Neurobiology of Disease

Alzheimer's Disease Peptide Epitope Vaccine Reduces Insoluble But Not Soluble/Oligomeric Aβ Species in Amyloid Precursor Protein Transgenic Mice

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Active vaccination of elderly Alzheimer's disease (AD) patients with fibrillar amyloid-β peptide (Aβ$_{42}$), even in the presence of a potent Th1 adjuvant, induced generally low titers of antibodies in a small fraction (~20% responders) of those that received the AN-1792 vaccine. To improve the immunogenicity and reduce the likelihood of inducing adverse autoreactive T-cells specific for Aβ$_{42}$, we previously tested in wild-type mice an alternative approach for active immunization: an epitope vaccine that selectively initiate B cell responses toward an immunogenic self-epitope of Aβ in the absence of anti-Aβ T cell responses. Here, we describe a second generation epitope vaccine composed of two copies of Aβ$_{1-11}$ fused with the promiscuous nonself T cell epitope, PADRE (pan human leukocyte antigen DR-binding peptide) that completely eliminates the autoreactive T cell responses and induces humoral immune responses in amyloid precursor protein transgenic 2576 mice with pre-existing AD-like pathology. Based on the titers of anti-Aβ$_{1-11}$ antibody experimental mice were divided into low, moderate and high responders, and for the first time we report a positive correlation between the concentration of anti-Aβ$_{1-11}$ antibody and a reduction of insoluble, cerebral Aβ plaques. The reduction of insoluble Aβ deposition was not associated with adverse events, such as CNS T cell or macrophage infiltration or microhemorrhages. Surprisingly, vaccination did not alter the levels of soluble Aβ. Alternatively, early protective immunization before substantial neuropathology, neuronal loss and cognitive deficits have become firmly established may be more beneficial and safer for potential patients, especially if they can be identified in a preclinical stage by the development of antecedent biomarkers of AD.

Key words: immunotherapy; Alzheimer's disease; epitope vaccine; antibody; PADRE; β-amyloid

Introduction

The age-related accumulation of amyloid-β (Aβ) in the CNS has been hypothesized to play a central role in a cascade of events that eventually induces neuronal loss in affected brain regions in Alzheimer's disease (AD) (Selkoe, 1991, 1994; Hardy and Higgins, 1992; Price and Sisodia, 1994; Esler and Wolfe, 2001; Hardy and Selkoe, 2002). Previously, major support for the amyloid cascade hypothesis emerged from Aβ-immunotherapy studies demonstrated that anti-Aβ antibodies were capable of reducing AD-like pathology (Schenk et al., 1999) and improving behavior in amyloid precursor protein (APP) transgenic (Tg) mice (Chen et al., 2000; Janus et al., 2000; Morgan et al., 2000). First, immunother-

apy clinical trial in AD patients using the AN-1792 vaccine containing B and T cell "self epitopes" of Aβ$_{42}$ was halted during Phase IIa, when ~6% of the participants developed aseptic meningoencephalitis (Schenk, 2002; Weiner and Selkoe, 2002; Hock et al., 2003; Nicoll et al., 2003; Orgogozo et al., 2003; Ferrer et al., 2004; Bayer et al., 2005; Fox et al., 2005; Gilman et al., 2005; Masliah et al., 2005; Patton et al., 2006). Importantly, only vaccinated participants (n = 18) developed meningoencephalitis, whereas none of the control patients (n = 72) injected with placebo developed adverse events (Orgogozo et al., 2003). Data from these trials suggest that the aseptic meningoencephalitis may have been caused by a T cell-mediated autoimmune response (Nicoll et al., 2003; Ferrer et al., 2004), and that anti-Aβ antibodies were not responsible for the observed adverse effects after active vaccination. In fact, low/moderate titers of anti-Aβ antibodies generated in a small subset of immunized patients (19.7%) were capable of reducing parenchymal amyloid pathology (Nicoll et al., 2003, 2006; Ferrer et al., 2004; Masliah et al., 2005; Patton et al., 2006; Boche et al., 2007; Nitsch and Hock, 2007) and diminishing progressive cognitive decline associated with the disease (Hock et al., 2003; Gilman et al., 2005). However, ~80% of the immunized subjects failed to develop anti-Aβ antibody titers ("nonresponders"), indicating that the Aβ self-antigen in the

AN-1792 vaccine was not a strong immunogen, thus suggesting that alternative immunotherapeutic strategies should be pursued.

Based on the results generated in mouse models of AD (Bard et al., 2000; DeMattos et al., 2001; Dodart et al., 2002), a new clinical trial, AAB-001 (Elan and Wyeth Pharmaceuticals, http://elan.com/investorrelations/events/elanwyethsymposium_adpd.asp), has been initiated by using passive transfer of a humanized monoclonal anti-Aβ antibody (bapineuzumab) in an attempt to avoid the problems associated with active immunization of elderly AD patients. However, the design of this new trial is associated with additional challenges such as multiple injections of high concentrations of anti-Aβ antibody every 13 weeks, the high cost of this monoclonal humanized antibody as well as possible side effects of passive vaccination, including microhemorrhages observed in passively immunized very old APP Tg mice (Pfeifer et al., 2002; Wilcock et al., 2004; Racke et al., 2005). This suggests that development of safe active immunotherapeutic strategies may still be desirable.

Previously, we engineered and tested a first generation epitope vaccine in wild-type mice (Agadjanyan et al., 2005), and here we report the development and testing the safety and efficacy of therapeutic vaccination of APP Tg 2576 mice with pre-existing AD-like pathology with a second generation epitope vaccine composed of two copies of the B cell epitope, Aβ_{1-11} in tandem with pan human leukocyte antigen DR-binding peptide (PADRE), a synthetic, foreign promiscuous T cell epitope [pre-existing AD-like pathology implies the accumulation of soluble oligomeric forms of amyloid-beta peptide leading to the impairment of cognitive functions in $\geq$6-month-old APP Tg 2576 mice (Lesne et al., 2006)].

Materials and Methods

Mice, epitope vaccine, peptide immunogens, and experimental protocol. Aged ($\sim$9.4 months old) female APP Tg 2576 mice were bred and provided by the animal facility associated with the University of California at Irvine (UCI) Alzheimer's Disease Research Center. All animals were housed in a temperature and light-cycle controlled facility, and their care was under the guidelines of the National Institutes of Health and an approved Institutional Animal Care and Use Committee protocol at University of California at Irvine.

To engineer an epitope AD vaccine, we synthesized the N terminus of an immunodominant B cell epitope of Aβ_{1-11} (McLaurin et al., 2002; Bard et al., 2003; Cribbs et al., 2003) in tandem with a promiscuous foreign T cell epitope, so called pan-DR epitope, PADRE (Alexander et al., 1994). The peptide 2Aβ_{1-11}-PADRE was synthesized as a multiple antigenic peptide (MAP), containing a core matrix of 4 branching lysines (Tam, 1988; Chai et al., 1992) to generate 2Aβ_{1-11}-PADRE-MAP (Invitrogen, Carlsbad, CA). Aβ_{42} peptide was synthesized at the Peptide Core Facility at the Institute for Brain Aging and Dementia at UCI by solid-phase Fmoc amino acid substitution and purified by reverse-phase high-pressure liquid chromatography.

Mice were immunized with 2Aβ_{1-11}-PADRE-MAP or fAβ_{42} as described previously (Cribbs et al., 2003; Petrushina et al., 2003; Agadjanyan et al., 2005). Briefly, 2Aβ_{1-11}-PADRE-MAP (500 μg/ml) or the fibrillar (Schenk et al., 1999; Cribbs et al., 2003) Aβ_{42} peptide (500 μg/ml) were mixed with Quil A, a Th1-type conventional adjuvant, and mice were injected with 50 μg of antigen subcutaneously. Experimental mice at age 9.4 $\pm$ 0.9 months old were immunized with 2Aβ_{1-11}-PADRE-MAP ($n = 21$) or fAβ_{42} ($n = 12$), whereas the control group of APP Tg 2576 mice ($n = 11$) was injected with an adjuvant only. All mice were boosted at monthly intervals. Cellular immune responses were analyzed in five mice from each group killed at 9 d after the fourth immunization. We continued to boost the remaining mice at monthly intervals and sera was collected at 8–10 d after the third, fourth, sixth and 10th immunizations and used for detection of anti-Aβ antibodies. At the end of the study, after 10 vaccinations when animals were 19.4 $\pm$ 0.9 months old, neuropathological changes were compared across groups in response to treatment. In these animals, cellular immune responses also were analyzed.

T cell proliferation. The analysis of T cell proliferation was performed in splenocyte cultures from individual animals, as described previously (Cribbs et al., 2003; Agadjanyan et al., 2005). In addition, CD4$^+$ T cell proliferation was assessed using FACS assay according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Briefly, to detect antigen-specific proliferation of CD4$^+$T cells, we stained splenocyte cultures by 1 μM succinimidyl ester of carboxyfluorescein diacetate (CFSE; Invitrogen) for 10 min at 37°C. After washing, the cells were incubated for 3 d in culture media alone or with PADRE (5 μM). After incubation, the cultures were stained with phycoerythrin (PE)-labeled rat anti-mouse CD4 monoclonal antibodies (BD Biosciences). Because dead cells might fluoresce nonspecifically, these cells were excluded from the assay using a nucleic acid dye (7-amino actinomycin D from BD PharMingen, San Diego, CA), and proliferation of viable cells was analyzed by FACS-can flow cytometer (BD Biosciences) as described by the manufacturer. CD4$^+$ population was separately analyzed using CellQuest software (BD Biosciences).

Production of cytokines by immune splenocytes. The same splenocytes used to assess T cell proliferation were used for detection of Th1 (IFNγ) or Th2 (IL4) lymphokines by ELISPOT (BD PharMingen) as described previously (Cribbs et al., 2003; Agadjanyan et al., 2005). In addition, the FACS method was used for detection of specific cytokines by CD4$^+$ T cells (Pala et al., 2000). Briefly, the cultures of splenocytes from experimental and control animals were restimulated for 3–4 d with PADRE peptide (5 μM), and then for 4–6 h with PMA (phorbol 12-myristate 12-acetate) and ionophore (ionomycin) (both from Sigma, St. Louis, MO). In addition, we used Brefeldin A (BD Biosciences) to block cytokine secretion, which increases intracellular accumulation. Surface staining was performed using FITC-labeled anti-mouse CD4 monoclonal antibodies (MoAb; BD PharMingen). Cells were washed, fixed, permeabilized, and CD4$^+$T cell subset producing IL-4 or IFNγ was detected using the appropriate PE-labeled anti-mouse cytokine antibodies (BD PharMingen).

Detection of anti-Aβ antibodies by ELISA. Total anti-Aβ_{42} antibodies were detected as described previously (Cribbs et al., 2003; Petrushina et al., 2003) with small modifications: to develop the color reaction we used the 3,3',5,5' tetramethylbenzidine (TMB) peroxidase substrate (Pierce, Rockford, IL), and plates were analyzed spectrophotometrically at 450 nm. The standard curve for determination of anti-Aβ antibody concentrations in the sera was based on different known concentrations of monoclonal antibody 20.1 kindly provided by Dr. Van Nostrand (Stony Brook University, Stony Brook, NY). The concentrations of antibody were recorded in micrograms per milliliter. To determine the specific isotypes, pooled sera from mice were diluted 1:2500 and tested in duplicate. As we reported previously, mice of H2^{bxs} immune haplotype (APP Tg 2576) do not express IgG2a, producing IgG2c anti-Aβ antibodies instead (Petrushina et al., 2003). Therefore, in our experiments we used anti-IgG2a^b-specific antibodies (BD PharMingen) along with anti-IgG1-, IgG2b- and IgM- specific antibodies (Zymed, San Francisco, CA).

Detection of Aβ plaques in human brain tissues. Sera from immunized mice were also screened for the ability to bind to Aβ plaques in the human brain as we described previously, using immunohistochemistry (Ghochikyan et al., 2003; Agadjanyan et al., 2005). A digital camera (Olympus, Tokyo, Japan) was used to capture images of the plaques at 20$\times$ magnification. The binding of antisera (dilution 1:1000) to the β-amyloid plaques was blocked by preabsorption of the sera with 5 μM Aβ_{1-15} peptide (1 h, 37°C).

Immunohistochemistry. To analyze the effect of active immunization with the epitope vaccine on neuropathological changes in APP Tg 2576 mice (19.4 $\pm$ 0.9 months old), the brains were processed for immunohistochemistry and histochemistry by previously published methods (Ghochikyan et al., 2003; Agadjanyan et al., 2005). Animals were killed under deep Nembutal sodium solution (150 mg/kg, i.p.) anesthesia. To ensure proper fixation and immunostaining of brain tissues, mice were exsanguinated by transcardial perfusion with normal saline. Then brains

were removed and bisected in midsagittal plane. The right hemisphere was snap frozen for biochemical analysis, whereas the left hemisphere was fixed in 4% paraformaldehyde for immunohistochemical analysis. Forty-micrometer-thick free-floating coronal sections of fixed hemibrains were collected using a vibratome. To assess the extent of neuropathology and neuroinflammation that occurs in the brains of mice, the following primary antibodies were used. $A\beta$ deposits were detected with anti-$A\beta_{42}$ (dilution, 1:2000; Invitrogen) and anti-$A\beta_{40}$ (1:10000; Invitrogen). Activated microglia were detected with the anti-I-A/I-E [marker of major histocompatibility complex (MHC) II alloantigens; 1:200; BD PharMingen] and anti-CD45 (1:300; Serotec, Raleigh, NC) antibodies. Astrocytes were labeled with anti-glial fibrillary acidic protein (GFAP; 1:3000) antibodies (Eng et al., 2000). Infiltration of T cells and macrophages were analyzed using anti-CD3-ε (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD4, anti-CD8 (1:250; Novocastra Laboratories, Newcastle, UK), and anti-F4/80 (1:50; Serotec) antibodies, respectively. The tissues from all animals within a given experimental group were processed in parallel. Sections to be immunostained with anti-$A\beta$ antibodies were pretreated in 90% formic acid for 4 min to enhance $A\beta$ staining (Kitamoto et al., 1987). Sections for the staining with anti-CD45 and anti-I-A/I-E (anti-MHC II) were pretreated with proteinase K (0.03 mg/ml) for 5–7 min at room temperature. Hydrogen peroxide-quenched and blocked sections were incubated with primary antibody overnight at 4°C. Sections were then washed and incubated with appropriate biotinylated secondary antibodies (1 h at room temperature). After multiple washes, the tissues were incubated in ABC for 1 h, and color development was performed using DAB (3,3′-diaminobenzidine) substrate kit (Vector Laboratories, Burlingame, CA). Sections were mounted on Vectabond-coated slides (Vector Laboratories), dehydrated, and covered using DPX (BDH Laboratory Supplies, Poole, UK). Fibrillar $A\beta$ deposits were visualized using Thioflavin S (ThS) as described by (Schmidt et al., 1995). Briefly, mouse brain sections were washed with Tris buffer and stained for 10 min with a solution of 0.5% ThS in 50% ethanol. Finally, sections were washed in 50% ethanol and Tris buffer, then dried and covered using Vectashield (Vector Laboratories).

Quantitative image analysis. NIH imaging was used to analyze the area occupied by β-amyloid and glial reactivity as described previously (Head et al., 2001). Immunostaining was captured using a Sony (Tokyo, Japan) high-resolution CCD video camera (XC-77) and NIH image 1.59b5 software. For every animal, 12 images (525 × 410 μm each) of the frontal parietal region in the cortex at approximately the same plane (0.74 to −2.9 mm with respect to bregma) of two adjacent sections were captured with a 20× or 40× objective. The samples included six images from the superficial layer and the remaining six from the deep layer. NIH imaging was used to analyze the area occupied by β-amyloid ($A\beta$ load) relative to the background, and expressed as the percentage of area occupied. The threshold for detection of immunoreactivity was established and then held constant throughout the image analysis. ThS-positive plaques were counted by visual inspection of cortical region of all stained sections although blind with respect to treatment condition; a mean semiquantitative score was independently determined for each slide by two observers.

Prussian blue staining for microhemorrhage. Staining for hemosiderin deposits was performed on duplicate adjacent coronal sections of the mice brains containing similar regions located at approximately −1.5 bregma point, 50 μm thick, collected from immunized and naive, age-matched APP Tg 2576 mice. The sections were stained with Prussian blue working solution (equal parts of freshly made 5% potassium ferrocianide and 5% hydrochloric acid) for 30 min at room temperature, washed in deionized water, and counterstained with Nuclear fast red. Possible hemorrhage events in the form of the number of Prussian blue-positive profiles were counted in the brains of each mouse on all sections by two independent observers, and the average number of hemosiderin deposits was calculated per each brain hemisphere.

Biochemical analysis. Biochemical analysis of the brain tissue was processed as described previously (Kawarabayashi et al., 2001), except that cortices of right hemispheres of brains were used. Briefly, frozen cortices were thawed, minced and then homogenized in 50 mM Tris-HCl buffer containing 2% SDS, pH 8.0, and a mixture of protease inhibitors (MP Biomedicals, Solon, OH). Homogenates were centrifuged (100,000 × g, 1 h, 4°C) and supernatants were stored at −70°C for additional analysis of soluble β-amyloid. Seventy percent formic acid was added to the pellets for extraction of SDS-insoluble β-amyloid. After sonication samples were centrifuged (100,000 × g, 1 h, 4°C) and supernatants were stored for analysis of insoluble β-amyloid. After neutralization of formic acid with 1.0 M Tris-base/0.5 M NaH$_2$PO$_4$, concentrations of insoluble and soluble $A\beta_{40}$ and $A\beta_{42}$ were analyzed using β-amyloid ELISA kits (Invitrogen) according to the manufacturer's recommendations. Plates were analyzed spectrophotometrically at 450 nm via a microplate reader, and the concentrations of $A\beta_{40}$ and $A\beta_{42}$ were calculated using standard curves for $A\beta_{40}$ and $A\beta_{42}$ peptide by comparing the sample's absorbance with the absorbance of known concentrations of a standard. Using the wet weight of cortex region in the original homogenate, the final values of $A\beta$ were expressed as micrograms per gram wet weight of cortex.

Dot blot assay and combination of IP and WB. Soluble fractions from cortical homogenates of experimental and control mice used in ELISA were also used for dot blot assay and for both immunoprecipitation (IP) and Western blot (WB). Total protein concentration in the homogenates was determined using bicinchoninic acid assay (Pierce) and adjusted to 4 mg/ml with PBS.

Dot blot. Two microliters of sequential dilutions of homogenates were applied to nitrocellulose membrane (GE Healthcare, Piscataway, NJ), air-dried, and blocked with 5% fat-free dry milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Oligomers were detected by using HRP-conjugated A11 polyclonal antibody (kindly provided by Dr. C. Glabe, University of California, Irvine, Irvine, CA). Blots were developed with ECL detection system (Santa Cruz Biotechnology). Autoradiograms were scanned, and densitometry of $A\beta$ oligomer spots was performed with NIH Image J software, version 1.36b. The relative optical density was calculated and presented as average value ±SD for each group.

IP and WB. Aliquots of homogenates were pooled into four groups (based on low, moderate, high anti-$A\beta$ antibody responders and control nonimmunized mice, 200 μg of total protein in each pooled aliquot), diluted to 500 μl with PBS and incubated (overnight at 4°C) with anti-$A\beta$ 20.1 monoclonal antibody immobilized on protein G-sepharose. The beads were washed three times in PBS, and proteins were eluted in 20 μl of SDS-PAGE loading buffer by boiling. Samples were subjected to electrophoresis on 12.5% SDS-Tris polyacrylamide gel and proteins were electrotransferred to polyvinylidene difluoride membrane (GE Healthcare). The membrane was boiled for 2 min and blocked with 5% fat-free dry milk followed by detection of $A\beta$ oligomers using anti-$A\beta$ biotinylated 20.1 monoclonal antibody. Three experiments with the same homogenates were performed and Western blots were scanned and converted into digital files. Densitometry of $A\beta$ oligomer bands were performed using NIH Image J software, version 1.36b and data (average ± SD) were presented from three experiments.

Statistical analysis. All statistical parameters (mean, SD, significant difference, etc.) used in experiments were calculated using Prism 3.03 software (GraphPad Software, San Diego, CA). Statistically significant differences were examined using an ANOVA and *post hoc* comparisons were done using Tukey's test ($p < 0.05$ was considered as statistically different).

Results

The second generation epitope vaccine stimulates CD4$^+$IFNγ^+ T cells specific to PADRE without activation of autoreactive anti-$A\beta$ T helper cells

To circumvent the side effects of the AN-1792 vaccine against AD, we engineered a vaccine in which a foreign T helper cell epitope was incorporated with two copies of the B cell epitope of $A\beta_{42}$. APP Tg 2576 mice vaccinated with 2$A\beta_{1-11}$-PADRE-MAP induced T cell responses directed against the foreign antigenic determinant, PADRE, but not against self $A\beta$ antigen. More specifically, splenocytes isolated from vaccinated animals proliferated after re-stimulation with PADRE, but not $A\beta_{40}$ (Fig. 1*A*). In

contrast, control APP Tg2576 mice vacci-nated with fibrillar $A\beta_{42}$ antigen ($fA\beta_{42}$) that has self B and T cell antigenic deter-minants induced only autoreactive T cells activated after restimulation with $A\beta_{40}$ (Fig. 1*A*).

Direct measurement of activation of PADRE-specific CD4$^+$ T helper cells in vaccinated APP Tg 2576 mice by a FACS assay confirmed these results. CD4$^+$ T cells from mice vaccinated with the pep-tide epitope vaccine induced strong prolif-eration of this subset of T cells after re-stimulation with nonself PADRE peptide, but not $A\beta_{40}$ (Fig. 1*B*). In contrast, vacci-nation with $fA\beta_{42}$ formulated in QuilA ad-juvant induced proliferation of CD4$^+$ T cells activated after re-stimulation with self-antigen $A\beta_{40}$, but not with nonself T cell epitope PADRE. Of note, CD4$^+$ T cells from mice injected with adjuvant did not proliferate after restimulation either with PADRE or $A\beta_{40}$ peptides (Fig. 1*B*).

To further investigate T cell responses to vaccination, we analyzed Th1 (IFNγ) and Th2 (IL-4) cytokine production by splenocytes and CD4$^+$ T cells from im-munized and control APP Tg 2576 mice using ELISPOT and FACS assays, respec-tively (Fig. 1*C*,*D*). In these experiments, splenocytes from mice vaccinated with epitope vaccine were restimulated with PADRE, whereas splenocytes isolated from animals immunized with $fA\beta_{42}$ were restimulated with $A\beta_{40}$. Groups of APP Tg 2576 mice injected with the epitope vaccine induced a strong IFNγ (Th1) response based on the number of splenocytes producing this lym-phokine, whereas mice immunized with $fA\beta_{42}$ had low re-sponses. Because we used a Th1 adjuvant in both the epitope and $fA\beta_{42}$ vaccinations, it was not surprising that both groups of mice generated less IL-4 (Th2) than IFNγ (Th1), whereas splenocytes from naive mice did not generate either IL4 or IFNγ cytokines (Fig. 1*C*). We confirmed these results by measuring the produc-tion of IL-4 or IFNγ cytokines in CD4$^+$ T helper cells. We found that the number of PADRE-specific CD4$^+$IFNγ^+ T helper cells in mice immunized with the epitope vaccine was higher than the number of anti-Aβ CD4$^+$IFNγ^+ T cells in mice immunized with $fA\beta_{42}$ (Fig. 1*D*). The same was true for IL-4-producing anti-PADRE and anti-Aβ CD4$^+$ T cells, although both groups had significantly lower number of CD4$^+$T cells producing IL-4 than IFNγ (Fig. 1*D*). Thus, CD4$^+$T cells and splenocytes isolated from both vaccinated groups predominantly produced a Th1-type cytokine, IFNγ, consistent with antigens being formulated in a Th1-type adjuvant, Quil A. Cellular immune responses ana-lyzed after 4 (Fig. 1) and 10 (data not shown) immunizations showed the same specificity and profile. Collectively, the analyses of T cells demonstrated that the epitope vaccine did not activate autoreactive T cells, but stimulated nonself PADRE-specific CD4$^+$IFNγ^+T lymphocytes.

Anti-PADRE-specific CD4$^+$IFNγ^+ T lymphocytes help B cells to produce therapeutically potent anti-Aβ_{1-11} specific antibody in vaccinated APP Tg 2576

To determine the ability of activated PADRE-specific T cells to stimulate anti-Aβ B cells, we measured antibody concentrations
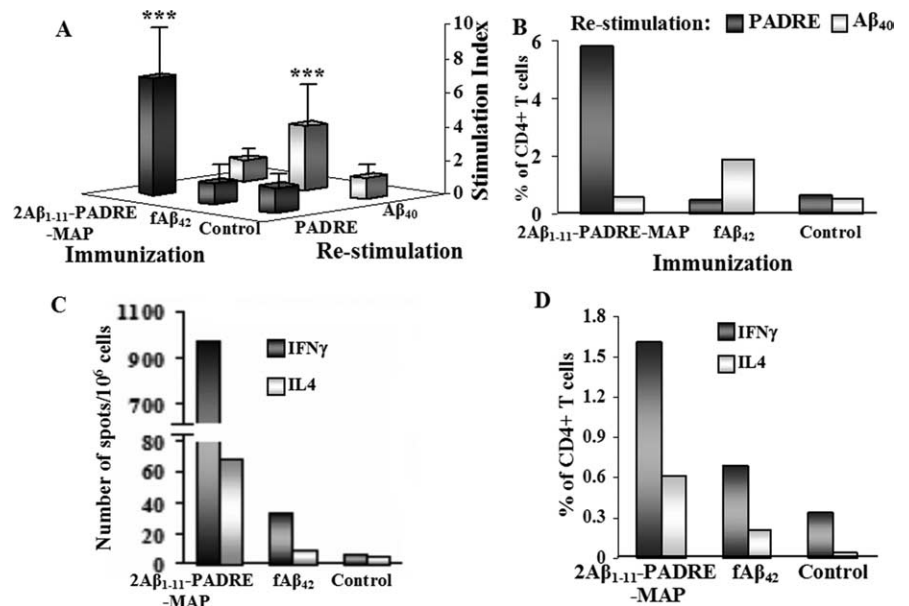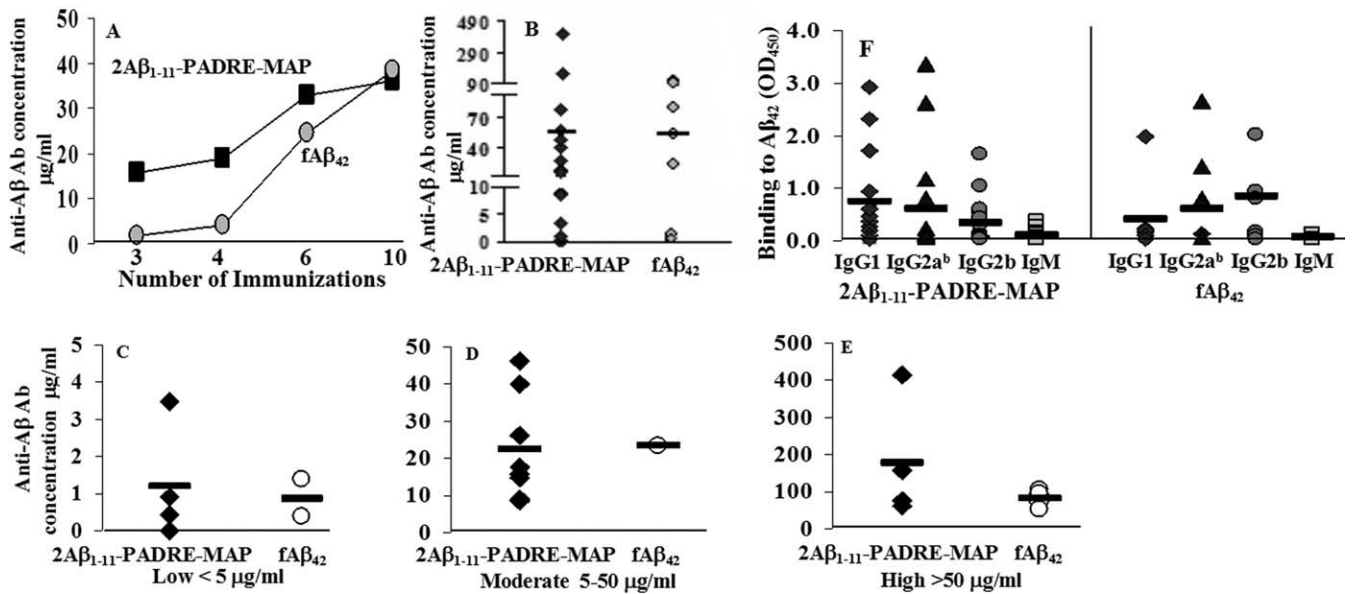

Figure 1. The second generation peptide epitope vaccine induces T cell response specific to promiscuous and nonself epitope, PADRE. Splenocytes were isolated from individual mice ($n = 5$) after fourth immunization and restimulated *in vitro* with PADRE or Aβ_{40} peptides. ***A***, Proliferation of splenocytes was detected by ^{3}[H] thymidine incorporation. ***B***, ***D***, Proliferation of CD4$^+$T cells (***B***) and production of cytokines by this T cell subset (***D***) were detected by flow cytometry in pooled splenocyte cultures. Production of IFNγ and IL4 cytokines by pooled immune splenocytes was detected by ELISPOT assay (***C***). The experiment was repeated after the 10th immunization with similar results. ***$p < 0.001$.

in the sera pooled from each group of animals after three, four, six, and 10 immunizations. After three injections of the epitope vaccine the concentrations of anti-Aβ antibodies in the sera were higher than that in mice immunized with $fA\beta_{42}$. However, after the sixth immunization this difference in anti-Aβ antibody titers steadily diminished, becoming equal after the last two boosts in both groups (Fig. 2*A*). Of note, mice immunized with the Quil A adjuvant alone did not induce anti-Aβ antibodies (data not shown).

As mentioned above, these data were generated with sera pooled from each of the group; however, in individual animals we found significant variability in anti-Aβ antibody responses (Fig. 2*B*). Concentrations of anti-Aβ_{1-11} antibodies after 10 immuni-zations were high in four mice immunized with the epitope vac-cine (176.8 ± 163.56 μg/ml), whereas an additional eight and four vaccinated animals induced moderate (22.2 ± 14.16 μg/ml) and low levels (1.2 ± 1.5 μg/ml) of anti-Aβ_{1-11} antibodies, re-spectively (Fig. 2*C–E*). This individual variability in humoral im-mune responses was also detected in the group of mice immu-nized with $fA\beta_{42}$ antigen (Fig. 2*C–E*): four mice generated high (82.65 ± 22.68 μg/ml), one mouse had moderate (23.3 μg/ml), and two animals had low concentrations (0.9 ± 0.7 μg/ml) of anti-Aβ_{42} antibodies. Thus, the epitope vaccine was at least as effective as $fA\beta_{42}$ in induction of antibodies after 10 immuniza-tions, but was significantly more effective at initiating the anti-body immune responses (Fig. 2*A*).

To characterize the types of humoral immune responses from each vaccination group, we measured the production of IgG1, IgG2a^b, IgG2b, and IgM anti-Aβ antibodies in the sera collected from individual animals after a total of 10 injections of the epitope vaccine or $fA\beta_{42}$. All mice vaccinated with the epitope vaccine generated comparable amounts of IgG1, IgG2a^b, and as a result, the average of IgG1/IgG2a^b ratio was close to 1 (Fig. 2*F*). This ratio implied that the epitope vaccine formulated in Th1

Figure 2. The second generation epitope vaccine selectively initiates B cell responses toward an immunogenic epitope of $A\beta_{1-11}$, whereas T cell help is provided by a genetically linked nonself T cell epitope, PADRE. **A, B,** Concentration of anti-$A\beta$ antibody detected in the pooled sera after three, four, six, and 10 immunizations (**A**) or in individual animals after 10 immunizations (**B**). **C–E,** APP Tg 2576 mice immunized with epitope or $fA\beta_{42}$ vaccines generated low (**C**), moderate (**D**), or high (**E**) concentrations of anti-$A\beta$ antibody. **F,** Detection of IgG1, IgG2a[b], IgG2b, and IgM isotypes of anti-$A\beta$ antibody.
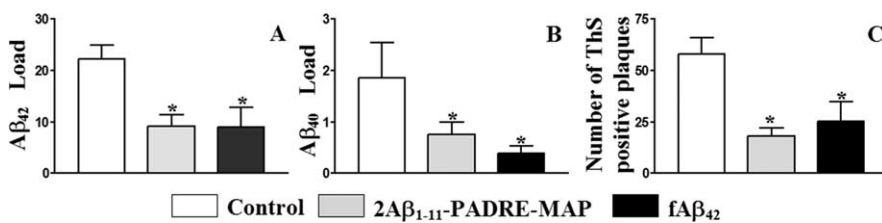
Figure 3. **A–C,** The second generation epitope vaccine inhibits $A\beta$ deposition in the brains of 19.4 ± 0.9-month-old APP Tg 2576 mice. Image analysis of $A\beta_{42}$ (**A**), $A\beta_{40}$ (**B**), and Thioflavin S (**C**) staining in cortex of mice vaccinated with epitope vaccine, $fA\beta_{42}$, or control mice. Vaccinated mice showed a significant reduction in $A\beta_{42/40}$ load (cored and diffuse) and in the number of ThS-positive (cored) plaques (*$p < 0.05$) compared with the control animals. Error bars represent the average $\pm$ SE for $n = 6$ in the group of control mice, and $n = 16$ and $n = 7$ in the groups of mice vaccinated with epitope and $fA\beta_{42}$ vaccines, respectively.
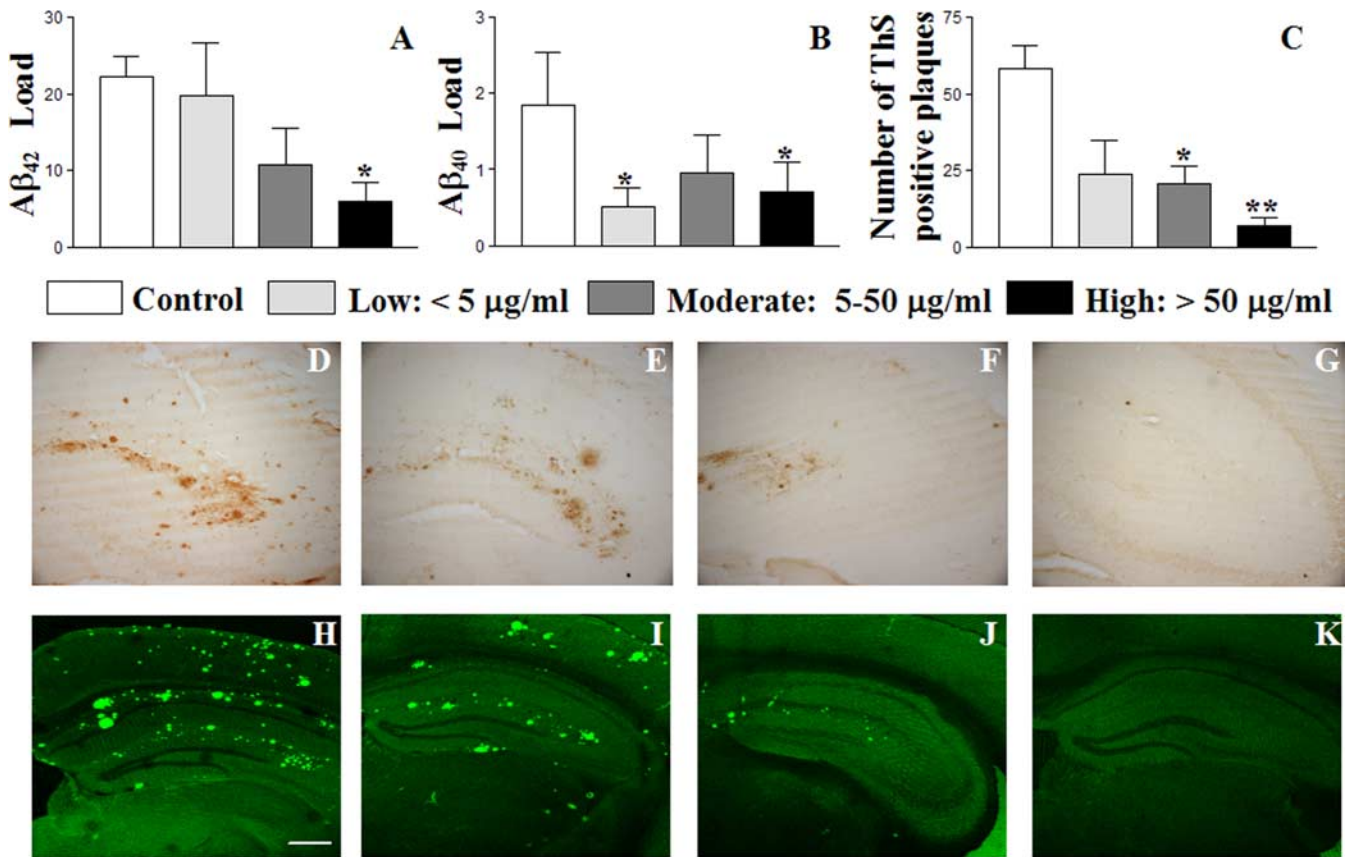
with the epitope vaccine or $fA\beta_{42}$, we detected significantly less $A\beta_{42}$ or $A\beta_{40}$ load (diffuse and cored plaques) than in brains of control animals (Fig. 3 A, B). Additionally, we demonstrated significant reduction of ThS-positive $A\beta$ deposits (cored plaques) in the brains of experimental mice versus controls (Fig. 3C). Collectively, these results demonstrate that immunization with epitope vaccine is effective in reducing cerebral $A\beta$ burden in APP Tg 2576 mice, which were ~9 months old at the start of the study.

As described previously, data from the first human clinical trial indicated that AD patients responding to the AN-1792 vaccine and producing anti-$A\beta$ antibody titers over 1:2200 showed some slowing of cognitive decline and localized reduction of plaques (Hock et al., 2003; Gilman et al., 2005; Masliah et al., 2005; Nicoll et al., 2006). To address this question in our preclinical trials, we grouped all mice vaccinated with the epitope vaccine based on the concentrations of generated anti-$A\beta$ antibodies (Fig. 2C–E), and analyzed the levels of soluble and insoluble $A\beta$ in the brains of experimental and control animals. The rationale for using $A\beta$ as an outcome measure reflecting possible functional improvements was based on previous work showing that $A\beta$ is correlated with behavior in APP Tg 2576 mice (Hsiao et al., 1996; Westerman et al., 2002) and that vaccination of APP Tg mice with $fA\beta_{42}$ leads to behavioral improvements and reduced $A\beta$ (Chen et al., 2000; Janus et al., 2000; Morgan et al., 2000; Das et al., 2001, 2003). We observed a positive correlation between the concentration of anti-$A\beta_{1-11}$ antibodies and reduction of cortical $A\beta_{42}$ load in the brains of vaccinated APP Tg 2576 mice (coefficient of correlation is -0.83, $p < 0.05$). As shown in Figure 4A, there are no differences in $A\beta_{42}$ load between the group of mice with low concentrations of anti-$A\beta_{1-11}$ antibodies and control mice. Although $A\beta_{42}$ load is noticeably reduced in the brains of

adjuvant induced a Th1 type humoral response. Similar results were observed in mice immunized with $fA\beta_{42}$; immunization in these animals induced anti-$A\beta_{42}$ antibodies of all IgG isotypes tested, and IgG1/IgG2a[b] ratio was <1 (Fig. 2F). Antibodies generated by the epitope vaccine recognized human $A\beta$ deposits when used in immunohistochemical experiments in an AD case, and this binding was blocked by preabsorption of sera with $A\beta_{42}$, $A\beta_{1-11}$, or $2A\beta_{1-11}$ peptides equally well (data not shown). Thus, as was expected, the epitope vaccine induced antibodies specific to the $A\beta_{1-11}$ sequence of $A\beta_{42}$.

The link between anti-$A\beta_{1-11}$ antibody concentrations and clearance/reduction of AD-like neuropathology in the brains of APP Tg 2576 mouse model of AD

To demonstrate the therapeutic efficacy of anti-$A\beta$ antibodies generated in response to the peptide epitope vaccine, we analyzed neuropathological changes in aged (19.4 ± 0.9 months old) experimental and control APP Tg 2576 mice (after 10 injections). Figure 3 shows a significant decrease in cortical plaque burden in APP Tg 2576 mice immunized with both the epitope vaccine and the $fA\beta_{42}$ antigen, compared with the control adjuvant-only injected group. More specifically, in brains of mice immunized

Figure 4. **A–C**, The anti-Aβ antibody concentration positively correlates with the reduction of Aβ_{42} and Thioflavin S, but not Aβ_{40} load in the cortex areas of APP Tg 2576 mice immunized with epitope vaccine. Error bars represent the average $\pm$ SE for control mice ($n = 6$) and mice with low ($n = 4$), moderate ($n = 8$), and high ($n = 4$) anti-Aβ antibody concentrations. *$p < 0.05$; **$p < 0.01$. **D–K**, The same results were obtained after staining of hippocampal regions with anti-Aβ_{42}-specific monoclonal antibody (**D–G**) or Thioflavin S (**H–K**). Representative images of hippocampal regions of control mice (**D, H**) and mice with low (**E, I**), moderate (**F, J**), and high (**G, K**) anti-Aβ antibody concentrations. Original magnifications, 4$\times$. Scale bar, 500 μm.

mice with moderate concentration, this reduction was not significant, whereas in the mice with a high concentration of serum anti-Aβ_{1-11} antibodies Aβ_{42} load was reduced significantly ($p = 0.018$). Immunostaining of adjacent brain sections with anti-Aβ_{40}-specific antibodies revealed a marked reduction of Aβ_{40} load in all groups of experimental mice versus the controls, independent of serum antibody concentrations (Fig. 4B). However, ThS staining also showed positive correlation between the concentrations of anti-Aβ_{1-11} antibodies and reduction of cortical cored plaques (Fig. 4C), which in APP Tg 2576 mice contain both A$\beta_{40/42}$ peptides (Kawarabayashi et al., 2001). Similar effects were observed in the hippocampal regions of vaccinated APP Tg 2576 mice stained with anti-Aβ_{42}. Representative images of hippocampal regions stained with anti Aβ_{42}-specific monoclonal antibodies (D-G) or ThS (H-K) are shown in Figure 4D–K.

To further confirm an association between serum antibody concentrations and differential effects on reducing Aβ_{40} or Aβ_{42}, we measured insoluble Aβ_{40} and Aβ_{42} in cortical homogenates of experimental and control mice by sandwich ELISA. Insoluble Aβ_{40} and Aβ_{42} concentrations were significantly lower in the cortex of mice vaccinated with the epitope vaccine or fAβ_{42}, compared with control animals (Fig. 5A,B) ($p < 0.001$ and $p < 0.05$, respectively). However, when we compared Aβ levels in groups with low, moderate, and high titers of antibodies, we found a significant reduction of Aβ_{40} and Aβ_{42} only in mice with anti-Aβ_{1-11} antibody levels >50 μg/ml (Fig. 5C,D) ($p < 0.05$ and $p < 0.001$, respectively). Collectively, these results demonstrate that high concentrations of anti-Aβ antibody are critical for effective

reduction of insoluble Aβ in APP Tg 2576 mice with pre-existing AD-like pathology.

A potential problem of immunotherapy is that reduction of insoluble Aβ may lead to increased levels of soluble forms of this peptide (Patton et al., 2006), possibly oligomers, which are known to be more neurotoxic and can impair cognitive function (Klein et al., 2001; Gong et al., 2003; Cleary et al., 2005; Klyubin et al., 2005; Lesne et al., 2006). Accordingly, we measured soluble Aβ_{40} and Aβ_{42} levels in detergent-extracted samples of cortical homogenates of control mice and animals vaccinated with epitope vaccine or fAβ_{42} by capture ELISA (Fig. 5A,B). There were no statistically significant differences between experimental and control mice. In addition, we evaluated the levels of Aβ oligomers in the cortical homogenate by dot blot using A11 anti-oligomeric antibodies (Kayed et al., 2003). All available homogenates from low ($n = 3$), moderate ($n = 7$), and high ($n = 3$) responders as well as control ($n = 5$) mice were used in these experiments. No differences were found between all three groups of immunized mice as well as between vaccinated and control animals (Fig. 5E,F). Of note, no oligomers were detected in brain homogenates from wild-type mice (data not shown). To confirm these results and to measure the relative levels of high molecular weight (>5- to 6-mers) oligomers, the cortical homogenates were subjected to IP followed by WB. The levels of Aβ 5-mers, 9-mer, and 12-mer oligomers in detergent-extracted samples of cortical homogenates obtained from experimental and control mice did not significantly differ, and wild-type mice did not deposit Aβ-reactive oligomers (Fig. 5G,H). Therefore, the level of
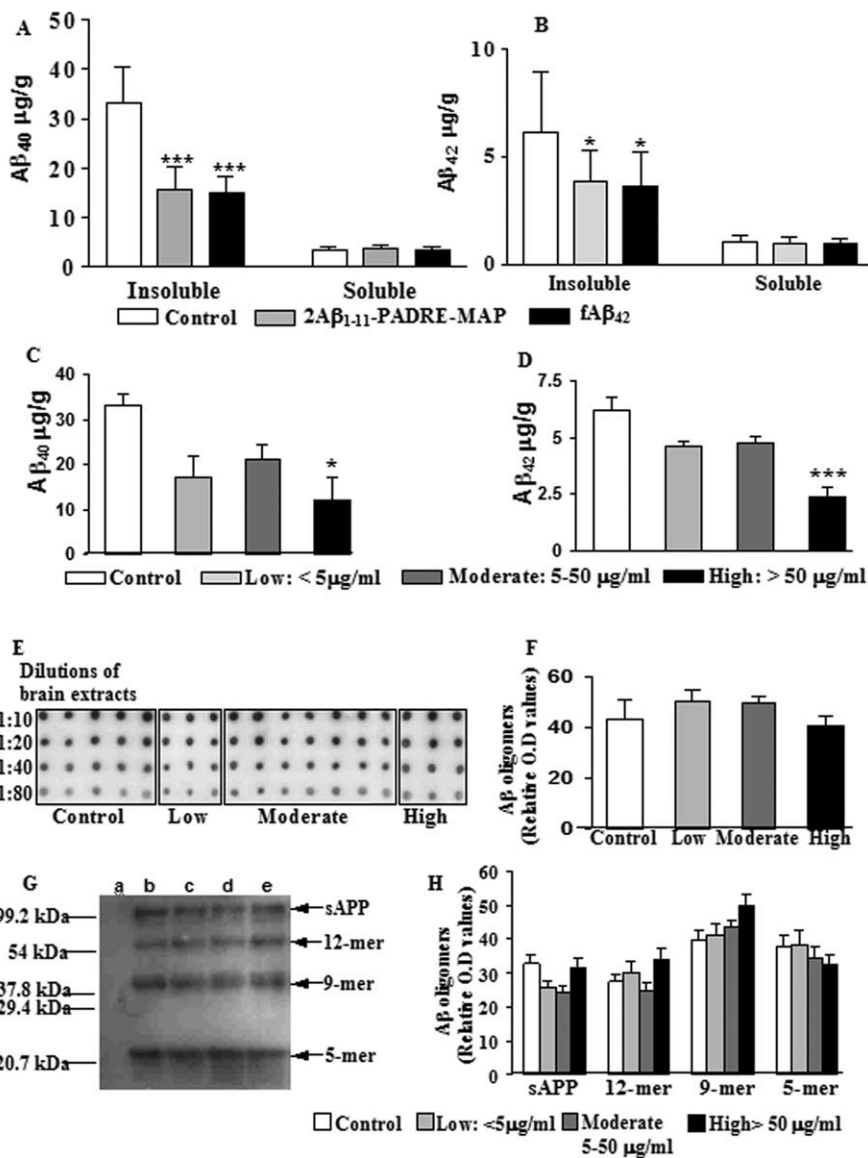
Figure 5. The reduction of insoluble, but not soluble $A\beta_{40}$ and $A\beta_{42}$ levels are positively correlated with the concentrations of anti-$A\beta_{1-11}$ antibody. ***A***, ***B***, A significant decrease in the total (parenchymal and vascular) levels of insoluble $A\beta_{40}$ and $A\beta_{42}$ in cortical homogenates was observed after epitope vaccine and $fA\beta_{42}$ immunization. ***C***, ***D***, The significant reductions in insoluble $A\beta_{40}$ and $A\beta_{42}$ levels were observed only in groups with high titers of antibodies. *$p < 0.05$ and ***$p < 0.001$. However, the levels of soluble $A\beta_{40}$ and $A\beta_{42}$ in cortical homogenates did not differ between experimental and control groups (***A***, ***B***). ***E–H***, The level of oligomeric forms of $A\beta$ detected in cortical homogenates using dot blot (***E***, ***F***) or by combination of IP with WB (***G***, ***H***) also was not changed after vaccination. In dot blot assay, homogenates from individual animals were diluted and analyzed using A11 oligomer-specific antibody (***E***), and relative optical density (***F***) was presented as the average ± SD. Pooled homogenates of each group of mice were analyzed by IP/WB (***G***), and no significant difference between the density and thickness of oligomeric bands (***H***) was detected (with the average ± SD calculated from three independent experiments). Lanes in WB: a, wild-type; b, control; c, low, <5 μg/ml; d, moderate, 5–50 μg/ml; e, high, >50 μg/ml.

oligomeric, toxic forms of $A\beta$ is not reduced in the brain of APP Tg 2576 mice at least when vaccination was initiated in mice with pre-existing AD-like pathology. These differences between reducing insoluble and soluble $A\beta_{42}$ *in vivo* are not connected to the differences in binding affinity of anti-$A\beta_{1-11}$ antibodies to these forms of β-amyloid as we previously demonstrated (Mamikonyan et al., 2007).

The effect of vaccination on microglial activation, lymphocyte infiltration, and microhemorrhages

To detect inflammation-related pathology in the brains of animals immunized with the epitope vaccine, the same brain regions used for $A\beta$ burden studies were evaluated for microglial activation (MHC class II, CD45), astrocyte hypertrophy (GFAP), and microhemorrhages in blood vessels (Prussian blue). Quantitative image analysis of MHC class II (I-A[b]/I-E[b]) positive cells demonstrated significantly decreased microglial activation in APP Tg 2576 mice vaccinated with the epitope vaccine, and this decrease was comparable with that seen in the cerebral cortex of mice immunized with $fA\beta_{42}$ (Fig. 6A). Representative MHC class II immunoreactivity is shown for mice vaccinated with the epitope vaccine for comparison with control animals (Fig. 6B–E). These data are similar to the results obtained after immunostaining of the brain tissues with anti-CD45 antibodies (Fig. 6F–I). Thus, immunization with the epitope vaccine decreased microglial activation in the brains of aged APP Tg 2576 mice.

Astrocytes in both experimental and control mice were detected using the astrocyte-specific marker (GFAP) and a quantitative image analysis indicated that vaccinated mice had a lesser degree of astrocytosis compared with the control group. However, immunization with epitope vaccine led to a 14% reduction, whereas immunization with $fA\beta_{42}$ resulted in ~53% reduction in GFAP immunoreactivity (Fig. 6J). Importantly, immunization with the epitope vaccine reduced astrocytosis to ~50% of the mice with high sera concentrations of antibodies and this decrease is statistically significant (Fig. 6K).

Previously, it was reported that very old APP Tg mice receiving passive transfer of anti-$A\beta$ monoclonal antibodies developed cerebral microhemorrhages (Pfeifer et al., 2002; Wilcock et al., 2004). More recently, it was shown that cerebral amyloid angiopathy-associated microhemorrhages are increased in ~20-month-old APP+presenilin 1 (PS1) transgenic mice immunized eight times with $fA\beta_{42}$ formulated in complete Freunds adjuvant (CFA)/incomplete Freunds adjuvant (IFA) (Wilcock et al., 2007). To assess this potential adverse side effect of epitope vaccine, we selected adjacent coronal sections of mouse brains located at −1.5 bregma and visualized microhemorrhages by detecting hemosiderin (byproduct of degradation of hemoglobin, occurring at the sites of previous microhemorrhages) deposits using Prussian blue staining. Characteristic blue hemosiderin-positive profiles were observed primarily in the neocortical, leptomeningeal, hippocampal and thalamic areas of the brain. These microhemorrhages were detected at a rate of 2.8 ± 0.8 per hemibrain in control nonimmunized mice and 2.6 ± 1.33 in animals immunized with the epitope vaccine. However, vaccination of a
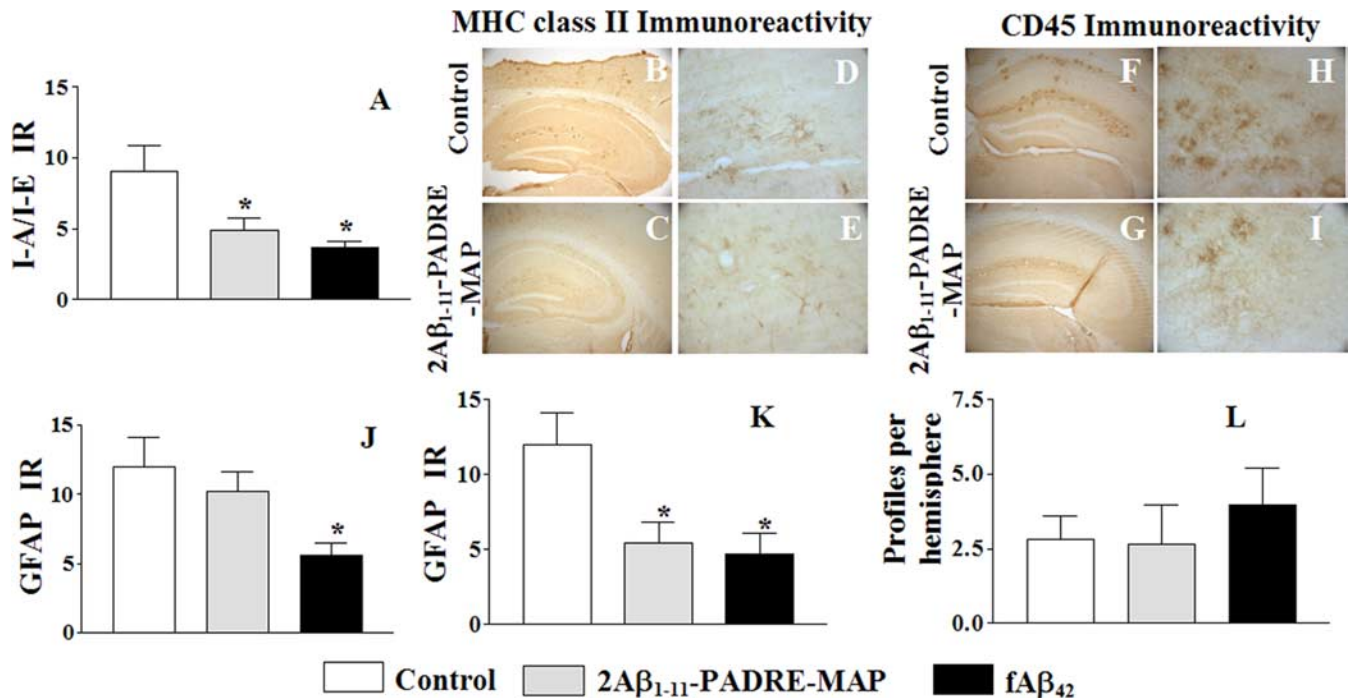
Figure 6. The second generation epitope vaccine reduces glial activation without increasing brain microhemorrhages. **A**, Image analysis of cortex areas from vaccinated or control mice was performed after staining with anti-I-A/I-E antibody. Vaccination either with the epitope vaccine, or fAβ_{42} decreased microglial activation in cortical region of immune mice (*$p < 0.05$) compared with that in control mice. **B–I**, Representative pictures of brain sections stained with anti-I-A/I-E (**B–E**) and anti-CD45 (**F–I**) antibodies showing a decreased immunoreactivity also in the hippocampal brain regions in mice immunized with the epitope vaccine (**C, E, G, I**) compared with control animals (**B, D, F, H**). Original magnifications: **B, C, F, G**, 5×; **D, E, H, I**, 40×. **J, K**, Image analysis of cortical regions from vaccinated or control mice was performed after staining with anti-GFAP antibody. Astrocytosis was significantly (*$p < 0.05$) decreased in mice immunized with fAβ_{42}. A reduction in GFAP load was slightly reduced in all mice vaccinated with the epitope vaccine, but it was significant only in mice with high concentration anti-Aβ antibody. **L**, The number of Prussian blue-positive profiles in the brain hemispheres of experimental and control mice did not differ significantly.

group of APP Tg 2576 mice with fAβ_{42} had resulted in 3.97 ± 1.24 hemosiderin-positive profiles. Although not statistically significant, there was nonetheless approximately a 41% increase compared with the control group, and these data resemble the results generated by (Wilcock et al., 2007) with APP+PS1 aged mice. Thus, immunization with the second generation epitope vaccine did not increase the incidence of cerebral microhemorrhages in ~19-month-old APP Tg 2576 mice (Fig. 6L). Although there is only one unconfirmed report about lymphocyte infiltration detected in the brains of wild-type mice immunized with Aβ_{42} formulated in CFA/IFA followed by injection with pertussis toxin (Furlan et al., 2003), we also investigated whether immunization with the epitope vaccine formulated in Th1 adjuvant might also induce T cell infiltration into the brain. Our standard methodology for detecting T cells in brain vessels of mice (Ghochikyan et al., 2003) did not identify CD3-, CD4-, and CD8-positive T cells or F4/80-positive macrophages in the brains of mice immunized with our second generation epitope vaccine (data not shown). Thus, reduction of insoluble Aβ deposition in immunized APP Tg mice was not associated with deleterious side effects, including cerebral inflammation and microhemorrhages.

Discussion

Published results from the recently halted AN-1792 clinical trial in patients with AD suggested that aseptic meningoencephalitis was linked to an adverse T cell-mediated autoimmune response (Nicoll et al., 2003; Ferrer et al., 2004). Presence of anti-Aβ antibodies correlated with effective reduction of Aβ pathology in patients that came to autopsy (Nicoll et al., 2003; Ferrer et al.,

2004; Masliah et al., 2005), suggesting a possible therapeutic benefit of vaccination (Bayer et al., 2005; Fox et al., 2005; Gilman et al., 2005). We analyzed the effect of different concentrations of anti-Aβ antibodies in serum on neuropathological changes in the brains of actively immunized APP Tg 2576 mice in the absence of an autoreactive anti-Aβ T cell response. We have obtained results somewhat similar to data described in the AN-1792 clinical trial: (1) the higher the concentrations of anti-Aβ antibody specific to the N terminus of Aβ_{42}, the larger the therapeutic effect. The NTB composite z-scores were regressed on the geometric mean antibody titers, although this correlation was not significant (Gilman et al., 2005). (2) AN-1792 vaccine reduced the number of Aβ plaques, but not the level of soluble Aβ in the brains (Patton et al., 2006). To circumvent the side effects of the AN-1792 vaccine and to reduce the potential for cell-mediated autoimmune toxicity in AD patients, we engineered the first (Agadjanyan et al., 2005) generation of epitope vaccine and tested it in wild-type mice. Here, we report on design and testing of the second generation peptide epitope vaccine in APP Tg 2576 mouse model (Hsiao et al., 1996; Kawarabayashi et al., 2001; Westerman et al., 2002), and demonstrated that this prototype AD vaccine induced strong anti-PADRE cellular and anti-Aβ humoral responses (Figs. 1, 2). If this proves to be predictive for human trials and vaccinated individuals induce strong anti-Aβ antibody responses without generating potentially harmful autoreactive T helper cells, our epitope vaccine approach may represent a reasonable alternative to a passive vaccination strategy with humanized anti-Aβ antibody.

The AN-1792 clinical trial data demonstrated significant individual variability in anti-Aβ antibody responses in vaccinated

AD patients (Bayer et al., 2005; Gilman et al., 2005). As mentioned by Patton et al. (2006), only 59 individuals in the immunized cohort induced desirable antibody titers to immunization with $fA\beta_{42}$. Importantly, some of these AD patients showed a trend toward slowing of cognitive decline associated with the disease (Hock et al., 2003), improvement in the memory domain of the NTB and the decreased CSF tau levels (Gilman et al., 2005). Collectively, these data suggested that higher titers of antibodies might be beneficial for AD patients. Because the concentrations of anti-Aβ antibodies in individual APP Tg 2576 mice vaccinated with our epitope vaccine were not uniform either (Fig. 2B) we were able to analyze the association between the neuropathological changes in vaccinated APP Tg 2576 mice with the concentration of anti-Aβ antibodies in the sera of these animals. We observed that the concentration of anti-Aβ antibodies was associated with a therapeutic effect in APP Tg 2576 mice (Fig. 4). Our data also indicated that high concentrations of anti-Aβ antibodies are critical, selectively for the reduction of $A\beta_{42}$ deposits, whereas $A\beta_{40}$ deposits and cored plaques are more responsive to Aβimmunotherapy overall and can be cleared even with low levels of anti-Aβantibodies (Fig. 4). Previous data with APP Tg mice have demonstrated that although Aβ plaques were reduced, the total level of soluble and insoluble Aβ did not differ between immunized and control mice (Janus et al., 2000). Clinical studies demonstrated that immunizations with AN-1792 vaccine mobilize parenchymal plaques and reduce the level of insoluble Aβ, but increase the level of vascular and soluble β-amyloid (Masliah et al., 2005; Nicoll et al., 2006; Patton et al., 2006). These data suggest that the increased level of soluble Aβ may produce conditions that favor formation of toxic oligomeric species of Aβ. To address this issue, we analyzed the levels of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in cortex homogenates obtained from immunized and control APP Tg 2576 mice and demonstrated that vaccination significantly decreased the levels of insoluble $A\beta_{40}$ and $A\beta_{42}$. Importantly, vaccination did not increase the levels of the more toxic soluble Aβ in mouse model of AD (Fig. 5A,B) in contrast to data reported with the AN-1792 vaccine (Patton et al., 2006). Additionally, we demonstrated that only the high concentrations of anti-Aβ antibodies significantly decrease both insoluble $A\beta_{40}$ and $A\beta_{42}$ in cortical tissues of experimental mice (Fig. 5C,D). Thus, our vaccine did not affect the levels of soluble $A\beta_{40}$ and $A\beta_{42}$ in the brains of mice with pre-existing AD-like pathology. Previously it was demonstrated that Aβ neurotoxicity requires insoluble fibril formation (Loo et al., 1993; Lorenzo and Yankner, 1994). However, emphasis has shifted to soluble oligomers as pathological species of $A\beta_{42}$ (Gong et al., 2003; Cleary et al., 2005; Klyubin et al., 2005; Lesne et al., 2006), although aggregation-related toxicity was reported almost a decade earlier (Pike et al., 1991). In APP Tg 2576 mice, memory deficits are first detected in 6-month-old mice accumulating 12-mer oligomers (Lesne et al., 2006) and before overt plaque deposition. Accordingly, we analyzed the levels of oligomers in the brains of APP Tg 2576 mice using an anti-oligomeric antibody (A11), and have demonstrated that immunization with the epitope vaccine did not induce a reduction in immunized compared with control animals (Fig. 5E,F). We further confirmed these by dot blot experiments using Aβ fractions immunoprecipitated from cortical homogenates and then detecting oligomers by Western blotting. Of note, in these experiments we used 12.5% SDS-Tris polyacrylamide gel that allowed the detection of oligomers $\geq$20 kDA. The levels of Aβ oligomers in the brains of experimental and control mice were not significantly different and no oligomers were detected in wild-type mice (Fig. 5G, lane a). These results suggest that either

higher concentration of anti-Aβ antibodies are needed to significantly reduce oligomeric Aβ in brains of immunized mice, or more likely that immunization should be initiated at an earlier age, in mice either without pre-existing Aβ pathology or with early-stage AD-like pathology. Whereas our ongoing prevention immunization studies with the DNA epitope vaccine may allow us to address these hypotheses, we need to mention that multiple transcutaneous immunizations of young PSAPP mice without pre-existing AD-like pathology with $fA\beta_{42}$ and cholera toxin generated high titers of anti-Aβ antibodies ($>$250 μg/ml) that reduced the levels of both insoluble and soluble cerebral Aβ detected in aged mice (Nikolic et al., 2007). Interestingly, analysis from two cases from the AN-1792 clinical trial report also suggest that "anti-amyloid immunization may be most effective not as therapeutic or mitigating measures, but as a prophylactic measure when Aβ deposition is still minimal" (Patton et al., 2006).

It is difficult to predict the effects of frequent passive delivery of high concentrations of humanized anti-Aβ antibodies in ongoing passive immunotherapy trials with AD patients because this treatment approach may still induce undesirable side effects, for example microhemorrhages. Although these clinical studies are important for the future of Aβ immunotherapy, an effective active immunization approach is still feasible providing that the AD vaccine is safe, induces an adequate antibody response to important B cell epitope of Aβ, and is free of harmful autoreactive T cell responses. The second generation epitope vaccine described in this study induced peripheral anti-PADRE-specific Th1-type proinflammatory responses without infiltration of T cells or macrophages in the brains of vaccinated APP Tg2576 mice. It is known that AN-1792 vaccine caused an increase in cerebral vasculature deposition of Aβ (Masliah et al., 2005; Nicoll et al., 2006; Patton et al., 2006). Previously it was also shown that active $A\beta_{42}$ vaccination of double transgenic (APP+PS1) mice resulted in significantly increased cerebral amyloid angiopathy and associated microhemorrhages (Wilcock et al., 2007). Although we did not directly investigate the effect of epitope vaccine on Aβ deposition in cerebral vasculature, we demonstrated that anti-$A\beta_{1-11}$ antibodies did not increase the incidence of cerebral microhemorrhages in the brains of immunized mice (Fig. 6L). Thus, we suggest that an epitope vaccine could be used as a safe and effective measure for the treatment of people with early preclinical stage AD especially if they can be diagnosed by measuring Tau/Aβ and pTau/Aβ ratio in CSF (de Jong et al., 2006; Fagan et al., 2007a,b) and/or detecting of accumulation of Aβ in the brains using Pittsburgh Compound-B positron emission tomography scan (Klunk et al., 2004).

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