

**Table 4.** Continued

Ab transfer	Inflammation		Fibrosis		
	Dose	Incidence	Grade	Incidence	Grade
—		2/2	2	n.e. <sup>†</sup>	n.e.
—		0/5	0	n.e.	n.e.
—		2/3	0.8 ± 0.6	0/3	0
5× dilution (1 ml) × 2/week × 5 weeks		3/3	3.3 ± 0.3 <sup>§</sup>	3/3	3.3 ± 0.2 <sup>¶</sup>
5× dilution (1 ml) × 2/week × 5 weeks		4/4	3.4 ± 0.3 <sup>§</sup>	4/4	3.3 ± 0.3 <sup>¶</sup>
5× dilution (1 ml) × 2/week × 5 weeks		4/4	1.5 ± 0.5 <sup>§</sup>	4/4	1.6 ± 0.4 <sup>¶</sup>
5× dilution (1 ml) × 2/week × 5 weeks		0/3	0	0/3	0

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# Nonviral A $\beta$ DNA vaccine therapy against Alzheimer's disease: Long-term effects and safety

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Edited by Hugh O. McDevitt, Stanford University School of Medicine, Stanford, CA, and approved May 10, 2006 (received for review February 6, 2006)

It was recently demonstrated that amyloid  $\beta$  (A $\beta$ ) peptide vaccination was effective in reducing the A $\beta$  burden in Alzheimer model mice. However, the clinical trial was halted because of the development of meningoencephalitis in some patients. To overcome this problem, anti-A $\beta$  antibody therapy and other types of vaccination are now in trial. In this study, we have developed safe and effective nonviral A $\beta$  DNA vaccines against Alzheimer's disease. We administered these vaccines to model (APP23) mice and evaluated A $\beta$  burden reduction. Prophylactic treatments started before A $\beta$  deposition reduced A $\beta$  burden to 15.5% and 38.5% of that found in untreated mice at 7 and 18 months of age, respectively. Therapeutic treatment started after A $\beta$  deposition reduced A $\beta$  burden to  $\approx$ 50% at the age of 18 months. Importantly, this therapy induced neither neuroinflammation nor T cell responses to A $\beta$  peptide in both APP23 and wild-type B6 mice, even after long-term vaccination. Although it is reported that other anti-A $\beta$  therapies have pharmacological and/or technical difficulties, nonviral DNA vaccines are highly secure and easily controllable and are promising for the treatment of Alzheimer's disease.

amyloid  $\beta$ -peptide | DNA vaccination

Alzheimer's disease is a chronic neurodegenerative disorder that is the most common cause of progressive impairment of memory and cognitive function in aged humans. The etiology of the disease is thought to be the result of an imbalance between amyloid  $\beta$  (A $\beta$ ) production and clearance (amyloid cascade hypothesis) (1, 2). On the basis of this hypothesis, Schenk *et al.* (3) developed an A $\beta$ -peptide vaccine, immunized amyloid precursor protein (APP)-transgenic mice with the peptide in complete Freund's adjuvant (CFA), and demonstrated a marked amyloid reduction in the brain. Repetitive intranasal administration of A $\beta$ -peptide and adjuvant (4) and the passive transfer of anti-A $\beta$  antibodies were also effective in reducing amyloid deposits (5). Moreover, vaccinated mice showed an improvement in memory loss (6, 7). Thus, A $\beta$  peptide vaccine therapy has been shown to be effective in animal models, and human clinical trials were started with Betabloc (AN-1792), composed of synthetic A $\beta$ 1-42 and QS21 as an adjuvant (8). However, the phase II clinical trial was halted because of the development of acute meningoencephalitis that appeared in 18 (6%) of 298 vaccinated patients (9). Importantly, it was later demonstrated by autopsy that there was a significant reduction of amyloid deposition and disappearance of degenerative axons in a treated patient (10). At the same time, T cell-dominant meningeal encephalitis was present in the cerebral cortex. These findings suggest that the vaccine therapy is a promising strategy for human Alzheimer's disease if excessive immune reactions are minimized to avoid unwanted neuroinflammation.

Recently, it was reported that naked plasmid DNAs encoding proteins are taken into cells and produce the proteins in a small amount for a relatively long period when injected into the muscle or skin (11). Then, the proteins that are released in the extracellular space induce antibodies against the proteins (12, 13). Thus, gentle and quiet immune reactions could be obtained by DNA vaccine administration. In our and other's laboratories,

immune therapies with DNA vaccines have been examined in autoimmune disease models (14–17) and have been found to be effective in preventing the diseases without the use of adjuvants. Here, we developed nonviral A $\beta$  DNA vaccines and were able to reduce the amyloid burden in the cerebral cortex and hippocampus of Alzheimer's disease model (APP23) mice by vaccination. Importantly, the side effects, such as T cell proliferation and neuroinflammation, were absent even after long-term administration of the vaccines in both APP23 and wild-type B6 mice.

## Results

**Preparation and Characterization of Nonviral A $\beta$  DNA Vaccines.** We prepared three types of nonviral A $\beta$  DNA vaccines using a mammalian expression vector, pTarget. The sequence of A $\beta$ 1-42 and additional sequences were inserted in the plasmid, as shown in Fig. 1A. The first one contains only the A $\beta$ 1-42 sequence with the Kozak sequence at the 5' end (referred to as K-A $\beta$  vaccine) (Fig. 1A1). To the second, the Ig $\kappa$  signal sequence of mouse Ig was added to improve the secretion ability (IgL-A $\beta$  vaccine) (Fig. 1A2), and the third possesses the Fc portion of human Ig at the 3' end to maintain stability (A $\beta$ -Fc vaccine) (Fig. 1A3). Before *in vivo* administration, these DNA vaccines were transfected to HEK293T cells, and the secretion of A $\beta$ 1-42 peptide into the culture supernatant was assayed with Western blotting (Fig. 1B). The production of intracellular A $\beta$ 1-42 peptide was confirmed in all three vaccines by ELISA (data not shown). It was clearly demonstrated that the supernatants of cultured cells that had been transfected with IgL-A $\beta$  and A $\beta$ -Fc vaccines contained translated proteins (4.5 and 35 kDa, respectively), whereas K-A $\beta$ -transfected cells did not secrete the peptide into the extracellular space. These findings indicate that the addition of the leader sequence is important for transportation of the protein to the extracellular space as reported in ref. 18 and that this event is critical for the effects of DNA vaccines (see below).

**Reduction of Amyloid Burden by A $\beta$  DNA Vaccination.** We used two types of regimens to examine the effect of A $\beta$  DNA vaccination, i.e., prophylactic and therapeutic. For the prophylactic protocol, vaccine administration was started at 3–4 months of age, before the appearance of amyloid deposition. APP23 mice received 6 weekly and subsequent biweekly injections of the vaccines and were examined at 7, 9, 12, 15, and 18 months of age (Fig. 1C1). The paraffin-embedded sections of the brain were stained immunohistochemically with 6F/3D against A $\beta$ 8-17, and the area of amyloid depositions was quantitated as the total sum of the pixels with NIH IMAGE software.

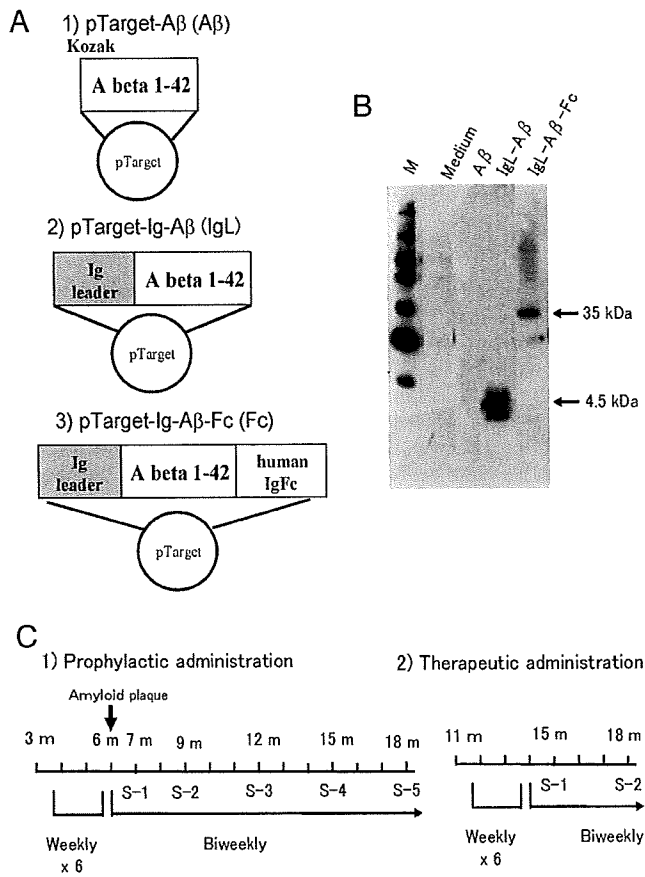
Conflict of interest statement: M.S. is employed by and a shareholder of Novartis Institutes of Biomedical Research.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: A $\beta$ , amyloid  $\beta$ ; A $\beta$ -Fc, IgL-A $\beta$ -pTarget, the Fc portion of immunoglobulin; APP, amyloid precursor protein; CFA, complete Freund's adjuvant; IgL-A $\beta$ , immunoglobulin leader sequence-A $\beta$ -pTarget; K-A $\beta$ , Kozak-A $\beta$ -pTarget; Th, T helper.

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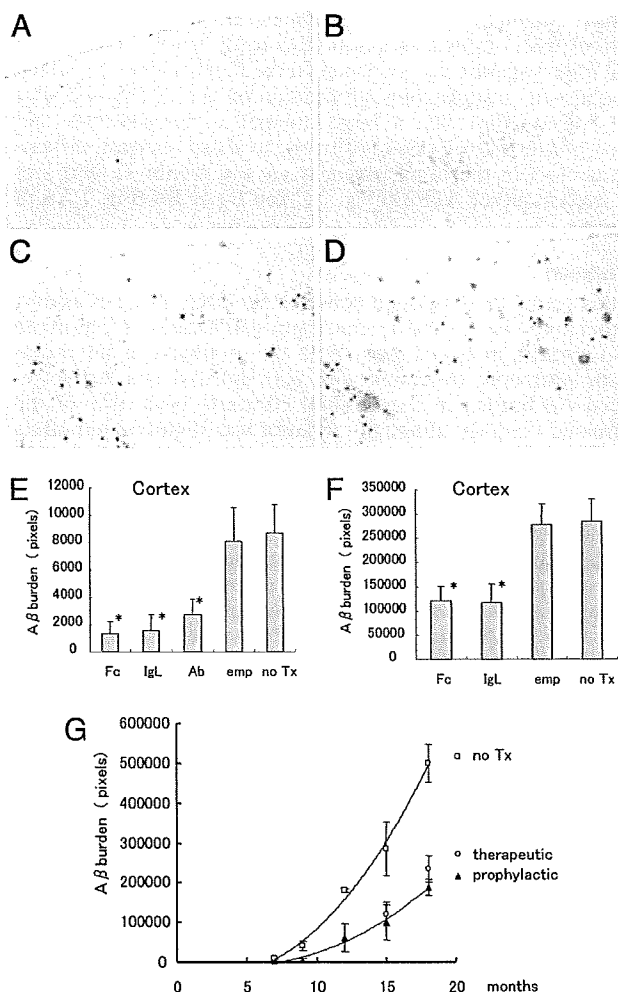
**Fig. 1.** Construction of DNA vaccines (A), *in vitro* characterization (B) and the treatment protocol (C). (A) Three DNA vaccines were produced by using a mammalian expression vector. DNA encoding the Aβ1-42 sequence was inserted in XhoI/KpnI site of the plasmid (K-Aβ vaccine) (A1). In the second vaccine, the signal sequence of mouse Igκ is added to the 5' end to improve the secretive efficiency (IgL-Aβ vaccine) (A2). The third vaccine possesses the Fc portion of human immunoglobulins to improve the stability of the secreted protein (Aβ-Fc vaccine) (A3). (B) Western blot analysis revealed that translated Aβ proteins were detected in supernatants of cultured cells transfected with IgL-Aβ and Aβ-Fc vaccines. (C) The protocol of vaccine treatment. To examine the prophylactic effect of DNA vaccines, the vaccines were administered to APP23 mice starting from 3–4 months of age, before the appearance of amyloid depositions. The mixture of one of the vaccines (100 μg) and bupivacaine (0.25 mg) was injected intramuscularly on a weekly basis for the first 6 weeks. Then, the vaccine without bupivacaine was injected every 2 weeks thereafter. Mice were sampled at 7, 9, 12, 15, and 18 months of age (C1). For therapeutic treatment, the vaccines were administered to APP23 mice starting from 12 months of age, after the appearance of amyloid plaques. Samplings were performed at 15 and 18 months of age (C2). m, months of age.

In the first series of the prophylactic treatment, mice were analyzed at 7, 9, and 12 months of age (Table 1, Exp. 1). At 7 months of age, granular amyloid depositions were recognized in the frontal cortex in the control groups (empty-vector-administered and untreated mice) (Fig. 2B). At this stage, Aβ plaques were not detected in the hippocampus (data not shown). In sharp contrast, cortical Aβ depositions in mice treated with Aβ-Fc (Fig. 2A), IgL-Aβ, and Aβ (data not shown) vaccines were significantly reduced ( $P < 0.01$ ). The Aβ burden was reduced to ~15–30% of the untreated groups (Fig. 2E). At 12 months of age, amyloid depositions in untreated mice were increased, and some of them became large ( $>50 \mu\text{g}$ ) in the frontal cortices of the untreated mice (Fig. 4C, which is published as supporting information on the PNAS web site). Cortical

**Table 1. Summary of experimental data on nonviral Aβ DNA vaccine therapy against Alzheimer model mice**

Exp.	Group	Strain	No. of animals	Start of Tx, mo	Sampling, mo	Duration of Tx, mo	Protocol										Inflammation					
							Weekly	Biweekly	Monthly	Fc	IgL	Aβ	K-	Emp	Tx	No	Fc	IgL	Aβ	Emp	Tx	No
1	1	Tg	15	4	7	3	Prophylactic	X6	X3	15.5	18.2	31.4	93.1	100	100	N.T.	N.T.	N.T.	Neg	Neg	Neg	
	2	Tg	32	4	9	5	Prophylactic	X6	X8	12.7	13.3	28.1	103	100	100	N.T.	N.T.	N.T.	Neg	Neg	Neg	
	3	Tg	17	4	12	8	Prophylactic	X6	X12	33.7	28.6	51.3	103	100	100	22	26	65.3	114	100	Neg	Neg
2	1	Tg	20	3	15	12	Prophylactic	X6	X24	30.6	37.2	N.T.	91.2	100	100	32.5	38.4	N.T.	96	100	Neg	Neg
	2	Tg	16	3	18	15	Prophylactic	X6	X29	38.5	46.2	N.T.	91.3	100	100	36.4	44.8	N.T.	99.5	100	Neg	Neg
3	1	Tg	23	12	15	3	Therapeutic	X6	X4	42.4	41.6	N.T.	98.1	100	100	40.3	43.5	N.T.	100	100	Neg	Neg
	2	Tg	19	12	18	6	Therapeutic	X6	X10	47	49.9	N.T.	96.7	100	100	38	46	N.T.	86.6	100	Neg	Neg
4	1	Tg	18	7	15	8	Therapeutic	X6	X9	67.6	81.7	N.T.	90.6	100	100	69.4	73.9	N.T.	95.6	100	Neg	Neg
	2	Tg	16	7	18	11	Therapeutic	X6	X12	79.6	88.1	N.T.	91.7	100	100	74.8	75.6	N.T.	92	100	Neg	Neg
5	1	B6	25	4	7	3	-	X6	X4	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	Neg	Neg
	2	B6	25	4	9	5	-	X6	X9	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	Neg	Neg
	3	B6	24	4	12	8	-	X6	X14	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	Neg	Neg

The vaccines were administered for the indicated duration to a large number of mice (176 APP23 and 74 B6 mice). In Exp. 1, the mice were divided into five groups as follows: Aβ-Fc vaccine administration, IgL-Aβ vaccine administration, K-Aβ vaccine administration, empty-vector administration, and no treatment. In Exps. 2–5, the K-Aβ vaccine administration group was excluded from the analysis because of its lower efficacy than other two vaccines. Each group consisted of 4–6 mice. Prophylactic effects were examined in Exps. 1 and 2. Therapeutic effects were examined in Exps. 3 and 4. All APP23 mice were analyzed immunohistochemically with mAb (6F/3D) against Aβ7-18. Then, the reduction of amyloid plaque was quantified, as shown in Materials and Methods. In Exp. 5, DNA vaccines were administered to B6 mice to know whether the vaccines induce neuroinflammation. Tissues from three mice in each group were immunohistochemically stained with mAbs against CD5 and Mac-3. Neg., negative finding, i.e., no neuroinflammation; N.T., not tested; Exp., experiment; Tx, treatment; Tg, transgenic; Emp, empty vector.



**Fig. 2.** Reduction of A $\beta$  burden in APP23 mice after DNA vaccination. (A) In mice vaccinated with A $\beta$ -Fc vaccine, amyloid plaques in the frontal cortex were reduced after 3 months of prophylactic treatment. (B) Immunohistochemical examinations revealed that granular amyloid depositions were detected in the frontal cortices of untreated mice at 7 months of age. (C) Amyloid plaques were reduced in mice treated with A $\beta$ -IgL vaccine. (D) At 15 months of age amyloid plaques of variable size were detected in the frontal cortices of untreated mice. (E) Quantitative analysis demonstrated that the cortical A $\beta$  burden at 7 months was significantly decreased ( $P < 0.01$ ) after the prophylactic treatment with A $\beta$ -Fc (15.5% of untreated controls), IgL-A $\beta$  (18.2%), and A $\beta$  vaccine (31.4%) compared with those found in untreated and empty-vector-vaccinated mice. (F) Therapeutic treatment with A $\beta$ -Fc and IgL-A $\beta$  vaccines significantly reduced ( $P < 0.01$ ) cortical A $\beta$  burden at 15 months. The overall quantitative analysis is depicted in G. The amyloid deposition was first detected in untreated mice at 7 months of age and rapidly increased after 15 months of age (open squares). Prophylactic administration of Fc-A $\beta$  vaccine prevented the A $\beta$  deposition to 10–30% of that in untreated animals before 12 months of age and to 40–50% after 15 months (filled triangles). The effects of therapeutic administration were almost the same as those of prophylactic administration (open circles). Tx, treatment; emp, empty vector. Original magnification,  $\times 62$  (A and B);  $\times 24$  (C and D).

A $\beta$  depositions were significantly reduced ( $P < 0.01$ ) to  $\approx 30$ –50% of the untreated group (Fig. 4D) after A $\beta$ -Fc (Fig. 4A) and IgL-A $\beta$  (Fig. 4B) vaccine treatment. A $\beta$  depositions in the hippocampus were also decreased equally ( $P < 0.01$ ) (Table 1, Exp. 1, Group 3). It was shown that the suppressive effect of A $\beta$ -Fc vaccine was almost equal to IgL-A $\beta$  vaccine. However, K-A $\beta$  vaccine was less effective than the former two (Figs. 2E and 4D) and was not used in subsequent experiments. The

second part of the prophylactic treatment analyzed the mice at 15 and 18 months of age (Table 1, Exp. 2). At these time points, the plaques in untreated groups had rapidly increased. Untreated APP23 mice showed an age-dependent increase of amyloid plaques in the cerebral cortex (Fig. 2G, open squares) and hippocampus (data not shown). The prophylactic protocol, using A $\beta$ -Fc vaccine, revealed that the final reduction rate of A $\beta$  burden in the cerebral cortex at 18 months of age was  $\approx 38.5\%$  of untreated groups (Table 1, Exp. 2 and Fig. 2G, closed triangles). These results demonstrated that two of the three vaccines produced in this study were effective in prophylactic treatment.

When considering the clinical applications, it is critical to know the effects of the vaccines in therapeutic application. For this purpose, the vaccination was started at 12 months of age, 6 months after the start of A $\beta$  deposition, and the brains were examined at 15 (Fig. 2) and 18 (Fig. 5, which is published as supporting information on the PNAS web site) months of age. In therapeutic treatment, amyloid plaques in the cortex were significantly decreased ( $P < 0.01$ ) (Fig. 2F) by A $\beta$ -Fc and IgL-A $\beta$  vaccination (Fig. 2D) compared with the controls (Fig. 2C). A $\beta$  depositions in the hippocampus were also decreased ( $P < 0.01$ ) (Table 1, Exp. 3). Although the therapeutic protocol (Fig. 2G, open circles) seemed to be less effective than the prophylactic one (Fig. 2G closed triangles), the difference was not significant. It should be noted that APP23 mice treated with the therapeutic protocol received DNA vaccines for only 3 and 6 months, respectively (Table 1, Exp. 3). Thus, A $\beta$  DNA vaccines had sufficient effects, even if the vaccines were administered after amyloid depositions appeared.

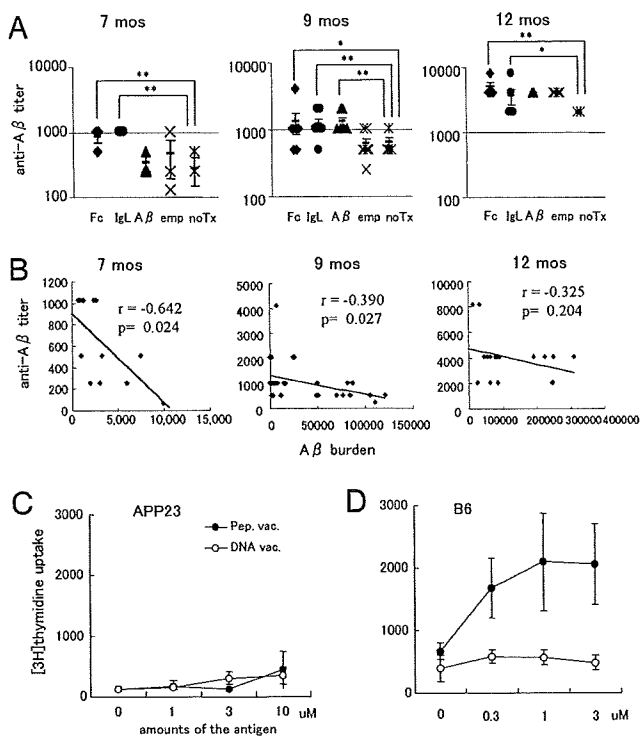
Recently, it was reported that the intracellular A $\beta$  deposition in cortical pyramidal neurons is the first neurodegenerative event in Alzheimer's disease development (19). Therefore, we counted the number of neurons containing intracellular A $\beta$  depositions in the cortices of A $\beta$ -Fc-vaccine-administered and control mice. A $\beta$ -deposited neurons were significantly decreased with both the prophylactic (50.2% of untreated control,  $P < 0.01$ ) and therapeutic (59.54%,  $P < 0.05$ ) treatments at 15 months of age (Fig. 6, which is published as supporting information on the PNAS web site).

#### Change in the Serum A $\beta$ Antibody Titer After Vaccine Administration.

The titers of serum anti-A $\beta$  antibodies after the prophylactic treatment (Table 1, Exps. 1 and 2) were determined by ELISA. The levels of anti-A $\beta$  antibodies were significantly increased (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ) 2- to 4-fold compared with the untreated and empty-vector-vaccinated mice (Fig. 3A). The titers showed an age-dependent increase in both treated and untreated mice, because the antibody production was induced in untreated APP23 mice by high levels of A $\beta$  in the sera of aged mice. In contrast, the anti-A $\beta$  antibodies in the sera of wild-type B6 mice were below the detection limit (data not shown). We also analyzed the relationships between the amounts of amyloid depositions and anti-A $\beta$  titers (Fig. 3B). The amounts of amyloid depositions were significantly smaller in mice with high antibody titers. A significant correlation between the antibody titer and the reduction of A $\beta$  burden was observed at 7 months ( $r = -0.642$ ) (Fig. 3B, 7 months) and 9 months ( $r = -0.38965$ ) (Fig. 3B, 9 months) of age by the CORREL function. The difference became less clear at a later stage (Fig. 3B, 12 months).

#### T Cell-Proliferation Assay After Vaccine Administration.

To determine whether DNA vaccination induces the T cell activation and proliferation that are key steps for the development of neuroinflammation, APP23 and B6 mice were injected with DNA vaccines. A group of mice were also immunized with A $\beta$  peptide/CFA. Three weeks after the first injection, lymphocytes were isolated and cultured with A $\beta$  peptide (0–10  $\mu$ M) for 3



**Fig. 3.** B and T cell responses of mice treated with A $\beta$  DNA vaccines. (A and B) Titration of anti-A $\beta$  antibodies in vaccinated APP23 mice. The anti-A $\beta$  antibody titer in treated mice was significantly increased (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ )  $\approx$ 2- to 4-fold compared with the untreated group. The titer levels were increased in untreated APP23 as well as treated mice at the same age period (A). There was significant correlation between the serum anti-A $\beta$  antibody titer and the reduction of amyloid depositions at 7 months of age (CORREL function  $r = -0.642$ ,  $t_0 = 2.648 > t_{10} = 2.228$ ) (B). At 9 months of age, a significant correlation was present, but the difference was less marked compared with that at 7 months ( $r = -0.38965$ ,  $t_0 = 3.10 > t_{22} = 2.086$ ). A significant difference was not noted at 12 months of age ( $r = -0.325$ ,  $t_0 = 1.3309 < t_{17} = 2.110$ ). (C and D) T cell responses in APP23 (C) and B6 (D) mice after immunization with A $\beta$  peptide/CFA or DNA vaccination. Lymphocytes isolated from two strains were incubated with A $\beta$  peptide (0–10  $\mu$ M) for 3 days. Incorporation of [ $^3$ H]thymidine was measured by liquid scintillation spectrometry. In APP23 mice, neither T cells from peptide-immunized mice nor those from DNA-vaccinated mice were activated in the presence of A $\beta$ 1-42 (C). In contrast, A $\beta$  peptide immunization, but not DNA vaccination, induced a significant T cell response in B6 mice (D). All of the data are the mean values  $\pm$  SD, and the representative results from three different experiments are shown. Tx, treatment; emp, empty vector.

days. Incorporation of [ $^3$ H]thymidine was measured by using liquid scintillation spectrometry. T cells from APP23 mice did not react with A $\beta$  peptide after both A $\beta$  DNA vaccine and A $\beta$  peptide administration (Fig. 3C). In sharp contrast, T cells from B6 mice responded significantly to A $\beta$  peptide after immunization with A $\beta$  peptide, but not after A $\beta$  DNA vaccination (Fig. 3D). Notably, A $\beta$  DNA vaccination did not induce T cell proliferation in either APP or B6 mice after three injections (Fig. 3C) and 5-month treatment (data not shown). These findings suggest that T helper (Th)1 cells in APP23 mice are in an immunologically tolerant state against A $\beta$ 1-42, probably because of long-term exposure to a high level of A $\beta$ 1-42.

**Pathological Examination of the Brains of Vaccinated Mice.** The presence or absence of neuroinflammation in the brain was examined immunohistochemically after the long-term administration of DNA vaccines. Because the T cell assay demonstrated the significant differences between APP23 and B6 mice, patho-

logical examinations were performed in both strains. Brain sections were stained with anti-CD5 mAb against T cells and with Mac-3 against macrophages. In the thymus (Fig. 7A, which is published as supporting information on the PNAS web site) and spleen (Fig. 7B), a large number of lymphocytes and macrophages were stained positively. In sharp contrast, inflammatory foci in the brain parenchyma and meninges were not detected in either APP23 (Fig. 7C and D) or B6 (Fig. 7E and F) mice.

### Discussion

In this study, we developed nonviral A $\beta$  DNA vaccines against Alzheimer's disease and demonstrated satisfactory effects in the A $\beta$  reduction in model mice. With the prophylactic and therapeutic protocols, treatment with both IgL-A $\beta$  and A $\beta$ -Fc reduced A $\beta$  burden in the cerebral cortex to  $\approx$ 40–50% of the untreated controls, although the latter was slightly more effective than the former. It should be noted that mice killed at 18 months of age received DNA vaccines for only the last 6 months. These findings suggest that relatively short-term vaccination is sufficient for the reduction of A $\beta$  burden. Because it was demonstrated in the other experimental setting that 50% reduction of A $\beta$  burden resulted in full recovery of the cognitive disturbance (6), the suppressive effects of DNA vaccines demonstrated in this study is satisfactory. Furthermore, it was reported that A $\beta$  immunotherapy reduces not only extracellular A $\beta$  plaques but also intracellular A $\beta$  accumulation and, most notably, leads to the clearance of early tau pathology by using the triple-transgenic model of Alzheimer's disease (20–22). Taken together, the outcome of DNA vaccine therapy is promising when applied to human Alzheimer's disease.

The elevation of anti-A $\beta$  antibodies was also detected after DNA vaccination. However, the antibody elevation was mild to moderate ( $\approx$ 2- to 4-fold) compared with that found in mice that had received A $\beta$  peptide (10,000-fold) (3). The adjuvant in peptide vaccines (23) may activate Th1 type T cells (10), which induce the rapid increase of antibody titers as a result. As demonstrated in this study, DNA vaccination was able to be performed without adjuvants, resulting in the absence of obvious T cell proliferation in both APP23 and B6 mice (Fig. 7) and did not cause neuroinflammation, even after long-term DNA vaccination (Fig. 7, which is published as supporting information on the PNAS web site). Importantly, mild elevation of the antibody titers induced by DNA vaccines could reduce amyloid deposits, probably because DNA vaccination constantly induces the antibody production at a low titer for a long period. Thus, the maintenance of high anti-A $\beta$  antibody titer levels is not necessary for effective treatment with DNA vaccines.

To minimize excessive immune reaction in mice and patients, we should recognize the difference in immunological reactions against A $\beta$  between the Alzheimer model and wild-type mice. As clearly demonstrated here, there was no Th1 cell response to A $\beta$  peptide in APP23 mice after A $\beta$  peptide/CFA injection, whereas, in B6 mice, the same immunization protocol induced a significant T cell response against A $\beta$  peptide (Fig. 3D). These findings strongly suggest that autoreactive Th1 cells in model mice are in a state of immune tolerance because of a high A $\beta$  expression from an early stage of life. In contrast, Th2 cells helping the antibody production seem to be working, as evidenced by the fact that vaccinated animals possessed elevated levels of anti-A $\beta$  antibodies. Similar findings were reported by Qu *et al.* (24), with gene-gun administration of A $\beta$  plasmid DNA. Monsonego *et al.* (25) also reported that the immune responses of T and B cells of model mice are low compared with those of wild-type mice. In contrast, a significant T cell reactivity to A $\beta$  peptide was detected in patients with Alzheimer's disease (23). Thus, strong immune induction is dangerous for patients with Alzheimer's disease.

The CpG motif, which exists in plasmid DNAs, is reported to induce Th1-type immunity and up-regulates IFN- $\gamma$  production under certain circumstances (26–28). However, this phenomenon is observed only when a relatively high dose of CpG oligonucleotides is used (29). In contrast, empty vectors containing the CpG motif ameliorated the clinical and histological severity of the autoimmune encephalomyelitis that is thought to be a Th1-mediated disease, as shown in previous studies by us (30) and others (31, 32).

Recently, A $\beta$  DNA vaccines were developed by using virus vectors (33, 34). Although, these vaccines effectively decreased A $\beta$  depositions in the brains of model mice, the possibility of viral replication could not be completely excluded. The plasmid vector is safe and has no possibility of viral infection and transformation because it exists as an episome without being built into the chromosome in eukaryotic cells (12, 13). Another important factor is related to technology. When DNA vaccines are in clinical use, large amounts of vaccines are necessary for treatment of a large number of patients who would be treated for a long period. Nonviral DNA vaccines have an advantage because they can be mass-produced with a high purity at a low price.

In summary, we demonstrated that nonviral A $\beta$  DNA vaccines are highly effective and safe in reducing the A $\beta$  burden in model mice and, thus, are promising as a vaccine therapy against human Alzheimer's disease.

### Materials and Methods

**Animals.** The APP23 transgenic mice used in this study express human APP751 cDNA with Swedish double mutation under the control of the neuron-specific mouse Thy-1 promoter (35, 36). The Thy-1 expression cassette lacks intron 3, which contains the elements required for expression in the thymus. RT-PCR and Western blot analysis of APP23 mice have confirmed the lack of detectable expression of the transgene in the thymus and spleen (D. Abramowski, C. Sturchler-Pierrat, and M.S., unpublished data). This finding is in agreement with a report by Moechars *et al.* (37), who used the same expression cassette and also found that the exogenous transgene with this promoter sequence is expressed only in the brain but not in the thymus. APP23 mice were initially established on a B6D2 background and have been continuously backcrossed to C57BL/6J (B6). In APP23 mice, amyloid depositions appear from 6 months of age, predominantly in the neocortex and hippocampus. A $\beta$  plaques have most of the characteristics of human Alzheimer's disease plaques, including fibrillary A $\beta$  cores, and are surrounded by dystrophic neuritis and activated glial cells. Region-specific amyloid-associated neurodegeneration, including neuron loss, synapse deficits, and cholinergic alterations, are present in these mice (38). Wild-type B6 mice were purchased from Charles River Breeding Laboratories (Kanagawa, Japan). All animal experiments were approved by the institute committee and performed in accordance with institutional guidelines.

**The Development of DNA Vaccine.** We prepared three A $\beta$  DNA vaccines using a pTarget mammalian expression system (Promega, Tokyo) (Fig. 1A). The DNA fragment, encoding A $\beta$ 1–42, was made to anneal two oligonucleotides covering the entire A $\beta$ 1–42 sequence. The Kozak sequence was inserted at the 5' end of the A $\beta$ 1–42 sequence (referred to as K-A $\beta$  vaccine). In the second vaccine, the signal sequence of mouse Ig $\kappa$  was added to the 5' end of the A $\beta$ 1–42 sequence to improve the secretory efficiency of A $\beta$  peptide (IgL-A $\beta$  vaccine). The third vaccine was made by adding the Fc portion of human immunoglobulins to the 3' end of the A $\beta$ 1–42 sequence to stabilize the secreted protein (A $\beta$ -Fc vaccine). To prevent unwanted disulfide bonds, three cysteines in the sequence were substituted with serine.

**Transfection of DNA Vaccines and Western Blot Analysis.** Three DNA vaccines were transfected to HEK293T cells and the amounts of produced A $\beta$  peptide were measured by using ELISA. HEK293T cells in 60–70% confluence were prepared on 6-well plates (Costar, Cambridge, MA). The cells were first cultured in serum-free RPMI medium 1640 for 2 h with one of three DNA vaccines (K-A $\beta$ , IgL-A $\beta$ , or A $\beta$ -Fc) and Lipofectamine PLUS reagent (Invitrogen). Then, the cells were cultured overnight in RPMI medium 1640 supplemented with 5% FBS for cellular stabilization and growth. For the Western blot analysis, the cells were again cultured in serum-free medium for an additional 8 h to remove unnecessary proteins.

Culture supernatants and cell pellets were harvested and run on NuPAGE 12% Bis-Tris gel (Invitrogen) and transferred to the PVDF membrane (Immobilon-P; Millipore). After blocking with 10% nonfat milk, the blots were incubated with 6E10 (anti-human A $\beta$ 1–17 antibody, 1:100; Abcam, Cambridge, U.K.) at 4°C for 1 h, followed by incubation with biotin-conjugated anti-mouse IgG (1:1,000; Vector Laboratories) for 1 h and with ABC-HRP (Vector Laboratories) for 1 h. The blots were developed by enhanced chemiluminescence reagents (Immunostar kit; Wako Biochemicals) according to the manufacturer's instructions.

**Administration of the Vaccines.** DNA vaccines (100  $\mu$ g) and bupivacaine (0.25 mg) in 100  $\mu$ l was administered i.m. on a weekly basis for 6 weeks (39). The vaccines without bupivacaine were injected biweekly thereafter. To examine the prophylactic effect, vaccination was started at 3–4 months of age, before amyloid plaque appearance. The therapeutic treatment was started at 12 months of age, after amyloid plaque appearance.

**Immunohistochemistry.** Mice were killed under deep anesthesia, and the brains were removed and immersion-fixed in 4% paraformaldehyde. Paraffin-embedded sections (6  $\mu$ m) were stained immunohistochemically with mAb (6F/3D) against A $\beta$ 8–17 (DAKO), anti-CD5 mAb against T lymphocytes (BD Biosciences Pharmingen) and Mac-3 against mononuclear phagocytes (BD Biosciences Pharmingen). For 6F/3D staining, the sections were pretreated in formic acid for 3 min. The sections were then incubated in the primary antibody at a 1:200 dilution. After washing, the sections were incubated with biotinylated horse anti-mouse IgG (Vector Laboratories), followed by a horseradish peroxidase (HRP)-labeled Vectastain Elite ABC kit (Vector Laboratories). HRP-binding sites were detected in 0.005% diaminobenzidine and 0.01% hydrogen peroxide. CD5 (1:25) and Mac-3 (1:25) stainings were performed similarly, with overnight incubation of the primary antibodies.

**Quantitative Analysis of A $\beta$  Burden.** A $\beta$  burdens were quantitated in the cerebral cortex and hippocampus, according to the method described in ref. 40. All of the procedures were performed by an individual blind to the experimental condition of the study. The images under an Olympus Vanox microscope were captured with a 3 charge-coupled device Olympus digital camera. The amyloid load was measured in 10 fields from the cingulate to retrosplenial cortex in the left hemispheres of the mice (600  $\times$  400  $\mu$ m each), chosen randomly. Analysis in the hippocampus was performed on the entire hippocampus in a similar manner. A $\beta$  depositions that occupied the field were expressed as pixels by using the NIH IMAGE software.

**ELISA.** Microtiter plates were coated with 2  $\mu$ g/ml human A $\beta$ 1–40 (Peptide Institute, Osaka) in 0.1 M sodium carbonate buffer (pH 9.5) at 4°C overnight. After washing three times, plates were incubated for 2 h with serial dilutions of plasma samples in PBS in 12 rows of wells starting with 4-fold-diluted plasma (the greatest dilution tested was 1:213). The plates were washed and

incubated with a 1,000-fold dilution of biotinylated anti-mouse IgG (Vector Laboratories), followed by incubation with 2-fold dilutions of Vectastain ABC-kit solution (Vector Laboratories). Bound antibodies were detected by using SIGMA FAST (Sigma-Aldrich), and the absorbance at 450 nm was read on an automated plate reader (Model 550; Bio-Rad). The antibody titer was defined as the reciprocal of the greatest dilution of plasma that gives half-maximal binding to A $\beta$ , which was determined by dividing the highest OD<sub>450</sub> value in the dilution range of each sample by 2.

**T Cell-Proliferation Assay.** The proliferative responses of draining lymph node cells were assayed in microtiter plates (Costar, Cambridge, MA) by the uptake of [<sup>3</sup>H]thymidine. A $\beta$ 1-42 peptide (50  $\mu$ g) emulsified with CFA (twice) and Fc-A $\beta$  vaccine

(three times) was injected into APP23 mice or B6 mice, and then the drainage lymph nodes were taken 3 weeks after the first injection. Lymph node cells ( $2 \times 10^5$  cells per well) were cultured with 0.3–10  $\mu$ M A $\beta$ 1-42 peptide for 3 days and subsequently pulsed for 18 h with 0.5  $\mu$ Ci (1 Ci = 37 GBq) of [<sup>3</sup>H]thymidine (Amersham Pharmacia Biotech). Incorporation of [<sup>3</sup>H]thymidine was measured by liquid scintillation spectrometry.

**Statistical Analysis.** Student's *t* test or Mann–Whitney's *U* test was used for the statistical analysis. Correlations between the antibody titer and the reduction of A $\beta$  burden was estimated by the CORREL function.

We thank Y. Kawazoe for technical assistance. This study was supported, in part, by Grants-in-Aid from the Ministry of Education, Japan, and a grant from Novartis Institutes of Biomedical Research.

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