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Evidence for peripheral clearance of cerebral Aβ protein following chronic, active Aβ immunization in PSAPP mice

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Abstract

Immunization with amyloid-β (Aβ) peptide in mouse models of Alzheimer's disease has been reported to decrease cerebral Aβ levels and improve behavioral deficits. Several mechanisms have been proposed, including antibody-induced phagocytosis of Aβ by cerebral microglia and increased efflux of Aβ from the brain to the periphery. The latter mechanism was suggested in mice undergoing acute, passive transfer of an Aβ monoclonal antibody. Here, PSAPP transgenic mice were actively immunized by a single intraperitoneal injection of synthetic Aβ followed by chronic intranasal administration of Aβ with the mucosal adjuvant, *Escherichia coli* heat-labile enterotoxin, LT, twice weekly for 8 weeks. Serum from Aβ-immunized mice had an average of 240 μg/ml of anti-Aβ-specific antibodies; these antibodies had epitope(s) within Aβ1-15 and were of immunoglobulin (Ig) isotypes IgG2b, IgG2a, and IgG1. Immunization led to a 75% decrease in plaque number ($P < 0.0001$) and a 58% decrease in Aβx–42 levels ($P < 0.026$) in brain, and gliosis and neuritic dystrophy were diminished. No pathological effects of the immunization were observed in kidney, spleen, or snout. Serum Aβ levels increased 28-fold in immunized mice (53.06 ng/ml) compared to controls (1.87 ng/ml). Most of the Aβ in the serum of the immunized mice was bound to antibodies. We conclude that following active immunization, anti-Aβ antibodies sequester serum Aβ and may increase central nervous system to serum Aβ clearance.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease but there is no effective treatment or cure. Amyloid-β protein (Aβ) has become a therapeutic target for the treatment of AD because of its presence in neuritic plaques, its neurotoxicity in vitro and in vivo, and increased levels in humans with familial AD mutations in the amyloid precursor protein (APP) and presenilin (PS1, PS2) genes, Aβ42, in particular, is increased in the presence of PS mutations (Selkoe, 1999). Therapeutic strategies seek to inhibit the Aβ generating proteases (β- and γ-secretases), prevent Aβ aggregation, increase Aβ clearance, prevent Aβ deposition into cerebral plaques, and inhibit the inflammatory response to Aβ deposition.

A number of studies have shown that Aβ immunization results in a significant reduction in cerebral Aβ levels and, in some studies, improvement in cognitive deficits in APP transgenic (tg) mice. Schenk and colleagues first reported the beneficial effects of Aβ immunization; chronic intra-

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peritoneal (i.p.) injections of synthetic human Aβ1–42 peptide with complete (CFA) or incomplete Freund's adjuvant almost completely prevented plaque deposition when given prior to initiation of plaque formation and dramatically lowered cerebral Aβ levels if given after the initiation of plaque deposition in PDAPP tg mice (Schenk et al., 1999). Similar effects were achieved by chronic passive transfer of certain Aβ antibodies. Data from these experiments suggested a mechanism that involved the passage of Aβ antibodies across the blood–brain barrier (BBB), binding of the antibody Fc regions to Fc receptors on microglial cells, and the induction of Aβ phagocytosis by the microglia (Bard et al., 2000).

Several reports have since confirmed and extended these results. We reported that weekly intranasal (i.n.) administration of Aβ1–40 between 5 and 12 months of age in PDAPP mice led to a significant (~56%) reduction in cerebral Aβ burden and low titers of anti-Aβ antibodies that recognized an epitope in the N-terminus of Aβ and were mostly IgG1 and IgG2b (Weiner et al., 2000; Lemere et al., 2000). We have since obtained 12-fold higher anti-Aβ antibody titers in wild-type B6D2F1 mice by adding the mucosal adjuvant, *Escherichia coli* heat-labile enterotoxin, LT (Lemere et al., 2002). In two studies, protection from cognitive deficits was added to the beneficial effects of Aβ immunization in AD tg mice (Janus et al., 2000; Morgan et al., 2000). Multiphoton imaging in the brains of live PDAPP mice after local Aβ antibody application directly demonstrated the clearance of Aβ plaques (Backsai et al., 2001). Newer formulations of Aβ as an immunogen have been reported and include using genetically engineered filamentous phages displaying the Aβ3–6 epitope (EFRH) (Frenkel et al., 2000), a soluble, nonamyloidogenic, nontoxic homolog of Aβ (Sigurdsson et al., 2001), and microparticle-encapsulated Aβ (Brayden et al., 2001).

The mechanisms for the Aβ lowering effects of Aβ immunization are not well understood. Antibody-induced phagacytosis of Aβ by microglia in the brain has been proposed (Bard et al., 2000). Recently, DeMattos and coworkers demonstrated that passive transfer of a monoclonal Aβ antibody, m266 (against Aβ epitope 13–28), altered central nervous system (CNS) and plasma Aβ levels in acute studies in PDAPP transgenic mice (DeMattos et al., 2001). A very substantial (1000-fold) increase in plasma Aβ was detected several days after intravenous injection of m266 and suggested that the Aβ antibody, m266, in the periphery was acting as a "sink" by enhancing clearance of Aβ from the brain to the peripheral compartments. Here, we show that following active Aβ immunization of PSAPP mice for 8 weeks, there is a significant decrease in cerebral Aβ levels and a concurrent marked increase in serum Aβ. Our results support the possibility that following passive as well as active Aβ immunization one mechanism for reducing cerebral Aβ involves antibody-enhanced clearance of soluble Aβ from the brain to the periphery.

Materials and methods

Animal treatments

PSAPP mice (Holcomb et al., 1998) were derived from a cross between mutant $APP_{K670N,M671L}$ tg mice (Tg2576 (Hsaio et al., 1996)) and mutant $PS1_{M146L}$ mice (line 6.2 (Duff et al., 1996)). The mice were housed in a mouse facility that is fully compliant with the PHS Policy on Humane Care and Use of Laboratory Animals. Twelve 5-week-old male and female mice were divided into two treatment groups: Aβ immunized and controls. Aβ immunized mice ($n = 5$) received a single i.p. injection of 100 μg Aβ + 50 μg CFA followed by i.n. delivery of 100 μg Aβ + 5 μg of the mucosal adjuvant, LT, two times per week for 8 weeks. Control mice were left untreated ($n = 5$) or received a single injection of 50 μg CFA followed by i.n. water + 5 μg LT ($n = 2$). A cocktail of human Aβ peptides [three parts Aβ1–40, 1 part Aβ1–42, Biopolymer Laboratory, CND, Boston, MA] was diluted at 4 mg/ml in distilled water, incubated overnight at 37°C. Congo red staining showed minimal presence of Aβ fibrils. Aliquots were frozen at −80°C and defrosted just prior to use. Native LT was mixed with Aβ just prior to i.n. immunization. Intranasal Aβ was delivered as previously described (Lemere et al., 2002). All mice were bled from the tail prior to and weekly throughout the 8 weeks of treatment. Serum was collected (Lemere et al., 2002) and frozen at −20°C. One hemibrain was fixed in 10% NBF and processed for paraffin sectioning and immunohistochemistry (IHC) while the other was frozen at −80°C. Kidney, spleen, and snout tissues were collected, fixed, and processed for paraffin sectioning.

A subsequent study was conducted using J20 APP transgenic mice (Mucke et al., 2000), bearing human mutant APP_{V717F} and $_{K670M/N671L}$, to further investigate the rise in Aβ levels in the periphery (i.e., blood) following the same Aβ immunization protocol as described above but increasing the number of vehicle control animals. There were six Aβ-immunized mice and six vehicle controls; however, one mouse from the Aβ treated group died 6 weeks into the study. The mice were treated for 16 weeks; plasma was collected every other week instead of weekly.

Anti-Aβ ELISA

Aβ antibody titers were determined by ELISA, as previously described (Lemere et al., 2002). The OD reading at 450 nm was obtained for each plate using a Benchmark Microplate Reader (Bio-Rad, Hercules, CA). Results were recorded in micrograms per milliliter. Aβ antibody titers were compared using alternate Welch's t test, which assumes Gaussian populations with unequal standard deviations (SD).

Aβ protein ELISA

Aβ ELISAs were performed on guanidine hydrochloride extracted brain homogenates as previously described (Weiner et al., 2000; Johnson-Wood et al., 1997). Mouse sera were diluted 1:10–1:200 and quantified by an $A\beta_{total}$ ELISA with Mab 266 to capture and biotinylated 3D6 (Aβ1–5) for detection. Control experiments used wild-type B6D2F1 mouse sera from Aβ-immunized (Lemere et al., 2002) or untreated mice. All ELISA antibodies were a gift of Elan Pharmaceuticals (So. San Francisco, CA).

Immunohistochemistry (IHC)

Anti-Aβ antibodies were used to immunolabel AD cortical plaques on paraffin-embedded, formalin-fixed sections as previously described (Wiener et al., 2000; Lemere et al., 1996) using immunized mouse sera diluted 1:100 in TBS (150 mM NaCl, 50 mM Tris) as primary antibodies. The Vector Elite horseradish-peroxidase ABC kit (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine (Sigma Chemical Co., St. Louis, MO) were used to visualize immunoreactivity. For epitope mapping of the mouse anti-human Aβ antibodies, six overlapping 15-residue fragments of human Aβ peptide (Aβ1–15, Aβ6–20, Aβ11–25, Aβ16–30, Aβ21–35, Aβ26–42; Biopolymer Laboratory, CND) and Aβ1–42 were incubated with mouse sera at 1:100 in TBS; 20 μg of peptide was added and shaken for 3 h at RT. Sera were then centrifuged for 2 min at 8000 rpm. Mouse sera (absorbed and unabsorbed) were used as primary antibodies on human AD brain sections. For immunoglobulin (Ig) isotyping, biotinylated anti-mouse IgG (Dako Corp., Carpenteria, CA), IgG1, IgG2a, IgG2b (IGN Biomedicals Inc., Aurora, OH), IgA (Sigma Chemical Co.), and IgM (Vector Laboratories, Inc.) were diluted 1:222 and used as secondary antibodies in the immunostaining protocol.

Paraffin sections of formalin-fixed mouse brain were examined by IHC using Aβ polyclonal antibody, R1282 (Selkoe Lab), to determine Aβ plaque burden. Plaque numbers were counted for the entire sagittal section by visual inspection under a microscope; a total of seven sections at roughly equal planes were counted for each mouse, the identity of which was unknown. Rat anti-mouse CD45 antibody (1:5000; Serotek, Inc., UK) was used to detect microglia in the brain while a GFAP Mab (1:500; Sigma Immunochemical Co.) was used to detect reactive astrocytes. A phosphorylated τ Mab, AT8 (Innogenetics, Belgium), and an APP Mab, 8E5 (gift of Elan Pharmaceuticals), were used to detect neuritic dystrophy. Paraffin sections of formalin-fixed kidney, spleen, and snout were examined by IHC for Aβ (R1282) and macrophages (CD45); snout tissue was also stained with hematoxylin and eosin.

Denaturing acid/urea polyacrylamide gels

Aβ/antibody complexes were detected by acid/urea polyacrylamide gel electrophoresis and Western blot analysis, as previously described (DeMattos et al., 2001). To enhance the Ig affinity of the anti-Aβ antibodies for protein G, 5 μg of unconjugated anti-mouse IgG (Vector Laboratories) was added to 20 μl of mouse serum and incubated at 4°C O.N. Prior to denaturation, sera were incubated with 20 μl protein G beads (Pierce Chemical Co., Rockford, IL) for 3 h at 4°C, spun at 10,000*g* for 5 min, and washed three times in 50 mM Tris. Protein G beads were eluted with 98% formic acid to a final concentration of 80%. Sera were denatured with formic acid (to 80%), reduced with β-mercaptoethanol (1%), and run on a 4–10–22% acid/urea step gel (6 M urea, 5% acetic acid). Gels were neutralized, transferred to 0.2 μm nitrocellulose, probed for Aβ with mAb 6E10 (Aβ1–17), and developed using ECL Plus (Amersham Pharmacia Biotech UK Ltd., England). In control experiments, serum from untreated or Aβ-immunized WT B6D2F1 mice (containing 816 μg/ml anti-Aβ antibodies) was spiked with a 1:1 ratio of Aβ1–40 (5 ng) and Aβ1–42 (5 ng).

Results

Aβ antibody characterization

At 5 weeks of age, PSAPP tg mice received a single i.p. injection of Aβ + CFA followed by 8 weeks of intranasal administration of Aβ + LT. All five Aβ-immunized mice generated anti-human Aβ antibodies, and Aβ antibodies were not detected in any of the seven control mice. Serum from Aβ-immunized mice had an average of 241 μg/ml of anti-Aβ-specific antibodies (range 70–611 μg/ml) (Table 1). Mouse sera were used as primary antibodies for IHC on human AD brain sections; numerous Aβ plaques were labeled. Mouse serum was then incubated with one of six overlapping 15-residue Aβ peptides prior to IHC on AD brain sections. Only Aβ1–15 and full-length Aβ peptides abolished plaque IR (Fig. 1a), indicating that the Aβ antibodies recognized an epitope(s) within the first 15 residues of Aβ. Immunoglobulin isotyping was conducted using Ig isotype-specific biotinylated secondary antibodies for IHC with mouse serum on human AD brain sections. The Aβ antibody isotypes were predominantly IgG2b, IgG2a, and IgG1 and included very low levels of IgA (Fig. 1b).

Cerebral Aβ levels and pathological findings

Aβ immunization of PSAPP mice significantly reduced the levels of cerebral Aβ compared to those of nonimmunized (control) PSAPP mice. Plaque IR using Aβ antibodies revealed discrete, punctate plaques in the 13-week-old PSAPP mice (Fig. 2a), making it feasible to count plaques across entire sagittal sections of brain. As previously re-

Table 1
Summary of results

PSAPP mouse ID	ELISA: brain Aβx–42 (pmol/g)	ELISA: brain Aβx–40 (pmol/g)	Plaque No. Ctx + HC	ELISA: serum Aβ antibodies (μg/ml)	ELISA: serum Aβ (ng/ml)
Aβ immunized					
1	169.68	163.99	21	138.75	31.76
2	217.46	181.77	48.5	259.17	59.72
3	228.72	215.73	24.5	611.50	31.45
4	322.55	329.81	24.5	124.75	55.64
5	42.40	67.49	2	70.22	86.74
Mean (± SD)	196.16 (±102.26)	191.76 (±94.80)	24.12 (±16.54)	240.88 (±218.37)	53.06 (±22.95)
Controls					
1[a]	93.98	68.40	29	0	1.90
2[a]	532.55	373.57	76	0	0
3	485.54	320.09	126.5	0	2.86
4	660.94	369.35	105	0	0
5	536.31	311.94	127.5	0	0
6	717.93	364.71	108.5	0	3.43
7	208.11	157.44	119.5	0	4.93
Mean (±SD)	462.19 (±229.50)	280.79 (±119.95)	98.86 (±35.46)	0	1.87 (±1.97)
Two-tailed alternate Welch's *t* test	$P < 0.0265$*	$P < 0.1853$	$P < 0.0012$*		$P < 0.0076$*
Fisher's exact test				$P < 0.001$*	

[a] vehicle controls.
* Significant difference between Aβ-immunized mice and control mice.

ported for other AD mouse models (Schenk et al., 1999; Bard et al., 2000) plaque burden varied. Nonetheless, a significant 75% decrease in Aβ-IR plaque number was observed in Aβ-immunized compared to control mice ($P < 0.0012$, Table 1, Fig. 2a). Reduced Aβ levels were also detected by ELISA on total guanidine HCl-solubilized brain extracts. Decreases in both Aβx–42 and Aβx–40 were detected in the immunized mice; however, only the 58% decrease in Aβx–42 levels in brain reached significance ($P < 0.0265$), whereas the 32% decrease in Aβx–40 did not (Table 1, Fig. 2c).

Mouse brains were examined for gliosis and neuritic dystrophy. For Aβ-immunized mice, GFAP-IR astrocytes, CD45-IR microglia, and APP- and τ-IR dystrophic neurites were decreased overall, and these changes were primarily associated with the remaining compacted plaques (Fig. 3). Control mouse brains contained many more plaques (Fig. 3e) and showed more glial (Fig. 3g and h) and neuritic (Fig. 3f) immunolabeling compared to Aβ-immunized mice (Fig. 3a–d).

Serum Aβ levels

Aβ ELISA revealed a significant 28-fold increase in serum Aβ levels in Aβ-immunized compared to control PSAPP mice ($P < 0.0076$, Table 1, Fig. 4c). To rule out potential confounding effects of the mouse serum Aβ antibodies in the Aβ ELISA, control experiments were performed using serum from untreated or Aβ-immunized wild-type B6D2F1 mice (treatments described by Lemere et al., 2002). Human Aβ was undetected in WT mouse serum, as expected. After spiking synthetic human Aβ peptide (100 ng/ml) into untreated and Aβ-immunized mouse sera, approximately 25% of the spiked peptide was detected by $Aβ_{total}$ ELISA (Fig. 4a). Nonspecific adsorption of Aβ peptide to the tube may account for the lack of full recovery of the peptide; spiking of Aβ peptide into TBS yielded even less Aβ (E. Spooner, unpublished observation). The presence of anti-human Aβ antibodies in WT mouse serum (in the absence of human Aβ protein) did not produce artifactually high Aβ readings (Fig. 4a).

To rule out the effects of the adjuvant LT alone on peripheral Aβ levels, J20 APP mice were immunized with Aβ peptide using the identical protocol and reagents as those administered to the PSAPP mice. Although the absolute levels of peripheral Aβ were much lower in J20 mice (picograms per milliliter) compared to PSAPP mice (nanograms per milliliter), 16-week immunized J20 mice had a significant seven-fold increase in plasma Aβ compared to vehicle control mice ($P < 0.017$; Mann–Whitney *U* test) as shown in Table 2. Aβ-immunized, but not vehicle control J20 mice, generated anti-Aβ titers (Table 2). However, even though all Aβ-immunized J20 mice began generating Aβ antibodies by 4 weeks of treatment, after 16 weeks, the antibody levels averaged slightly less than half of those generated in PSAPP mice after 8 weeks of treatment.

Next, acid/urea gel analysis was used to confirm the presence of Aβ40 and Aβ42 in mouse serum and to determine how much, if any, of the Aβ was bound to antibodies, using protein G beads to capture antibodies and their binding partners. Separate bands for Aβ40 and Aβ42 were detected (Fig. 4c and d, lanes 1 and 2). In control experiments, synthetic Aβ peptide spiked into serum from untreated and Aβ-immunized WT B6D2F1 mice was detected

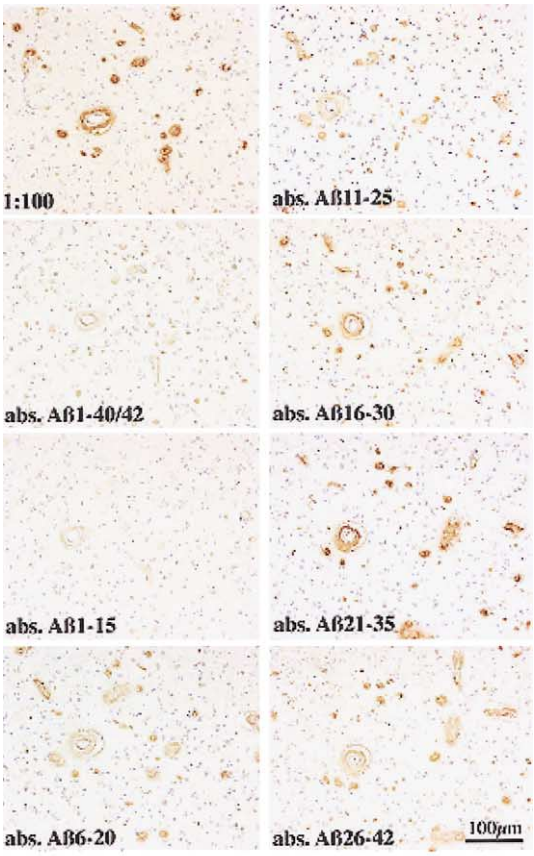

Fig. 1. Characterization of Aβ antibodies. a. Anti-human Aβ antibodies recognized epitopes within the first 15 residues of Aβ as revealed by the lack of plaque immunoreactivity in serial frontal cortical brain sections from a female AD patient (92 years old) using serum from an Aβ-immunized PSAPP mouse diluted 1:100. Absorption of the serum with Aβ1–15 or full-length Aβ1–40/42 peptide abolished plaque immunoreactivity. Absorption of the same serum with 5 other overlapping 15-mer Aβ peptides, or omission of peptide, allowed detection of AD plaques by the Aβ antibodies present in the mouse serum, indicating a lack of binding of the Aβ antibodies to these regions of Aβ. b. Immunohistochemistry using serum from Aβ-immunized PSAPP mice and biotinylated Ig isotype-specific secondary antibodies on serial frontal sections from a 92-year-old female AD patient revealed the presence of IgG2b, IgG2a, and IgG1 immunoglobulins, with much lower amounts of IgA. Plaque immunoreactivity was not detected using an IgM biotinylated secondary antibody for IHC. An Aβ Mab, 6E10 (Aβ1–15), was used with a pan-specific (IgG, IgM, IgA) biotinylated secondary antibody (Dako Corp., Carpenteria, CA) as a positive control to determine the number of plaques in the section. Scale bars, 100 μm.

(Fig. 4c, lane 3). The spiked Aβ peptide did not bind to protein G beads in the absence of Aβ antibodies (Fig. 4c, lane 5), while it bound to antibodies in Aβ-immunized mouse sera (Fig. 4c, lane 7). PSAPP mouse serum was also analyzed in the same assay. A strong Aβ40 band was detected in the mouse serum following denaturation by formic acid (Fig. 4d, lane 3). Prior to denaturation, sera from Aβ-immunized PSAPP mice were incubated with protein G beads. The beads were pelleted, and both the pellet and the supernatant were extracted with formic acid. Very little Aβ was detected in the supernatant, whereas a distinct Aβ40 band was observed in the pellet, indicating that most of the Aβ in the serum of Aβ-immunized mice was bound to IgG.

Pathological examination of kidney, spleen, and snout

Immunohistochemical analysis of formalin-fixed, paraffin sections of kidney, spleen, and snout revealed no Aβ IR and no histopathological changes (data not shown). Macrophage IR was similar in the kidney, spleen, and liver of Aβ-immunized and control PSAPP mice. The structural integrity of the nasal epithelium was indistinguishable between Aβ-immunized and control mice; no significant lymphocyte infiltration was observed.

Discussion

Here, we report for the first time that active Aβ immunization of PSAPP mice with synthetic human Aβ peptide from 5 (prior to plaque deposition) to 13 weeks of age (3–4 weeks after plaque deposition begins) results in a significant decrease in cerebral Aβ burden and a concommitant marked increase in serum Aβ. Use of a dual-immunization protocol in which a single i.p. injection of Aβ + CFA was followed by chronic i.n. Aβ + adjuvant LT greatly enhanced the production of anti-Aβ antibodies (mean: 241 μg/ml) compared to those seen in our earlier mucosal Aβ immunization study in PDAPP mice (mean: 26 μg/ml) in which mice received only Aβ intranasally and without adjuvant (Weiner et al., 2000; Lemere et al., 2000). This “prime/boost” strategy was used based on previous studies in our laboratory to

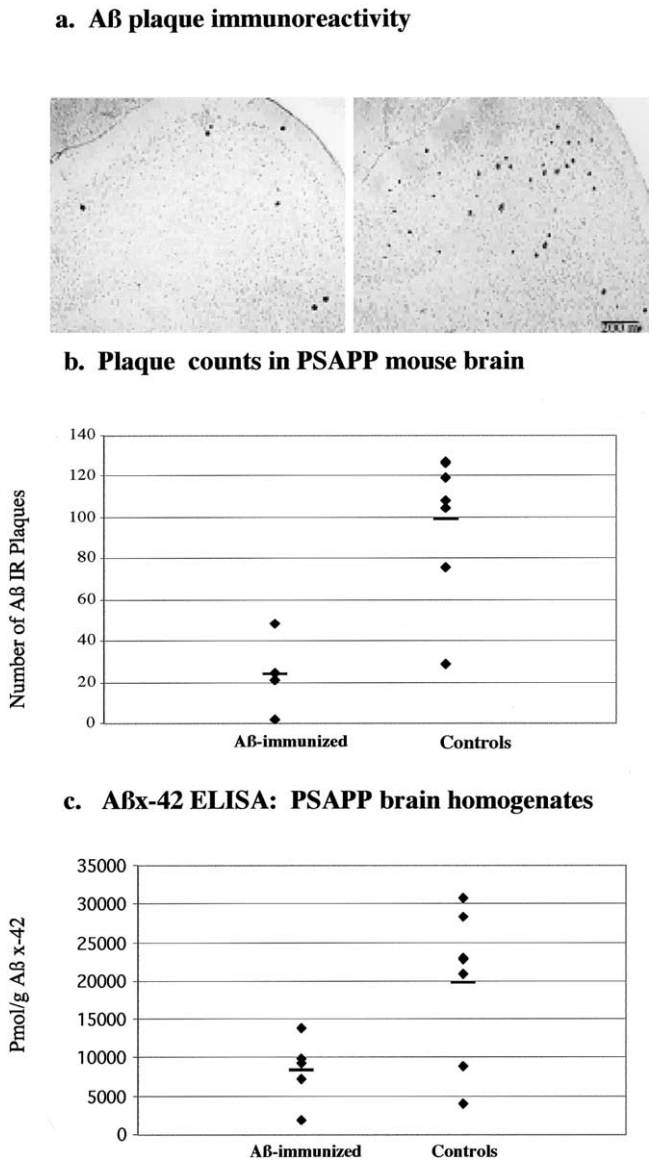

Fig. 2. Cerebral Aβ levels in PSAPP mice were reduced following Aβ immunization. a. Plaques were detected using Aβ polyclonal antibody, R1282, for immunohistochemistry on sagittal brain sections from Aβ immunized (left) and control (right) PSAPP mice. The presence of discrete, compacted plaques made it feasible to count the plaques in the entire section. b. Plaques were counted by visual inspection under the microscope for each of seven sagittal sections at equal planes of section for each mouse. The identity of each mouse was unknown at the time of counting. Aβ-immunized mice showed a 75% decrease in plaque burden compared to control mice. c. Guanidine HCL-extracted cerebral Aβ levels were quantified by ELISA. A 58% decrease in Aβx–42 was observed following Aβ immunization. Exact numbers of plaques and Aβ ELISA levels per mouse brain are reported in Table 1.

optimize Aβ immunization protocols; one i.p. injection of Aβ + CFA followed by chronic intranasal boosting with Aβ + LT more than doubled the anti-Aβ titers after 8 weeks compared to those generated by a single i.p. injection of Aβ + CFA (data not shown). In the present study, the plaque burden in Aβ-immunized mice was decreased 75% over that in control mice; higher Aβ antibody titers seen here may account for an even greater reduction in plaque burden compared with that observed in PDAPP hippocampus (60%) in our earlier study. In both studies, gliosis and neuritic dystrophy were frequently observed in association with remaining compacted plaques. Fewer plaques and therefore less gliosis and neuritic dystrophy were observed in the brains of the Aβ-immunized PSAPP mice. Serum Aβ levels were strikingly increased by 28-fold in the Aβ-immunized mice and the detectable serum Aβ was almost entirely bound to antibodies. No abnormal pathological findings or Aβ deposition were found in the kidney, spleen, or snout of Aβ-immunized PSAPP mice.

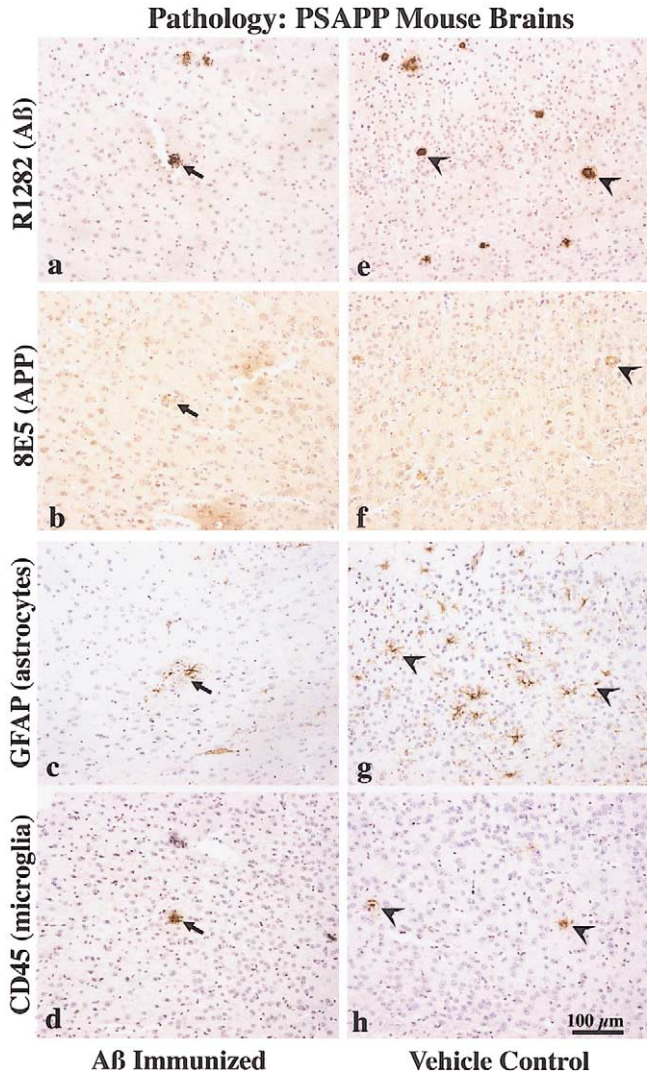

Fig. 3. Cerebral Aβ deposition, plaque-associated gliosis, and neuritic dystrophy were reduced in PSAPP mice following Aβ immunization (left). Fewer plaques were observed in adjacent serial sections of Aβ-immunized (a; see arrow for example) PSAPP mice, as detected by R1282, compared to vehicle controls (e; see arrowheads for examples). Aβ-immunized mice showed a corresponding reduction in neuritic plaques (b), plaque-associated reactive astrocytes (c), and microglia (d) compared to vehicle controls (f, g, and h). Scale bar: 100 μm.

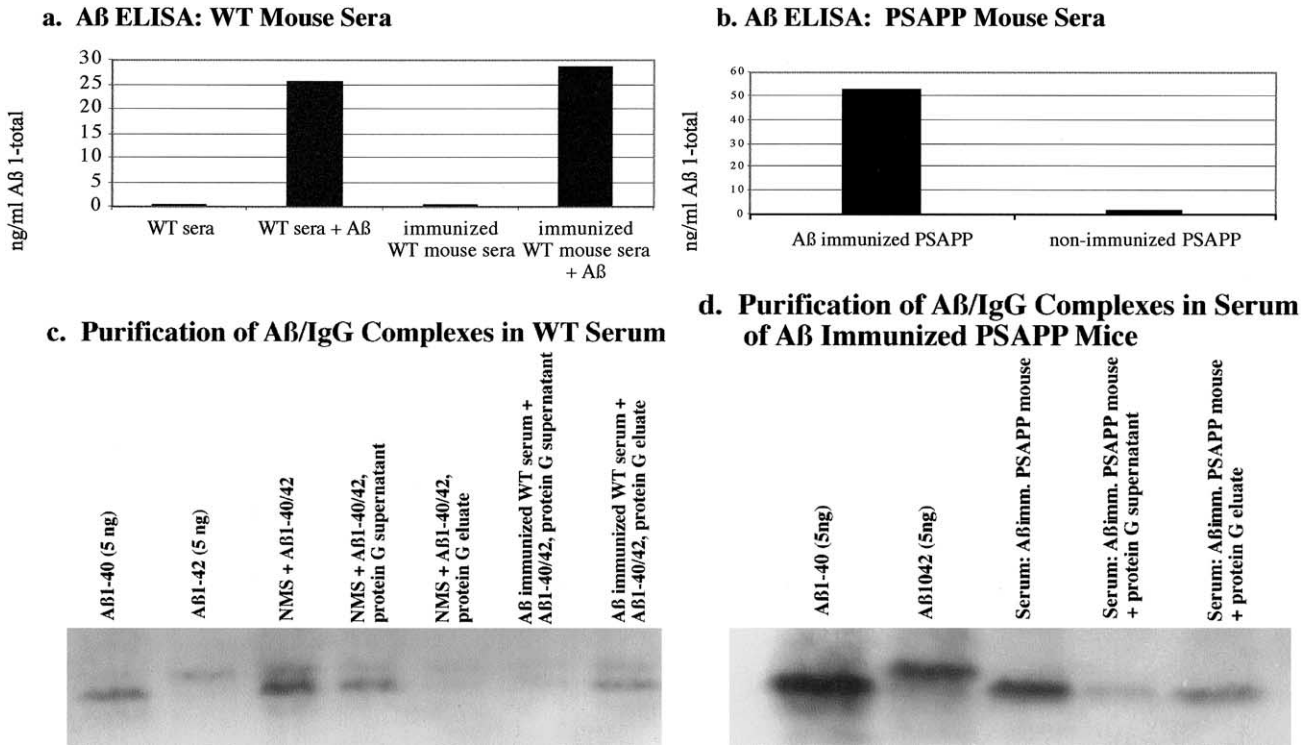

Fig. 4. Serum Aβ levels were significantly increased following Aβ immunization in PSAPP mice. a. A control experiment was performed to rule out artifactual detection of Aβ in immunized mouse serum. Serum from untreated or Aβ-immunized wild-type (WT) B6D2F1 was tested by ELISA for the presence of human Aβ before and after spiking with 50 ng Aβ (1:1 ratio of Aβ1–40 and Aβ1–42) in a 500-μl volume. Human Aβ was not detected in untreated or Aβ-immunized WT mouse serum. The addition of synthetic human Aβ to serum of untreated or Aβ-immunized WT mice allowed detection of Aβ by ELISA; Aβ levels were similar for each mouse and did not vary according to immunization status. b. $A\beta_{total}$ ELISA revealed a 28-fold increase in serum Aβ in Aβ-immunized vs untreated PSAPP mice. Exact values for each mouse are provided in Table 1. c. Acid/urea step-gradient gel analysis allowed visualization of Aβ. Aβ1–40 (lane 1) and Aβ1–42 (lane 2) were clearly discernible. [Note: Aβ (~4 kDa) runs a little high (6.5–7.5 kDa) on the acid/urea step-gradient gel.] In a control experiment, Aβ1–40/42 (5 ng each) spiked into WT serum (normal mouse serum, NMS) was detected (lane 3). Protein G beads were added to the mixture to bind antibodies. In the absence of Aβ antibodies in serum from WT untreated mice, Aβ peptide was detected in the supernatant and did not bind to protein G beads (lanes 4 and 5). Serum from Aβ-immunized WT mice showed the opposite effect. The spiked Aβ peptide was not observed in the supernatant, but instead was eluted from the protein G beads, indicating that the peptide was bound to Aβ antibodies in Aβ-immunized mouse serum (lanes 6 and 7). d. PSAPP mouse serum was also run on the acid/urea step-gradient gel. A strong Aβ40 band was detected in the mouse serum following denaturation by formic acid (lane 3). Prior to denaturation, serum from a Aβ-immunized PSAPP mouse was incubated with protein G beads, presumably capturing all antibodies present in the serum. The beads were spun down and the supernatant exposed to formic acid. Very low levels of Aβ40 were detected in the supernatant. However, when the beads were eluted with formic acid, a distinct, moderately intense Aβ40 band was observed, indicating that the majority of Aβ in the serum of Aβ immunized PSAPP mice was bound to antibodies.

Our results support the findings of DeMattos and colleagues (deMattos et al., 2001) and provide further evidence that one mechanism by which Aβ vaccination results in its effects may be via antibody-mediated clearance of soluble Aβ from the brain to the periphery. This is the first evidence that active immunization dramatically increases serum Aβ, consistent with antibodies enhancing Aβ clearance from brain in an AD mouse model. The 28-fold Aβ increase we observed in serum is substantially lower than the 1000-fold increase in plasma Aβ observed by DeMattos et al. several days after passive transfer with Mab 266. Several issues could explain this discrepancy. First, we measured Aβ in serum, whereas DeMattos et al. measured it in plasma; Aβ levels may be higher in plasma, and some Aβ may be lost when the blood clot is spun down to collect serum. Second, many different anti-Aβ antibodies are generated following active immunization and each of the antibody–Aβ complexes are likely to be cleared over time with a specific $t_{1/2}$. The gradual induction of Aβ antibody production in our chronic, active immunization protocol may allow the progressive, differential clearance of antibody–antigen complexes. In previous experiments with Mab 266, only one antibody–Aβ complex was assessed. Several properties of this antibody may be different from that of others. Third, the experiments were performed in two different AD mouse models using two very different immunization protocols. Baseline plasma $A\beta_{total}$ in PDAPP mice is ~10- to 20-fold lower (100–300 pg/ml) than that found in PSAPP mice (~2 ng/ml). The total amount of Aβ that we found in serum following active immunization of PSAPP mice was actually quite similar (~50 ng/ml) to

Table 2
Plasma anti-Aβ titers and Aβ levels in J20 mice

Treatment group (J20 APP tg)	Mouse ID	Age at start (mos)	Plasma Aβ levels (pg/ml)	Plasma anti-Aβ titers (μg/ml)
Aβ immunized	310	12.5	284.63	19.4
	325	12.4	350.63	56.2
	344	10.7	2800.0	357.5
	348	10.7	1697.7	9.3
	378	10.1	193.57	69.2
Average (SD)		11.28 (1.10)	1065.31* (1149.92)	102.32 (144.8)
Vehicle control	303	13.4	229.87	0
	311	12.5	177.62	0
	318	12.4	142.51	0
	339	10.7	25.59	0
	343	10.7	276.11	0
	388	10.1	53.24	0
Average (SD)		11.63 (1.31)	150.82 (97.92)	0

* $P \leq 0.017$, Mann–Whitney U test.

that seen in plasma following passive administration of Mab m266 (~100 ng/ml). It was the relative increase that differed.

In an additional study to test the effects of the adjuvant on peripheral Aβ levels and to confirm the correlation between the presence of anti-Aβ antibodies and increased Aβ levels in blood, we treated J20 APP transgenic mice with the identical protocol used for the PSAPP mice (albeit longer). Anti-Aβ titers and peripheral Aβ levels were measured in both the Aβ immunized and the vehicle control mice. After 16 weeks, there was a significant increase in plasma Aβ in the immunized mice compared to the vehicle controls; only the Aβ-immunized mice generated antibodies. In general, J20 mice have less human Aβ than PSAPP mice and Aβ deposition occurs much later. This may partially explain why the titers at 16 weeks in J20 mice were half of those in PSAPP mice after 8 weeks. Regardless, a significant elevation in plasma Aβ was correlated with the presence of anti-Aβ titers and not with the use of the adjuvant.

Another mechanism proposed for the reduction in cerebral Aβ following Aβ immunization is phagocytosis of Aβ by microglia following antibody binding to Fc receptors on microglia in brain (Bard et al., 2000). However, we have been unable to detect mouse Ig associated with plaques in paraffin brain sections of PSAPP (here) or PDAPP mice (CAL, unpublished data). In addition, DeMattos and coworkers were unable to detect Aβ Mab 266 in association with plaques in the brain following acute, passive transfer with Aβ (DeMattos et al., 2001). Tissue fixation and staining reagents may account for these differences. It is unlikely that such a microglial-mediated mechanism would lead to markedly increased Aβ levels in the periphery, but such analysis has not been reported. Another possibility is that anti-Aβ antibodies keep Aβ soluble and prevent its aggregation, as suggested by experiments in which monoclonal antibodies recognizing the amino-terminus of Aβ prevented the formation of Aβ fibrils in vitro (Solomon et al., 1996) and bound preformed Aβ fibrils, causing them to disaggregate (Solomon et al., 1997). Whether enough antibody enters the CNS after peripheral administration or generation to result in these effects has not been clarified.

While it is possible that multiple mechanisms are responsible for the lowering of cerebral Aβ following Aβ immunization, the efflux of soluble Aβ from brain to periphery, leading to increased levels of Aβ/anti-Aβ complexes in the periphery, appears to play an important role. Recent behavioral and other data support this possibility. First, it was found that following the peripheral administration of the anti-Aβ antibody m266 to PDAPP mice there was a rapid improvement (by 24 and 72 h) observed in two tests of spatial memory (Dodart et al., 2002). These behavioral changes correlate with the rapid changes observed in plasma Aβ. Second, it was found that baseline plasma Aβ in 12- to 13-month-old PDAPP mice did not correlate with plaque burden; however, peripheral administration of anti-Aβ antibody m266 to these mice resulted in a rapid increase in plasma Aβ that strongly correlated with plaque burden (DeMattos et al., 2002). Since plasma Aβ in PDAPP mice is derived from the CNS, this result strongly argues that this anti-Aβ antibody not only sequesters plasma Aβ and alters its clearance but also increases net CNS to plasma Aβ efflux. Understanding the details of these effects will likely provide important insights into how Aβ is normally transported between the brain and the periphery. It is possible that when Aβ antibodies bind Aβ outside the BBB (e.g., plasma), it shifts the distribution of Aβ to the periphery, where the complex is targeted for opsonization by complement C1q and cleared in the spleen or liver. High peripheral anti-Aβ titers could continuously bind up any Aβ that normally effluxes from the brain to the cerebral spinal fluid to the venous sinuses (blood), thus continuously sequestering Aβ in blood and preventing it from reentering the CNS. Future investigations are aimed at determining the fate of the Aβ/anti-Aβ complex in the periphery. Redistributing

Aβ to the periphery may provide an effective therapeutic strategy for treatment and/or prevention of AD in humans.

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