

# Reduction of $\beta$ -amyloid plaques in brain of transgenic mouse model of Alzheimer's disease by EFRH-phage immunization

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## Abstract

Antibodies to the epitope EFRH, representing residues 3–6 within the  $\beta$ -amyloid ( $A\beta$ ) sequence, were previously shown to affect the solubility and disaggregation of  $A\beta$  fibrils in vitro. Here, we describe a novel method of immunization, using as antigen the EFRH peptide displayed on the surface of the filamentous phage. The EFRH phage evoked effective auto-immune antibodies in amyloid precursor protein [V717I] (APP[V717I]) transgenic mice that recapitulate the amyloid plaques and vascular pathology of Alzheimer's disease (AD). The immunization provoked a considerable reduction in the number of  $A\beta$  amyloid plaques in the brain of the transgenic mice and may serve as the basis for anti- $A\beta$  vaccine.

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## 1. Introduction

The amyloid hypothesis remains the most attractive explanation as the underlying cause of Alzheimer's disease (AD), based on the general occurrence of amyloid pathology in the brains of all AD patients. In strong support are mutations in familial cases of early onset AD in the genes coding for amyloid precursor protein (APP) and presenilins (PS) [1,2]. Problems do remain. The precise involvement of amyloid in sporadic cases of AD, and the formation of neurofibrillary tangles [3] should be explained. The hypothesis of a "cascade" has exerted considerable attraction since that process would allow for intervention at multiple and different points to slow or halt the disease process [4]. The most effective treatment for AD is accepted to be directed towards (i) decreasing  $\beta$ -amyloid ( $A\beta$ ) production, (ii) increasing  $A\beta$  removal, or (iii) preventing  $A\beta$  fibril formation. Modulating events or mechanisms that are downstream of  $A\beta$  formation and deposition, e.g. free radicals, inflammation, calcium homeostasis, excitotoxicity, apoptosis, etc. would be less fundamental or even symptomatic [5].

A most promising approach in AD therapy is vaccination with  $A\beta$  peptides [6] which has been proposed and ap-

pears to act by increasing the elimination of  $A\beta$  peptides, most likely as immune complexes through endocytosis or phagocytosis by microglia and astrocytes. Recent findings in transgenic mouse models for Alzheimer's disease have demonstrated that an immune response against  $A\beta$  reduced the amyloid plaques and associated dystrophic neuritis [6]. Although very encouraging, the findings in these and in other studies [7–9] also raised a number of drawbacks and problems of the procedures, i.e. aspects of adjuvant, auto-immune response, blood–brain barrier, soluble  $A\beta$  peptide levels, among others [10].

We recently demonstrated that monoclonal antibodies (mAbs) specific for N-terminal epitopes of the  $A\beta$  peptide disaggregated preformed  $A\beta$  fibrils and neutralized their neurotoxic effects [11]. Moreover, the same or similar antibodies prevented or reduced amyloid formation in vitro [12] and in vivo [7]. Using a filamentous phage-peptide library displaying random combinatorial peptides, we have defined the EFRH residues located at positions 3–6 of the  $A\beta$  peptide to be the major epitope for anti-aggregating antibodies [13]. Blocking the EFRH-epitope with specific antibodies affected the dynamics of the  $A\beta$  peptides by preventing self-aggregation and promoting solubilization of preformed aggregates [11].

Here, we report the development of an EFRH-phage and its use as a novel immunization procedure rapidly evoking

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anti-aggregating A $\beta$  antibodies without requiring any additional adjuvant. This approach elicited a specific immune response and reduced the amyloid plaques in APP[V717I] transgenic mice, a mouse model that recapitulates the plaque and vascular amyloid pathology of Alzheimer's disease patients [14–16].

## 2. Materials and methods

### 2.1. Antibody binding to EFRH-phage

Binding of mAbs against different regions of A $\beta$ , as indicated, and of an anti-EFRH-phage anti-serum, was analyzed by ELISA. Wells of microtiter plates (Maxisorb, Nunc) were coated with rabbit anti-phage serum diluted 1:1000 in 0.1 M NaHCO<sub>3</sub>, pH 8.6, and incubated overnight at 4 °C. The wells were treated with PBS containing bovine serum albumin (1.5 mg/ml) and hemoglobin (1.5 mg/ml) for 2 h at 37 °C, to block all non-specific binding sites, and rinsed three times with PBS containing 0.05% Tween 20. EFRH phages (10<sup>10</sup> per well in 50  $\mu$ l) were incubated for 1 h at 37 °C. After washing, the diluted antibodies (1  $\mu$ g/ml) were added and allowed to saturate all specific binding sites by incubation overnight at 4 °C. Binding was measured as described [13,17].

### 2.2. Immunization protocols

The antigen used was filamentous phage carrying the EFRH-epitope within surface protein VIII, prepared as described [17]. APP[V717I] transgenic mice (age 16 months) were immunized by intraperitoneal (i.p.) injection of 10<sup>11</sup> phages in PBS without adjuvant. The mice received a total of six injections over a 4.5-month period, i.e. four injections every 2 weeks, followed by two boosts at 1-month interval. A group of six APP[V717I] mice were injected with PBS and served as controls. One week after the sixth injection, blood samples were collected and the brains quickly perfused with PBS, respectively, for determination of anti-EFRH titers [17] and for amyloid plaque load [16].

### 2.3. Electron microscopy

Negatively stained amyloid fibrils were prepared on carbon-coated grids floating on aqueous peptide solutions (0.5 mg/ml). Amyloid peptide fibrils were incubated alone or with anti-serum raised against the EFRH-phage (diluted 1:20) for 12 h at 37 °C. After negative staining with uranyl acetate (20 mg/ml) the grids were analyzed by EM (JEOL-1200 EX operated at 80 kV).

### 2.4. Immunofluorescence detection of $\beta$ -amyloid plaques

Brain sections from AD patients (Buxton Laboratories, Liverpool, UK) and from APP[V717I] transgenic mice were

fixed in 4% paraformaldehyde for 2 h, and then kept in 10% formalin saline for 2 days at room temperature. After embedding in paraffin, thin sections (4  $\mu$ m) were cut, collected on glass slides, and blocked with 3% milk in PBS for 30 min. After incubation with mouse polyclonal anti-serum against EFRH (1:10, 1 h at 37 °C) the sections were washed three times for 5 min each in PBS, again treated with blocking buffer (5 min, RT) before reaction with the secondary antibody (Cy<sup>TM</sup> three-labeled donkey anti-mouse IgG, 1:500, 1 h, RT). Finally, the preparations were washed three times in PBS and observed by confocal fluorescence microscopy.

### 2.5. Quantitative analysis of amyloid plaque load

The APP[V717I] mice were euthanized with chloroform and immediately decapitated. The right cerebral hemisphere was immersion-fixed with paraformaldehyde (4% in PBS, overnight, RT). Coronal vibratome sections were cut from the occipital two thirds of the right hemisphere. Quantitative analysis of vascular and plaque amyloid in the brain was performed after thioflavin-S staining. Two well-defined coronal sections at bregma –1.94 and –3.52 mm, respectively, were selected for quantification of the amount of amyloid load in plaques. Images (magnification 200 $\times$ ) from these sections were collected from a 3CCD color video camera and analyzed with appropriate software (AIS/C; Imaging Research). The total amyloid dense core load in plaques was expressed as an area in pixels as measured by the software.

## 3. Results

### 3.1. Antibodies raised against the EFRH-phage bind to A $\beta$ peptides

The epitope for anti-aggregating mAbs, localized using a combinatorial phage-displayed peptide library, exhibited the consensus sequence EFRH [17]. We determined that mAbs that disaggregate A $\beta$  in vivo and in vitro bind to the EFRH-phage similarly as a polyclonal serum against the EFRH-phage. Indeed, mAb 3D6 reduced amyloid plaques in the APP[V717I] transgenic mice [7] and specifically recognized the EFRH-epitope displayed on phage, while antibodies that failed to disaggregate plaques also failed to bind to the EFRH-phage (Fig. 1).

### 3.2. Immunization of APP[V717I] transgenic mice with EFRH-phage

APP[V717I] transgenic mice [14–16] were immunized with the engineered EFRH-phage by repeated intraperitoneal administration of 10<sup>11</sup> phages (Fig. 2). Seven days after each injection serum titers were determined for antibodies against A $\beta$  by a specific ELISA. Specific anti-A $\beta$  reactivity was obtained after the first i.p. injection of EFRH-phage, while

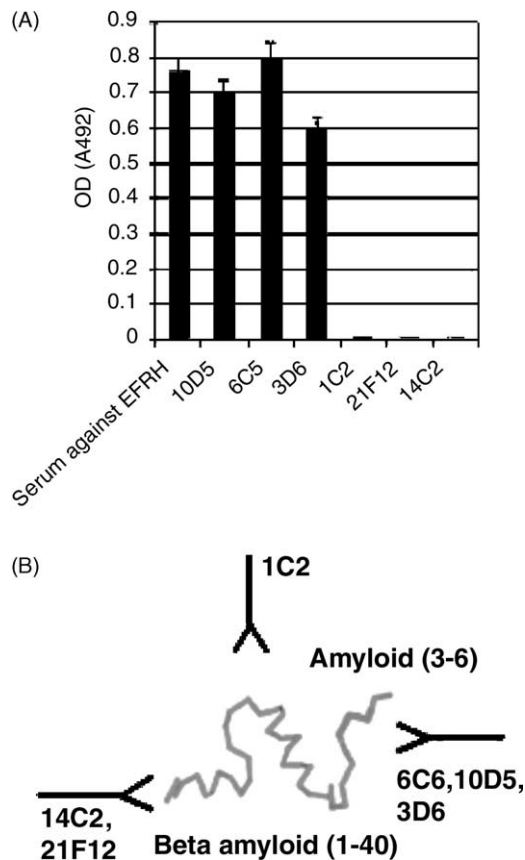


Fig. 1. ELISA for binding to f88-EFRH phage of anti-EFRH serum and of different mAbs-specific for A $\beta$ . (A) Antibodies were added to EFRH-phage-coated wells and binding was determined as described in Section 2. The polyclonal sera was preincubated with wild-type filamentous phage to deplete from it all non-EFRH anti-phage antibody specificities. (B) Schematic representation of the A $\beta$  peptide with the approximate location of the epitopes of the mAbs used in (A).

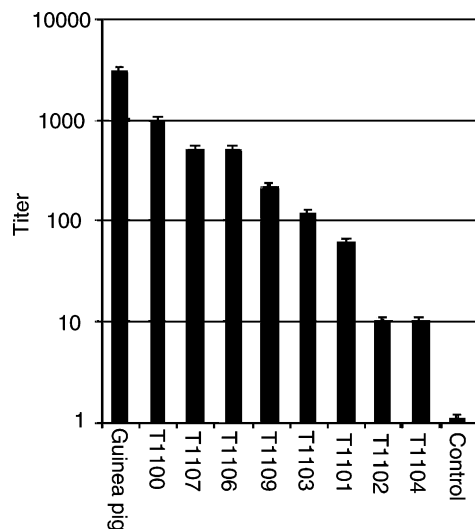


Fig. 2. Anti-serum titers (IgG) of the APP-transgenic mice immunized with EFRH-phage. The titer was measured after six i.p. injections of  $10^{11}$  EFRH-phages. Guinea pigs were similarly immunized as described before [18] and are included for comparison.

the titers increased to reach about 1:1000 after six injections (Fig. 2).

### 3.3. Anti-serum to EFRH-phage converted fibrillar to non-fibrillar A $\beta$ and decorates amyloid plaques

A $\beta$  fibrils were prepared and incubated with control mouse serum and with anti-EFRH serum taken from immunized APP-transgenic mice. Electron microscopy of the negatively stained A $\beta$  fibrils and the immune complexes with anti-EFRH-phage demonstrated that even at low concentrations of anti-serum the A $\beta$  fibrils were disaggregated into amorphous material (Fig. 3). Depositions of amyloid plaques in the hippocampus of APP[V717I] transgenic mice and of AD patients were demonstrated to be specifically decorated by the anti-EFRH anti-sera (Fig. 4).

### 3.4. Reduction of $\beta$ -amyloid plaques in APP[V717I] transgenic mice brains following EFRH-phage administration

APP[V717I] transgenic mice (16 months old) were immunized with the EFRH-phage and analyzed at age 21 months. At this age, the amyloid plaque pathology is maximally and stably established. Age-matched APP[V717I] transgenic mice were injected with vehicle only and served as untreated controls. All mice received four i.p. injections every 2 weeks followed by two booster injections 1 and 2 months later, or a total of six injections over 4.5 months.

Five out of eight (65%) APP[V717I] transgenic mice that were immunized with the EFRH-phage developed and maintained serum titers of antibodies against A $\beta$  that varied between 1:100 and 1:1000. Three (35%) of the APP[V717I] transgenic mice developed only a low titer ( $\sim$ 1:10) over the 4-month immunization period. The amyloid burden in the brain was significantly reduced in the immunized APP[V717I] transgenic mice that developed anti-A $\beta$  titers of at least 1:100 (Fig. 5), suggesting a dose–response relationship between antibody-titer and reduced amyloid load.

The total amyloid dense core load in plaques was expressed as an area in pixels as measured by the software, as mentioned in Section 2. The data are shown as percentages of the total investigated surface, including hippocampus, cortex. Distribution in each group was as equal as possible. By comparison, regarding the amount of plaque in the brain, we directly related the plaque between each group.

## 4. Discussion

Generation of amyloid peptides and the deposition of amyloid plaques and vascular amyloid is the main pathological hallmark of AD. Previous in vitro studies on A $\beta$  fibrils assembled from synthetic peptides identified specific monoclonal antibodies, i.e. 6C6 and 10D5, that (i) recognize the epitope EFRH, (ii) solubilize and disaggregate A $\beta$  fibrils,

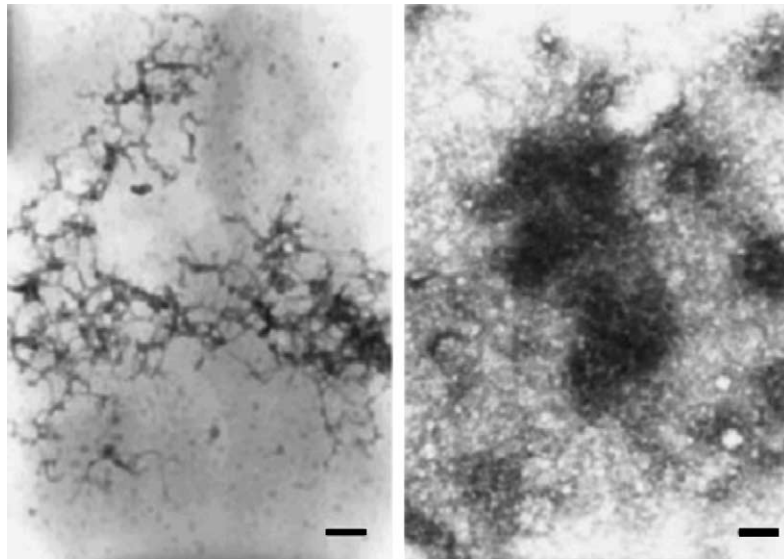


Fig. 3. Electron micrographs of A $\beta$  aggregates in the absence (left) or presence (right) of anti-serum raised against the EFRH-phage. Negatively stained amyloid fibrils were prepared by floating carbon-coated grids on aqueous peptide solutions (0.5 mg/ml). After air-drying the proteins were negatively stained and examined by EM (scale bar, 100 nm).

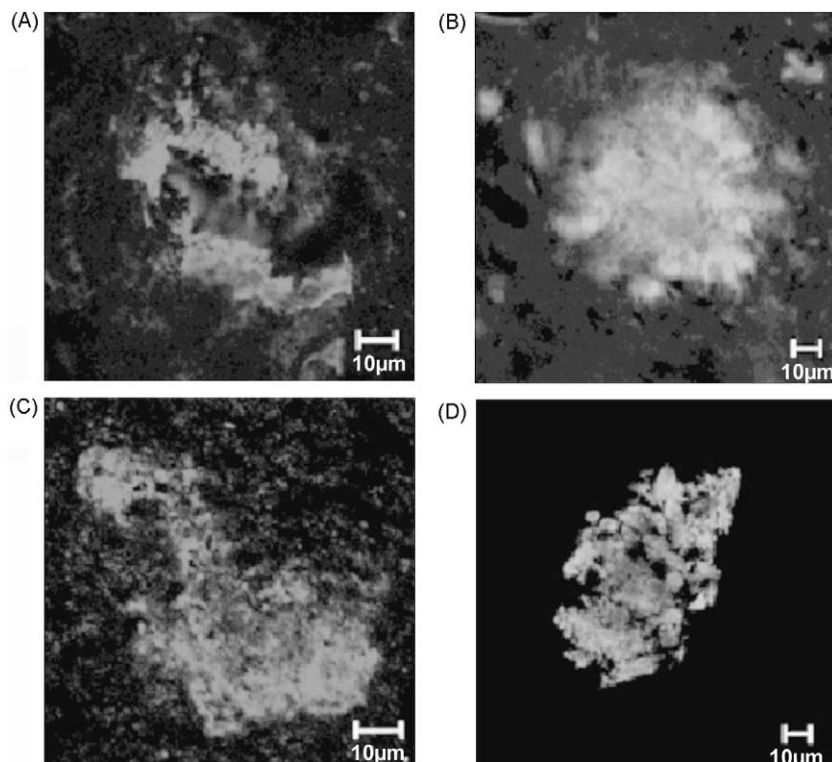


Fig. 4. Immunofluorescent detection of amyloid plaques by serum raised against the EFRH-phage. Brain sections of APP[V717I] transgenic mouse (A) and AD patients (C) were stained with EFRH-sera and compared to Congo red staining ((B) and (D), respectively). Staining was visualized by confocal microscopy (magnification 66 $\times$ ).

and (iii) reduce A $\beta$  neurotoxic effects on PC12 cells [11,12]. These findings contributed considerably to attempts to use A $\beta$  vaccination as a major therapeutic approach in AD [6].

Recently, reported experimental studies [6,7] demonstrated that active immunization with the A $\beta$  peptide, as

well as passive immunization with anti-A $\beta$  antibodies, attenuated aspects of AD pathology, i.e. reduced amyloid burden in transgenic PDAPP mice. Clearly, only some specified antibodies against A $\beta$  were effective, e.g. those that recognize epitopes located in the N-terminal region of A $\beta$ .

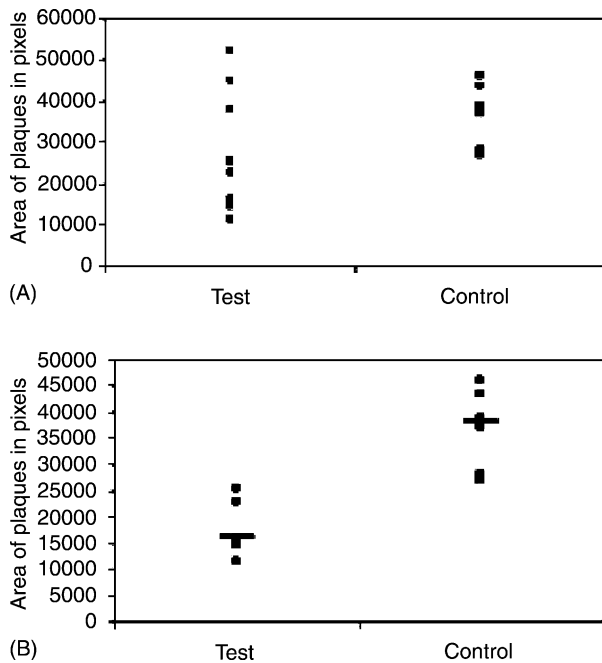


Fig. 5. Amyloid plaque load in APP[V717I] transgenic mice immunized with EFRH-phage. The test group was immunized with the engineered EFRH-phage by repeated intraperitoneal administrations of  $10^{11}$  for a total of six injections, while the control group was immunized with PBS. All the immunized mice, including those which exhibit a titer less than 1:100 against  $\beta$ -amyloid are shown in (A). In (B), we showed statistical evaluation omitting the mice with a lower titer than 1:100. Using student's *t*-test, the two-tailed *P*-value is  $\sim 0.10019$ . The test compared each pair from the two groups to the mean of each group.

The three antibodies that were most effective in vitro and/or in vivo [7,11] specifically recognize the EFRH-epitope of  $A\beta$  (Fig. 1).

For an immunization strategy to be effective it needs not only to identify the specific nature of the antigen or of the epitope, but also to address the formulation and method of delivery of the antigen as a major and critical parameter. We are engaged in an effort to analyze this problem and to optimize an immunization vehicle and protocol. To our knowledge this is the first attempt to use antibodies against a defined "anti-aggregating" epitope in combination with the recombinant phage as a vaccine. This unique approach is aimed at treatment of a unique disease that is caused by abnormal conformational changes or folding of a peptide, as it is presented in Alzheimer's disease, and eventually for other neurodegenerative disorders or amyloidoses.

We have shown here that immunization with a filamentous bacteriophage that carries about 300 copies of the EFRH-epitope elicited high titers of IgG antibodies of high affinity against the  $A\beta$  peptide in a relatively short period of time. These antibodies were operationally similar or identical in their in vitro and in vivo anti-aggregating properties to monoclonal antibodies against peptide (1–28), corresponding to the N-terminal region of  $A\beta$ , raised by direct injection with fibrillar  $A\beta$  in the presence of adjuvant [17,18].

We investigated the effect of the serum raised against EFRH phage on disruption of the toxic  $A\beta$  fibril, using the ThT reagent that binds specifically to fibrillar amyloid structures. Sera raised against EFRH peptide at dilutions of 1:5 and 1:20 disrupted the fibril structure of  $\beta$ -amyloid with extensive deterioration of fibril morphology with a 75% and 50% decrease, respectively, in ThT fluorescence. The unrelated serum used as control did not significantly inhibit fibril formation [17]. Moreover, serum raised against EFRH phage exhibits a protective effect in preventing  $A\beta$  mediated neurotoxicity toward pheochromocytoma PC12 cells. Diluted serum (1:5) prevented the neurotoxicity of  $A\beta$  (80% cell viability) [18]. Analysis by electron microscopy of negatively stained  $A\beta$  fibrils previously inoculated with such antibodies visualized how anti-EFRH serum, even in low concentrations, disaggregated the amyloid fibrils into an amorphous form (Fig. 3).

In addition, the anti-EFRH-phage anti-sera were demonstrated to label the amyloid plaques effectively, both in brain sections of human AD patients and in brain sections of the transgenic mice (Fig. 4).

In an APP[V717I] transgenic mouse model that recapitulates the amyloid pathology of AD [14–16], in vivo immunization with the EFRH-phage demonstrated its effectiveness in eliciting antibody titers, at least 1:100 dilution, thereby reducing the amyloid plaque load. Our in vitro data confirmed that disaggregation of the plaque measured by ThT assay occurred at various ratios of antibody and amyloid peptide. It is significant that even at a molar ratio of  $\beta$ -peptide/antibody 1:100 [12] there is a reduction of ThT fluorescence which corresponds to fibrillar aggregation, suggesting the dose-dependent effect of the antibody. It is not clear at the moment why two of the APP-transgenic mice that were immunized with the EFRH-phage did not develop a proper immune response or high titers.

Although additional studies are needed and are ongoing, this finding might become the basis of an effective index of the humoral immune response and indeed also of the actual effect on amyloid plaque load in brain.

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