Aβ peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer’s disease

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Much evidence indicates that abnormal processing and extracellular deposition of amyloid-β peptide (Aβ), a proteolytic derivative of the β-amyloid precursor protein (BAPP), is central to the pathogenesis of Alzheimer’s disease (reviewed in ref. 1). In the PDAPP transgenic mouse model of Alzheimer’s disease, immunization with Aβ causes a marked reduction in burden of the brain amyloid
dysfunction in murine models of Alzheimer’s disease would support the hypothesis that abnormal Aβ processing is essential to the pathogenesis of Alzheimer’s disease, and would encourage the development of other strategies directed at the ‘amyloid cascade’. Here we show that Aβ immunization reduces both deposition of cerebral fibrillar Aβ and cognitive dysfunction in the TgCRND8 murine model of Alzheimer’s disease without, however, altering total levels of Aβ in the brain. This implies that either a ~50% reduction in dense-cored Aβ plaques is sufficient to affect cognition, or that vaccination may modulate the activity/abundance of a small subpopulation of especially toxic Aβ species.

To explore the behavioural consequences of Aβ immunization, we used the TgCRND8 murine model of Alzheimer’s disease that expresses a mutant (K670N/M671V and V717F) human Aβ transgene under the regulation of the Syrian hamster prion promoter on a C3H/B6 strain background (M.A.C. et al., manuscript in preparation). TgCRND8 mice have spatial learning deficits at 3 months of age that are accompanied by both increasing levels of SDS-soluble Aβ and increasing numbers of Aβ-containing amyloid plaques in the brain. Age- and sex-matched TgCRND8 mice and non-Tg littermates in three cohorts were vaccinated at 6, 8, 12, 16 and 20 weeks with either Aβ42 or islet-associated polypeptide (IAPP), which has similar biophysical properties to Aβ but is associated with a non-central nervous system (CNS) amyloidosis. Both immunogens were in β-pleated-sheet conformation at the
time of injection (Fig. 1) and induced detectable antibody titres in all mice by 13 weeks of age (as measured by enzyme-linked immunoabsorbent assay (ELISA) using fibrillar forms of the respective immunogen). These titres increased by a further ~2–3-fold at 23 weeks (Aβ42 titres in Aβ42-immunized mice (mean ± s.e.m.): 1:3,640 ± 470 at 13 weeks; 1:7,500 ± 1,712 at 23 weeks; IAPP titres in IAPP-immunized mice: 1:3,833 ± 1,167 at 13 weeks; 1:11,500 ± 3,661 at 23 weeks). The sera from Aβ-immunized mice intensely decorated extracellular, dense-core plaque deposits when applied as an immunohistochemical reagent to sections from brain from TgCRND8 mice containing abundant amyloid plaques (which predominantly display Aβ in a β-sheet conformation) or to formic-acid-treated sections (which also display additional non-β-sheet Aβ-epitopes) (Fig. 1). However, these sera reacted only very weakly with diffuse, non-fibrillar Aβ deposits, which can be readily detected in these tissues by anti-Aβ monoclonal antibodies such as 4G8 (data not shown). The Aβ-immune sera did not stain normal neurons, indicating limited crossreactivity with BAPP holoprotein.

In contrast, sera from non-immunized or IAPP-immunized mice did not stain any structures. Together, these data indicate that in this strain of mice immunization with Aβ42 protofibrillar assemblies induced antibodies directed primarily towards Aβ in a β-sheet conformation.

The mice were tested longitudinally in a reference memory version of the Morris water maze test at 11, 15, 19 and 23 weeks (Fig. 2). At each age of testing, the hidden platform was placed in a different quadrant of the pool. These data were analysed for the entire test period using a mixed model analysis of variance (ANOVA), with immunogen (Aβ42 versus IAPP) and genotype (TgCRND8 versus non-Tg) as a between-subject factor, and age-of-testing (11, 15, 19 and 23 weeks) as a within-subject factor. This longitudinal design and mode of analysis, which simulates longitudinal human clinical trails, revealed that Aβ42-immunized TgCRND8 mice performed significantly better than IAPP-immunized TgCRND8 mice (P < 0.05), with 31% of the performance variance being due to the effects of the immunogen. However, the improvement was partial—the Aβ42-immunized TgCRND8 mice did not perform as well as their non-Tg littermates (P < 0.01).

Because the experimental design involved testing of naive mice at 11 weeks of age, followed by a series of reversal tests at 15, 19 and 23 weeks, an additional analysis carried out on the reversal tests confirmed improved performance for the Aβ-immunized Tg mice (P < 0.02) during this phase of testing as well. The overall conclusion that Aβ42 immunization ameliorates the cognitive deficit of TgCRND8 mice was robust, regardless of whether the analysis assessed latency to reach the hidden platform or swim path length (a measure that is less sensitive to swim speed and floating).

The improved performance of Aβ42-immunized TgCRND8 mice was not due to a nonspecific effect of immunization or to an effect on other behavioural, motor, or perceptual systems. Control studies
in non-Tg mice established that immunization with Freund's Adjuvant plus phosphate buffered saline, Freund's plus Aβ12, Freund's plus IAPP, IAPP alone, or Aβ12 alone had no effect on performance in the Morris water maze test (P > 0.1). During non-spatial pre-training at 11 weeks, the latency of random search for a hidden platform, as well as swim speed, and swim path length to a visible platform were not affected by genotype or immunization (P > 0.05). Similarly, at 23 weeks, performance during a visible platform test and spontaneous exploration of the open field were not significantly influenced by immunization (P > 0.05). Gender effects were not significant between the TgCRND8 and IAPP- or Aβ12-immunized non-Tg littermates (P > 0.05).

Analysis of performance in the probe trials with the platform removed (annulus crossing index, passes over platform site or dwelling in the target quadrant) revealed no significant differences owing to immunization or genotype, or interactions between these two factors (each P > 0.05). This probably arises both because the probe trials were conducted within 30 min of the final training trial on day 5 (by which point most mice had learned), and because repeated administration of probe trials (at 15, 19 and 23 weeks) makes them a less effective measure of spatial memory.

Because the main analysis revealed significant immunogen × genotype (P < 0.01) and immunogen × genotype × age (P < 0.05) interactions, as well as significant main factor effects for immunogen, genotype and age of testing (P < 0.01 for all), post hoc analyses were carried out for each age of testing. These showed markedly improved performance in Aβ12-immunized TgCRND8 mice at 11 and 23 weeks, with Aβ12-immunization accounting for large portions of the variance (ω²) (19% at 11 weeks; 42% at 23 weeks). Analyses at 15 and 19 weeks did not show statistically significant differentiation of the two immunogens. However, this probably reflects the effects of previous test experience ('carrying-over' effect). Reduction of impairment during re-testing in the water maze has been reported in another study of a murine model of Alzheimer's disease, and similar re-test effects are commonly seen in placebo-treated patients in human trials. The subsequent re-emergence of impaired cognition in the IAPP-immunized TgCRND8 mice at 23 weeks probably reflects the advance of Alzheimer's-disease-related phenotypes, which are clearly progressive over this epoch in TgCRND8 mice. Thus, increased Aβ levels are first detectable by western blot or ELISA analysis in brain homogenates at 8.5–17 and 10 weeks, respectively, but thereafter rise progressively. Similarly, cortical dense-cored Aβ-plaques are first detectable immunohistochemically at 7–10 weeks, but increase by a further 18-fold at 19–27 weeks (M.A.C. et al., manuscript in preparation).

In agreement with previous reports, Aβ12 immunization caused ~50% reduction in the number and size of Aβ-positive dense-cored plaques (Fig. 3) without affecting steady-state levels of βAPP holoprotein, amino-terminal secreted fragments (βAPPs), or carboxy-terminal fragments in the brain (see Supplementary Information). However, in contrast to the previous reports, Aβ12 immunization had no significant effect on the levels of formic-acid-extractable Aβ in brain at 13 weeks (P > 0.1) or 25 weeks (IAPP immunized (n = 7): Aβ40 = 11,475 ± 1,567 fmol per mg protein, Aβ42 = 39,983 ± 4,387; Aβ12 immunized (n = 6): Aβ40 = 12,276 ± 2,386; and Aβ42 = 50,457 ± 8552, mean ± s.e.m., P = 0.27 two-tailed t-test). Sandwich ELISAs using two other detection antibodies (directed at Aβ residues 1–4 and 17–24) yielded similar results, arguing against a selective effect of immunization on Aβ species differing in their amino terminus identity or in post-translational modification.

One explanation for our results is that dense-cored cerebral amyloid plaques are the toxic moiety, and the ~50% reduction caused by Aβ12 vaccination is sufficient to prevent or reverse the behavioural deficits. Although this cannot be excluded, the density of such plaques correlates poorly with the ante-mortem severity of dementia in most human studies. Furthermore, a dissociation between plaque deposition and cognitive and/or neuronal dysfunction has been described in other TgAPP mice. An alternative explanation is that immunization affects Aβ either in a particular conformation (for example, β-sheet forms in protofilibrils) or in a restricted compartment. The former is more likely because we used oligomeric assemblies of Aβ in β-sheets (‘protofilibrils’) as an immunogen, and the resultant antisera preferentially recognized β-sheet forms of Aβ. This is significant because monoclonal antibodies raised to Aβ epitopes that initiate fibril aggregation inhibit assembly of synthetic Aβ oligomeric protofilibrils in vitro. It is possible, therefore, that the antibodies induced in the current strain of mice may bind to β-sheet oligomeric aggregates and inhibit further assembly. This Aβ species is especially neurotoxic, a critical intermediary in fibrillogenesis and an accurate predictor of neurodegeneration. Consequently, small amounts of such antibodies that cross the blood–brain barrier (0.1% of serum levels) might be sufficient to attenuate both the behavioural deficits caused by this neurotoxic form of Aβ and the further aggregation of these species into fibrillar Aβ in dense-cored plaques. Because this pool of Aβ is small, and because antibodies to this form of Aβ might need only intermolecularly of Aβ fibrils to have a functional effect, these antibodies need not necessarily cause large changes in total cerebral Aβ. It is also possible that the antibodies redistribute Aβ from dense-cored plaques to diffuse Aβ deposits. Either might explain the divergent effects on levels of dense-cored plaques and Aβ measured biochemically (although high levels of cerebral Aβ in the presence of relatively few plaques are seen in humans and in transgenic mouse models).

It is conceivable, however, that immunization might modulate Aβ metabolism through several distinct mechanisms, including destruction of Aβ by microglial phagocytosis. Such different effects, which might reflect variations in antigen presentation or in strain-specific immune response, might also explain the disparity between the effects of Aβ immunization on total Aβ levels in this and the previous studies. If correct, such variations could complicate the use of active immunization in humans. In addition to clinical caveats raised previously, it is also important to emphasize that, although Aβ12 immunization had a strong effect on behaviour and neuropathology in this mouse model, it did not fully reverse these features. This might reflect either inefficient ingress of antibodies to the CNS and/or the possibility that other βAPP proteolytic fragments may be involved in the pathogenesis of Alzheimer's disease. These issues will need to be addressed directly by future studies. Nonetheless, our data support the hypotheses that Aβ plays a central role in Alzheimer's disease and that procedures that modulate its production, assembly and/or removal might be used as treatments.

**Methods**

**Mice**

The TgCRND8 mice (M.A.C. et al., manuscript in preparation) were maintained in a hybrid C3H/B6 background. Experimental groups derived from crosses to B6 mice were matched for gender and weight, and transgene identity was unknown to experimenters at all stages of the study. Sixty-eight 6-week-old mice were immunized (TgCRND8, n = 28, non-Tg, n = 40); 60 entered the behavioural testing at 11 weeks (Aβ immunized: TgCRND8, n = 12; non-Tg, n = 20; and IAPP immunized: TgCRND8, n = 9; non-Tg, n = 19). Thirteen mice were killed at 13 weeks, and their brains analysed (Aβ immunized: TgCRND8, n = 3; non-Tg, n = 4; IAPP immunized: TgCRND8, n = 2, non-Tg = 4). All remaining mice were longitudinally tested until 23 weeks of age (Aβ immunized: TgCRND8, n = 9; non-Tg, n = 16; IAPP immunized: TgCRND8, n = 7; non-Tg, n = 15). Thirteen non-immunized mice (TgCRND8, n = 5; non-Tg, n = 8) were included as controls in the test conducted at 11 weeks of age.

**Immunization procedures**

Synthetic Aβ38 and IAPP peptides were isolated by reverse phase high performance liquid chromatography on a C18 phenylapak column, with purity determined using mass spectrometry and amino-acid analyses. Before preparation of the vaccine, the secondary structure and fibre morphology of the peptides were assessed using circular dichroism spectroscopy and by electron microscopy. The immunization protocol and schedule have been described. Antibody titres were assessed in triplicate by ELISA (see
Supplementary Information for more details in serum samples (200 µl of blood) collected at 13 and 25 weeks.

Behavioral tests and data analysis

The water maze apparatus, mouse handling and general testing procedures have been described23. Before the first spatial learning test at 11 weeks, all mice underwent non-spatial pre-training (NSP) to assess swimming abilities and to accustom mice to the test4,23 (see Supplementary Information). Two days after the NSP phase, all mice underwent a reference task in a hidden platform placed in the centre of one quadrant of the pool for 5 days, with four trials per day. After the last trial of day 5, the platform was removed from the pool and each mouse received one 60-s swim probe trial. Escape latency (s), length of swim path (cm), swim speed (cm s−1), % of time of escape and 5 less time then 5 cm3, % of time in outer zone (near the pool wall), and % of time and path in each quadrant of the pool were recorded using an on-line HIS image video tracking system 23 (see Supplementary Information).

For the probe trials, an annulus-crossing index was calculated that represents the number of passes over the platform site, minus the mean of passes over alternative sites in other quadrants. The index expresses the spatial place preference and controls for alternative search strategies or place preferences, such as circular search paths23. All mice were re-tested at 15, 19 and 23 weeks of age, one week before the next immunization. At each re-testing, the platform was placed in the centre of a different, semi-randomly chosen pool quadrant for all five sessions of training. At the end of the experiment, all mice were given a cue (visual platform) learning test. This was followed by the open-field test to investigate spontaneous locomotor exploration. Behavioural data was analyzed using a mixed model of factorial ANOVA. Degrees of freedom were adjusted by Greenhouse–Geisser epsilon correction for heterogeneity of variance. A Bonferroni Inequality correction was applied for multiple comparisons. Omega squared (ω2) was used as a measure of effect size caused by different factors.

Analysis of βAPP and amyloid burden in brain

Three 5-µm sections at 25-µm intervals from one cerebral hemisphere were immunostained with a mixture of 6F/3G anti-ββ monoclonal antibody to residues 8–17 (which is primarily reactive against dense-cored plaques) with 4G8 (ref. 28), or with sera from immunized mice, and counterstained with haematoxylin and resin mounted as described (M.A.C. et al., manuscript in preparation). For some samples the formic-acid treatment step was omitted. End products were visualized with diaminobenzidine. Amyloid plaque burden was assessed using Leica IA 3001 image analysis software interfaced with a Leica microscope and a Hitachi KP-M110 CCD video camera. The quantitative analysis was performed at ×25 magnification, and the image frame and guard size was set to 0,639,479 (307,200 µm2) for each slide. The brain area (cortex or hippocampus) was outlined using the edit plane function, and the area and number of plaques in the outlined structure were recorded. Data were pooled for all three sections.

Cerebral Aβ levels were assayed from formic-acid-extracted, hemi-brain sucrose homogenates using an ELISA method (see Supplementary Information) in which Aβ was trapped with either monoclonal antibody to Aβ (IRF/Ab40/10) or Aβ42 (IRF/Ab42/26) and then detected with horseradish peroxidase (HRP)- conjugated IRF/Ab17. The dilution of IRF/Ab17 antibodies were optimized to detect Aβ in the range of 50 to 800 fmol ml−1. ELISA signals are reported as the mean ± s.e.m. of four replica wells in fmol Aβ per mg total protein (determined with the BioRad DC protein assay), based on standard curves using synthetic Aβ1–40 and Aβ1–42 peptide standards (American Peptide Co. Sunnyvale, CA). Cerebral βAPP levels were analysed in supernatant of brain as described24.

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βAPP peptide vaccination prevents memory loss in an animal model of Alzheimer’s disease


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Vaccinations with amyloid-β peptide (AB) can dramatically reduce amyloid deposition in a transgenic mouse model of Alzheimer’s disease1. To determine if the vaccinations had deleterious or beneficial functional consequences, we tested eight months of AB vaccination in a different transgenic model for