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Original Paper

Active Immunization of Mice with an *Aβ-Hsp70* Vaccine

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Abstract

Heat-shock proteins are highly immunogenic. Complexed with an antigen, they act as adjuvants, inducing a humoral and cellular immune response against both the antigen and the chaperone. In this study, we produced an Hsp70-supported vaccine to induce the generation of antibodies against amyloid-β (Aβ) peptides, the major constituent of β-amyloid plaques in Alzheimer's disease. The vaccine consisted of synthetic human $A\beta_{42}$ covalently cross-linked with DnaK, an Hsp70 homolog of *Escherichia coli*. Active immunization of mice with this vaccine resulted in the generation of antibodies against Aβ, that were detectable in sera after the first booster immunization. Antibody titers varied markedly with the genetic background of the mice. Prophylactic short-term immunization of transgenic mice (APP tg2576) before the onset of plaques, however, did not prevent amyloid plaque deposition. There were no differences in the plaque load and in the level of Triton X-100-soluble Aβ peptides in the brains of immunized and control-treated transgenic mice. Unexpectedly, the level of formic-acid soluble Aβ peptides tended to be higher in immunized mice. The reason for the increase may be an enhanced deposition of Aβ in the small cerebral blood vessels. These data emphasize the need for anti-Aβ antibodies that remove Aβ peptides from the central nervous system without negative side effects.

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Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. Its neuropathological characteristics include neuronal loss, neurofibrillary tangles, β-amyloid plaques and often congophilic angiopathy [1, 2]. The β-amyloid deposits consist mainly of amyloid-β peptides (Aβ) of 40 or 42 amino acid residues [3]. For the formation of plaques, the highly amyloidogenic peptide $A\beta_{42}$ is essential [4]. Aβ peptides originate from the amyloid precursor protein (APP) through cleavage by β- and γ-secretases. In AD, the production of Aβ exceeds its removal from brain tissue resulting in elevated brain levels of soluble or aggregated $A\beta_{42}$ and $A\beta_{40}$ [5]. Finally, the Aβ aggregation leads to the formation of β-amyloid fibrils that are

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deposited in extracellular plaques and in walls of blood vessels. Detergent-insoluble Aβ aggregates that appear at the onset of the disease have been suggested to exert negative effects on long-term potentiation [6] and on memory [7]. Increased levels of brain Aβ are proposed to be the primary cause of AD [8–10]. This hypothesis is supported by the finding that Aβ fibrils precede and accelerate the formation of neurofibrillary tangles [5, 11]. Due to the central role of Aβ peptides in AD pathology, research focuses on inhibiting the formation of Aβ or on facilitating its removal from the brain (i.e. on β- and γ-secretase inhibitors [12], β-sheet inhibitors [13] or vaccines against $A\beta_{42}$ [14]).

In a previous experimental vaccination, mice expressing an AD-related mutated human APP gene that are characterized by amyloid pathology similar to AD (PDAPP) have been immunized against Aβ by peripheral injections of aggregated $A\beta_{42}$ [14] and initial human studies showed the generation of antibodies against β-amyloid in patients with AD [15]. Immunization of the mice has been shown to ameliorate the AD-like pathology. The experiment has been repeated by many other groups in a variety of transgenic mouse models [16, 17]. Nasal administration of Aβ peptides has also been proven effective [18]. In addition, a soluble non-amyloidogenic, non-toxic Aβ-homologous peptide [19] or segments of the Aβ peptide [20] have served as immunogens. Active vaccinations, as well as passive immunizations [18, 21–23], have resulted in a reduction in the extent of β-amyloid plaques, or prevented plaque formation. The remaining plaques have been found to be decorated with antibodies and surrounded by activated microglia containing phagocytosed Aβ [14, 22]. These phenomena may be explained by blocking fibril formation, clearing Aβ aggregates and disaggregation of amyloid fibrils [24–26] by microglial F_c-receptor mediated phagocytosis [14, 22] or by drainage of Aβ from the cerebrospinal fluid into the blood plasma [27, 28]. The transgenic mice were partially protected from cognitive impairment when continuously vaccinated before the onset of the disease; mice vaccinated after the onset of disease showed an improved cognitive performance [16, 17, 29].

In our study, we induced antibodies against $A\beta_{42}$ by using the heat-shock protein (Hsp) DnaK, an Hsp70 homolog of *Escherichia coli*, as adjuvant and compared this vaccine with the conventional procedure using complete and incomplete Freund's adjuvant (CFA and IFA, respectively). Hsp are highly immunogenic and major immunological targets during bacterial and parasitic infections [30]. When complexed with another peptide or protein, Hsp can induce humoral and cellular immune responses against both the coupled antigen and themselves without the need of a classic adjuvant. This feature of Hsp is known for several experimental vaccines against both infections and tumors [31, 32]. The mechanism underlying this activity of Hsp in the immune system is hypothetical: Hsp act in a general way by inducing cytokine release and expression of antigen-presenting and co-stimulatory molecular complexes on the antigen-presenting cells (APC) [33, 34]. More specifically, the Hsp-antigen complex may be internalized into APC with the help of specific cellular surface receptors. After cleavage of the substrate and translocation of the fragments into the endoplasmic reticulum, the fragments might be displayed on MHC class I at the surface of APCs and be recognized by cytotoxic T cells [35]. Moreover, the antigens as exogenous proteins are presented to helper T cells through MHC class II complexes.

Materials and Methods

Mice and Materials

Female C57BL/6 mice (Harlan Laboratories, Horst, The Netherlands) or mice propagated from tg2576 mice expressing human APP under the control of the prion promoter (SwAPP) [36] with an at least 50% FvB background were used in this study (kindly provided by Dr. Karen Hsiao, Minneapolis, Minn., USA). Breeding and genotyping of SwAPP mice were performed as described previously and according to an approved study protocol [37]. DnaK was prepared as described elsewhere [38]. Antibodies 6E10 (recognizes segment 1–17 of human Aβ) and 4G8 (recognizes amino acid residues 17–24 of human and murine Aβ) were from Signet Laboratories (Dedham, Mass., USA). 6H1 (recognizes amino acid residues 1–17 of human Aβ), 22C4 (recognizes amino acid residues 30–42 of human and murine Aβ) and 9G10 (recognizes amino acid residues 17–42 of human and murine Aβ) were from Evotec Neurosciences (Hamburg, Germany). Polyclonal rabbit anti-DnaK antibodies were a gift from Dr. Hans-Joachim Schönfeld, Hoffmann-La Roche, Basel, Switzerland. Peptide $A\beta_{42}$, the DC Protein Assay, Tricine 10–20% gels and Tris-glycine 8% gels were purchased from Bachem (Bubendorf, Switzerland), BioRad (Munich, Germany) and Invitrogen (Basel, Switzerland), respectively.

Vaccine Preparation and Immunization Procedure

Before each set of intraperitoneal injections, human $A\beta_{42}$ was freshly dissolved in 4.1% (v/v) aqueous triethylamine (final concentration 1 mg $A\beta_{42}$/600 µl) and lyophilized to remove contaminating ammonia. This procedure was performed twice. Phosphate-buffered saline (PBS; 137 m*M* NaCl, 2 m*M* KCl, 12 m*M* sodium phosphate, pH 7.4) was added to the lyophilized $A\beta_{42}$ (final concentration 444 µ*M*). The suspension was vortexed, incubated overnight at 37°C and ultrasonicated 4 times for 30 s. Glutaraldehyde (0.02% v/v) was then added. After 2 h of gentle shaking at room temperature, the suspension was again 4 times ultrasonicated. DnaK and PBS were added to adjust both DnaK and $A\beta_{42}$ to a final concentration of

75 μM. The suspension was gently shaken at room temperature for 2 h (vaccine *DnaK-Aβ₄₂*). The yield of cross-linking was examined after ultracentrifugation at 100,000 *g* for 1 h at 4°C. Maximally 20% of the total DnaK remained in the supernatant. BSA plus Aβ₄₂ and Aβ₄₂ alone were cross-linked in the same manner (vaccine *BSA-Aβ₄₂* and *Aβ₄₂*, respectively). For the preparation of the vaccine containing Aβ₄₂ and Freund's adjuvant, aggregated and cross-linked Aβ₄₂ was emulsified 1:1 (v/v) with CFA for priming, followed by boosts in IFA. The mice received an intraperitoneal injection containing 100 μg Aβ₄₂ in 300 μl PBS at the age of 6, 8 and 12 weeks. Blood samples were taken at 10, 14, 18, 24, 30 and 40 weeks of age.

Determination of Antibody Titers in Sera

Blood was taken by retrobulbar punction and centrifuged in Microtainer Brand Serum Separator Tubes (Becton Dickinson, Franklin Lakes, N.J., USA) during 10 min with 2,000 *g* at 4°C. The sera were kept at –80°C. All measurements were carried out in duplicate.

For determination of anti-Aβ₄₂ antibody titers, 96-well Nunc MicroWell plates were coated by incubation with 200 ng Aβ₄₂ in 100 μl PBS per well overnight at 4°C. The plates were washed 4 times with washing solution (PBS, 0.05% Tween 20), blocked with 250 μl blocking buffer (PBS, 0.1% Tween 20, 5% (w/v) dried milk powder) for 2 h at 37°C. After 4 washes, sera were diluted through 1:3 dilution steps after a 1:33.3 dilution in blocking buffer up to a dilution of 1:72,900. Each well was incubated with 200 μl of diluted serum for 2 h at 37°C and then washed 5 times with washing solution. Sheep anti-mouse-Ig-HRP (Amersham; 200 μl per well, 1:5,000 diluted in blocking buffer) was added, the titer plate incubated for 1 h at 37°C, and washed 5 times with washing solution. Soluble BM-Blue POD substrate (100 μl; Roche) was used for the color reaction. The absorption at 405 nm was measured after 20 min incubation at room temperature. The titers were defined as the dilution of antiserum that produced 50% of the maximum signal.

Determination of the anti-DnaK antibody titer was performed in a similar way. Nunc MicroWell plates (96 wells) were coated with 100 ng DnaK in 100 μl PBS during 1 h at room temperature. The wells were washed 4 times, blocked with 250 μl blocking buffer for 30 min at room temperature and washed 4 times with washing solution. Mouse and control sera were prediluted 1:33.3 or 1:100 times in blocking buffer. These diluted sera were serially diluted; at each dilution, 200 μl was incubated in the wells during 1 h at room temperature. After washing 5 times with washing solution, sheep anti-mouse-Ig-HRP (Amersham; 200 μl per well, 1:5,000 diluted in blocking buffer) was added to each well; the plates were incubated for 1 h at 37°C, and washed 5 times with washing solution. Soluble BM-Blue POD substrate (100 μl; Roche) was added to each well and the absorption at 405 nm measured after 10 min at room temperature. Titers were defined as above.

Homogenization of Brain Tissue

Frontal lobes of mice (~40 mg) were triturated 15 times, first with 22-gauge and then with 26-gauge needles in 150 μl lysis buffer (250 mM sucrose, 10 mM Tris-HCl, 1% Triton X-100, pH 8.0) containing 6 μl of 25× complete proteinase inhibitor solution (Roche). The homogenates were centrifuged at 20,000 *g* during 20 min at 4°C. The supernatant was collected and protein concentration was determined with a DC Protein Assay (Bio-Rad) and directly used for Western blotting.

Alternatively, the frontal lobes were added to 10 volumes (w/v) of 70% (v/v) formic acid. The brains were triturated as above. The homogenates were ultracentrifuged with 200,000 *g* during 1 h at 4°C. The pellet was discarded and the supernatant neutralized through 21-fold dilution with 1 M Tris-HCl (pH 11.2). The solutions were divided into 500-μl samples and stored at –20°C. The homogenates were then either used for ELISA or for Western blotting analysis. For Western blotting, formic acid extracts were concentrated by precipitation in 10% trichloroacetic acid at –20°C. After centrifugation at 20,000 *g* during 10 min at 4°C and discarding the supernatant, the pellet was washed 3 times during 30 s with 200 μl acetone (–20°C). These washing procedures were repeated twice. The pellet was dried with Speed Vac, dissolved and boiled in 25 μl 1× Lämmli buffer for Western blotting.

Aβ ELISA

Nunc MicroWell plates (96 wells) were coated with 150 μl 20 μg/ml of monoclonal mouse antibody 22C4 directed against the COOH termini of Aβ₄₂ and Aβ₄₀ in PBS at 4°C overnight. The plates were blocked during 4 h at 37°C with 250 μl blocking buffer (100 mM Tris-HCl, 5 mM EDTA, 1% BSA (Sigma), 1% gelatin (Sigma), 0.1% Tween 20, pH 7.6). After 5 times washing with PBS containing 0.02% Tween 20, the plates were stored at 4°C until further use. Samples of 120 μl of the neutralized formic acid extracts were loaded into each well of the ELISA plate and incubated overnight at room temperature in 120 μl ELISA buffer (0.1 M Tris-HCl, 0.05 M EDTA, 0.05 M NaCl, 1.5% Tween 20, pH 7.2). If necessary, the samples were adjusted to the linear range of the standard curve by dilution with ELISA buffer. As a negative control, one sample was loaded into an uncoated well. A standard curve was prepared with ELISA buffer containing 0, 6.25, 12.5, 25, 50 and 100 ng Aβ₄₂ per ml. After washing 5 times with PBS, the wells were incubated with 150 μl of biotinylated anti-Aβ antibody 6E10 (1:1,000 diluted in blocking buffer) during 20 h at room temperature. The plates were then washed 5 times with PBS and the color reaction performed with a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA) and tetramethylbenzidine (Sigma). After 30 min, the reaction was stopped by adding sulfuric acid and the optical density was measured at 450 nm. In parallel, the concentrations of Aβ₄₂ were determined using a commercially available kit (Innogenetics, Gent, Belgium) according to the provider's instructions.

Western Blotting

Samples of cellular extracts were diluted 1:1 with 2× Lämmli buffer [39] and loaded onto a Novex 10–20% Tricine gel or an 8% Tris-glycine gel. Electrophoresis was performed at 20 mA in 1× Tris/Tricine (12.1 g Tris-HCl, 17.9 g Tricine, 1 g SDS per liter, pH 8.3). MultiMark® multicolored Standard LC5725 (Invitrogen) served as marker. The proteins were blotted overnight at 4°C and 25 mA onto a Protran BA 79 membrane (0.45 μm) from Schleicher & Schuell using Towbin buffer (25 μM Tris-HCl, 192 μM glycine, 0.02% SDS, 20% methanol, pH 8.5). The membrane was cooked for 5 min in PBS and then blocked for 6 h at room temperature with Tris-buffered saline (TBST; 100 mM Tris-HCl, 0.9% NaCl, 0.05% Tween 20, pH 8.0) containing 5% (w/v) milk powder. Incubation with the first antibody (either an anti-Aβ₄₂ or the anti-DnaK antibody), diluted in blocking buffer, was performed overnight at 4°C. The membrane was washed 3 times with TBST and once with TBST containing 5% (w/v) milk powder and then incubated with sheep anti-mouse-Ig-HRP (1:2,000 diluted in blocking buffer; Amersham)

Fig. 1. Time course of antibody titers after immunization of C57BL/6 mice with the *DnaK-Aβ42* vaccine. Mice were immunized at 6 weeks of age and boosted 2 and 6 weeks thereafter. **A** Anti-Aβ42 antibody titer. **B** Anti-DnaK antibody titer. Hollow circles represent titers of single mice, full circles represent the average. Note that the average titers of anti-DnaK antibodies were, with the exception of the first time point, about 10-fold higher than the anti-Aβ42 antibody titers.
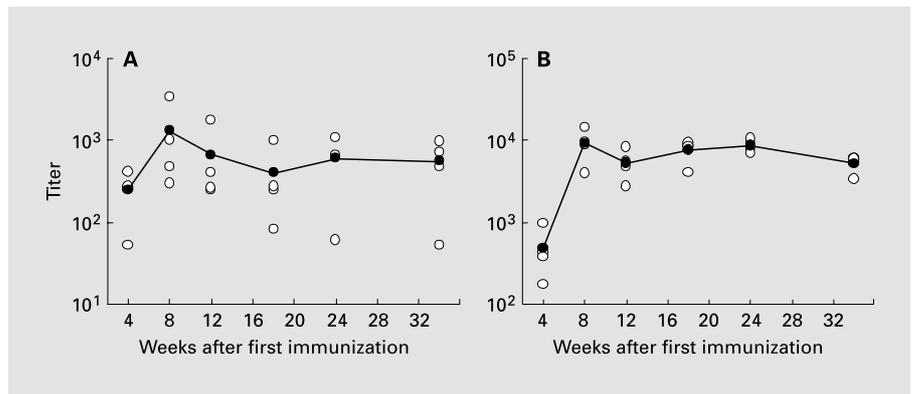
for 2 h at room temperature. After washing 3 times with TBST, detection was carried out with ECL™ Western blotting detection reagents RPN 2106.

Immunohistology and Stereological Analysis of Amyloid Burden in Brain

Mice were perfused with PBS. After cutting off the frontal lobe, the remaining brain was fixed in 4% (v/v) paraformaldehyde for 48–72 h and then embedded into paraffin. Serial 5-μm coronal sections were cut throughout the neocortex. Every eighth section was immunostained with antibody 4G8 yielding a total of 5–7 sections per mouse with a 40-μm distance between the individual sections. As negative controls, sections were stained with the secondary antibody only (goat-anti-mouse IgG). Amyloid plaque burden was then estimated by sampling through the neocortical region at 20× magnification and counting the percentage of points on an overlaid grid which were over plaques. This analysis was performed with Stereologer Software (SPA, Alexandria, Va., USA).

Results

Generation of Antibodies

The titers of anti-Aβ42 and anti-DnaK antibodies in sera of immunized mice were determined at different times after immunization. In a first set of experiments, C57BL/6 mice which had been treated with the *DnaK-Aβ42* construct (n = 4) showed a measurable antibody titer against Aβ42 (fig. 1A). The average titers of both anti-Aβ42 and anti-DnaK antibodies (fig. 1B) increased after the second boost to a maximum of 1,300 and 9,200, respectively, and then slowly decreased to 560 and 5,200, respectively, at the age of 40 weeks, i.e. 26 weeks after the last booster injection. In all control groups, i.e. mice immunized with aggregated and cross-linked Aβ42 in CFA/IFA (n = 5), *BSA-Aβ42* (n = 5) DnaK alone (n = 5) and PBS (n = 5), no humoral response against Aβ42 could be detected. Anti-DnaK antibodies were generated only
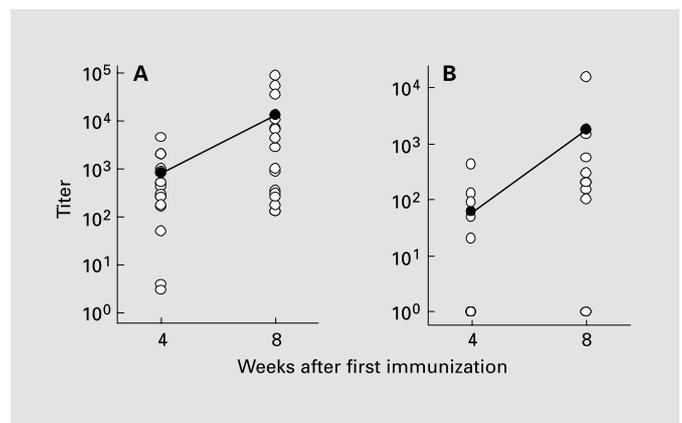

Fig. 2. Time course of antibody titers after immunization of SwAPP mice with the *DnaK-Aβ42* vaccine. Mice were immunized at 6 weeks of age and boosted 2 and 6 weeks thereafter. Anti-Aβ42 and anti-DnaK antibody titers were determined in randomly selected animals to compare the immune responses between non-transgenic and transgenic mice. **A** Anti-Aβ42 antibody titer of non-transgenic mice. **B** Anti-Aβ42 antibody titer of transgenic mice. Hollow circles represent titers of single mice, full circles represent the average. Note that the anti-Aβ42 antibody titers in wild-type animals were about 10-fold higher than in transgenic animals.

in mice immunized with *DnaK-Aβ42* or with DnaK alone.

After the successful induction of anti-Aβ42 antibodies in C57BL/6 mice, the vaccination procedure was applied in transgenic and non-transgenic SwAPP mice. As in the case of C57BL/6 mice, anti-Aβ42 antibodies were only detected in animals vaccinated with the *DnaK-Aβ42* vaccine and the anti-Aβ42 antibody titers increased after the second boost in both non-transgenic and transgenic SwAPP mice (fig. 2). In transgenic mice, the titers of both
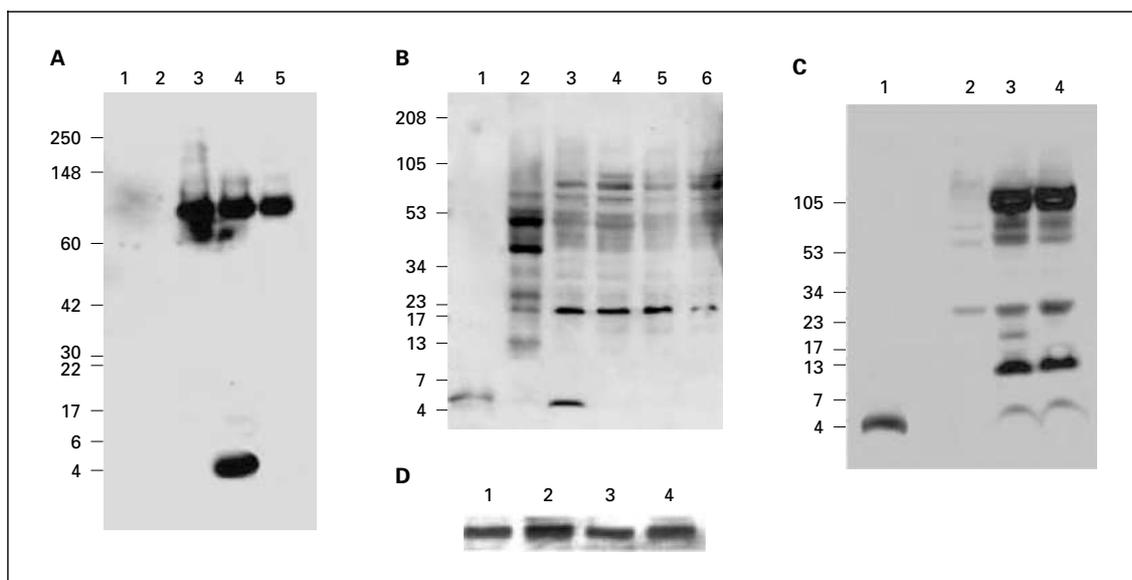
Fig. 3. $A\beta_{42}$ in brain homogenates of 6- to 9-month-old mice detected by Western blotting. **A** Detection of $A\beta_{42}$ with antibody 6E10 (specific for human $A\beta$) after SDS-PAGE of formic acid extracts of mouse brains on a 8% Tris/glycine gel and Western blotting. Lane 1, non-immunized non-transgenic mouse; lane 2, non-transgenic mouse immunized with *DnaK-Aβ42*; lane 3, non-immunized transgenic mouse; lane 4, transgenic mouse immunized with *DnaK-Aβ42*; lane 5, transgenic mouse immunized with *BSA-Aβ42*. **B** Detection of $A\beta_{42}$ with antibody 9G10 (recognizes both human and murine $A\beta$) of formic acid extracts of mouse brains on a 10–20% Tricine gel and Western blotting. Lane 1, $A\beta_{42}$ standard (5 ng); lane 2, transgenic mouse immunized with *BSA-Aβ42*; lane 3, transgenic mouse immunized with *DnaK-Aβ42*; lane 4, non-immunized transgenic mouse; lane 5, non-transgenic mouse immunized with *DnaK-Aβ42*; lane 6, non-immunized non-transgenic mouse. **C** Detection of $A\beta_{42}$ with antibody 6E10 of Triton X-100-treated mouse brains on a 10–20% Tricine gel and Western blotting. Lane 1, $A\beta_{42}$ standard (5 ng); lane 2, non-immunized non-transgenic mouse; lane 3, non-immunized transgenic mouse; lane 4, transgenic mouse immunized with *DnaK-Aβ42*. **D** Detection of Hsp70 in formic acid extracts of mouse brains by Western blotting with anti-DnaK antibody. Lane 1, transgenic mouse immunized with *DnaK-Aβ42*; lane 2, non-immunized transgenic mouse; lane 3, wild-type mouse immunized with *DnaK-Aβ42*; lane 4, non-immunized wild-type mouse.

anti-$A\beta_{42}$ (1,800) and anti-DnaK antibodies (3,700) were one order of magnitude lower than in non-transgenic littermates 2 weeks after the second booster injection (13,100 and 44,000, respectively). At the age of 12–14 months, when the mice were sacrificed, none of the *DnaK-Aβ42*-immunized transgenic mice (n = 9) showed a measurable anti-$A\beta_{42}$ titer (with one exception, a titer of 50) in contrast to the *DnaK-Aβ42*-immunized wild-type SwAPP mice (n = 7) which showed, at the same time, a mean titer of 230.

Plaque Burden of the Transgenic Mice

No correlation between treatment and plaque load in transgenic mice could be observed. At the age of 12–14 months, the majority of animals showed a low plaque burden; in both the untreated (n = 4) and *DnaK-Aβ42*-immunized (n = 6) SwAPP transgenic mouse groups, there was 1 animal with a plaque load of ~ 3%, whereas the other members showed a level below 0.3%.

Brain Levels of $A\beta_{42}$

The $A\beta$ content in formic acid extracts of brains of 6- to 9-month-old non-transgenic and transgenic immunized or control mice was analyzed with Western blotting. At that point in time, no or few single plaques were seen on the histological sections of the brains. For Western blotting, antibody 6E10, specific for human $A\beta$ (fig. 3A), or 9G10, recognizing both human and murine $A\beta$ (fig.3B), were used. *DnaK-Aβ42*-immunized transgenic mice showed a large band at 4 kDa corresponding to $A\beta$, whereas no increase in $A\beta$ was observed in the other groups. No changes in full-length APP levels were visible. Western blots for $A\beta$ in Triton X-100 extracts of the same brains showed no difference in the $A\beta$ levels between the immunized and non-immunized transgenic mouse groups (fig. 3C). In the formic acid extracts, Hsp70 levels were similar in all mouse groups (fig. 3D). The increase in human $A\beta$ in brains of *DnaK-Aβ42*-treated transgenic mice was quantified with ELISA (fig. 4A). The $A\beta$ levels
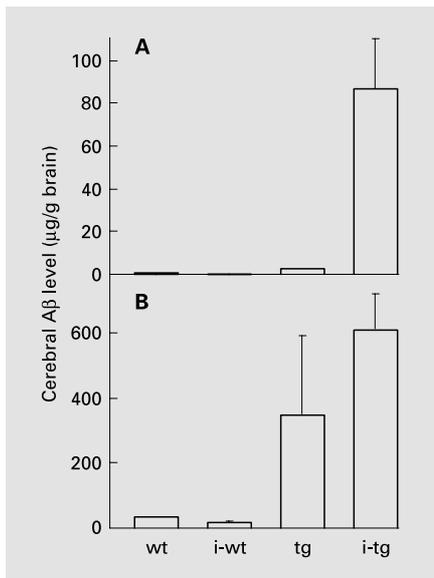
Fig. 4. Cerebral amyloid load determined with ELISA. Formic acid-extracted cerebral Aβ-levels of non-immunized non-transgenic (wt) and transgenic (tg) mice, as well as of *DnaK-Aβ42*-immunized non-transgenic (i-wt) and transgenic (i-tg) mice. **A** 6- to 9-month-old mice. wt, n = 2; i-wt, n = 2; tg, n = 2; i-tg, n = 3. **B** 12- to 14-month-old mice. wt, n = 3; i-wt, n = 4; tg, n = 4; i-tg, n = 6. Mean ± SEM are shown.

in non-transgenic non-immunized and immunized mice, as well as the Aβ-levels in non-immunized transgenic mice, were near the background (<3 μg/g brain tissue), whereas the Aβ content of the immunized transgenic animals was about 90 μg/g wet brain tissue.

Total Aβ in formic acid extracts of brains was quantitatively determined by ELISA with SwAPP mice sacrificed at the age of 12–14 months where all mice exhibited amyloid plaques. The cerebral levels of formic acid-extracted total Aβ (fig. 4A, B) and Aβ42 (not shown) were higher in the immunized transgenic mice than in the non-immunized transgenic mice. The measurements showed clearly higher values for the animals with high plaque load (>3%; 1 animal in each group), than for the 3–5 mice per group with low plaque load (<0.3%). Whereas in untreated transgenic SwAPP mice, Aβ was – in addition to plaques – mainly deposited in the walls of the large cerebral blood vessels, in the immunized transgenic mouse with a plaque load of 3%, Aβ was also found to be deposited in the small cerebral blood vessels (fig. 5). The difference in Aβ levels of formic acid extracts between immunized and non-

immunized transgenic mice did not reach statistical significance.

To ensure that the increase in Aβ42 was not mediated by DnaK activity or anti-DnaK antibodies, we repeated the immunization procedure with DnaK (n = 4), Aβ42 (n = 3) and *DnaK-Aβ42* (n = 2) as vaccines and determined the amount of total Aβ in formic acid brain homogenates after sacrificing the mice at the age of 5–7 months. The Aβ amount in animals immunized with DnaK and Aβ42 was 1.1- and 0.9-fold the level of the age-matched non-immunized transgenic control, respectively. In the mice injected with *DnaK-Aβ42*, the total Aβ level was 2.2-fold higher than in the transgenic control, parallel with the effect of immunization described above.

Discussion

This is the first study reporting the generation of antibodies against Aβ with DnaK, an Hsp70 homolog in *E. coli*, as adjuvant. The vaccine was obtained by cross-linking Aβ42 and DnaK with glutaraldehyde following our previously published procedure for developing antibodies against prion protein (PrP) in wild-type mice [40]. SwAPP mice were immunized 3 times with this vaccine several months before the onset of β-amyloid plaque formation.

Antibodies against Aβ42 in wild-type C57BL/6 mice were detected after the first booster injection, whereas in control mice immunized with Aβ42 in Freund's adjuvant no antibodies were detectable even after the second booster injection. Therefore, in our study, the *DnaK-Aβ42* vaccine was clearly superior to classic immunization with Aβ42 emulsified in Freund's adjuvant.

The titers of both the anti-Aβ42 and anti-DnaK antibodies in the C57BL/6 mice were, on average, about 10 times lower than in non-transgenic C57BL/6 × FvB hybrid mice, suggesting a strong influence of the genetic background on the immune response. For mice generated by SwAPP breeding, the titers of the transgenic mice were close to 10 times lower than those of non-transgenic littermates. These results parallel reports demonstrating lower antibody titers in transgenic mice compared with the corresponding wild-type animals after three inoculations of an Aβ vaccine [41]. This observation may be explained by a tolerance phenomenon due to the overproduction of human APP in the transgenic animals, resulting in hyporesponsiveness to Aβ of the immune system [42]. This hyporesponsiveness seems to be due to an impaired T-cell response, which has also been reported in AD patients [43], rather than to B-cell tolerance. This finding was sug-
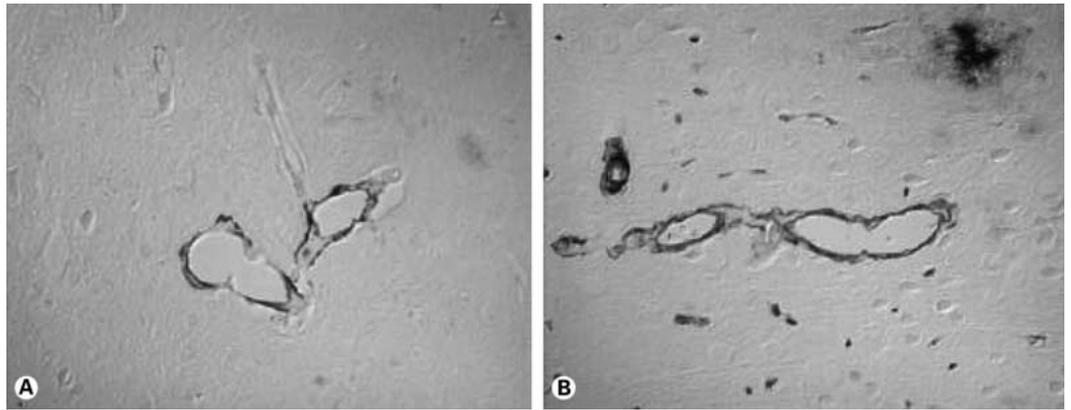
Fig. 5. Deposition of Aβ in the walls of cerebral blood vessels. Coronal section ($40\times$ magnification) stained with 4G8 against Aβ of a non-treated transgenic (**A**) and an immunized transgenic (**B**) mouse. Both mice have a plaque load of 3% and were sacrificed at 12 months of age.

gested to be of relevance for the immunization of humans who have elevated cerebral levels of Aβ.

Despite generation of antibodies by immunization at 6, 8 and 12 weeks of age, we could not observe a protection against later plaque formation. In previous studies, prophylactic vaccinations led to successful prevention of plaque development [14, 16, 17]. In these studies, however, mice were continuously immunized until the appearance of plaques in the control mice, whereas in our study we aimed to establish a vaccination procedure, following many commonly used vaccination protocols in humans, with only three immunizations long before the onset of plaque development. This procedure was shown to be effective in generating antibodies against murine prion protein [40]. To our knowledge, no successful prophylactic immunization against β-amyloid with only three injections has been reported to date.

The rapid induction of antibodies against Aβ$_{42}$ with our protocol is due to the specific role of Hsp in the immune system. Despite their high structural inter-species similarity [44], Hsp are highly immunogenic and may serve as carrier and adjuvant when coupled with an antigen [45, 46]. The high degree of sequence identity includes the risk that Hsp-containing vaccines might lead to cross-reactivity with the host Hsp and autoimmune disease. However, healthy individuals are routinely and very early in life primed to non-self Hsp through infections and immunizations with vaccines containing Hsp – like those containing *Bacille Calmette-Guérin* against tuberculosis or attenuated *Bordetella pertussis* against whooping cough – which lead to autoimmunity but not to autoimmune disease [47–51].

Upon active or passive vaccination, the brain levels of Aβ have been reported to be reduced [14, 19], unchanged [16] or even increased [52]. The reasons for the discrepancies may include different immunogenetic backgrounds of the mice [20]. In our study, we observed no change in Triton X-soluble Aβ, but an increase in formic acid-extractable Aβ indicating that Aβ aggregates increased in response to *DnaK-Aβ$_{42}$* vaccination. This trend was apparent in 6- to 9-month-old mice without β-amyloid plaque pathology, and less marked in the 12- to 14-month-old mice. In untreated transgenic SwAPP mice, formic acid-soluble Aβ begins to increase at this age, and is associated with decreased detergent-soluble forms of Aβ. These detergent-insoluble forms can inhibit the formation of long-term potentiation and impair learning and memory functions [6, 7]. This change in brain Aβ deposition is reported to be accompanied with only minimal Aβ deposits seen with immunohistochemistry [53].

In agreement with other studies, the amount of full-length APP was not changed upon immunization [14, 16, 22]. The levels of murine Hsp70 remained unchanged in all groups. The mechanism leading to the observed increase in Aβ levels is unknown. It is neither due to DnaK nor to anti-DnaK antibodies, as transgenic mice immunized with DnaK only did not show an increase in brain levels of Aβ; likewise, mice immunized with Aβ only did not show increased Aβ levels. Thus, our results indicate a crucial impact of the adjuvant on the biological consequences of Aβ vaccination. With the *DnaK-Aβ* vaccine, we did not observe changes in plaque burden. However, we found an Aβ deposition in the small cerebral blood vessels in the *DnaK-Aβ$_{42}$*-immunized transgenic mouse

with the highest plaque load (3%) in contrast to the non-immunized transgenic littermate with the same plaque load (fig. 5). This anecdotal finding may explain the observed increased Aβ levels after immunization and reflect increased Aβ aggregates in cerebral amyloid angiopathy lesions as observed in the brain blood vessels of APP23 transgenic mice [54]. Further experimentation is required to determine the role of various adjuvants on the biological responses to Aβ vaccination.

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