The Journal of Immunology

Papillomavirus-Like Particles Are an Effective Platform for Amyloid-β Immunization in Rabbits and Transgenic Mice

Eduardo Zamora, Alessandra Handisurya, Saeed Shafti-Keramat, David Borchelt, Gay Rudow, Katherine Conant, Christopher Cox, Juan C. Troncoso and Reinhard Kirnbauer

J Immunol 2006; 177:2662-2670; ;
doi: 10.4049/jimmunol.177.4.2662
http://www.jimmunol.org/content/177/4/2662

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Papillomavirus-Like Particles Are an Effective Platform for Amyloid-β Immunization in Rabbits and Transgenic Mice[1]

Eduardo Zamora,[2]* Alessandra Handisurya,[2†] Saeed Shafti-Keramat,[†] David Borchelt,[‡] Gay Rudow,* Katherine Conant,* Christopher Cox,[§] Juan C. Troncoso,[3]* and Reinhard Kirnbauer[†]

Immunization with amyloid-β (Aβ) prevents the deposition of Aβ in the brain and memory deficits in transgenic mouse models of Alzheimer's disease (AD), opening the possibility for immunotherapy of AD in humans. Unfortunately, the first human trial of Aβ vaccination was complicated, in a small number of vaccinees, by cell-mediated meningoencephalitis. To develop an Aβ vaccine that lacks the potential to induce autoimmune encephalitis, we have generated papillomavirus-like particles (VLP) that display 1–9 aa of Aβ protein repetitively on the viral capsid surface (Aβ-VLP). This Aβ peptide was chosen because it contains a functional B cell epitope, but lacks known T cell epitopes. Rabbit and mouse vaccinations with Aβ-VLP were well tolerated and induced high-titer autoAb against Aβ, that inhibited effectively assembly of $A\beta_{1-42}$ peptides into neurotoxic fibrils in vitro. Following Aβ-VLP immunizations of APP/presenilin 1 transgenic mice, a model for human AD, we observed trends for reduced Aβ deposits in the brain and increased numbers of activated microglia. Furthermore, Aβ-VLP vaccinated mice also showed increased levels of Aβ in plasma, suggesting efflux from the brain into the vascular compartment. These results indicate that the Aβ-VLP vaccine induces an effective humoral immune response to Aβ and may thus form a basis to develop a safe and efficient immunotherapy for human AD. *The Journal of Immunology,* **2006, 177: 2662–2670.**

Immunization with amyloid-β (Aβ)[4] (4) peptide decreases the deposition of Aβ in the brain of transgenic (tg) mouse models of human Alzheimer's disease (AD). This effect, first reported by Schenk et al. (1), has been confirmed by several laboratories, including ours (2–6). Passive transfer of Ab directed against Aβ also has resulted in reduced Aβ load in brain and improved behavioral performance of tg mice (7). Based on these observations, Aβ immunotherapy has become a leading mechanism-based approach for the therapy of AD. The only trial of Aβ immunotherapy conducted in humans using $A\beta_{1-42,}$ was complicated by meningoencephalitis in some patients (8) and consequently the study was halted. Autopsies in two patients who suffered from encephalitis showed infiltration of the brain with T lymphocytes, but also extensive areas of the neocortex that were largely devoid of Aβ plaques (9, 10). These observations suggest that the immune response generated against the peptide vaccine elicited the clearance of Aβ plaques, but also T cell-mediated toxicity. Moreover, a follow-up study of 30 vaccinees suggests that Aβ-autoAb can slow down cognitive decline in AD patients (11).

Our aim is to develop an improved Aβ vaccine that effectively induces a B cell response capable of abrogating Aβ deposition without inducing a T cell-mediated encephalitis. A potential problem is that Aβ is a self-Ag to which the immune system is normally tolerant and thus might not respond or do so inefficiently. However, potentially self-reactive B-cells may become activated by molecular mimicry of microbial Ags in the pathogenesis of autoimmune diseases (12, 13). Several attempts have been made to provide vaccines that are able to overcome B cell tolerance, e.g., by linkage of self-Ags to foreign Th epitopes (14), or by coapplication of strong adjuvants. Recently, antigenic peptides have been fused or cross-linked to the major capsid protein L1 of papillomaviruses (PV), providing self-assembling empty viral capsids or virus-like particles (VLP), that express the foreign peptide on the particle surface in a repetitive and ordered array (15–17). Immunizations with VLP have induced high-titer and high-avidity (auto)-reactive IgG Ab even without coadministration of adjuvants. The induced autoAb were functionally active in vitro, long-lasting, and could be successfully boosted. Importantly, no adverse effects or induction of autoimmune disease have been observed. In phase III vaccine trials in young women, a human PV type 16 (HPV16) L1 VLP vaccine has proven safe and effective in preventing genital HPV16 infection and associated neoplasias (18), indicating the usefulness of PV capsids as a vaccine platform in humans.

In the present study, we have generated a chimeric fusion protein by incorporating the N terminus of Aβ (1–9 aa) into a hypervariable region of the L1 major capsid protein of bovine PV type 1 (BPV1). Following expression and self-assembly in insect cells, the Aβ-epitope is exposed at high density (360 times) on the surface of an assembled Aβ-VLP (19). The N-terminal peptide comprising

*Department of Pathology, Division of Neuropathology, Johns Hopkins University, Baltimore, MD 21205; †Laboratory of Viral Oncology, Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna, Vienna, Austria; ‡Santa Fe Health Alzheimer's Disease Research Center, Department of Neuroscience, McKnight Brain Institute, University of Florida, Gainesville, FL 32611; and §Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205

Received for publication July 19, 2005. Accepted for publication May 4, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This investigation was supported by Alzheimer's Association Grant 11RG-013097), Johns Hopkins Alzheimer's Disease Research Center Grant NIAAG 05146, and European Commission Grant QLK2-CT-2002-01500 (to R.K.).

[2] E.Z. and A.H. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Juan C. Troncoso, Department of Pathology, Division of Neuropathology, Johns Hopkins University, 558 Ross Building, 720 Rutland Avenue, Baltimore, MD 21205-2196. E-mail address: troncoso@jhmi.edu

[4] Abbreviations used in this paper: Aβ, amyloid-β; tg, transgenic; AD, Alzheimer's disease; PV, papillomavirus; VLP, virus-like particle; HPV, human papillomavirus; BPV1, bovine papillomavirus type 1; NZW, New Zealand White; APP, amyloid precursor protein; PS1, presenilin 1; wt, wild type; TEM, transmission electron microscopy; MHC-II, MHC class II; BBB, blood-brain barrier; RT, room temperature.

0022-1767/06/$02.00

1–9 aa of Aβ was chosen as Ag because it contains a B cell epitope, but it does not include known T cell epitopes (20). Aggregation of Aβ-peptide is a key event in the pathogenesis of AD.

Immunization of a New Zealand White (NZW) rabbit with Aβ-VLP generated Aβ-specific antisera, that prevented the aggregation of Aβ in an in vitro assay of fibrillogenesis. Furthermore, we have examined the effect of immunization on Aβ deposition in vivo, using the amyloid precursor protein (APP)/presenilin 1 (PS1) tg mouse model of AD. Importantly, high-titer autoAb were induced, that cross-reacted with Aβ plaques when tested on human AD brain sections. Trends for reduced amyloid burden in brain were observed, that were associated with increased microglial activation around Aβ plaques.

Materials and Methods

Generation of recombinant baculovirus expressing the Aβ epitope DAEFRHDSG-BPV1 L1 fusion protein and purification of chimeric Aβ-VLP

To generate recombinant baculoviruses expressing the Aβ epitope DAE FRHDSG (corresponding to 1–9 aa of the human Aβ protein) on a predicted surface loop of L1 VLP as a fusion protein, oligonucleotides encoding the epitope were inserted by inverse-touchdown PCR into an immunogenic region (between 133/134 aa) of the BPV1 L1 major capsid protein using pEVmod transfer vector as previously described (17). The forward and reverse primer sequences, respectively, were as follows: 5′-GACATGACTCAGGAACCCAAACAACAGATGAC-3′and 5′- GGAA TTCTGCATCGGTGACTTTTCTATTCAC-3′. Final clones were verified by restriction enzyme digest and by nucleotide sequencing of the inserted sequence and the junctional L1 region. By cotransfection of Sf9 insect cells with transfer vector and linearized baculovirus DNA (BaculoGold; BD Pharmingen), recombinant baculoviruses were generated by standard methods (21). Sf9 insect cells were infected with baculovirus stocks and lysed, and high-molecular-mass structures were separated by density gradient ultracentrifugation. VLP-containing bands were collected and dialyzed against PBS/0.5M NaCl/0.05% NaN_3 (17, 22). BPV1 L1/L2-VLP consisting of wild type (wt) L1 major plus L2 minor capsid proteins were generated in a similar manner (21). Purified chimeric Aβ-VLP were negatively stained and analyzed by transmission electron microscopy (TEM) for self-assembly of L1 and integrity of the VLP (23).

Western blot analysis

Purified Aβ-VLP, wt BPV1 L1-VLP, or lysates of Sf9 cells infected with baculoviruses were analyzed for the presence of Aβ or BPV1 L1 epitopes. Briefly, samples were denatured in sample buffer containing 2% 2-ME, electrophoresed on 10% SDS-PAGE, immunoblotted, and probed with mAb 7B6 (1/100) raised against Aβ 1–12 aa, or mAb AU-1 (1/5000) (BabCo) recognizing a linear BPV1 L1 epitope. Finally, blots were incubated with peroxidase-labeled goat-anti-mouse Ab (Jackson ImmunoResearch Laboratories) and developed using the ECL system.

VLP-ELISA

Purified Aβ-VLP or wt BPV1 L1-VLP were used as Ag in ELISA as described (24). Briefly, native VLP were coated overnight at 4°C onto 96-well microtiter plates (100 ng per well). mAb 6, 9, and 5B6 are BPV1-neutralizing mouse mAb directed against conformation-dependent and type-specific L1 epitopes (25), mAb AU-1 recognizes an internal, linear BPV1 L1 epitope (DTYRYI). Serial dilutions of mAb were added to triplicate wells, developed by peroxidase-labeled goat-anti-mouse Ab, and the peroxidase substrate ABTS, and ODs were determined at 405 nm. Replication variation in the assays was <5%.

Vaccination of animals with Aβ-VLP

A NZW rabbit was inoculated with 100 μg of Aβ-VLP each four times, at 2- to 4-wk intervals, using complete Freund's adjuvant for the prime, and IFA for the boost inoculations. Serum samples were collected before, and 10–14 days after the third and the final immunization. A control rabbit had received wt BPV1 L1-VLP in a similar manner.

APP/PS1 tg mice were derived from bigenic mice line 85, created by coinjecting separate APP and PS1 transgenes. The two constructs encode chimeric mouse/human APP harboring the Swedish mutation K670/M671L (APPswe), and human PS1 encoding exon 9 deletion mutation (PS1E9), each driven by its own mouse prion protein promoter element.

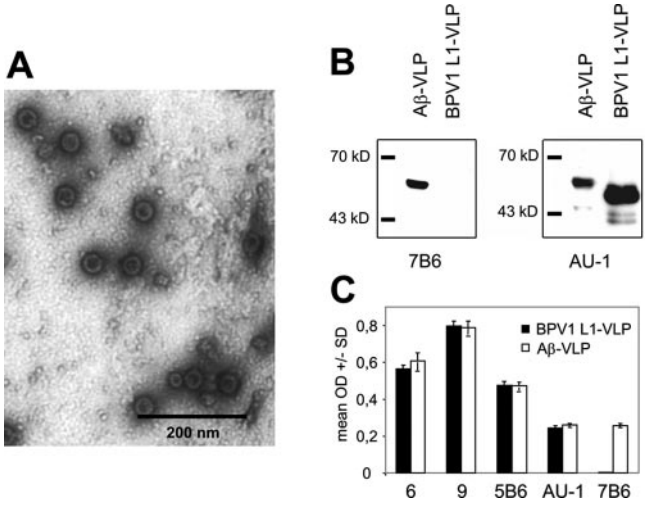

FIGURE 1. *A*, Self-assembly of Aß-BPV1-L1 protein into VLP. Chimeric Aβ-BPV1-L1 protein was expressed in Sf9 insect cells and high-molecular weight complexes were isolated by density gradients. Aliquots of Aβ-VLP were absorbed onto glow-discharged carbon-coated copper grids, negatively stained with 1% uranylacetate and visualized by TEM (magnification ×30,000). *B*, Aβ-VLP express the inserted Aβ epitope DAEFRHDSG. Aliquots of purified Aβ-VLP or wt BPV1 L1-VLP were separated by SDS-PAGE and analyzed by Western blot using mAb 7B6 raised against the N-terminal 12 aa of Aß (*left panel*), or mAb AU-1 directed against BPV1 L1 (*right panel*). *C*, Self-assembled Aβ-VLP display neutralization epitopes of wt BPV1 L1-VLP and the Aβ-epitope on the particle surface. Wt BPV1 L1-VLP (■) and Aβ-VLP (□) were examined by ELISA using conformation-dependent, BPV1-neutralizing mAb 6, 9, and 5B6. MAb 6 and 5B6 bind to pentamers as well as completely assembled VLP, whereas mAb 9 recognizes only complete VLP. MAb 7B6 is directed to the N terminus of Aβ. Dilution of all Ab was 1/100. Bars represent mean OD values of triplicate wells (± SD).

These mice develop Aβ plaques in cerebral cortex and hippocampus at 6 mo that increase in size and number with age. All tg mice were heterozygous and had a C57BL/6 background (26). The animal experiments described in this study were reviewed and approved by Johns Hopkins University School of Medicine Animal Care Committee.

APP/PS1 mice were divided into experimental and control groups. Controls were divided into immunized and nonimmunized animals. Mice in the experimental group ($n = 6$; three males and three females) received 10 μg of Aβ-VLP i.m. in the absence of adjuvant. Mice in the immunized control group received either 10 μg of wt BPV1 L1/L2-VLP ($n = 6$; three males and three females) or PBS ($n = 1$). The nonimmunized control group consisted of age-matched APP/PS1 (two males and two females). All immunized mice (experimental and controls) received the first injection at 3 months of age, before they developed Aβ plaques, followed by a booster 2 wk later, and nine additional monthly injections.

ELISA measurement of autoAb to Aβ peptide in rabbit sera

Rabbit sera were tested by ELISA for reactivity to synthetic Aβ peptide 1–9 aa (DAEFRHDSG), full-length Aβ peptide 1–42 aa, or irrelevant peptide (AVLPPVP) as a specificity control. Streptavidin-coated 96-well plates (Nunc) were coated with 1 μg of peptide, linked at the N terminus via two alanin residues to biotin (JPT Peptide) in 100 mM Tris-HCl (pH 7.5)/150 mM Nacl/0.1% Tween 20 coating buffer overnight at 4°C and blocked with PBS/1% nonfat dry milk. Serial dilutions of rabbit sera were added in triplicates, and after incubation, plates were developed as described above using goat-anti-rabbit peroxidase-labeled Ab (Kjerkegaard & Perry KPL). Replication variation in these assays was <5%. Immune serum dilutions with mean OD_{405} values greater than twice the mean OD value (± SD) of preimmune sera were considered positively immunoreactive.

To further examine specificity, serum of Aβ-VLP immunized rabbit was preabsorbed with either $Aβ_{1–9}$ (DAEFRHDSG) or irrelevant control peptide (AVLPPVP) by incubation in streptavidin-coated plates coated with 5 μg of the respective biotinylated peptide. Subsequently, absorbed sera were analyzed for remaining immunoreactivity to Aβ peptide by ELISA.

Inhibition of Aβ fibrillogenesis by Aβ autoAb

Synthetic human Aβ peptide (1–42 aa) was obtained from Biosource, and an aqueous stock solution prepared at 1 mg/ml. Formation of fibrillar Aβ was induced by incubation at 37°C for 1 wk as previously described (27). To examine the effects of sera raised against Aβ-VLP on fibril formation, rabbit serum was added in various amounts to the dissolved peptide and incubated at 37°C. After 1 wk, samples were applied to carbon-coated grids, negatively stained with uranylacetate, and analyzed by TEM. Fibril formation was quantified using the AxioVision LE software program (Zeiss).

Histology and immunohistochemistry

Mice were euthanized at 12 mo of age by transcardiac perfusion with PBS. Brains were removed and bisected sagittally. One hemibrain was frozen; the other half was fixed in 4% paraformaldehyde for 24 h, cryoprotected in sucrose 30%, frozen, and coronally sectioned at 40-μm intervals. Every eighth section was treated with 88% formic acid for 5 min, followed by a 30 min incubation in 3% H_2O_2 (1 ml of 30% H_2O_2, 8 ml of TBS, 1 ml of methanol), washed in TBS, and blocked for 1 h with 3% normal goat serum in 0.1% Triton X-100 in TBS. Sections were incubated overnight with primary Ab at 4°C, incubated for 1 h with secondary Ab at RT (Vectastain ABC; Vector Laboratories), developed in diaminobenzidine, and counterstained with cresyl violet, following standard histological procedures.

The following primary Ab were used: 6E10 for human Aβ (1/5,000; Signet Laboratories), F4/80 (1/500; Serotec), mac-1 (1/1,000; Serotec) for microglial, IA/IE for MHC class II (MHC-II) of activated microglia (1/1,000; BD Pharmingen), glial fibrillary acidic protein for astroglia (1/500; Dako), CD45R for B lymphocytes (B220, 1/50; BD Pharmingen), and CD3 for T lymphocytes: (1/100; Oncogene Research Products).

We also examined Aβ Ab in the serum of vaccinated mice for binding to Aβ plaques in autopsy brain tissue sections from cases of AD. Four percent of paraformaldehyde fixed-frozen 10-μm sections were blocked in 3% normal goat serum in TBS, incubated overnight with mice sera at 4°C diluted in PBS (1/500 dilution), washed in TBS, incubated for 1 h with secondary Ab, and developed by a DAB kit (Vectastain) as described by manufacturer.

ELISA measurement of autoAb to Aβ 1–42 in mouse plasma

Blood was collected in tubes containing 5 μl of 0.5 M EDTA and immediately centrifuged. Plasma was stored at −80°C until assayed by ELISA. The 96-well plates (U96 Maxisorp; Nalgene) were coated with 100 μl of $Aβ_{1-42}$ peptide (2.5 μg/ml in 50 mM carbonate buffer, pH 9.6), and incubated overnight at 4°C with gentle agitation. Plates were washed four times in PBS/Tween (PBST) and blocked in 5% BSA in PBST for 2 h at room temperature (RT), and 100 μl of the diluted samples were added to each well (at 1/1,000, 1/5,000, and 1/10,000) overnight at 4°C. Plates were washed in PBST and 100 μl of secondary Ab (biotinylated affinipure goat anti-mouse; Jackson Immunoresearch Laboratories) was added (1:20,000) for 2 h at RT. Finally, 1/10,000 neutravidin-HRP (Pierce) was added and the plates developed by SureBlue reagent (KPL). The reaction was stopped by addition of phosphoric acid (J. T. Baker), and plates were read at OD_{450} nm. The anti-$Aβ_{1-42}$ Ab titer was defined as the dilution of antiserum yielding 50% of the maximum signal (1). Qualitative isotyping of Ab was performed using a commercial mouse mAb isotyping kit (IsoStrip; Roche) as described by the manufacturer.

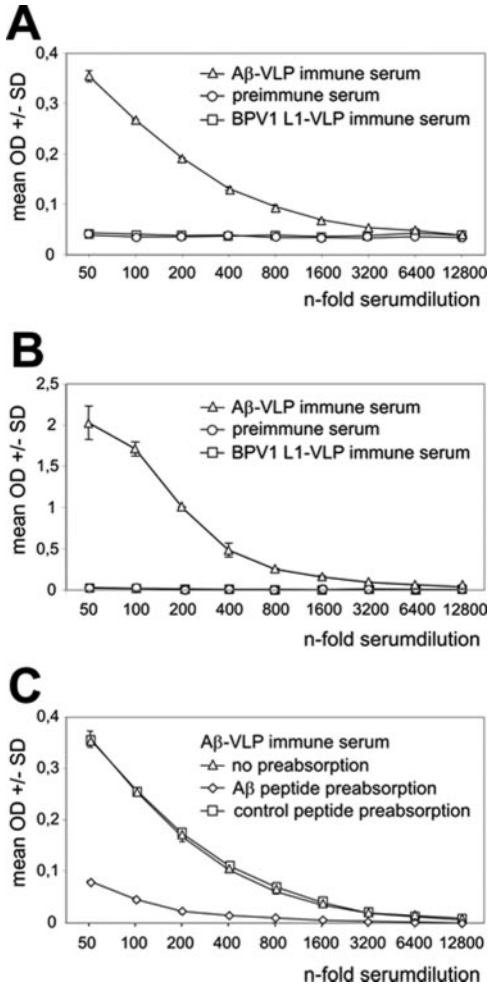

FIGURE 2. Immunization with Aβ-VLP induces specific autoAb against the Aβ epitope. *A* and *B*, Immune serum of an Aβ-VLP immunized NZW rabbit (△) was tested by ELISA using synthetic peptide DAE FRHDSG (*A*) or full-length Aβ 1–42 peptide (*B*) as the capture antigen. Serum of a rabbit immunized with wt BPV1 L1-VLP (□) or rabbit preimmune serum (○) served as controls. *C*, Aβ-VLP-immunized rabbit serum was absorbed by overnight incubation with either Aβ peptide DAE FRHDSG (◇), control peptide AVLPPVP (□), or mock-absorbed (△) prior to analysis by ELISA using the Aβ 1–9 peptide as antigen. Each data point represents the mean OD (± SD) of triplicate wells. Shown is a representative of three experiments.

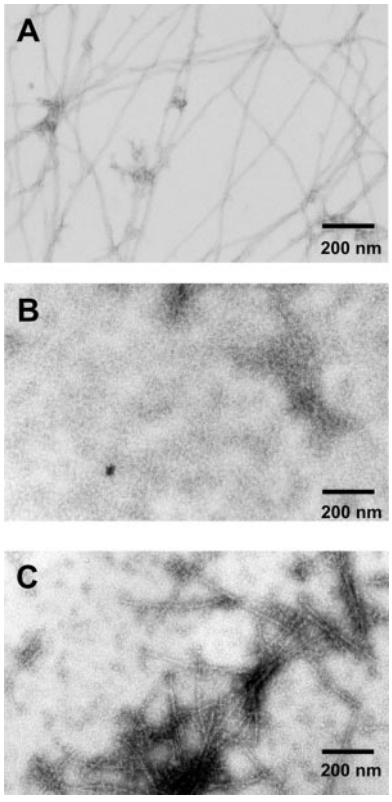

FIGURE 3. Aβ-VLP immune serum inhibits Aβ fibril formation. In aqueous solution, synthetic human Aβ peptide 1–42 aa forms fibrillar aggregates when kept at 37°C for 1 week (*A*). Aβ-VLP-immunized rabbit serum (*B*) or control rabbit serum (*C*) was added to the Aβ peptide in a volume ratio of 1/6 and coincubated at 37°C for 1 week. Samples were absorbed onto carbon-coated copper grids, stained with uranylacetate, and visualized by TEM (magnification ×30,000).

ELISA measurements of Aβ in brain homogenates and plasma

The frozen hemibrain was weighed and homogenized by sonication in 1 ml of PBS, 10 mM EDTA, and 10 μl of 1× protease inhibitor (Sigma-Aldrich). A 200-μl aliquot of each PBS brain homogenate was removed. Protein aggregates were denatured with 300 μl of 8.2 M guanidine-HCl/50 mM, pH was adjusted to 8.0, and 5 μl of protease inhibitor was added to each tube. The samples were mixed for 4 h at RT. 50 μl of sample were then diluted in 950 μl of reaction buffer (1× DPBS plus 5% BSA plus 0.03% Tween 20) and centrifuged at 14,000 rpm at 4°C for 20 min. The supernatant was collected and kept at −80°C until use. $A\beta_{1-40}$ and $A\beta_{1-42}$ in brain and plasma samples were detected by ELISA using a BioSource kit (28).

Measurement of Aβ load in mouse brain by stereology

To assess the Aβ load, the fixed-frozen left cerebral hemisphere was serially sectioned in the coronal plane at 40 μm and all sections were collected. For each brain, a systematic random sample of sections was selected at eight-section intervals. This sampling scheme yielded 10–14 sections that spanned the entire cortex and hippocampus of each hemibrain. Using a light microscope interfaced with the Microbrightfield Stereology System (MBF Bioscience), we compared the area fraction (29) of immunoreactivity for Aβ (sections immunostained with 6E10) in the cortex and hippocampus as described previously (2).

Results

Chimeric Aβ-BPV1-L1 protein self-assembles into VLP that express the Aβ-DAEFRHDSG epitope on the particle surface

Multimeric Ags, particularly on particle surfaces such as viruses or VLP, can often directly stimulate B cell proliferation and differentiation into Ab-secreting plasma cells by efficient cross-linking of the B cell receptor, responses which can be T cell independent. To generate chimeric proteins that display an Aβ-B cell epitope on the surface of a self-assembled viral particle, the N-terminal (1–9 aa) peptide DAEFRHDSG of Aβ was genetically engineered into an immunogenic region of L1 major capsid protein of BPV1 (17).

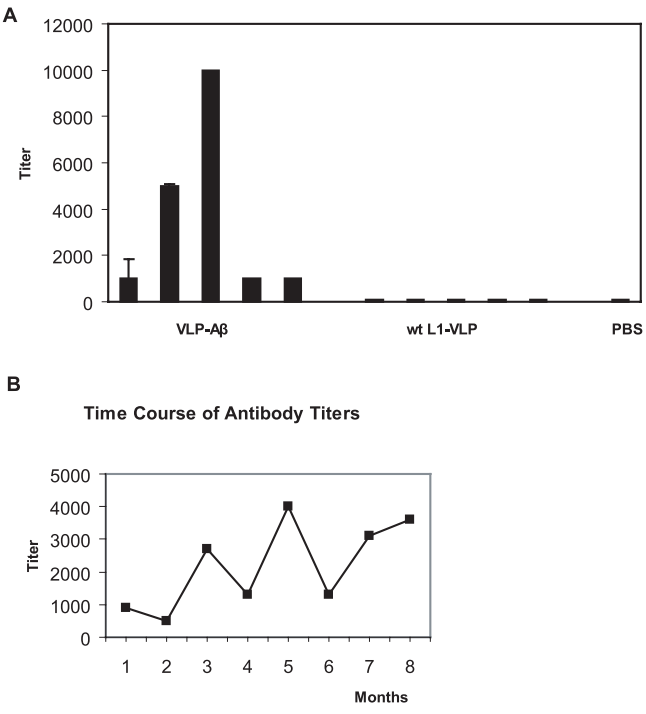

FIGURE 4. *A*, ELISA measurement of autoAb to Aβ following the last immunization. Mice immunized with Aβ -VLP (n =5) developed a specific $A\beta_{1-42}$ autoAb response, that was absent in control animals injected with wt L1-VLP or PBS (n = 6; p = 0.05). Columns represent mean Ab titer of triplicate measurements for each animal ± SE. *B*, Time course of Ab titers in mice immunized with Aβ-VLP (n = 5). Each point represents the mean Ab titer of the five immunized animals.

Particles were purified from Aβ-L1 recombinant baculovirus-infected cells by density gradients (17). Analysis by TEM revealed circular structures of ~50- to 55-nm diameter (Fig. 1*A*), indicating that chimeric Aβ-BPV1-L1 protein self-assembed into full-size VLP that are structurally similar to wt BPV1 L1-VLP (30). In addition, smaller particles and capsomers (the pentamer subunits of VLP, each consisting of five L1 molecules) were observed (15, 21).

To verify antigenicity of the fusion protein, in particular expression of the predicted Aβ epitope DAEFRHDSG, purified Aβ-VLP were analyzed by immunoblotting. The mAb 7B6, directed against the N terminus of Aβ, recognized a band of ~55 kD, corresponding to the expected MW of the Aβ-L1 fusion protein (Fig. 1*B*, *left panel*). Specific immunoreactivity was absent with parental wt BPV1 L1-VLP. In contrast, mAb AU-1 (Fig. 1*B*, *right panel*), which is directed against a linear epitope of BPV1 L1, reacted with both Aβ-L1 and wt BPV1 L1. As expected, Aβ-L1 migrated slower than wt BPV1 L1 at ~60 kD, consistent with the higher MW of the Aβ-L1 fusion protein.

To determine whether Aβ-VLP retain antigenic surface structures of wt L1-VLP, immunoreactivity was examined by ELISA under nondenaturing conditions, using conformation-dependent and neutralizing mAb directed against the BPV1 L1 capsid protein. mAb 6 and 5B6 bind to both intact VLP as well as pentamer subunits, whereas mAb 9 requires assembly into the higher-ordered structure of VLP for binding (17, 25, 31). In contrast, the nonneutralizing mAb AU-1 recognizes an internal, linear epitope of L1. As shown in Fig. 1*C*, reactivity patterns of mAb 6, 9, and 5B6 with chimeric Aβ-VLP (□) were comparable to wt VLP (■), indicating correct assembly of chimeric Aβ-BPV1 L1 protein into capsomers subunits that form complete VLP similar to wt L1. mAb AU-1 reacted with both protein preparations, demonstrating the presence of small amounts of nonassembled L1 protein as observed regularly in purified VLP preparations. In addition, expression of the inserted Aβ peptide by native Aβ-VLP was analyzed by ELISA using mAb 7B6. As shown in Fig. 1*C*, mAb 7B6 reacted specifically with Aβ-VLP, but not with parental BPV1 L1-VLP, indicating display of the Aβ epitope on the VLP surface.

Immunization with Aβ-VLP induces a high-titer autoAb response to the Aβ peptide

We next sought to determine whether immunizations with Aβ-VLP are capable of inducing an autoAb response against Aβ peptide and thus breaking B cell tolerance to this self-Ag. NZW rabbits were chosen for vaccinations, because the $A\beta_{1-9}$ peptide DAEFRHDSG, displayed by the Aβ-VLP vaccine, has 100% amino acid sequence identity between rabbit and human, thus representing a useful model to test for autoAb induction. Rabbits were inoculated four times s.c. with 100 μg of Aβ-VLP, or parental L1-VLP as a control, prepared in complete Freund's (prime) or IFA (boosts), and sera were obtained before and after the final immunization. No signs of toxicity or adverse effects have been observed during a 3-mo period.

To determine the humoral autoimmune response, rabbit sera were tested by ELISA using $A\beta_{1-9}$ peptide DAEFRHDSG as the capture Ag. The synthetic Aβ peptide was linked via a 2-alanin spacer to biotin, to ensure accessibility following attachment to streptavidin-coated microtiter plates. Immunization of a NZW rabbit with Aβ-VLP induced Aβ-autoAb with a titer of 1600 (Fig. 2*A*). In contrast, no specific reactivity was observed testing preimmune serum or serum of a wt L1-VLP immunized rabbit. In addition, reactivity of the Aβ-VLP immunized rabbit sera was absent when an irrelevant control peptide of similar length (AVLP PVP) was used as ELISA Ag (data not shown). Rabbit sera were

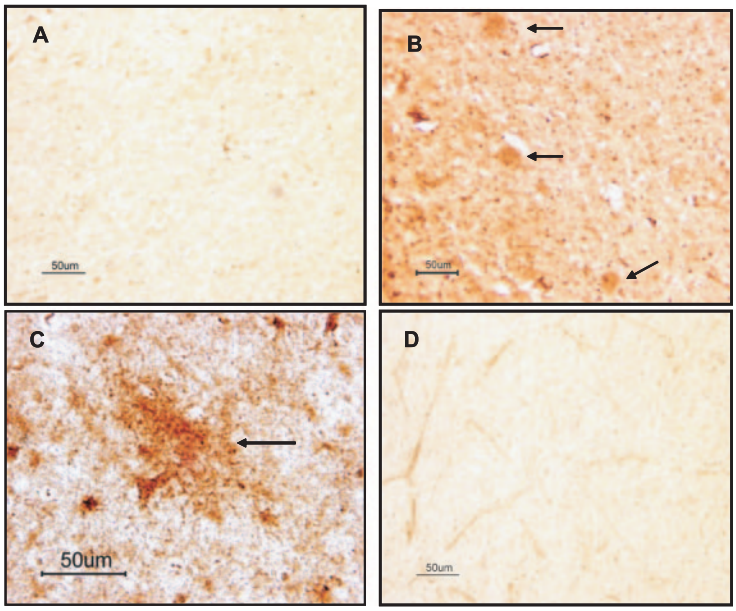

FIGURE 5. Cerebral cortex from an AD case immunostained with mice sera. *A*, Aβ-VLP preimmune sera; *B*, postimmune sera (after third dose). Arrows show areas of plaques. *C*, Higher magnification of an amyloid plaque (arrow) immunostained with Aβ-VLP postimmune sera (after third dose). *D*, wt L1-VLP postimmune sera control (after third dose).

also tested for immunoreactivity to full-length $A\beta_{1-42}$, demonstrating similar results as compared with those obtained with $A\beta_{1-9}$ peptide (Fig. 2*B*).

To further confirm specificity of these results, the rabbit Aβ-VLP immune serum was absorbed with $A\beta_{1-9}$ peptide (DAE FRHDSG) or control peptide (AVLPPVP) (Fig. 2C). In comparison to untreated serum, absorption with Aβ peptide reduced immunoreactivity to Aβ to a large extent (>80% at the lowest serum dilution), whereas preabsorption with the control peptide had no effect. As expected, Aβ-VLP immunization of rabbit induced high titer Ab (>600,000) directed against the wt VLP carrier (data not shown).

Antisera to Aβ-VLP inhibit Aβ fibrillogenesis in vitro

Prolonged incubation of soluble full-length $A\beta_{1-42}$ peptide induces spontaneous aggregation into amyloid fibrils with neurotoxic activity (27). To assess the protective potential of Aβ-VLP-induced autoAb, we determined the effect of immune sera on the formation of amyloid fibrils in vitro. Antisera of either Aβ-VLP immunized, or wt L1-VLP immunized control rabbits, were added to an aqueous solution of $A\beta_{1-42}$ peptide (1 mg/ml) and incubated at 37°C for 1 wk. In the absence of added serum, numerous fibrillar aggregates were visualized by TEM of negatively stained samples (Fig. 3*A*). Addition of Aβ-VLP immune serum at a 1:6 v/v ratio of Aβ peptide resulted in complete inhibition of Aβ fibril formation (Fig. 3*B*). Specific inhibition was observed with decreasing volume ratios up to 1:60 (data not shown). Unspecific inhibition was observed, when serum raised against wt L1-VLP was added. The number of fibrils was reduced as compared with untreated Aβ peptides, although to a much lesser extent, compared with anti-Aβ-VLP serum-treated preparations (Fig. 3*C*).

To quantify the specific effect of Aβ-VLP antisera, fibril number and length were measured using micrographs taken from different independent grids. In the absence of added serum the mean number of fibrils was 212.5 with a mean length of 50304.6 nm. Addition of Aβ-VLP immunized rabbit serum decreased the fibril number by 98.6% (mean, three fibrils) and length by 92.8% (mean, 3622.9 nm). The addition of control serum at the same dilution resulted in a mean fibril number of 64.3 and mean fibril length of 22,828 nm (reduction by 69.7 and 54.6%, respectively).

Induction of autoAb to Aβ in a tg mouse model of AD

APP/PS1 tg mice derived from bigenic mice line 85 were used in this experiment. These mice develop Aβ plaques in the cerebral cortex and hippocampus at 6 mo that increase in size and number with age. APP/PS1 tg animals vaccinated with Aβ-VLP tolerated the immunization well and developed specific autoAb to $A\beta_{1-42}$ as determined by ELISA, with titers in the range of 1,000 to 10,000, compared with control animals immunized with wt L1-VLP (or PBS) (Fig. 4*A*) ($p = 0.05$; mean, 3600). Ab titers were low following the initial immunizations, rose significantly after the third injection, and remained detectable throughout the whole period of vaccination (Fig. 4*B*). Isotyping of the $A\beta_{1-42}$ specific Ab detected the presence of IgG1, IgG2a, IgG2b, and IgG3 (data not shown).

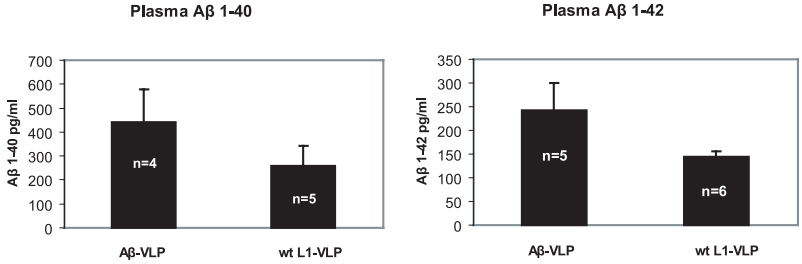

FIGURE 6. ELISA measurement of Aβ peptide in plasma. Immunization of tg mice with Aβ-VLP resulted in a trend for increased levels of $A\beta_{1-40}$ ($n = 4$; $p = 0.115$) and $A\beta_{1-42}$ ($n = 5$; $p = 0.115$) in plasma, compared with wt L1-VLP vaccinated controls ($n = 5$ and 6, respectively). Columns indicate mean Aβ levels (pg/ml; ± SE).

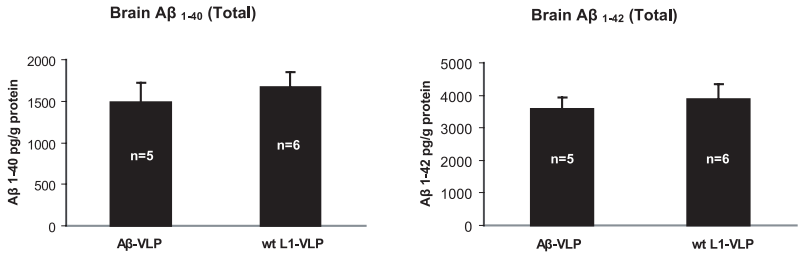

FIGURE 7. ELISA measurements of Aβ in brain homogenates. APP/PS1 mice immunized with Aβ-VLP showed a trend for decrease in total brain $A\beta_{1-40}$ ($n = 5$; $p = 0.565$) and $A\beta_{1-42}$ ($n = 5$; $p = 0.997$) in comparison to wt L1-VLP-immunized controls (n = 6 and 6, respectively), as assessed by ELISA. Columns indicate mean Aβ levels (pg/g of protein; ± SE).

We also examined Aβ Ab in the serum of vaccinated mice for their capacity to bind to Aβ plaques in autopsy brain tissue sections from cases of AD. Sera from Aβ-VLP-vaccinated mice recognized amyloid plaques in brain tissue sections from an AD case. In contrast, preimmune sera from the same mice as well as sera from wt VLP-immunized mice did not show any immunoreactivity (Fig. 5).

Levels of Aβ in plasma of APP/PS1 tg mice

We next examined whether circulating autoAb were associated with increased Aβ plasma levels, which would indicate efflux of Aβ from the brain into the plasma, as proposed by the peripheral sink hypothesis (32, 33). When measured by ELISA, we observed a trend for elevated plasma levels of $A\beta_{1-40}$ (71.02%, $p = 0.115$), and $A\beta_{1-42}$ (68.71%, $p = 0.115$) in animals immunized with Aβ-VLP, compared with control mice injected with wt VLP or PBS (data not shown) (Fig. 6).

Levels of total Aβ in brain homogenates

To examine whether Aβ-VLP immunizations decrease the levels of Aβ in the brain, we measured total Aβ in brain homogenates. Trends for lower levels of total Aβ in brain tissues from Aβ-VLP immunized tg mice as compared with wt VLP immunized controls were detected (Fig. 7), for $A\beta_{1-40}$ between 5.72 and 54% ($p = 0.565$), and for $A\beta_{1-42}$ between 2.68 and 24.86% ($p = 0.997$).

To assess a possible effect of Aβ immunization on Aβ plaque burden in the brain, the fractional area of Aβ was measured by stereology. Aβ-VLP-immunized animals showed trends for reduced Aβ fractional area both in cerebral cortex (23.07%; $p = 0.154$) and hippocampus (28.83%; $p = 0.343$) (Fig. 8). Interestingly, we observed the greatest reduction of Aβ burden in the animal with the highest Ab titer, i.e., 48.07% in the cortex and 97.8% in the hippocampus.

Activation of microglia in the brain of control and Aβ-VLP-immunized APP/PS1 mice

Microglia cells have been shown to become activated following active or passive Aβ immunization, both in humans and mice (9, 34, 35). For this reason, we examined microglia in control and Aβ-VLP-immunized mice by immunostaining for MHC-II, a marker of cell activation. By measuring the fractional area of MHC-II positivity, we observed a trend for increase in MHC-II immunoreactivity in Aβ-VLP-vaccinated mice compared with controls, both in cortex ($p < 0.061$) and hippocampus ($p < 0.085$), (Fig. 9). Both groups of animals showed reactive microglia, however, a larger number of MHC-II-positive microglial cells were observed in "clusters" at the periphery of amyloid plaques in Aβ-VLP immunized mice, compared with control animals immunized with wt VLP (or PBS; data not shown) (Fig. 10). Morphologically, MHC-II-positive microglial cells demonstrated enlargement of the soma, and thickening and shortening of their processes. These changes appeared more often in Aβ-VLP vaccinated mice, compared with wt BPV1 L1-VLP controls (Fig. 10). Most importantly, immunostains of brain sections with Ab for T (CD3) and B (CD45) lymphocytes were negative in both groups of vaccinated animals (data not shown), indicating the lack of a brain-infiltrating cellular autoimmune response.

Discussion

The aim of this study is to develop a safe and effective vaccine for human AD, that generates a strong Ab response against the Aβ self-Ag, but avoids induction of a T cell response, to minimize the risk of cell-mediated autoimmune encephalitis (8). In this study, we have generated a new type of VLP-based vaccine directed against the N terminus of Aβ. The Aβ-VLP vaccine is comprised of an Aβ B cell epitope inserted into a PV major capsid protein L1. This chimeric Aβ-L1 protein has the intrinsic capacity to self-assemble into a VLP that directs expression of the Aβ epitope at high density, and in a repetitive and regularly spaced manner, to the particle surface. This vaccination elicits high-titer of Aβ-specific autoAb in two animal species. Importantly, immune sera inhibited the formation of neurotoxic Aβ aggregates in vitro, indicating that autoAb targeting $A\beta_{1-9}$ peptide are functionally active to prevent fibrillogenesis. Because the Aβ epitope is 100% conserved between rabbit and human, this approach has therapeutic potential for the prevention or therapy of AD.

The degree of Ag organization is an important discriminator by B-cells for self/nonself. Highly organized Ags such as VLP can induce T cell-independent, extremely efficient B cell responses and may eventually break B cell unresponsiveness to self-Ags (16, 17, 36). In addition, immunization with PV-VLP-like particles have been shown to mediate T cell proliferative (CD4 and CD8) and cytokine responses (37), and thus may provide additional help for induction of efficient Ab responses. These data indicate that VLP

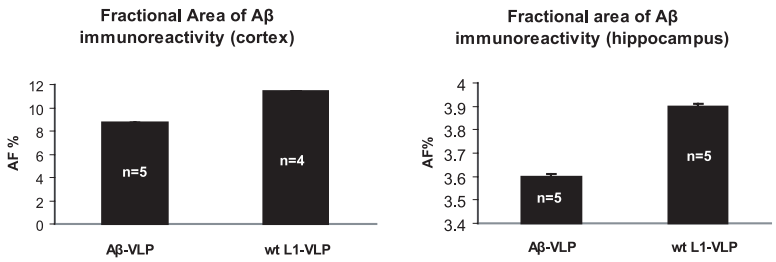

FIGURE 8. Brain measurement of Aβ load by stereology. Immunoreactivity was measured in the cortex ($n = 5$; $p = 0.061$) and hippocampus ($n = 5$; $p = 0.085$) of mice vaccinated with Aβ-VLP, compared with wt VLP-immunized control mice ($n = 4$ and 5, respectively). Bars indicate SE.

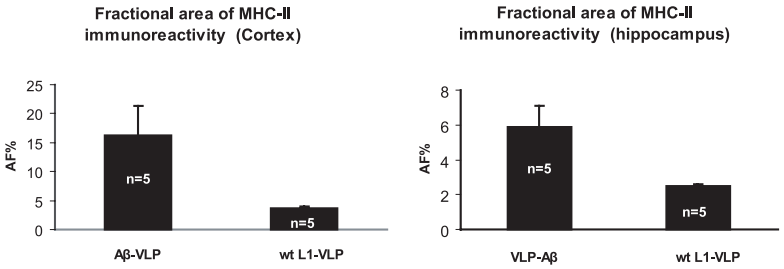

FIGURE 9. Fractional area of MHC-II (marker of activated microglia). A trend for an increase in the fractional area (AF %) of Aβ immunoreactivity was measured in the cortex ($n = 5$; $p = 0.154$) (*A*) and hippocampus ($n = 5$; $p = 0.343$) (*B*) of mice vaccinated with Aβ-VLP ($n = 5$), compared with wt L1-VLP-immunized control mice ($n = 5$). Bars indicate SE.

vaccination induces both the innate and adaptive arms of the immune system, consistent with the activation of APCs, such as monocytes and dendritic cells, or granulocytes. Each Aβ-VLP as described in this study carries 360 copies of the Aβ B cell epitope in a closely spaced and highly ordered (para-cristalline) array on immunodominant capsid surface loops. Vaccination efficiently induced potentially protective autoAb directed against the Aβ target peptide, which is a major regulatory site involved in formation of Aβ plaques (38). Aβ-VLP that incorporate Aβ peptide$_{1-9}$ are a significant technological advance over previous full-length Aβ vaccine formulations, as they appear to carry a low risk of inducing cell-mediated encephalitis due to the avoidance of predicted Aβ T cell epitopes. In addition, Aβ-VLP have the flexibility to incorporate larger peptides of Aβ, which might increase immunogenicity even further. Recently, a VLP-based vaccine has been generated, in which Aβ$_{1-40}$ has been chemically linked to BPV1 L1-VLP. Immunizations with this type of vaccine demonstrated the ability to induce autoAb against Aβ in APP tg mice, and VLP vaccination was superior to peptide vaccination (39). However, unfavorable results from the ELAN study preclude further use of full-length Aβ as a vaccine candidate in humans.

As shown previously, Ab that effectively reduce pathology in AD brain (40) recognize the N-terminal 16 aa of the Aβ protein, presumably because it contains a dominant B cell epitope that is accessible to Ab either in solution and/or on the surface of the plaques (20). Similar to our results shown in this study, "effective" Ab also recognized amyloid plaques in brain sections of AD patients.

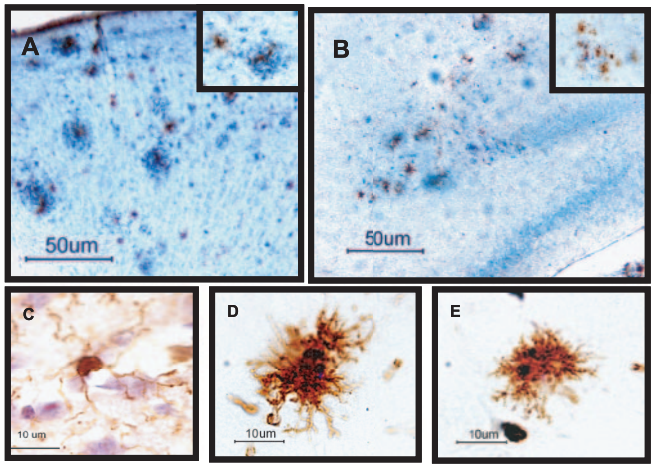

FIGURE 10. Coronal brain sections and wt L1-VLP-vaccinated mice (control) (*A*) and of Aβ-VLP-vaccinated mice (*B*). Double immunostaining was performed with Ab against the microglial activation marker MHC-II (IA/IE) (brown immunoreactivity) and against Aβ (6E10) (blue immunoreactivity). Aβ-VLP-vaccinated mice showed an increased number of MHC-II immunoreactive cells, compared with control mice. *C*, Shows F4/80 immunostaining of a "resting" microglial cell in the brain of a C57BL/6 control mouse. *D* and *E*, Demonstrate activated microglial cells immunostained with IA/IE (marker for MHC-II) in an Aβ-VLP vaccinated mouse. Note the increased size of the soma and the thickening and retraction of the cell processes.

Several potential mechanisms may underlie the efficacy of Aβ-autoAb to enhance the clearance of Aβ from the brain of APP/PS1 mice. These include the prevention of neurotoxic fibril formation (27), efflux of soluble Aβ from the brain to the plasma, also known as the peripheral sink mechanism (32), and microglia-mediated removal of Aβ (1, 7, 34, 35, 41). Results from our study indicate that all three mechanisms may be at work. First, Aβ-VLP vaccination induced immune sera that inhibit Aβ-fibrillogenesis in vitro. Importantly, relatively small amounts of antiserum (volume ratios of 1/60 vs peptide solution) were sufficient for efficient inhibition. Although only about one percent of Ab generated after immunization will cross the blood-brain barrier (BBB), this may suffice to prevent APP aggregation, and favor removal from the brain parenchyma (7). Second, we observed a decrease of Aβ levels in brain and a concomitant elevation of Aβ in the plasma of Aβ-VLP vaccinated animals compared with controls, suggesting an efflux of soluble Aβ species from the brain parenchyma across the BBB into the vascular compartment, where it is bound by circulating Ab (32). Third, a stronger activation of microglia associated with plaques in Aβ-VLP immunized mice was detected as compared with controls. This is in agreement with a hypothesis of brain Aβ removal by microglial FcR-mediated phagocytosis, following Ab binding to Aβ plaques (7, 34, 35). The above mechanisms are not mutually exclusive and may act synergistically to reduce Aβ burden in the brains of APP/PS1 mice. Although previous studies have demonstrated the independent existence of these mechanisms, ours is the first to present evidence in support of the three mechanisms acting simultaneously.

Stereological assessment of cerebral cortex and hippocampus revealed a trend for reduced Aβ deposits. Analysis of brain homogenates by ELISA showed lower levels of total Aβ$_{1-40}$ and Aβ$_{1-42}$ in Aβ-VLP-vaccinated, compared with control animals. The lack of a stronger and statistically significant effect of Aβ-VLP immunization on Aβ load may be attributed to several factors, including the affinity of the generated autoAb for Aβ, the strain and transgenes of the mice, and also the very small number of animals examined. However, measurement of Aβ load is based on the detection of deposited Aβ, and it is not a reliable indicator of the presence and concentration of soluble Aβ species. In the future, we plan to study larger groups of mice to examine efficacy of VLP vaccines that incorporate longer Aβ peptides.

Our neuropathological observations revealed a strong effect of Aβ immunization on microglial cells. In the brains of APP/PS1 mice immunized with Aβ-VLP, microglial cells appeared plump with short and thick processes, and a large number of these cells were localized predominantly in "clusters" at the periphery of the plaques, compared with controls. Measurements of fractional areas of MHC-II, a marker of microglial activation, showed a trend for

increase in MCH-II positive microglial cells in Aβ-VLP vaccinated mice compared with controls. These changes suggest that immunizations with Aβ-VLP of this specific strain of mice (APP/PS1, Line 85) activate microglia and recruit it to the periphery of plaques to remove Aβ. One possible mechanism of microglial activation could be the passage of specific anti-Aβ Ab across the BBB and into the brain parenchyma. Subsequently, specific anti-Aβ Ab would opsonize Aβ, bind to microglial FcR, and thereby promote microglia activation and Aβ phagocytosis (35). It should be noted that among mice vaccinated with Aβ-VLP, marked microglial activation was observed in three of the five animals examined.

Although vaccines using the whole Aβ 1–42 sequence (2) are more efficient than the Aβ-VLP vaccine used in this study in terms of reducing the Aβ load in the brain of tg mice, administration of the whole peptide has two problems. It requires the administration of an adjuvant, which may produce toxicity, and entails the injection of T cell epitopes, presumable responsible for cell-mediated encephalomyelitis that occurred in the human vaccination trial.

The general safety of autoAb induction as an approach to immunotherapy of AD must obviously be considered seriously. Notably, the immunized rabbit producing autoAb to Aβ appeared healthy over 5 mo. Importantly, we also did not observe systemic or cerebral toxicity in immunized mice. In particular, there was no evidence for infiltration of brains with B- or T cells, and there were no detectable mononuclear cells in perivascular cuffs or in the subarachnoid space. This suggested that the $A\beta_{1-9}$ Ag exposed on VLP did not elicit a T cell response directed against brain parenchyma, probably because most of the T cell epitopes of Aβ are located beyond Aβ 15-aa residue (20). As additional safety feature, vaccination with HPV L1-VLP has already been conducted in thousands of young adults in late phase trials and proven safe and effective (18, 42).

The use of VLP-based vaccines that induce autoAb specific to disease targets could be an effective alternative to passive administration of Ab or peptide vaccinations. Possible advantages are more constant Ab levels over time, the need for less frequent administration, and the absence of an inactivating Ab response to therapeutic Ab. In addition, peptide vaccinations frequently suffer from low immunogenicity resulting in low-titer and short-lived Ab levels, even when potent adjuvants are used. Thus, our observations of Aβ autoAb production suggest that Aβ-VLP could be considered as a possible formulation in future trials of Aβ immunization in humans.

Acknowledgments

We thank Dr. Joanna Jankowsky for her advice with the breeding of tg mice, and Audrey Horter and Karen Wall for preparation of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Schenk, D., R. Barbour, W. Dunn, G. Gordon, H. Grajeda, T. Guido, K. Hu, J. Huang, K. Johnson-Wood, K. Khan, et al. 1999. Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* 400: 173–177.
2. Vehmas, A. K., D. R. Borchelt, D. L. Price, D. McCarthy, M. Wills-Karp, M. J. Peper, G. Rudow, J. Luyinbazi, L. T. Siew, and J. C. Troncoso. 2001. β-Amyloid peptide vaccination results in marked changes in serum and brain Aβ levels in APPswe/PS1ΔE9 mice, as detected by SELDI-TOF- based ProteinChip technology. *DNA Cell Biol.* 20: 713–721.
3. Sigurdsson, E. M., H. Scholtzova, P. D. Mehta, B. Frangione, and T. Wisniewski. 2001. Immunization with a nontoxic/nonfibrillar amyloid-β homologous peptide reduces Alzheimer's disease-associated pathology in transgenic mice. *Am. J. Pathol.* 159: 439–447.
4. Morgan, D., D. M. Diamond, P. E. Gottschall, K. E. Ugen, C. Dickey, J. Hardy, K. Duff, P. Jantzen, G. DiCarlo, D. Wilcock, et al. 2000. A β-peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature* 408: 982–985.
5. McLaurin, J., R. Cecal, M. E. Kierstead, X. Tian, A. L. Phinney, M. Manea, J. E. French, M. H. Lambermon, A. A. Darabie, M. E. Brown, et al. 2002. Therapeutically effective antibodies against amyloid-β peptide target amyloid-β residues 4-10 and inhibit cytotoxicity and fibrillogenesis. *Nat. Med.* 8: 1263–1269.
6. Dodart, J. C., K. R. Bales, K. S. Gannon, S. J. Greene, R. B. DeMattos, C. Mathis, C. A. DeLong, S. Wu, X. Wu, D. M. Holtzman, and S. M. Paul. 2002. Immunization reverses memory deficits without reducing brain Aβ burden in Alzheimer's disease model. *Nat. Neurosci.* 5: 452–457.
7. Bard, F., C. Cannon, R. Barbour, R. L. Burke, D. Games, H. Grajeda, T. Guido, K. Hu, J. Huang, K. Johnson-Wood, et al. 2000. Peripherally administered antibodies against amyloid β-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat. Med.* 6: 916–919.
8. Senior, K. 2002. Dosing in phase II trial of Alzheimer's vaccine suspended. *Lancet Neurol.* 1: 3.
9. Nicoll, J. A., D. Wilkinson, C. Holmes, P. Steart, H. Markham, and R. O. Weller. 2003. Neuropathology of human Alzheimer disease after immunization with amyloid-β peptide: a case report. *Nat. Med.* 9: 448–452.
10. Ferrer, I., M. Boada Rovira, M. L. Sanchez Guerra, M. J. Rey, and F. Costa-Jussa. 2004. Neuropathology and pathogenesis of encephalitis following amyloid-β immunization in Alzheimer's disease. *Brain Pathol.* 14: 11–20.
11. Hock, C., U. Konietzko, J. R. Streffer, J. Tracy, A. Signorell, B. Muller-Tillmanns, U. Lemke, K. Henke, E. Moritz, E. Garcia, et al. 2003. Antibodies against β-amyloid slow cognitive decline in Alzheimer's disease. *Neuron* 38: 547–554.
12. Karlsen, A. E., and T. Dyrberg. 1998. Molecular mimicry between non-self, modified self and self in autoimmunity. *Semin. Immunol.* 10: 25–34.
13. Rose, N. R. 1998. The role of infection in the pathogenesis of autoimmune disease. *Semin. Immunol.* 10: 5–13.
14. Dalum, I., M. R. Jensen, P. Hindersson, H. I. Elsner, and S. Mouritsen. 1996. Breaking of B cell tolerance toward a highly conserved self protein. *J. Immunol.* 157: 4796–4804.
15. Chackerian, B., D. R. Lowy, and J. T. Schiller. 1999. Induction of autoantibodies to mouse CCR5 with recombinant papillomavirus particles. *Proc. Natl. Acad. Sci. USA* 96: 2373–2378.
16. Chackerian, B., D. R. Lowy, and J. T. Schiller. 2001. Conjugation of a self-antigen to papillomavirus-like particles allows for efficient induction of protective autoantibodies. *J. Clin. Invest.* 108: 415–423.
17. Slupetzky, K., S. Shafti-Keramat, P. Lenz, S. Brandt, A. Grassauer, M. Sara, and R. Kirnbauer. 2001. Chimeric papillomavirus-like particles expressing a foreign epitope on capsid surface loops. *J. Gen. Virol.* 82: 2799–2804.
18. Koutsky, L. A., K. A. Ault, C. M. Wheeler, D. R. Brown, E. Barr, F. B. Alvarez, L. M. Chiacchierini, and K. U. Jansen. 2002. A controlled trial of a human papillomavirus type 16 vaccine. *N. Engl. J. Med.* 347: 1645–1651.
19. Chen, X. S., R. L. Garcea, I. Goldberg, G. Casini, and S. C. Harrison. 2000. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Mol. Cell.* 5: 557–567.
20. Monsonego, A., and H. L. Weiner. 2003. Immunotherapeutic approaches to Alzheimer's disease. *Science* 302: 834–834.
21. Kirnbauer, R., F. Booy, N. Cheng, D. R. Lowy, and J. T. Schiller. 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. USA* 89: 12180–12184.
22. Kirnbauer, R., J. Taub, H. Greenstone, R. Roden, M. Durst, L. Gissmann, D. R. Lowy, and J. T. Schiller. 1993. Efficient self-assembly of human papillomavirus type 16 L1 and L1–L2 into virus-like particles. *J. Virol.* 67: 6929–6936.
23. Shafti-Keramat, S., A. Handisurya, E. Kriehuber, G. Meneguzzi, K. Slupetzky, and R. Kirnbauer. 2003. Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. *J. Virol.* 77: 13125–13135.
24. Kirnbauer, R., N. L. Hubbert, C. M. Wheeler, T. M. Becker, D. R. Lowy, and J. T. Schiller. 1994. A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. *J. Natl. Cancer Inst.* 86: 494–499.
25. Roden, R. B., E. M. Weissinger, D. W. Henderson, F. Booy, R. Kirnbauer, J. F. Mushinski, D. R. Lowy, and J. T. Schiller. 1994. Neutralization of bovine papillomavirus by antibodies to L1 and L2 capsid proteins. *J. Virol.* 68: 7570–7574.
26. Jankowsky, J. L., H. H. Slunt, T. Ratovitski, N. A. Jenkins, N. G. Copeland, and D. R. Borchelt. 2001. Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. *Biomol. Eng.* 17: 157–165.
27. Solomon, B., R. Koppel, E. Hanan, and T. Katzav. 1996. Monoclonal antibodies inhibit in vitro fibrillar aggregation of the Alzheimer β-amyloid peptide. *Proc. Natl. Acad. Sci. USA* 93: 452–455.
28. Monsonego, A., R. Maron, V. Zota, D. J. Selkoe, and H. L. Weiner. 2001. Immune hyporesponsiveness to amyloid β-peptide in amyloid precursor protein transgenic mice: implications for the pathogenesis and treatment of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 98: 10273–10278.
29. Gundersen, H. J., P. Bagger, T. F. Bendtsen, S. M. Evans, L. Korbo, N. Marcussen, A. Moller, K. Nielsen, J. R. Nyengaard, B. Pakkenberg, et al. 1988. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *Apmis* 96: 857–881.
30. Baker, T. S., W. W. Newcomb, N. H. Olson, L. M. Cowsert, C. Olson, and J. C. Brown. 1991. Structures of bovine and human papillomaviruses: analysis by cryoelectron microscopy and three-dimensional image reconstruction. *Biophys. J.* 60: 1445–1456.

31. Christensen, N. D., and J. W. Kreider. 1993. Monoclonal antibody neutralization of BPV-1. *Virus Res.* 28: 195–194.
32. DeMattos, R. B., K. R. Bales, D. J. Cummins, J. C. Dodart, S. M. Paul, and D. M. Holtzman. 2001. Peripheral anti-Aβ antibody alters CNS and plasma Aβ clearance and decreases brain Aβ burden in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 98: 8850–8855.
33. DeMattos, R. B., K. R. Bales, D. J. Cummins, S. M. Paul, and D. M. Holtzman. 2002. Brain to plasma amyloid-β efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. *Science* 295: 2264–2267.
34. Wilcock, D. M., G. DiCarlo, D. Henderson, J. Jackson, K. Clarke, K. E. Ugen, M. N. Gordon, and D. Morgan. 2003. Intracranially administered anti-Aβ antibodies reduce β-amyloid deposition by mechanisms both independent of and associated with microglial activation. *J. Neurosci.* 23: 3745–3751.
35. Wilcock, D. M., A. Rojiani, A. Rosenthal, G. Levkowitz, S. Subbarao, J. Alamed, D. Wilson, N. Wilson, M. J. Freeman, M. N. Gordon, and D. Morgan. 2004. Passive amyloid immunotherapy clears amyloid and transiently activates microglia in a transgenic mouse model of amyloid deposition. *J. Neurosci.* 24: 6144–6151.
36. Bachmann, M. F., R. M. Zinkernagel, and A. Oxenius. 1998. Immune responses in the absence of costimulation: viruses know the trick. *J. Immunol.* 161: 5791–5794.
37. Pinto, L. A., P. E. Castle, R. B. Roden, C. D. Harro, D. R. Lowy, J. T. Schiller, D. Wallace, M. Williams, W. Kopp, I. H. Frazer, J. A. Berzofsky, and A. Hildesheim. 2005. HPV-16 L1 VLP vaccine elicits a broad-spectrum of cytokine responses in whole blood. *Vaccine* 23: 3555–3564.
38. Solomon, B. 2002. Towards Alzheimer's disease vaccination. *Mini Rev. Med. Chem.* 2: 85–84.
39. Li, Q., C. Cao, B. Chackerian, J. Schiller, M. Gordon, K. E. Ugen, and D. Morgan. 2004. Overcoming antigen masking of anti-amyloid-β antibodies reveals breaking of B cell tolerance by virus-like particles in amyloidβ immunized amyloid precursor protein transgenic mice. *BMC Neurosci.* 5: 21.
40. Bard, F., R. Barbour, C. Cannon, R. Carretto, M. Fox, D. Games, T. Guido, K. Hoenow, K. Hu, K. Johnson-Wood, et al. 2003. Epitope and isotype specificities of antibodies to β-amyloid peptide for protection against Alzheimer's disease-like neuropathology. *Proc. Natl. Acad. Sci. USA* 100: 2023–2028.
41. Wilcock, D. M., M. N. Gordon, K. E. Ugen, P. E. Gottschall, G. DiCarlo, C. Dickey, K. W. Boyett, P. T. Jantzen, K. E. Connor, J. Melachrino, et al. 2001. Number of Aβ inoculations in $APP^{+}PS1$ transgenic mice influences antibody titers, microglial activation, and congophilic plaque levels. *DNA Cell Biol.* 20: 731–736.
42. Harro, C. D., Y. Y. Pang, R. B. Roden, A. Hildesheim, Z. Wang, M. J. Reynolds, T. C. Mast, R. Robinson, B. R. Murphy, R. A. Karron, et al. 2001. Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. *J. Natl. Cancer Inst.* 93: 284–292.