Filamentous phage as vector-mediated antibody delivery to the brain

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Communicated by Ephraim Katchalski-Katzir, Weizmann Institute of Science, Rehobot, Israel, January 15, 2002 (received for review July 27, 2001)

Early diagnosis of Alzheimer's disease is prevented by lack of means to visualize and target β amyloid plaques in the brains of affected people. There are many methods of detecting amyloid plaques by staining postmortem brain tissue, but none are available for monitoring in living patients. We propose anti-β amyloid antibodies as a highly specific probe to monitor amyloid plaque formation in living patients. Intranasal administration of filamentous phage as delivery vector of anti-β amyloid antibody fragment into Alzheimer's APP transgenic mice enables *in vivo* targeting of β amyloid plaques. The plaques were covisualized both by thioflavin-S and fluorescent-labeled antiphage antibodies in the olfactory bulb and the hippocampus region. The genetically engineered filamentous bacteriophage proved to be an efficient and nontoxic viral delivery vector to the brain, offering an obvious advantage over other mammalian vectors. The ability to image Aβ deposits *in vivo* would arguably provide the most useful diagnostic and monitoring test for early diagnosis of Alzheimer's disease.

The amyloid cascade hypothesis proposes that the onset and progression of Alzheimer's disease (AD) result from increased production of β amyloid peptides (AβP) and their progressive accumulation in senile plaques (reviewed in ref. 1). The clinical trials of novel techniques to decrease the burden of amyloid plaques (Aβ) (2, 3) required reliable and sensitive methods to monitor plaque burden in the brains of living AD patients. Accurate targeting of Aβ in brain regions could allow the preventative treatment and monitoring of drug efficacy during and after those trials.

There are different methods to detect plaques by staining postmortem brain tissue; however, none are yet available for monitoring living patients. Attempts to develop such probes have focused on Chrysamine G (CG), a derivative of Congo red that also binds Aβ *in vitro* and could be a potential probe for detecting plaques *in vivo* because it crosses the blood–brain barrier (BBB) of mice (4). Styrylbenzene derivative (BSB) is another probe recently designed to penetrate the BBB (5) and bind brain Aβ deposits, enabling radiological imaging of plaques in the brains of living animals. However, as aggregated fibrils with a β pleated structure are a common neuropathological feature of several neurodegenerative diseases, amyloid-binding probes such as BSB or CG are not specific only for AD. Injection by Wengenack *et al.* (6) of ^{125}I-PUT-Aβ 1–40 into the femoral veins of 6-mo-old transgenic mice resulted in *in vivo* labeling of some neuritic plaques.

Here we propose AβP antibodies displayed on filamentous bacteriophage as a highly specific probe to scan brain Aβ. The phage maintains the inert properties of the delivery vector and the ability to carry and preserve the biological activity of the antibodies. We demonstrated the usefulness of this Aβ-specific antibody for *in vivo* targeting of β amyloid deposition in live transgenic mice. Two complementary model systems are used to show how phages penetrate specific animal brain regions in a concentration-dependent manner, and how phages carrying such antibodies effectively label Aβ plaques *in vitro*.

Materials and Methods

Filamentous Phage. The bacteriophage vector, f88, used in this study was provided by G. Smith (University of Missouri, Columbia, MO), and the vector, which displays the single-chain antibody (scFv), was developed as described (7). Negatively stained filamentous phages were prepared by floating carbon-coated grids on aqueous phage solutions (10^{11} particles) and were air-dried. The untreated phage and the 2-min chloroform-treated phages (8) were negatively stained with aqueous solution of 2% uranyl acetate and then visualized by a JEOL model 1,200 EX electron microscope operated at 80 kV with a magnification of ×50,000. The correlation between the high permeability of the filamentous phage and its linear structure was measured after transfer of the phage through three lower orders of molecular mass cutoff membrane of 3 kDa (filamentous phage molecular mass is about several thousand kilodaltons). The integrity of the phages after chloroform treatment was measured by ELISA assays with polyclonal antibodies raised against coat proteins of the phages. 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 antiphage serum and incubated overnight at 4°C. The coated plates were washed three times with PBS/0.05% Tween 20, and 50 μl of 10^{10} phage particles was added to the wells and incubated for 1 h at 37°C. Wells were blocked with 3% milk in PBS for 2 h at 37°C, washed, and incubated with (1 μg/ml) antibody against phage envelope (this antibody recognizes spheroid phage as well as filamentous phage) for 1 h at 37°C. After washing, bound antibody was detected with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig (Sigma), diluted 1:5,000, for 1 h at room temperature. The bound antibody was monitored by the enzymatic activity of HRP, and the color developed was determined by microtiter plate reader at 495 nm.

Preparation of scFv. scFv was constructed from variable regions of heavy and light genes of the parental anti-AβP IgM 508 antibody. To clone the antibody 508 as a scFv in a phage display vector, mRNA was extracted from 10^8 hybridoma cells and was used as a source for antibody variable regions coding sequences. Reverse transcription–PCR was used to amplify the variable domains that were cloned into the phage display vector pCC-Gal6(Fv) (7).

Animals. The administration of the filamentous phage was performed both in 8-wk-old BALB/C mice and in transgenic mice containing a double mutation in the APP gene. The transgenic mice line contains both the APP London (APP[V717I]) and Swedish (K670M/N671L) mutations, marked as hAPP751 and generated at the laboratory of Eliezer Masliah (Department of Neurosciences and Pathology, University of California, San Diego, CA) as previously described (9). Under the regulatory control of the murine (m)Thy-1 gene, the mice show high levels of human APP expression in neurons in the neocortex, hip-

Abbreviations: AD, Alzheimer's disease; AβP, β amyloid peptides; Aβ, β amyloid plaques; scFv, single-chain antibody; ThS, thioflavin-S.

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pocampal, and olfactory bulb regions. At the age of 5–7 mo, those lines showed Aβ formation extending to the hippocampus, thalamus, and olfactory regions.

Phage Administration Protocols. *Mice.* Thirty BALB/C female mice were used in this experiment and were divided into three groups of 10 mice as follows: one group was immunized with filamentous phage, one with chloroform-treated phage, and the last group was kept as control. After single or three daily administrations of 10^{11} phage particles via intranasal instillation, the mice were killed and their brains taken for investigation.

Transgenic mice. Two hAPP751 transgenic mice (age 10 mo) were given 10^{11} filamentous phage carrying the 508F-scFv via intranasal administration. The mice received a total of three daily administrations. After 3 days, the mice were killed and their brains quickly perfused with PBS and taken for further investigation.

Detection of Filamentous Phage in Mice Brain Sections. Immediately after decapitation, brains were removed and cut in halves along the midsagittal sinus. Randomly, one brain half was fixed by immersion in 4% paraformaldehyde solution in 0.1 M phosphate buffer for 2 hr in 4°C and then immersed for cold protection in 4.5% sucrose in 0.1 M PBS overnight. The section was then moved to 30% sucrose for 2 h at 4°C. Sections of coronal blocks of the olfactory bulb and hippocampus were cut at thicknesses of 4 μm with a cryostat at −20°C and then applied to glass slides. These slides were used for phage detection by using an immunofluorescence technique. Slide sections blocked with 3% BSA in PBS for 30 min were incubated with rabbit polyclonal serum anti-M13 (1:100) for 1 h at 37°C. The cover slips were then washed three times for 5 min each in PBS, treated a second time with blocking buffer for 5 min at room temperature, and then reacted with secondary Cy 3 donkey anti-rabbit IgG.

***In Vitro* Detection of β-Aβ Using Anti-β-Amyloid scFv Displayed on Phage.** Brain sections from AD patients (Buxton Laboratories, Liverpool, U.K.) and from hAPP751 transgenic mice were fixed in 4% paraformaldehyde for 2 h and then kept in 10% formalin saline for 2 days at room temperature (RT). After embedding in paraffin, thin sections (4 μm) were cut, collected on glass slides, blocked with 3% milk in PBS for 30 min, and rabbit polyclonal serum anti-M13 (1:100) was added for an additional hour at 37°C. The sections were washed three times for 5 min each in PBS and again treated with blocking buffer (5 min, RT) before reaction with the secondary antibody (Cy 3-labeled goat anti-rabbit IgG, 1:500, 1 h, RT). Finally, the brain sections were washed three times in PBS and observed by confocal fluorescence microscopy.

***In Vivo* Targeting of Aβ Deposits in Transgenic Mice Using Phage-ScFv.** Ability of anti-β amyloid scFv displayed on phage to target Aβ *in vivo* was demonstrated as follows: 10^{11} particles of filamentous phage carrying the 508F-scFv were intranasal administrated to two hAPP751 transgenic mice (age 10 mo). Detection of the Aβ in the transgenic mice brain was performed both with thioflavin-S (ThS) staining and antiphage antibodies. Two well defined coronal sections at olfactory bulb and the hippocampus region were selected for visualizing amyloid in plaques. After staining with ThS, the slides were blocked with 3% milk in PBS for 30 min, then incubated with rabbit polyclonal serum as described above. Finally, the sections were washed three times in PBS and observed on a fluorescence microscope at a final magnification of ×10 or with a confocal fluorescence microscope at a final magnification of ×66. Staining with the ThS is represented by the yellow color, whereas staining with anti-β amyloid antibody is represented by red.

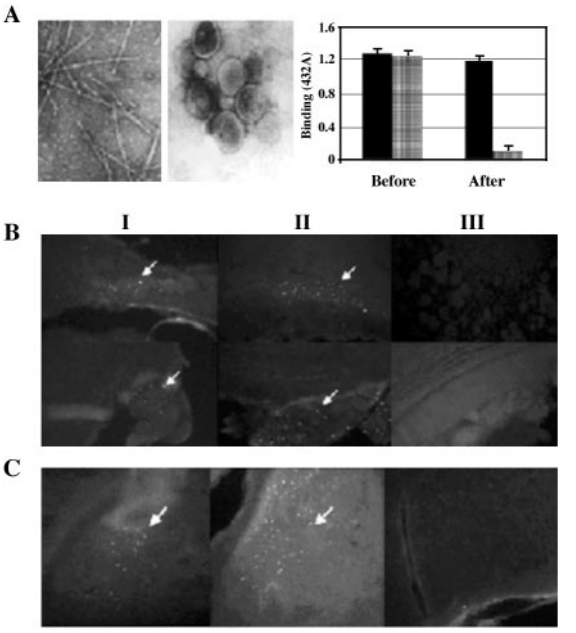

Fig. 1. Immunofluorescence detection of filamentous phage in BALB/C brain sections. (*A*) Electron micrographs of wild filamentous phage (*Left*) and spheroid phage after 2-min chloroform treatment (*Right*) were visualized by using a JEOL model 1200 EX electron microscope operated at 80 kV with a magnification of ×50,000. Their penetration properties through synthetic membrane with 3-kD cutoff were measured by ELISA assay as shown by immunological recognition of the wild phage (black) and chloroform treated phage (gray) before filtration (*Left*). Detection of the phages in filtrate is shown on the right. The presence of linear filamentous phage in mice olfactory bulb sections (*B*) and hippocampus region (*C*) were detected after one administration (I) and after three daily administrations (II) compared with administration with spheroid phage (III). The sections were observed on a fluorescence microscope at a final magnification of ×20.

Histological Test of Immunized Mice. The midsagittal brain half was used for preparing paraffin tissue sections for histology. The sections were fixed in 4% paraformaldehyde for 2 h followed by 10% formalin saline immersion for 2 days at room temperature and embedded in paraffin. Cuts of 4 μm were applied on glass slides. The slides were kept at room temperature until use.

The brain sections, prepared as mentioned before, were stained with hematoxylin and eosin. The stained sections were examined and photographed at a final magnification of ×20.

Results

Penetration of the Linear Filamentous Phage to Different Brain Regions of BALB/C Mice. Electron microscopy of negatively stained wild-type phage confirmed their linear structure (Fig. 1*A Left*). After 2 min of chloroform treatment, phage formed a spheroid structure (Fig. 1*A Right*). The correlation between the high permeability of the filamentous phage and its linear structure was measured after transfer of the phage through three lower orders of molecular mass cutoff membrane of 3 kDa (filamentous phage molecular mass is about several thousand kilodaltons). Filtration experiment showed slight reactivity of antiphage in filtrate of chloroform-treated phage, but the majority of the phages were detected on the membrane, despite the fact that removed proteins might penetrate the membrane (Fig. 1*A*).

To investigate the ability of the filamentous phage to enter the central nervous system, BALB/C female mice were challenged with single doses of 10^{11} filamentous phage particles via the intranasal route (of the 10 mice immunized with wild-type phage, five received a single dose and the other five three daily doses. Another 10 mice were immunized with spheroid phages

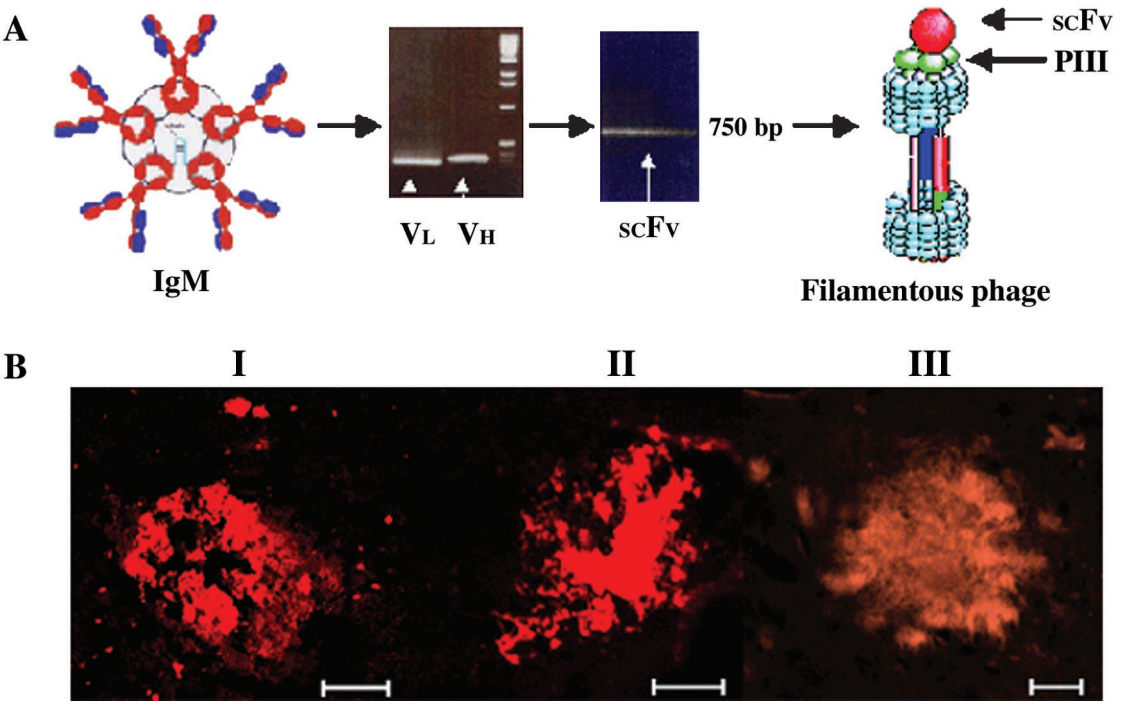

Fig. 2. Immunofluorescence detection of Aβ by scFv display on filamentous phage *in vitro*. (*A*) Scheme of preparation of a scFv DNA from variable regions of heavy and light genes of the parental anti-β amyloid IgM 508 antibody. The scFv DNA was encoded on the surface of protein III of the mature filamentous phage. (*B*) The presence of Aβ in the hippocampus sections of both APP transgenic mice (I) and human AD patients (II) were detected *in vitro* by using anti-β amyloid scFv displayed on mature filamentous phages and were compared with typical Congo red staining of β amyloid plaque (III). The sections were observed on a confocal microscope. (Bar = 5 μm.)

in the same distribution). We detected the presence of the filamentous phage both in the olfactory bulb and in the hippocampus region after administration of the wild-type phage (Fig. 1 *B* I, II, and *C* I, II, respectively). The amount of phage penetration depends on the number of intranasal administrations of the phage. We do not know yet whether the presence of phage in the hippocampus, but not in the cortex, is because of the small amount introduced under the experimental conditions used, or whether it is because of the olfactory tract in which the phages traveled from the nasal region to certain regions of the brain, like the hippocampus. No sign of the phage was found in the same regions of mice brains immunized with spheroid phage even after three daily doses (Fig. 1 *B* III and *C* III). We repeated the phage detection procedure in the brains of the five mice that received three daily doses of linear phage and killed them 28 days after the last administration. No phages were detected under the same experimental conditions in the specific brain regions investigated, compared with the brains of unimmunized mice.

Labeling of AD Plaques in Human and Transgenic Mice Brains. The ability of filamentous phages to display and stabilize therapeutic molecules such as scFv on their surface was examined by using an anti-β amyloid antibody fragment scFv-508F fused to a filamentous minor coat peptide pIII (Fig. 2*A*). The scFv phage specifically targeted Aβ from the hippocampus sections of both human Alzheimer's patients and old APP transgenic mice brains (Fig. 2*B* I and II, respectively). The comparison to Congo red-stained plaques from the same region confirmed the high specificity of the scFv phage for targeting the Aβ (Fig. 2*B* III).

Targeting of Alzheimer's Aβ with Phage Displaying ScFv. Two hAPP751 transgenic mice (age 10 mo) were given 10^{11} filamentous phage carrying the 508F-scFv via intranasal administration. The mice were exposed to three daily administrations of phages.

Fig. 3 shows a typical section out of several others scanned with the fluorescent confocal microscope. We found that the plaques were specifically decorated by anti-β amyloid antibody displayed as scFv on the phages in both the olfactory bulb (Fig. 3*A*) and hippocampus region (Fig. 3*C*). The individual plaques from the olfactory (Fig. 3*B*) and hippocampus (Fig. 3*D*) regions were visualized with scFv anti-β antibodies (column b) and ThS staining (column a) and covisualized with both probes (column c). As control, we used antibody 3F4 raised against region 1–16 of the AβP, which typically stained the plaques instead of scFv phage antibodies (first lines of Fig. 3 *B* and *D*, respectively).

Toxicity Studies. The filamentous phage seems to be an inert and specific carrier when presented with anti-β amyloid scFv, as shown in histology studies (Fig. 4). No visible toxic effects, because of phage administration, were detected in the brain by histology studies. The life span of more than 1 year, as well as the behavior of the challenged animals compared with untreated ones, suggests that phage administration is not harmful to the animals.

Discussion

There are no specific neuroimaging tests for AD to visualize Aβ plaques *in vivo*. In most cases, AD diagnosis depends on clinical signs in the late stages of the disease (10). The most important routinely used ancillary tests are computerized axial tomography and MRI scans. Brains of AD patients show global atrophy with enlarged ventricles and widened sulci. Fine-section coronal MRI of the medial temporal lobes reveals a disproportionate atrophy of the hippocampi. However, early in the disease the changes do not exceed those found in many mentally intact elderly people, and therefore one cannot rely on imaging procedures alone for diagnosis. Recent postmortem studies indicate that rises in the total quantity of Aβ in the hippocampus and several cortical regions correlate with the initial appearance of clinical impairment (11).

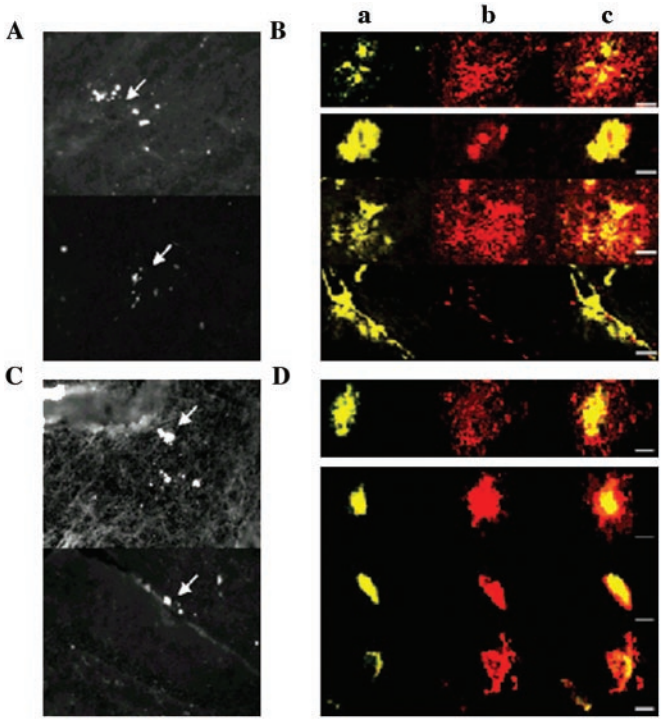

Fig. 3. Immunofluorescence detection of Aβ by scFv display on filamentous phage. The presence of filamentous phages in the olfactory bulb sections of APP transgenic mice (*A*) and in the hippocampus sections (*C*) were detected with anti-phage antibody staining. Typical brain sections were observed on a fluorescence microscope at a final magnification of $\times$20. The individual plaques from the olfactory (*B*) and hippocampus (*D*) regions were visualized with scFv anti-β antibodies (column b) and ThS staining (column a) and covisualized with both probes (column c). As control, we used antibody 3F4 raised against region 1–16 of the AβP, which typically stained the plaques instead of scFv phage antibodies (first lines of *B* and *D*, respectively).

Accurate targeting of Aβ plaques in these regions could allow for the selection of subjects for preventative treatment and the monitoring of drug efficacy during and after those trials.

Here we demonstrate the feasibility of a novel delivery approach of antibodies raised against the AβP displayed on filamentous bacteriophage to the central nervous system via intranasal administration, to image Aβ.

To demonstrate the ability of the anti-β amyloid scFv-phage to decorate Aβ *in vivo*, APP transgenic mice were exposed to intranasal administration of the filamentous phage with an anti-β amyloid antibody fragment. Aβ were visualized both by ThS and fluorescent labeled antiphage antibodies (Fig. 3). After intranasal phage administration of scFv in these mice, β amyloid brain plaques were specifically labeled in the two specific brain sections (olfactory and hippocampus) where most of the early Aβ are located (11). The stained plaques show high sensitivity, similar to those from human AD patients and transgenic mice detected *in vitro* by immunofluorescent techniques (Fig. 2). The filamentous phage maintains the biological activity of displayed foreign molecule of scFv 508F and efficiently penetrates biological membranes. After phage immunization via i.p. administration, no evidence of the phage was found in those specific brain regions, strongly indicating that the olfactory neuron route may target the plaques to specific regions.

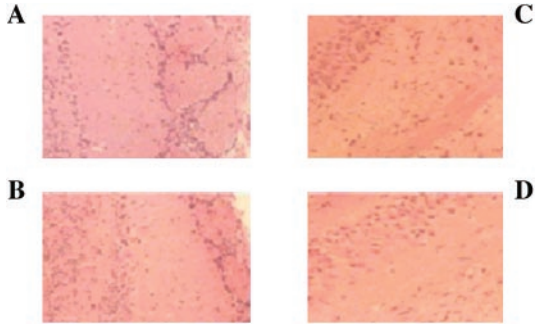

Fig. 4. Histology of brain sections after scFv-phage administrations. Brains slides of olfactory section (*A*) and hippocampus sections (*C*) after 3 days after phage administration were stained with hematoxylin and eosin, and compared with untreated mice brain sections (*B* and *D*, respectively). The stained sections were examined and photographed at a final magnification of $\times$20.

In this study, intranasal administration was chosen as a direct delivery route of vectors to the central nervous system (CNS) via the olfactory neuron system or by close neuron tissue. Olfactory receptor neurons are bipolar cells that reside in the epithelial lining of the nose, high in the nasal cavity. Their axons traverse the cribriform plate and project to the first synapse of the olfactory pathway in the olfactory bulb of the brain. They seem to form a highway by which viruses or other transported substances may gain access to the CNS (12).

In experiments previously reported (13), filamentous phages were intravenously injected into mice and were subsequently rescued from the different organs, showing that their integrity was not affected during membrane penetration to the organs. Filamentous bacteriophages offer an obvious advantage over other vectors. The filamentous phages, M13, f1, and fd, are well understood at both structural and genetic levels (14). They were genetically engineered to display both antigen and/or antibody and were used in different biological systems to present foreign proteins on their surfaces (15, 16).

This is the first demonstration, to our knowledge, that filamentous bacteriophage exhibits penetration properties to the central nervous system while preserving both the inert properties of the vector and the ability to carry foreign molecules. We showed a direct correlation between the number of applications and the amount of phage detected in the brain in both regions (Fig. 1 *B* and *C*). The linear structure of the phage is suggested to confer penetration properties via various membranes. No evidence was shown of spread of filamentous phage to other brain sections, strongly emphasizing the olfactory tract as the most probable path in this case. In a control experiment, we performed intranasal administration of chloroform treated spheroid phages to the mice under the same experimental conditions and no presence of phages was detected (Fig. 1).

Several hypotheses may be considered regarding the disappearance of the filamentous phage from the brain without inducing toxic effect, as shown in histology studies (Fig. 4), as well as the long life span of challenged animals. As in other reported cases, immune mechanisms may be involved that activate scavenger cells as microglia (17, 18).

Results confirmed the high specificity of scFv phage for *in vivo* targeting of Aβ. Nevertheless, not all Aβ were stained. Future experiments may be performed to radiolabel β amyloid antibodies with an isotope more suitable for diagnostic imaging, like ^{123}I, and to test their ability to label and detect amyloid deposits *in vivo* transgenic mice by using one of the same methods of diagnostic imaging as that used in humans, such as single-photon emission computed tomography. Additional studies are needed and are ongoing toward early diagnosis of the disease and therapeutic intervention before cognitive decline occurs

Future studies should determine whether coupling radiopharmaceuticals to phage antibodies would detect brain amyloid deposits *in vivo*, leading to an early and efficient diagnostic test for AD.

We express our thanks to Dr. Itai Benhar (University of Tel Aviv) for providing the vector and for assistance in 508F scFv preparation, to Dr. Manfred Windisch from JSW Research, Gratz, Austria, for providing the transgenic mice, and to Ingrid Reinprecht and Natalia Posternak for performing histological analysis. We express our thanks to Faybia Margolin for editing this work.

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