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Passive Immunization against Pyroglutamate-3 Amyloid-β Reduces Plaque Burden in Alzheimer-Like Transgenic Mice: A Pilot Study

Jeffrey L. Frost a, Bin Liu a, Martin Kleinschmidt b, Stephan Schilling b, Hans-Ulrich Demuth b, Cynthia A. Lemere a

a Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, Mass., USA; b Probiodrug AG, Halle (Saale), Germany

Key Words
Alzheimer's disease • Pyroglutamate-3 amyloid-β • Monoclonal antibody • Immunotherapy • Transgenic mice

Abstract
Background: N-terminally truncated and modified pyroglutamate-3 amyloid-β protein (pE3-Aβ) is present in most, if not all, cerebral plaque and vascular amyloid deposits in human Alzheimer’s disease (AD). pE3-Aβ deposition is also found in AD-like transgenic (tg) mouse brain, albeit in lesser quantities than general Aβ. pE3-Aβ resists degradation, is neurotoxic, and may act as a seed for Aβ aggregation. Objective: We sought to determine if pE3-Aβ removal by passive immunization with a highly specific monoclonal antibody (mAb) impacts pathogenesis in a mouse model of Alzheimer’s amyloidosis. Methods: APPswe/PS1ΔE9 tg mice were given weekly intraperitoneal injections of a new anti-pE3-Aβ mAb (mAb07/1) or PBS from 5.8 to 13.8 months of age (prevention) or from 23 to 24.7 months of age (therapeutic). Multiple forms of cerebral Aβ were quantified pathologically and biochemically. Gliosis and microhemorrhage were examined. Results: Chronic passive immunization with an anti-pE3-Aβ mAb significantly reduced total plaque deposition and appeared to lower gliosis in the hippocampus and cerebellum in both the prevention and therapeutic studies. Insoluble Aβ levels in hemibrain homogenates were not significantly different between immunized and control mice. Microhemorrhage was not observed with anti-pE3-Aβ immunotherapy. Conclusions: Selective removal of pE3-Aβ lowered general Aβ plaque deposition suggesting a pro-aggregation or seeding role for pE3-Aβ.

Introduction
Alzheimer’s disease (AD), the most common form of dementia, afflicts more than 30 million people worldwide. Amyloid-β protein (Aβ) is implicated in AD pathogenesis [1]. N-terminally truncated and pyroglutamate-modified Aβ peptide starting at residue 3 (pE3-Aβ) is abundant in cored and diffuse Aβ deposits as well as vascular amyloid in AD, presenilin-linked familial AD, and Down syndrome brain [2–6]. pE3-Aβ is formed upon removal of the first 2 N-terminal residues of Aβ followed by cyclization by glutaminyl cyclase (QC; isoQC) to convert the third residue, glutamic acid, to pyroglutamate [7]. Pyroglutamate formation makes Aβ peptide more hydro-
phobic, speeding up its aggregation; pE3-Aβ peptide resists degradation, favoring formation of stable, neurotoxic aggregates [8–11]. It is unclear if pE3-Aβ peptide is present in early plaque deposition or if it accrues later. However, a correlation has been reported between pyroglutamate-modified Aβ forms and severity of disease [12–14]. Taken together, these results indicate that pE3-Aβ peptide plays an important role in AD pathogenesis. Thereby, the N-terminal truncation and modification makes this peptide species a superior target for immunization. The primary advantage of such a strategy might be to capture and detoxify a particular Aβ molecule without affecting the potential physiological function of full-length Aβ. Here, we used passive immunization targeting pE3-Aβ in AD-like transgenic (tg) mice to determine if selective removal of this toxic peptide impacts AD pathogenesis.

Methods

Antibody Characterization

Western blot analysis was performed as described previously [15]. The cross-reactivity was determined by surface plasmon resonance using a Biacore 3000. Different pyroglutamate-modified peptides were immobilized covalently on CM5 chips. The binding onance using a Biacore 3000. Different pyroglutamate-modified

Animals

APPswe/PS1ΔE9 mice [16], harboring human APPswe (K595N/M596L) and PS1ΔE9 (deletion of exon 9), were obtained from Jackson Lab (Bar Harbor, Me., USA), and bred in our colony with C57BL/6 mice. All animal use was approved by the Harvard Standing Committee for Animal Use and was in compliance with all state and federal regulations. Cerebral Aβ plaque deposition and cerebral amyloid angiopathy are initiated at 5–6 months in this model [17].

Passive Immunization

Two trials were conducted in gender- and age-matched APPswe/PS1ΔE9 mice. A ‘prevention’ trial was initiated in 5.8-month-old mice (±0.38 SEM; anti-pE3-Aβ mAb, n = 6; PBS control, n = 3), during the early stage of plaque deposition, and continued for 32 weeks. A ‘therapeutic’ trial was undertaken in 23-month-old mice (±0.25 SEM; anti-pE3-Aβ, n = 4; PBS control, n = 4) with robust cerebral Aβ and pE3-Aβ plaque deposition and cerebral amyloid angiopathy, and continued for 7 weeks. Mice were vaccinated weekly by intraperitoneal injection of 200 μg of a new mouse IgG1 mAb specific for pE3-Aβ [mAb07/1; Probiodrug AG, Halle (Saale), Germany] or 100 μl of PBS (as a control).

Tissue Collection

Mice were sacrificed by CO2 inhalation 1 week after the final immunization. Blood was collected via cardiac puncture followed by perfusion with 20 ml PBS. The brain was removed and divided sagitally. One hemibrain was fixed for 2 h in 10% neutral buffered formalin and processed for paraffin embedding while the other was snap frozen and stored at –80°C for biochemical analysis.

Immunohistochemistry, Histology and Quantification

Ten-micrometer paraffin sections were immunolabeled using the ABC ELITE method (Vector Laboratories, Burlingame, Calif., USA) as previously described [18]. The following antibodies were used for immunohistochemical analysis: anti-CD45 (1:3,000, Serotec, Raleigh, N.C., USA), anti-Iba1 (1:500, Wako Chemicals, Richmond, Va., USA), anti-GFAP (1:1,000, DakoCytomation, Carpinteria, Calif., USA), rabbit polyclonal anti-Aβ R1282 (1:1,000, gift from D. Selkoe, Boston, Mass., USA) and anti-pE3-Aβ mAb07/1 (1:1,000, gift from Probiodrug AG, Halle (Saale), Germany). One-percent aqueous Thioflavin S (Sigma Aldrich, St. Louis, Mo., USA) was used to visualize fibrillar amyloid in plaques and blood vessels. Hemosiderin staining using 2% ferrocyanide (Sigma) in 2% hydrochloric acid was used to detect microhemorhages. Quantification of total R1282 immunoreactivity (IR), pE3-Aβ IR and Thioflavin S staining was performed using BIOQUANT image analysis (Nashville, Tenn., USA). The threshold of detection was held constant during analysis. The percent area occupied by R1282 IR, pE3-Aβ IR or Thioflavin S labeling in the entire hippocampus and cerebellum was calculated for 3 equidistant sagittal sections 300 μm apart per mouse. Vascular amyloid was evaluated semiquantitatively by 2 investigators (blinded to the treatment group) using the following criteria: ‘0’ = no positive blood vessels; ‘1’ = 1–5 positive blood vessels; ‘2’ = 6–10 positive blood vessels, and ‘3’ = 11 or more positive blood vessels.

Brain Homogenates and ELISAs

Snap-frozen whole hemispheres were homogenized in 5 volumes of Tris-buffered saline containing a protease inhibitor cocktail (Roche, Indianapolis, Ind., USA). Homogenates were spun at 175,000 g for 30 min at 4°C. The Tris-buffered saline pellet was resuspended in 10 volumes of guanidine buffer (5 M guanidine HCl, 50 mM Tris, pH 8.0). Samples were mixed for 4 h at room temperature and stored at –20°C. Aβ(3–42) and pE-Aβ(3–42) were quantified as previously described [19] using commercial ELISA kits (IBL, Hamburg, Germany).

Statistical Analyses

The Mann-Whitney U test (Prism 4.0 Software, GraphPad, San Diego, Calif., USA) was used to compare the results of immunized and PBS control mice. Significant differences were defined as p < 0.05.

Results

Anti-pE3-Aβ mAb Characterization

The specificity of antibody mAb07/1 was assessed by a combination of Western blot and surface plasmon resonance analysis (fig. 1). The antibody showed no cross-reactivity with full-length Aβ(1–x) or truncated, non-cyclized Aβ(3–x) in Western blot analyses. Moreover, pyroglutamate-modified neuropeptides or hormones were
not recognized by mAb07/1, suggesting a very high specificity of the molecule without potential of side effects. For immunization, the antibody was purified from cultures of mouse hybridoma cells and then sterile-filtered in PBS. The concentration range for injection was 2–2.5 mg/ml.

Passive Anti-pE3-Aβ Vaccination Lowered Total Aβ Deposition in a Prevention Trial

Aβ deposition begins in the hippocampus, neocortex and cerebellum at 5–6 months in APPswe/PS1ΔE9 mice and increases with age [17]. By 6 months, a small subset of mostly compacted plaques contains pE3-Aβ; this subset increases with age in proportion to general Aβ deposition (data not shown). In this study, mice were immunized weekly with the highly specific anti-pE3-Aβ mAb07/1 starting at 5.8 months of age, during the early stages of Aβ deposition. Following 32 weeks of vaccination, total Aβ deposition (including plaques and cerebral amyloid angiopathy) was reduced in the hippocampus and cerebellum in the approximately 14-month-old treated mice compared to age- and gender-matched PBS controls (table 1; fig. 2). In the hippocampus, pE3-Aβ and general Aβ (R1282) IR were reduced by 35% (p = 0.04) and 18% (p = 0.01), respectively, while Thioflavin-S-positive fibrillar amyloid was reduced by 50% (p = 0.02) in immunized mice when normalized to PBS controls. In the cerebellum, pE3-Aβ and general Aβ IR were lowered by 76% (p = 0.0004) and 52% (p = 0.005), respectively, while Thioflavin-S-positive fibrillar amyloid was reduced by 43% less (p = 0.13, n.s.) in immunized mice when normalized to the PBS control mice. The absolute values, listed in table 1, strongly suggest that passive immunization against pE3-Aβ reduced more than pE3-Aβ alone. For example, an absolute reduction of 2.4% in general Aβ (R1282) IR was observed in the hippocampus, whereas the absolute amount of pE3-Aβ in the PBS control group was much lower (0.61%; table 1). Similar reductions were observed in the cortex but were not quantified (data not shown). Semiquantitative analysis of vascular amyloid (scored 0–3) was similar in the hippocampus between PBS control and pE3-Aβ vaccinated mice (R1282: 0.89 ± 0.11 SEM vs. 0.59 ± 0.12, p = 0.11; pE3-Aβ: 0 vs. 0, p = n.s.). In the cerebellum, vascular amyloid was reduced by vaccination (R1282: 2.11 ± 0.26 vs. 1.47 ± 0.15, p = 0.03; pE3-Aβ: 0.33 ± 0.17 vs. 0.17 ± 0.09, p = 0.24). Microhemorrhages were absent in both groups of approximately 14-month-old mice.

No significant differences between immunized and control mice were observed in pE-Aβ(3–42) and Aβ(x–42) levels in guanidine HCl-extracted hemibrain homogenates; pE-Aβ(3–42) was approximately 0.2% of Aβ(x–42). Microgliosis (Iba-1 and CD45 IR) and astrocytosis (GFAP IR) appeared to be attenuated in the immunized mice compared to PBS controls.

Passive Anti-pE3-Aβ Vaccination Reduced Plaque Burden in the Absence of Microhemorrhage in a Therapeutic Trial

Twenty-three-month-old, plaque-bearing APPswe/PS1ΔE9 mice were passively immunized weekly for 7 weeks. The percent areas occupied by general Aβ IR and

Passive PyroGlu3-Aβ Immunotherapy in Mice

Fig. 1. Characterization of pE3-Aβ mAb07/1. a Western blot of Aβ(1–40), Aβ(3–40) and pE3-Aβ(3–40), detected with mAb07/1 or mAb6E10. Peptides (20 ng each) were separated in gels containing 8 M urea. The anti-pE3-Aβ mAb does not detect the truncated precursor or full-length Aβ. b Analysis of antibody binding using surface plasmon resonance. The peptides were covalently linked and the antibody applied in a buffered solution. Significant binding of mAb07/1 was only observed with pE3-Aβ(3–40) immobilized on the surface. Other peptides analyzed are: MCP-1 and 2; gastrin; GnRH; neurotensin; orexin; TRH; the N-terminus of collagen, and fibronectin.
**Cerebellum Hippocampus**

Therapeutic trial

were quantified by image analysis (Fig. 2).

**Hippocampus**

Prevention trial

were compared to that in PBS control mice. Immunohistochemical results (a, b) and Thioflavin S labeling were quantified by image analysis (b, d). Absolute values are provided in table 1. Scale bars, 200 μm. p values: * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. = nonsignificant (p = 0.089).

**Table 1.** Absolute values of percent labeling within the area of interest

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Aβ-pE3 vaccinated</th>
<th>Percent reduction</th>
<th>p value</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hippocampus</td>
<td></td>
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<tr>
<td>R1282 IR</td>
<td>13.0 ± 0.06</td>
<td>10.6 ± 0.88</td>
<td>18.20 ± 6.82</td>
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<td>pE3-Αβ</td>
<td>0.61 ± 0.02</td>
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<td>Thio S</td>
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<td>49.57 ± 8.11</td>
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<tr>
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<tr>
<td>R1282 IR</td>
<td>3.4 ± 0.32</td>
<td>1.7 ± 0.20</td>
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<td>pE3-Αβ</td>
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<td>0.1 ± 0.02</td>
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<td><strong>Therapeutic trial</strong></td>
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<tr>
<td>R1282 IR</td>
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<td>62.36 ± 7.20</td>
<td>0.0005</td>
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<td>pE3-Αβ</td>
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<td>0.6 ± 0.09</td>
<td>40.95 ± 8.90</td>
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<td>Thio S</td>
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<td>0.47 ± 0.06</td>
<td>38.34 ± 8.46</td>
<td>0.06</td>
</tr>
</tbody>
</table>

1 Percent reduction normalized to mean PBS control value.

**Fig. 2.** In a prevention study initiated during the early stage of plaque deposition, weekly passive immunization of APPsw/HET mice with anti-pE3-Αβ mAb07/1 from 5.8 to 13.8 months of age significantly reduced pE3-Αβ as well as general Aβ (R1282 IR) and fibrillar amyloid (Thioflavin S) deposition in the hippocampus (a, b) and cerebellum (c, d) compared to that in PBS control mice. Immunohistochemical results (a, c) and Thioflavin S labeling were quantified by image analysis (b, d).

Overall, therapeutic passive anti-pE3-Αβ immunization reduced both general Aβ and pE3-Αβ IR (table 1; fig. 3), similar to the reductions seen in the prevention study. In the hippocampus, pE3-Αβ and general Aβ (R1282) IR were reduced by 34% (n.s.) and 25% (p = 0.03), respectively, while Thioflavin-S-positive fibrillar amyloid was reduced by 49% (p = 0.04) in the immunized mice when normalized to the PBS control mice. In the cerebellum, pE3-Αβ and general Aβ IR were reduced by 41% (p = 0.03) and 62% (p = 0.0005), respectively, while Thioflavin S amyloid was lowered by 38% (p = 0.06, trend) in immunized mice when normalized to PBS control mice. Although we measured total deposition, reductions in plaque load accounted for the majority of differences in immunized compared to control mice; vascular amyloid deposits were unchanged relative to the age-matched control mice. Semiquantitative analysis of vascular amyloid (scored 0–3) was similar in the hippocampus between PBS control and pE3-Αβ vaccinated mice (R1282: 0.83 ± 0.11 SEM vs. 0.75 ± 0.13, p = 0.37; pE3-Αβ: 0.83 ± 0.17 vs. 0.75 ± 0.13, p = 0.41). In the cerebellum, vascular amyloid was reduced by vaccination (R1282: 2.5 ± 0.19 vs. 2.5 ± 0.15, p = 0.44; pE3-Αβ: 1.83 ± 0.21 vs. 1.83
Microhemorrhages were not observed in either the immunized or control mice at 24.7 months of age. Biochemical levels of Aβ(x–42) and pE-Aβ(3–42) were slightly reduced with immunization but did not reach significance (data not shown). As in the prevention study, microgliosis and astrocytosis appeared to be lower in the immunized mice compared to the PBS control mice, possibly reflecting the reduced plaque burden.

Discussion

APPsw/PS1ΔE9 mice showed increasing deposition of general Aβ, pE-Aβ, and fibrillar amyloid in the cortex, hippocampus and cerebellum with aging (i.e. from approx. 14 to 24.7 months of age), although pE-Aβ was detected in much lower quantities at both ages compared to general Aβ. In this pilot study, using a small number of mice per group, passive immunization with a highly specific anti-pE-Aβ mAb reduced all 3 types of deposits in the hippocampus and cerebellum in both a prevention study initiated in the early stage of plaque deposition and in a therapeutic study initiated in aged mice with robust Aβ deposition and gliosis. The percent of plaque reduction was similar between the prevention and the therapeutic trial, even though the absolute level of plaque deposition was higher in the older mice. However, even in the therapeutic study, the absolute amount of general Aβ and fibrillar amyloid deposits were reduced beyond the amount expected from clearance of pE-Aβ deposits alone as shown in table 1, suggesting that pE-Aβ removal may prevent new plaque formation. In both studies, Aβ deposit lowering by the pE-Aβ mAb was greater in the cerebellum than the hippocampus and cortex. Possibly, plaque deposition in the cerebellum appears later or the antibodies had more access to the cerebellum than other brain regions. It is still unclear, though, why the biochemical measurements of insoluble Aβ(x–42) and pE-Aβ(3–42) levels did not reflect the quantitative immunohistochemical results. Potential reasons are that the homogenates were derived from entire hemibrains, but the pathological analysis was done on small brain regions in thin sections, i.e. using a total of 3 equidistant brain sections per mouse. Moreover, different antibodies were used in the immunohistochemical and ELISA analyses, which may have contributed to the discrepancy we observed. For example, the ELISAs used here quantify Aβ(x–42) and pE-Aβ(3–42), including low levels of P3 fragments, but not Aβ ending...
at residue 40. Immunohistochemistry was performed using R1282 pAb that detects general Aβ and mAb07/1 (specific for Aβ starting at pyroglutamate at residue 3), both of which detect Aβ Ending at residues 40 and 42. Lastly, interanimal variability and the low numbers of animals used in these preliminary studies may have played a role as well.

Overall, our results confirm and extend the published recently by Wirths et al. [20] in which 6 weeks of passive immunization with their IgG2b pE3-Aβ oligomer-specific mAb, 9D5, led to reduced cerebral Aβ levels and plaque burden, and improved performance on the elevated plus maze test for anxiety in four 4.5-month-old 5XFAD tg mice compared to 4 control mice. Plans are underway to study the effects of passive immunization with the anti-pE3-Aβ mAb07/1 used in our study (that recognizes monomeric, oligomeric and fibrillar pE3-Aβ) on cognition, as well as AD pathogenesis, in a large cohort of AD-like tg mice.

What are the implications of these studies? Selective removal of pE3-Aβ peptides, especially early in AD pathogenesis, may lower deposition of pE3-Aβ, general Aβ, and fibrillar amyloid, as pE3-Aβ may play a role in the aggregation and/or deposition of multiple Aβ species in brain. Therefore, selective targeting of this particularly toxic Aβ species by immunotherapy may be an effective way to prevent or treat AD in humans in whom pE3-Aβ IR is present in most, if not all, cerebral Aβ deposits.

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