Development of Aβ terminal end-specific antibodies and sensitive ELISA for Aβ variant

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Abstract

Alzheimer’s disease (AD) is a neurodegenerative affliction associated with memory dysfunction. Senile plaques are a pathological hallmark of AD, and amyloid β (Aβ) peptides are the major component of these plaques. Aβ peptides are derived from proteolytic cleavage of the Aβ protein precursor (APP) by β- and γ-secretases to generate two principal species, Aβ1–40 and Aβ1–42. We have developed antibodies against the N- and C-termini of these peptides, and an ELISA for accurate and sensitive quantitative assessment. Sandwich ELISA composed of N-terminus (Aβ1) end-specific antibody, clone 82E1, and C-termini end-specific antibodies, and clones 1A10 and 1C3 for Aβ40 and Aβ42, respectively, detects full-length Aβ1–40 and 1–42 with a sensitivity in the sub single digit fmol/ml (equivalent to single digit pg/ml) range with no cross-reactivity to APP. A combination of C-termini antibodies and an antibody against the middle region of Aβ detects mouse Aβ in non-transgenic mouse brains.

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Alzheimer’s disease (AD) is a neurodegenerative affliction associated with memory dysfunction. Amyloid β (Aβ) peptides are the major component of senile plaques, a pathologic hallmark of AD. Aβ is generated from a larger precursor protein, the Aβ protein precursor protein (APP), by proteolytic processing at the N and C termini of the Aβ domain by β- and γ-secretases, respectively [1]. Modulating the APP processing cascade is a promising therapeutic approach, and β- and γ-secretase inhibitors are being aggressively pursued (for review [2–4]). To evaluate the efficacy of Aβ-lowering strategies, measurement of full-length Aβ1–40 and 1–42 is crucial. However, the majority of currently available Aβ ELISAs do not use N-terminus end-specific antibody, but rather an antibody against the middle, or N-terminal region (but not specific to the β-cleavage site). These ELISAs quantify Aβ1–40 and x–42, which are not the optimal measures for the evaluation of anti-Aβ therapeutic strategies. In this study, we have developed antibodies that specifically detect the N-terminus end (β-cleavage site, i.e., Aβ1) or the C-terminus ends (Aβ40 and 42) and have optimized ELISAs by combination of these antibodies. We have also developed ELISAs which can detect endogenous Aβ from mouse brain homogenate.

Materials and methods

Mice (BALB/c, Charles River, Japan) were immunized weekly with thyroglobulin conjugated Aβ peptides (50 μg/mouse). We used partial human Aβ peptide consisting of amino acid residues of 1–16
(DAEFRHDSGYEVHHQK), 11–28 (EVHHQKLFFAEDVGS NK), 35–40 (MVGGGVV), and 38–42 (GVVIA) as immunogens for Aβ N-terminus, middle region, and C-terminus-specific antibodies, respectively. The cysteine residue was combined in each immunogen beforehand for binding to the carrier protein, bovine thyroglobulin. After four to six immunizations, the spleen was isolated and fused with X63Ag8 myeloma cells. Epitopes and cross-reactivity to human and rodent Aβ of selected clones were determined by a microplate assay using various Aβ fragments (American Peptides, Sunnyvale, CA). A 96-well plate (Maxisorp, Nunc, Denmark) was coated with various Aβ fragments (human Aβ sequence unless otherwise indicated as “r”), such as –11 to –1, –4 to –8, –3 to –5, 1 to 5, 1 to 7, r1 to 9, 1 to 11, r1 to 15, 1 to 17, 1 to 40, r1 to 40, 1 to 42, 2 to 40, 3 to 7, 3 to 13, 3 to 40, 7 to 17, 10 to 20, 11 to 28, 13 to 19, 17 to 28, 20 to 29, 35 to 40, and 38 to 42 (100 ng/well) and blocked with Block Ace (Serotec) overnight at 4°C. The plates were incubated with selected antibodies for 4 h, then with an HRP-coupled anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL), and visualized by TMB substrate (Pierce, Rockford, IL).

For Western blotting analysis, we prepared various Aβ containing samples, such as fibril-free soluble and mixture of soluble and fibrill Aβ from synthetic peptides, culture medium from APP overexpressing HEK cells, and brain homogenate from APP overexpressing transgenic mice. Lyophilized Aβ was suspended in dimethyl sulfoxide and used as fibril-free Aβ. Aβ was loaded and incubated overnight at 4°C in 100 mM carbonate buffer, pH 9.6, containing 0.05% sodium azide. After blocking with 1% Block Ace in PBS overnight at 4°C, standards (human or mouse synthetic Aβ peptides 1–40 and 1–42) and samples were loaded and incubated overnight at 4°C. Human plasma specimens were obtained from the Mount Sinai Alzheimer’s Disease Research Center. Mouse brain Aβ was extracted in 0.4% dichrometane containing buffer, centrifuged, and the resultant supernatant was used. HRP-coupled detection antibody, either 82E1 or 12B2, was incubated for 4 h at room temperature and visualized using a TMB substrate.

Results and discussion

We selected clones that showed high and selective affinity to the Aβ peptide used for immunization. We identified four potential clones, 82E1, 12B2, 1A10, and 1C3, which react with different regions of Aβ peptide. The specific epitope for each of these clones was determined by plate assays using various Aβ fragments. We examined human and rodent cross-reactivity using human and rodent Aβ1–40 and 1–42. Clone 82E1 showed higher selectivity for human Aβ (approximately 10% cross-reactivity to rodent Aβ), probably because the human-specific 5th amino acid is a part of the epitope. Clones 12B2, 1A10, and 1C3 reacted with both human and rodent Aβ to a similar degree because their epitopes do not include a human-specific residue. Table 1 summarizes epitopecs, specificity, cross-reactivity to human and rodent Aβ, and applications of these antibodies.

Clone 82E1 reacted with both soluble and fibrillar Aβ to a similar degree (Fig. 1A). Clone 82E1 detected Aβ in

Table 1

<table>
<thead>
<tr>
<th>Clone</th>
<th>Selectivity, human/rodent cross-reactivity</th>
<th>Applications</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>WB</td>
</tr>
<tr>
<td>82E1 (IgG1)</td>
<td>N-terminus end specific (Aβ1–5)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>No cross-reactivity to non-cleaved APP fragments</td>
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<tr>
<td></td>
<td>Preference to human Aβ (≈10% cross-reactivity to mouse Aβ)</td>
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</tr>
<tr>
<td>12B2 (IgG1)</td>
<td>Middle region-specific (Aβ17–28)</td>
<td>+a</td>
</tr>
<tr>
<td></td>
<td>Cross-reactive to human and rodent Aβ at similar degree</td>
<td></td>
</tr>
<tr>
<td>1A10 (IgG1)</td>
<td>C-terminus end specific (Aβ40)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Cross-reactive human and rodent Aβ at similar degree</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;1.8% cross-reactivity to Aβ42</td>
<td></td>
</tr>
<tr>
<td>1C3 (IgG1)</td>
<td>C-terminus end specific (Aβ42)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cross-reactive human and rodent Aβ at similar degree</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.1% cross-reactivity to Aβ40</td>
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The utility of the antibodies for various applications is indicated as follows: ++++, very useful; +, useful with limitations; and –, not useful under our testing conditions.

a 12B2 did not work in tissue homogenates, but worked with synthetic Aβ peptides (>50 ng Aβ).
b 12B2 and 1C3 worked in IP but not as well as 82E1 and 1A10. WB, Western blotting; IHC, immunohistochemistry; IP, immunoprecipitation.
the SDS extract from APP-expressing transgenic mice (Tg2576), but did not detect non-cleaved APP (Fig. 1B). N-terminus specificity was further confirmed using HEK cells expressing Swedish mutant APP. 82E1 did not react with full-length APP (Fig. 1C, lane C), although a control antibody, 6E10 (epitope within 3–10), detected act with full-length APP (Fig. 1C, lane C), although a control antibody, 6E10 (epitope within 3–10), detected

C-terminus-specific antibodies, clones 1A10 and 1C3 (Aβ40 and Aβ42 specific, respectively), showed virtually no cross-reactivity to each other (<1.8 and <0.1%, respectively). The Aβ40-specific antibody, 1A10, worked well in immunoblotting (Figs. 2A and B), immunohistochemistry (Figs. 2C and 3B), immunoprecipitation, and ELISA, although Aβ42-specific antibody, 1C3, worked well only in immunohistochemistry (Fig. 3D) and ELISA.

We developed sandwich ELISA by a combination of these antibodies. We selected the C-terminus antibodies, either Aβ40 (1A10) or 42 specific (1C3), as the capturing antibody, and another antibody, either 82E1 or 12B2, as a detection antibody (reporter). We did not consider other combinations (82E1/12B2 for capturing and 1A10/1C3 for detection), because Aβ40 is much more abundant than Aβ42 in biological specimens and Aβ40 might monopolize the capturing antibody and reduce the sensitivity of Aβ42 detection. A combination of 1A10/82E1 and 1C3/82E1 gave a linear standard curve in the range of 1–1000 pg/ml (typical correlation factor >0.98) and detected human Aβ as low as 1.5 pg/ml (equivalent to sub fmol/ml range). We measured human Aβ in the plasma of AD patients and non-AD controls (Table 2). Previous publications showed that human plasma has a wide range of Aβ, and there is no significant difference between levels in AD and non-AD controls [7–11]. Our sandwich ELISAs using 1A10/82E1 and 1C3/82E1 similarly revealed no significant difference between AD and non-AD control cases (p = 0.442).

The epitope of clone 12B2 is 17–28 which is an identical sequence in human and rodent Aβ, and 12B2 reacted to human and rodent Aβ to a similar degree. We used this antibody as a reporter antibody and developed an ELISA to detect endogenous mouse brain Aβx-40 and x-42. A combination of 1A10/12B2 and 1C3/12B2 gave a linear standard curve in the range of 1–500 pg/ml (typical correlation factor >0.96). We measured...
endogenous brain and CSF Aβ in wild type (non-transgenic) mice, finding 25.2/4.8 fmol Aβ40/ml (mean ± SE, n = 4) and 817/73 fmol Aβ40/ml (mean ± SE, n = 2), respectively.

In conclusion, we have generated a series of anti-Aβ antibodies, including those that are specific both for the N-terminus β-cleavage site (clone 82E1) and for C-terminus γ-cleavage sites (clones 1A10 and 1C3). Because 82E1 is not cross-reactive with APP and β-cleavage site-specific, 82E1 is a critical tool in investigation of full-length Aβ. Combinations of these antibodies were used in ELISA to quantify full-length Aβ1–40 and 1–42, and rodent endogenous Aβx–40 and x–42. These assays will allow for very sensitive and accurate evaluation of anti-Aβ therapeutic approaches.

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**References**


