# Immunization against Alzheimer's β-amyloid plaques via EFRH phage administration

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**The epitope EFRH, corresponding to amino acids 3–6 within the human β-amyloid peptide (AβP), acts as a regulatory site controlling both the formation and disaggregation process of the β-amyloid fibrils (Aβ). Locking of this epitope by highly specific antibodies affects the dynamics of the entire AβP molecule, preventing self-aggregation as well as enabling resolubilization of already formed aggregates. Production of such antibodies by repeated injections of toxic human Aβ fibrils into transgenic mice suggests the feasibility of vaccination against Alzheimer's disease. Here, we report the development of an immunization procedure for the production of effective anti-aggregating β-amyloid antibodies based on filamentous phages displaying the EFRH peptide as specific and nontoxic antigen. Effective autoimmune antibodies were obtained by EFRH phage administration in guinea pigs, which exhibit AβP identical to the human AβP region. Moreover, because of the high antigenicity of the phage, no adjuvant is required to obtain high affinity anti-aggregating IgG antibodies after a short immunization period of 3 weeks. Availability of such antibodies opens up possibilities for the development of an efficient and long-lasting vaccination for the prevention and treatment of Alzheimer's disease.**

Alzheimer's disease | β-amyloid | vaccine | EFRH phage | autoantibodies

Amyloid plaques are thought to be responsible for the devastating mental decline in patients with Alzheimer's disease (1). The core of these plaques contains a peptide (AβP) of 40 to 42 amino acids comprising a fragment of the transmembrane and of the extracellular domains of the amyloid precursor protein (2). *In vitro*, these peptides tend to form fibrils that are toxic to cultured neuronal cells and lead to their death (3, 4). The toxicity has been linked to the aggregation and conformational status of the AβP (5, 6). We have recently shown that site-directed mAbs toward the N-terminal region of the human β-amyloid peptide bind to preformed β-amyloid fibrils, leading to their disaggregation and inhibition of their neurotoxic effect (7). Moreover, such antibodies were found to prevent the formation of fibrillar β-amyloid (8). Using a phage–peptide library composed of filamentous phage displaying random combinatorial peptides, we previously defined the EFRH residues located at positions 3–6 of the N-terminal AβP as the epitope of anti-aggregating antibodies within AβP (9, 10). Locking of this epitope by antibodies modulates the dynamics of aggregation as well as resolubilization of already formed aggregates (7, 8). Immunization with the fibrillar Aβ of the mouse model of Alzheimer's disease led to inhibition of the formation of amyloid plaques and the associated dystrophic neurites in the mouse brain (11), raising the possibility of vaccination against Alzheimer's disease. However, because of the low immunogenicity of the Aβ fibrils, repeated antigen administration is required to obtain high levels of anti-AβP antibody necessary to affect plaque formation. Moreover, immunizing with toxic fibrils may induce more accumulation of the toxic amyloid itself (12). Here, we report the development of a novel immunization procedure for raising of effective anti-aggregating AβP antibodies, using as antigen the filamentous phages displaying EFRH peptide that was found to be the main regulatory AβP site for fibril formation instead of the toxic amyloid fibrils.

## Materials and Methods

**Epitope Libraries.** The bacteriophage vectors Fuse 5 and f88, as well as the 15-mer phage–peptide library used in this study were generously provided by G. Smith (University of Missouri, Columbia, MO).

**Isolation of Phage Presenting EFRH from Peptide Library.** A library sample containing $10^9$ phage particles was subjected to three rounds of biopanning and amplification, as previously described (10). The selected phages were tested for their ability to bind to the antibody by ELISA assays. Wells of microtiter plates (Maxisorb, Nunc) were coated with 50 μl (at a dilution of 1:1,000 in 0.1 M $NaHCO_3$, pH 8.6) of rabbit anti-phage serum and incubated overnight at 4°C, as described (10). Positive phage clones were propagated, and their DNA were sequenced in the insert region by using an Applied Biosystems kit.

**Large-Scale Production of Recombinant Phage.** The recombinant phage carrying YYEFRH epitope within protein III or VHEPHEFRHVALNPV (amino acid sequence) as fusion to its major coat protein VIII were chosen and produced in large quantities for immunization. For this purpose, a 2-ml overnight culture of the corresponding *Escherichia coli* K91Kan recombinant colony was grown at 37°C in 2YT medium containing 20 μg/ml tetracycline and 50 μg/ml kanamicin. One-hundred microliters of this preculture were used to subculture 1 liter of 2YT/tet containing 2 mM isopropyl-D-thiogalactoside. After 16 h of incubation at 37°C, the culture was centrifuged at 7,500 × *g* for 30 min, and the supernatant with infectious phages was precipitated at 4°C for 2 h by the addition of 0.15 volume of a solution containing 16.7% polyethylene glycol-8000 and 3.3 M NaCl. After centrifugation, the phage pellet was resuspended in 40 ml of PBS and centrifuged again for bacteria contamination release; the supernatant was then reprecipitated and resuspended in less than 8 ml of PBS. The amount of phages was estimated spectrophotometrically (One OD at 269 nm represents $10^{11}$ phage/ml).

**Immunization Protocols.** *Mice.* BALB/c mice (38 weeks old) were immunized three times via intranasal (i.n.) or i.p. injections with doses of $10^{10}$ or $10^{11}$ phages per injection at 14-day intervals. The mice were challenged with the phages with or without Freund's adjuvants (Difco). Seven days after each injection, the mice were bled and their sera tested by ELISA

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ThT, thioflavin T.

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for a specific IgG reactivity for both phage and AβP. UV inactivation of the phages before immunization was performed as previously described (13).

*Guinea pigs.* Ten guinea pigs (weight 300 g each) were immunized four times s.c. or i.p. injections with doses of $10^{11}$ phages in PBS per injection at 14-day intervals. The guinea pigs were challenged with the phages without adjuvant. Seven days after the third and the fourth injections, the animals were bled and their sera tested by ELISA for both anti-phage and auto AβP immunoreactivity.

**Binding of Polyclonal Antibodies to Phages and β-Amyloid Peptide.** The specificity of the anti-phages and anti-EFRH polyclonal antibodies was analyzed by ELISA. Wells of microtiter plates (Maxisorb, Nunc) were coated with 50 μl of rabbit antiphage at 1:1,000 dilution (in 0.1 M NaHCO3, pH 8.6). Coated plates were washed three times with PBS/0.05% Tween 20 and 50 μl of enriched phage clones containing $10^9$ wild-type filamentous phages particles, which were then added to the wells and incubated for 1 h at 37°C. Wells were blocked with a mixture of 3% powdered milk in PBS for 2 h at 37°C, washed, and incubated with the serum antibody of different dilutions in 1% powdered milk in PBS for 2 h at 37°C. In experiments of antibody binding to β-amyloid peptide 1–16, 100 ng/well biotinylated β-amyloid peptides 1–16 were bound to streptavidin-coated microtiter plates for ½ h at room temperature. ELISA plates were previously coated with streptavidin for 16 h at 4°C. After washing, bound antibodies to phage and/or β-peptide were detected by incubation with horseradish peroxidase-conjugated antibodies, as previously described (10).

**Competitive Inhibition of Sera Binding to β-Amyloid Peptide by Various N-Terminal AβP Peptides.** The inhibition of sera binding to (1–16) AβP by various small peptides derived from the N-terminal of AβP was performed using 250 ng/well biotinylated β-amyloid peptides (1–16) bound to ELISA plates, as previously described. The various peptides derived from the N-terminal, as well as the whole AβP, were preincubated with sera (third or fourth immunization with f88-EFRH) for 30 min at 37°C before their addition to AβP-coated wells and left overnight at 4°C. After washing, bound antibody was detected by incubation with horseradish peroxidase-conjugated antibody as described above. The results were measured in $IC_{50}$, which is half the molar concentration of peptide that fully inhibits antibody binding. Peptides were synthesized by Applied Biosystems Synergy model 430A in the Unit for Chemical Services of The Weizmann Institute of Science (Israel) by solid-phase using fluorenylmethoxycarbonyl chemistry.

**Cell Culture and Cytotoxicity Assay.** Cultured PC12 cells were seeded into a 96-well plate at a density of $10^4$ cells/100 μl per well in a serum-free medium supplemented with 2 μM insulin. The prevention of Aβ neurotoxicity was measured as follows: 0.12 μM β-amyloid peptide, incubated for a week at 37°C to produce fibrils, was incubated with the third bleed sera of EFRH phage immunized animals and with sera of nonrelevant phage immunized animals at dilutions of 5:1 and 20:1 for 24 h. The reaction mixtures were added to the wells containing PC12 cells. The plates were incubated at 37°C for 2 days, after which cell viability was assessed by measuring cellular redox activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), as described (8).

**Thioflavin T Fluorimetry.** Aggregation of β-amyloid peptide AβP (1–40) was measured by the thioflavin T (ThT) binding assay, in which the fluorescence intensity reflects the degree of β-amyloid fibrillar aggregation (14). Aqueous solutions of 0.12 mM AβP (in 0.1 M Tris·HCl, pH 7.1) were incubated for a week at 37°C and reincubated in the presence of the third bleed serum raised against EFRH phage and the serum of irrelevant phage immunized animal at dilutions of 5:1 and 20:1 for 24 h. The fluorescence was measured after addition of 1 ml of ThT (2 μM in 50 mM glycine, pH 9) by LSB-50 Perkin–Elmer spectrofluorimeter.

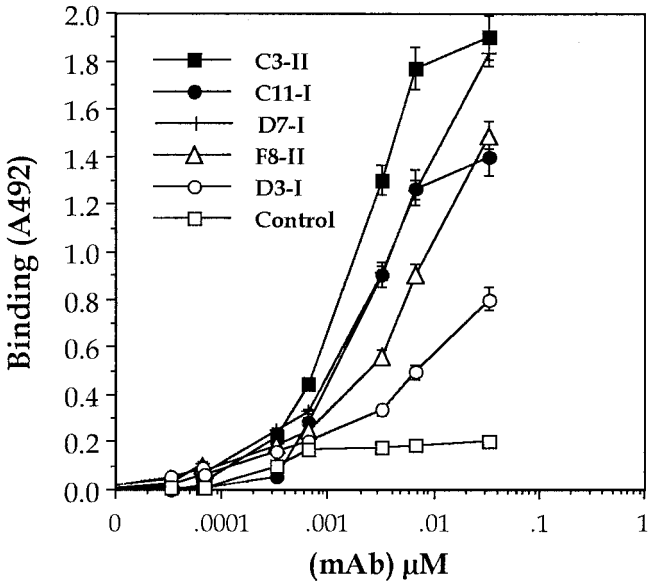


**Fig. 1.** Binding of the anti-aggregating mAb to ERFH-phage. Antibodies were added to the phage-coated wells, and binding was analyzed by ELISA, as described. △, KEPRHHIQHHERVIR (F8-II); ■, VHEPHEFRHVALNPV (C3-II); +, DTEFRHSSNNFSAVR (D7-I); ●, STEFRHQTTPLHPNS (C11-I); ○, SAADFRHGSPPISAF (D3-I); □, wild filamentous phage used as control.

## Results

**Raising AβP Anti-Aggregating Antibodies Through Immunization with EFRH Phage.** To select the phages containing EFRH peptide epitope, we screened the phage–epitope library with biotinylated antibody (9). After three cycles of panning and phage amplification, ELISA analysis revealed that of the phage clones, most (above 90%) bound specifically to the antibody. Six positive phage clones were propagated, and their DNA were sequenced in the insert region. The sequence EFRH appeared in four clones, and one additional clone had sequence EPRH with one residue substitution of phenylalanine with proline. In one additional clone, the inserted peptide bears the sequence of the three residues FRH but not of the glutamate residue. The half-maximal binding obtained from each selected phage was about $10^{-9}$ M (Fig. 1) which resembled the level of binding of these antibodies to the whole AβP. One specific f88-EFRH phage (C3-II) showed higher affinity compared with the others (Fig. 1), due to many copies of EFRH epitope on the phage surface.

Mice were immunized with genetically engineered filamentous phage C3-II through i.p. immunization (Fig. 2*A*). Seven days after each injection of $10^{10}$ phages displaying the epitope, the mice were bled and their sera tested by ELISA for antibody IgG reactivity against wild-type (wt) phage (not bearing the peptide EFRH on its surface) and against AβP. Highly specific antibodies (IgG) against the AβP were received after the first injection, and the titers increased with the second and the third injections to 1:50,000 (Fig. 2*B*). Moreover, we found that specific IgG and IgA antibodies against AβP anti-aggregation epitope EFRH were obtained 7 days after only one boost of $10^{11}$ phages via intranasal administration (Fig. 2*C*), offering new approaches for

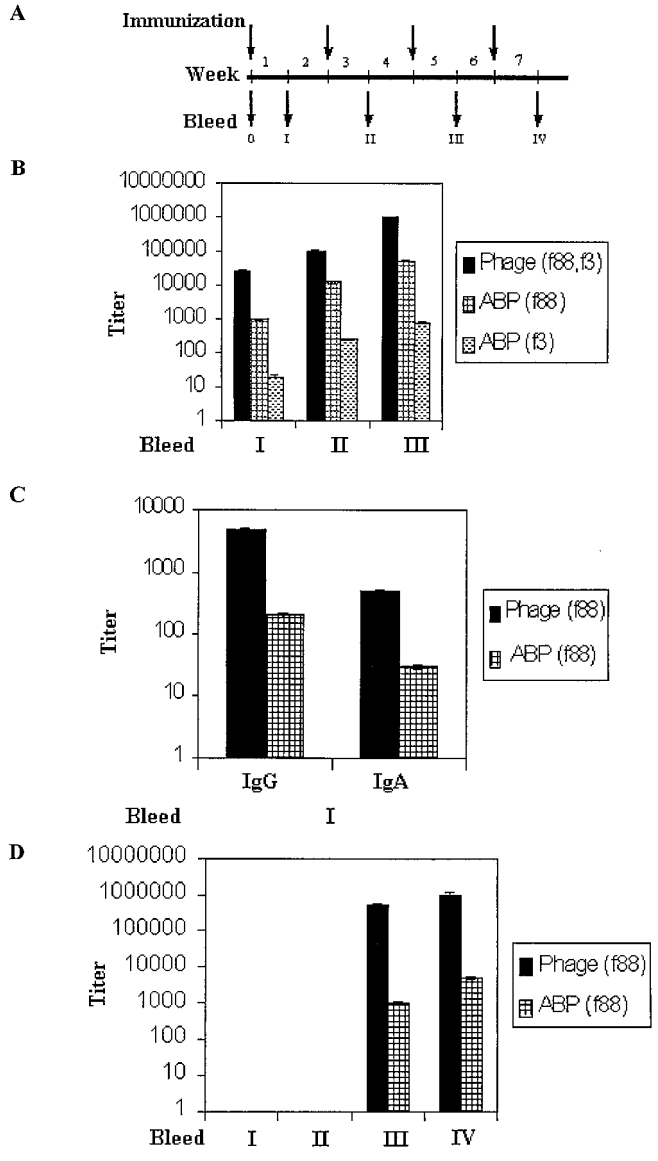


**Fig. 2.** Immunization with f88 or f3 filamentous phage displaying EFRH epitope. (*A*) Diagram of immunization schedule. (*B*) Serum IgG titer of different bleeds from mice immunized with f3-EFRH or f88-EFRH phage against wild phage coat proteins and the N terminus of β-amyloid peptide (1–16 amino acid) after three i.p. administrations or (*C*) after single i.n. administration. (*D*) Serum IgG titer of guinea pigs from animals s.c. immunized with EFRH phage was measured after the third and the fourth administrations.

simple and fast immunization. Similar results were obtained after immunization with $10^{11}$ phages per injection without the adjuvant with an additional administration of antigen (data not shown). For comparison, mice were immunized with filamentous phages carrying the peptide YYEFRH (amino acid sequence) fused to its minor coat peptide pIII (Fig. 2*B*). After the third injection, the low titer against AβP (1:750) may have been due to the low number of the insert copies of the gpIII on the phage envelope.

The ability of the phage display system to induce auto anti-AβP antibodies was demonstrated using guinea pigs. Whereas in mice there is a switch of one amino acid (glycine instead of arginine in position 5-EFGH), in guinea pigs the amino acids are identical to the human sequence. Doses of $10^{11}$ phages per injection were used to immunize s.c. at 14-day intervals. The

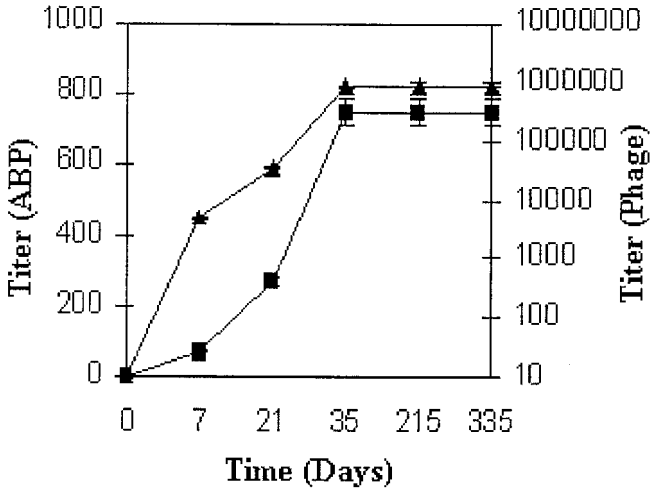


**Fig. 3.** Long-lasting immunization with f3-EFRH filamentous phage. Serum IgG titer of different bleeds from mice immunized against phage coat proteins and the EFRH epitope on the N terminus of AβP. ■, sera against AβP 1–16; sera against wild filamentous phage.

higher sera titer against AβP of 1:5000 was measured after the fourth s.c. injection (Fig. 2*D*).

Immunization through phage carrying epitope was found to be long-lasting (Fig. 3). No toxic effect due to autoimmune response against the self-epitope EFRH was detected in the histology test of different organs (including the brain) from the challenged animals following 3 months after the last administration (data not shown).

**Immune Specificity of the EFRH Phage Anti-Sera Toward Whole AβP.** The specificity of sera raised against f88-EFRH toward AβP was assayed by competitive inhibition experiments with small peptides derived from AβP: EFRH, DAEFRH, DAEFRHD, DAEFRHDSG, and AβP itself as shown in Table 1. All of the synthetic peptides that bear the motive EFRH inhibited binding of antibodies to the AβP, with $IC_{50}$ values of about $5 \times 10^{-6}$ M, similar to the antibody raised against the Aβ peptide 1–28. These data indicate that mouse and guinea pig antibodies recognized the same epitope, composed of four amino acid residues corresponding to positions 3–6 in the AβP, similar to the anti-aggregating antibodies previously investigated (9).

**Prevention of the β-Amyloid Neurotoxic Effect by Serum Antibody Raised Against EFRH Phage.** Serum raised against EFRH peptide exhibits a protective effect in preventing Aβ-mediated neurotoxicity toward pheochromocytoma PC12 cells. Diluted serum (1:5) prevented the neurotoxicity of Aβ (80% cell viability),

**Table 1. Competitive inhibition of sera binding to the AβP by peptides derived from its N terminus of animals immunized with f88-EFRH compared to amyloid anti-aggregating antibody, Frenkel *et al.* (9)**

| Peptide | Sera M | mAb 10D5 M |
|---|---|---|
| FRH (4–6) | $\sim 10^{-3}$ | $3 \times 10^{-3}$ |
| **EFRH** (3–6) | $6.0 \times 10^{-6}$ | $3 \times 10^{-6}$ |
| DA**EFRH** (1–6) | $3.0 \times 10^{-6}$ | $8 \times 10^{-7}$ |
| DA**EFRH**D (1–7) | $5.0 \times 10^{-6}$ | $9 \times 10^{-7}$ |
| DA**EFRH**DSG (1–9) | $5.0 \times 10^{-6}$ | $1 \times 10^{-6}$ |
| AβP (1–40) | $3.0 \times 10^{-6}$ | $8 \times 10^{-7}$ |
| WVLD | ND* | ND* |

*$IC_{50}$ value of less than $10^{-2}$ M, which cannot be detected by ELISA assay.

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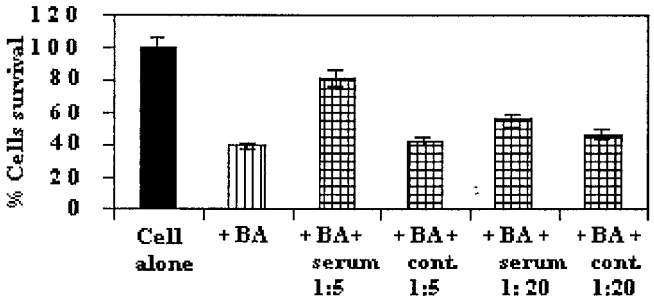


**Fig. 4.** Prevention of AβP-mediated toxic effect on PC12 cells by sera against f88-EFRH phage. Cells were incubated with fibrillar Aβ alone, or with fibrillar Aβ that had been incubated with serum from the third bleeding at different concentrations. Serum of unimmunized animal was used as negative control. The MTT assay was used to estimate cell survival.

whereas an unrelated serum showed no effect (Fig. 4) using the MTT test, as previously described (8).

**Disaggregation of β-Amyloid Fibril by the Sera Raised Against EFRH Phages.** To examine the effect of the serum raised against EFRH on disruption of the toxic Aβ fibril, we used the ThT reagent that binds specifically to fibrillar amyloid structures (13). Aliquots of AβP samples were incubated for a week at 37°C and then incubated with sera antibodies (Fig. 5). Sera raised against EFRH peptide at dilutions of 1:5 and 1:20 disrupted the fibril structure of β-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.

## Discussion

The display of short immunogenic determinants fused to a phage surface provides the basis for the development of a novel peptide vaccine against β-amyloid plaque. As recently reported, filamentous bacteriophages are excellent vehicles for the expression and presentation of foreign peptides in a variety of biological systems (15–17). Administration of filamentous phages induces a strong immunological response to the phage proteins in all animals tested, without any evidence of toxic effects (15–17). Phage proteins pIII and pVIII are proteins that have been often used for phage display. The recombinant filamentous phage approach for obtaining specific peptide antigens has a major advantage over chemical synthesis, as the products obtained are the result of the biological fidelity of translational machinery and are not subject to the 70–94% purity levels common in the solid-phase synthesis of peptides. The phage presents an easily renewable source of antigen, as additional material can be obtained by growth of bacterial cultures. We present here the use of genetically engineered filamentous phages as a means of obtaining both the antigen and the carrier for antibody production without the use of adjuvant. We have previously shown that the EFRH residues located at positions 3–6 of the N terminus of AβP represent the epitope of anti-aggregating mAbs within AβP. Moreover, the interaction of this epitope with such specific antibodies may interfere with pathological effects in the central nervous system, such as inflammatory events and other pathogenic mechanisms in Alzheimer's disease (18).

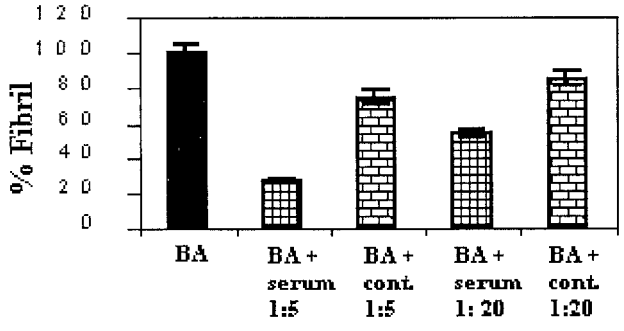


**Fig. 5.** Interference of immunized sera with fibrillar β-amyloid formation. Fluorescence of ThT correlates with the amount of fibrillar β-amyloid formed after reincubation of Aβ fibril with sera from immunized animal. As control, serum of unimmunized animal was added in the same experimental conditions.

Immunization with the EFRH phage may, in a short period (a few weeks), raise the high concentration of high affinity (IgG) anti-aggregation antibodies to prevent the formation of plaques and to minimize further toxic effects. The level of antibody in the sera was found to be related to the number of peptide copies per phage. Although pIII is present in four copies, pVIII is found in 2,700 copies, conferring more immunogenicity to the f88 vector (15).

The antibodies resulting from EFRH phage immunization are similar, in their anti-aggregating properties, to antibodies raised by direct injection with fibrillar toxic β-amyloid. Such antibodies are able to sequester the peripheral AβP, thus avoiding the passage through the blood–brain barrier and, as recently shown in a transgenic mouse model, to cross the blood–brain barrier and to dissolve already formed β-amyloid plaques (11).

The high immunogenicity of filamentous phages enables the raising of antibodies against self-peptides or antigens. Immunization of guinea pigs with EFRH phage as an antigen, in which the AβP sequence is identical to that in humans, resulted in the production of such antibodies. These antibodies recognize the full-length β-peptide 1–40 and exhibit anti-aggregating properties as antibodies raised against whole peptides and/or β-amyloid (7, 8).

The above data demonstrated that a recombinant bacteriophage displaying a self-epitope can be used as a vaccine to induce autoantibodies for disease treatment. Filamentous phages are normally grown by using a laboratory strain of *E. coli*, and although the naturally occurring strain may be different, it is reasonable to assume that delivery of phage into the gut will result in infection of the natural intestinal flora. We have found that UV-inactivated phages are as immunogenic as their infective counterparts. There is evidence of long-lasting filamentous phages in the guts of the immunized animals, which may explain the long-lasting immune response found in the pIII immunized mice (19).

Due to the high antigenicity of the phage, administration can be given by the intranasal route, which is the easiest way for immunization without any use of adjuvant. Intranasal administration seems to be an effective route for mice immunization in terms of timing and reproducibility of response (13). As olfactory changes are proposed to play a role in Alzheimer's disease (20), the advantages of mucosal immunization are both on the effective induction of specific AβP IgA antibodies in the saliva for preventing pathologic effect of the disease and on the simplicity of antigen administration.

Identification of an antiaggregating epitope displaced on recombinant phage surface may replace the use of whole antigen for immunization in the treatment of diseases acquired by abnormal conformational changes of a self-protein, such as Alzheimer's disease, prion diseases, and other amyloidoses diseases.

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1. Vines, G. (1993) *Trends Biotechnol.* **11,** 49–55.
2. Ashall, F. & Goate, A. M. (1994) *Trends Biochem. Sci.* **19,** 42–46.
3. Sherman, S. M. & Iversen, L. L. (1995) *J. Biochem. (Tokyo)* **311,** 1–16.
4. Cruz, L., Urbanc, B., Buldyrev, S. V., Christie, R., Gomez-Isla, T., Havlin, S., McNamara, M., Stanley, H. E. & Hyman, B. T. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 7612–7616.
5. Kirshenbaum, K. & Daggett, V. (1995) *Biochemistry* **34,** 7640–7647.
6. Lee, J. P., Stimson, E. R., Ghilardi, J. R., Mantyh, P. W., Lu, Y.-A., Felix, A. M., Llanos, W., Behbin, A., Cummings, M., Van Criekings, M., *et al*. (1995) *Biochemistry* **34,** 5191–5200.
7. Solomon, B., Koppel, R., Frenkel, D. & Hanan-Aharon, E. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4109–4112.
8. Solomon, B., Koppel, R., Hanan, E. & Katzav, T. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 452–455.
9. Frenkel, D., Balass, M. & Solomon, B. (1998) *J. Neuroimmunol.* **88,** 85–90.
10. Frenkel, D., Balass, M., Katchalski-Katzir, E. & Solomon, B. (1999) *J. Neuroimmunol.* **95,** 136–142.
11. Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., *et al.* (1999) *Nature (London)* **400,** 173–174.
12. Araujo, D. M. & Cotman, C. W. (1992) *Brain Res.* **569,** 141–145.
13. Galfre, G., Delmastro, P., Meola, A., Monaci, P. & Cortese, R. (1997) *Vaccine* **15,** 1276–1285.
14. Levine, H., III. (1993) *Protein Sci.* **2,** 404–410.
15. Willis, E. A., Perham, N. R. & Wraaith, D. (1993) *Gene* **128,** 79–83.
16. Meola, A., Delmastro, P., Monaci, P., Luzzago, A., Nicosia, A., Felici, F., Cortese R. & Galfre, G. (1995) *J. Immunol.* **154,** 3162–3172.
17. Bastein, N., Trudel, M. & Simard, C. (1997) *Virology* **234,** 118–122.
18. McGeer, P. L., Rogers, J. & McGeer, E. G. (1994) *Alzheimer's Dis. Assoc. Disord.* **8,** 149–158.
19. Zuercher, A. W., Miescher, S. M., Vogel, M., Rudolf, M. P., Stadler, M. B. & Stadler, B. M. (2000) *Eur. J. Immunol.* **30,** 128–135.
20. Murphy, C. (1999) *Physiol. Behav.* **66,** 177–182.