (mean age: 63 ± 5 years in AD [n = 47] and 62 ± 7 years in HC [n = 15]), confirming the significant decrease of Aβ1-42-AL in AD (p = .012). The distribution of the Aβ1-42-AL is presented in Figure 3. This effect was independent of the ApoE4 carrier status. Amyloid β1-42 autoantibody levels were significantly reduced in AD patients without the ApoE4 allele (p = .01) as well as in those carrying at least one ApoE4 allele (p = .001). There was no effect of ApoE4 genotype on Aβ1-42-AL in the AD patients (p = .48).

In the AD group, there was no correlation between Aβ1-42-AL and age (p = .112, r = .278) or MMSE score (p = .049, r = .651); however, increased Aβ1-42-AL were associated with female gender (p = .204, r = .05). In the HC group, no correlations between Aβ1-42-AL and age or gender were observed.

Table 1. Group Characteristics of Patients and Control Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (y)</th>
<th>Gender (n)</th>
<th>MMSE Score</th>
<th>ApoE4-Positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (Range)</td>
<td>Female/Male</td>
<td>Mean ± SD (Range)</td>
<td>(%)</td>
</tr>
<tr>
<td>Alzheimer’s Disease (n = 96)</td>
<td>70 ± 8 (50–86)</td>
<td>63/33</td>
<td>21 ± 5 (1–30)</td>
<td>60.3</td>
</tr>
<tr>
<td>Healthy Control Subjects (n = 30)</td>
<td>55 ± 9 (35–77)</td>
<td>14/16</td>
<td>29 ± 1 (27–30)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

MMSE, Mini-Mental State Examination; ApoE4, apolipoprotein Eε4; n.d., not determined.

*At least one ApoE4 allele.

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In receiver operating characteristic curve analysis, specificity was 46.7% when sensitivity was set at 81.3%. Sensitivity was 47.9% when specificity was set at 83.3%. This was confirmed in the age-matched subgroup (sensitivity 80.9% per specificity 46.7%; specificity 86.7% per sensitivity 46.8%).

Discussion

In this study, Aβ₁₋₄₂-AL were measured by a newly developed immunoprecipitation assay based on standard chloramine T labeling of Aβ₁₋₄₂-P. To our knowledge, this is the first immunoprecipitation assay described for Aβ₁₋₄₂. In contrast to the two enzyme-linked immunosorbent assays reported in the literature (Du et al 2001; Weksler et al 2002), this assay includes the use of HPLC-purified Aβ₁₋₄₂-P in liquid phase and the addition of selected protease inhibitors to the incubation buffer. Under these assay conditions, the antigen is presented purified and nondegraded, and all antigenic epitopes are readily accessible and not masked by a potential close proximity to plastic surface as observed with other antigens (Wilkin et al 1989). We demonstrated the specificity of this immunoprecipitation assay by blocking experiments with unlabeled Aβ₁₋₄₂-P, which could completely abolish the precipitation of the tracer.

Despite the differences in assay systems used, the reduced Aβ₁₋₄₂-AL in serum of AD patients are consistent with the results of two recent publications comparing serum Aβ₁₋₄₂-AL (Weksler et al 2002) and cerebrospinal fluid Aβ₁₋₄₀-AL (Du et al 2001) of AD patients with those of HC and showing a significant decrease in AD patients. This consistent reduction of mean Aβ-AL both in cerebrospinal fluid and serum suggests a disease-related effect of Aβ autoantibodies in AD patients; however, the reason Aβ autoantibodies are lowered in patients with AD compared with those of HC is still unknown.

Despite the significant group differences, variability of Aβ₁₋₄₂-AL was high within groups. Only a small part of this variability could be attributed to gender effects, with higher mean Aβ₁₋₄₂-AL occurring in women. Age, ApoEε4 carrier status, and MMSE score had no effect. Except for gender, these results are consistent with the results reported by Hyman et al (2001). The high variance leading to the overlap between AD patients and control subjects might be partly due to measurement variability, as well as to additional sources of Aβ autoantibodies in the serum outside of the central nervous system because it is still unknown from which source the measured Aβ autoantibodies in serum derive.

Consensus criteria for a suitable biomarker of AD (Gwinn et al 1998) could not be fulfilled. The measurement of Aβ autoantibodies for the monitoring of therapy effects, however, is supported by a recent study measuring significant change of Aβ autoantibody titers and Aβ titers in serum and cerebrospinal fluid of patients with neurologic diseases and AD after treatment with intravenous immunoglobulin (Dodel et al 2002).

In summary, we demonstrated significantly lower Aβ₁₋₄₂-AL in serum of AD patients, using a newly developed immunoprecipitation assay. Further investigations are needed to elucidate the pathophysiologic role of Aβ autoantibodies in AD and to

Table 2. Serum Amyloid Aβ₁₋₄₂ Autoantibody Levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Amyloid Aβ₁₋₄₂ Autoantibody (U) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s Disease (n = 96)</td>
<td>3.1 ± 3.28</td>
</tr>
<tr>
<td>Healthy Controls (n = 30)</td>
<td>5.88 ± 4.54</td>
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Figure 2. Optimization of the immunoassay. (A) Specific binding of amyloid autoantibodies to amyloid β(Aβ₁₋₄₂) tracer. Gray bars: without unlabeled Aβ₁₋₄₂. Black bars: in the presence of unlabeled Aβ₁₋₄₂. (B) Standard curve in arbitrary units of an Aβ₁₋₄₂ autoantibody–positive serum diluted in horse serum. (C) Precision profile of the assay. Technical sensitivity defined at an intra-assay coefficient of variation of 10%. All data below were considered as not detectable.
determine potential factors within and outside the central nervous system that alter the Aβ-AL in AD patients and HC. There is evidence for the therapeutic use of Aβ autoantibodies because emerging immunotherapeutic strategies suggest the measurement of Aβ autoantibodies as a potential marker of treatment effects and follow-up in AD.

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Figure 3. Box plots of amyloid β (Aβ)1-42 autoantibody levels of patients with Alzheimer’s disease (AD) and of healthy control subjects (HC). The line at 2 U represents the limit of the technical sensitivity.