For the determination of DRB1*1501, cDNA was amplified using a DR-2 specific primer pair (5′-TTCCTGTGGCAGCCTAAGAGG-3′ and 5′-CCGCTGCACTGTGGAGCTCT-3′). Then, the PCR products were examined for the DR2 allelic subtypes by dot blot analysis using four SSO. The SSO probes used in this study were as follows: DRB2813 (5′-GTTCCTGGACAGATACTT-3′), DRB7011 (5′-GACATCCTGGAGCAGGCG-3′), DRB8601 (5′-AACTACGGGGTTGGTGAG-3′), and DRB5706 (5′-GCCTGACGCTGAGTACTG-3′).

2.4. Interferon beta-1b (IFNB-1b) treatment

Eight million IU of IFNB-1b was injected subcutaneously every other day for 10 MS patients. Patients started this treatment either on admission to our hospital between August and December 2003 or before this admission. Five other MS patients were not treated with IFNB-1b because they refused treatment with this drug when their physicians provided information regarding the regimen. The treating physicians were blinded as to the HLA subtyping. Two NMO patients were also treated with IFNB-1b. However, their HLA were not analyzed because consent was not given by these two patients.

2.5. Statistical analysis

The frequencies of HLA DPB1*0501 and DRB1*1501 were analyzed using Fisher's exact probability method by a commercial software package (SPSS version 10.0J, SPSS Japan Inc, Tokyo, Japan).

3. Results

3.1. Japanese MS frequently carried HLA DPB1*0501 which is associated with NMO

We analyzed the frequency of HLA alleles in the MS and NMO groups (Fig. 1). Contrary to previous reports, the frequency of the DRB1*1501 allele, which is a major HLA allele of MS, did not differ between our MS and healthy subjects. Moreover, the DPB1*0501 allele, which is associated with NMO [12], was carried significantly more

Table 1
Clinical and genetic data of patients studied

Patient no.	Sex	Age, years	Clinical diagnosis	Disease duration, years	EDSS	DPB1*0501	DRB1*1501	Cerebral symptom	LESL	Blindness	CSF pleocytosis	Efficacy of IFNB-1b
1	M	50	RRMS	4	8.5	+	+	+	+	−	+	Relapse increased
2	F	45	RRMS	11	2.0	+	−	+	+	−	+	Relapse increased
3	F	54	RRMS	2	7.0	+	−	+	+	−	−	Skin ulcer
4	M	31	SPMS	8	7.5	+	+	+	−	+	−	Skin ulcer
5	F	52	SPMS	39	6.0	+	−	+	−	+	−	Relapse increased
6	M	26	RRMS	<1	2.0	+	−	+	−	−	−	Continued
7	M	47	SPMS	19	6.5	+	+	+	−	−	−	NA
8	M	37	SPMS	15	8.5	−	−	+	+	−	−	Continued
9	F	24	RRMS	<1	1.0	−	+	+	−	−	−	Continued
10	F	40	RRMS	4	2.0	−	−	+	−	−	−	Continued
11	M	33	RRMS	4	2.0	−	−	+	−	−	−	Continued
12	M	37	SPMS	11	6.0	−	+	+	−	−	−	NA
13	F	52	SPMS	8	7.0	−	+	+	−	−	−	NA
14	M	41	SPMS	26	7.5	−	−	+	−	−	−	NA
15	M	25	RRMS	<1	3.0	−	−	+	−	−	−	NA
16	F	77	NMO	2	7.5	+	−	−	+	+	−	NA
17	F	44	NMO	2	2.0	+	−	−	+	−	−	NA
18	F	36	NMO	9	7.0	+	+	−	−	−	−	NA
19	F	70	NMO	12	6.0	−	−	−	+	+	−	NA
20	F	62	NMO	9	2.0	−	−	−	+	−	+	NA
21	M	31	NMO	<1	6.0	−	−	−	+	−	−	NA
22	F	52	NMO	9	3.5	−	+	−	+	−	−	NA
23	F	57	NMO	16	1.0	−	+	−	+	−	−	NA
24	M	32	NMO	1	1.0	−	−	−	−	−	−	NA
25	F	41	NMO	22	2.0	−	+	−	−	−	−	NA
26	F	43	NMO	4	4.5	NT	NT	−	+	−	+	Skin ulcer
27	F	39	NMO	19	3.5	NT	NT	−	+	−	−	Skin ulcer

Disease duration = disease duration at entry to this study (August to December 2003).
LESL = longitudinally extensive spinal cord lesion.
IFNB-1b = interferon beta-1b.
NA = not administered.
Skin ulcer = patients who discontinued IFNB-1b due to skin ulcer.
Continued = patients who continued IFNB-1b because relapse did not markedly increase and there were no severe side effects.
NT = not tested.

Table 2
Positivity of severe optic-spinal characteristics and HLA allele, and effect of IFNB-1b in patients with MS and NMO

	"Supportive criteria" for NMO[a]	HLA				IFNB-1b treatment[b]
		DPB1*0501 (+) DRB1*1501 (−)	DPB1*0501 (+) DRB1*1501 (+)	DPB1*0501 (−) DRB1*1501 (+)	DPB1*0501 (−) DRB1*1501 (−)	
MS ($n=15$)	(+) ($n=6$)	3	2	0	1	Discontinued 5/6 (83%) Continued 1/6 (17%)
	(−) ($n=9$)	1	1	3	4	Discontinued 0/4 (0%) Continued 4/4 (100%)
NMO ($n=10$)	(+) ($n=7$)	2	0	2	3	NA
	(−) ($n=3$)	0	1	1	1	NA

[a] "Supportive criteria" for NMO include a longitudinally extensive spinal cord lesion, CSF pleocytosis and blindness.
[b] Cases/total administered (%).
IFNB-1b = interferon beta-1b.
NA = not administered.

frequently by MS patients ($p=0.03$) than by healthy subjects. Thus, our MS group was thought to consist of a heterogeneous population demonstrating genetic characteristics of both MS and NMO.

3.2. MS with DPB1*0501 showed severe optic neuritis, myelitis and CSF pleocytosis

We also analyzed the clinical characteristics of MS patients to determine whether some patients showed features of NMO. Of 7 patients of the MS group ($n=15$) who carried the DPB1*0501 allele, two patients (patients 1 and 2) showed both LESL and CSF pleocytosis (Table 1). One (patient 3) showed only LESL. Another two patients (patients 4 and 5) were blind. The remaining two (patients 6 and 7) did not show these manifestations of severe optic-spinal demyelination. In 8 MS patients without the DPB1*0501 allele, only one patient (patient 8) had LESL and none showed blindness or CSF pleocytosis. These findings demonstrate that MS patients with the DPB1*0501 allele, HLA associated with NMO, showed more severe optic neuritis, myelitis and CSF pleocytosis than MS patients without DPB1*0501.

3.3. IFNB-1b treatment was not successful in MS with genetic and clinical characteristics mimicking NMO

IFNB-1b treatment was discontinued in five MS patients for the following reasons (Table 1). Patient 1 developed a relapse with LESL after a few weeks of IFNB-1b administration and became confined to a wheelchair. IFNB-1b was discontinued for 6 months then restarted. Again, the patient developed another relapse with LESL. Patient 2 had experienced only three relapses during the past 9 years before IFNB-1b administration. Three months after IFNB-1b treatment, she developed two separate relapses in the cervical cord and optic nerve. Patient 5 showed secondary progression of the disease without marked relapse for 7 years. Then, within 2 years after IFNB-1b treatment, she developed continuous progression and 5 episodes of marked relapses in the optic nerves leading to blindness. Patients 3 and 4 had severe skin ulcers at the injection sites requiring surgical repair. The other 5 patients continued IFNB-1b because relapse did not markedly increase and there were no severe side effects. Flu-like symptoms appeared in patients 6, 9 and 11, but disappeared within 3 months. There were no relapses during the follow-up period in patients 6, 9 and 10. One relapse in the cerebrum occurred in patient 11. Secondary progression did not markedly speed up and there was no marked relapse in patient 8. Thus, all five DPB1*0501-positive MS patients who met the "supportive criteria" for NMO, i.e., LESL, CSF pleocytosis or blindness, showed a poor prognosis following IFNB-1b treatment (Table 2). IFNB-1b treatment rather exacerbated the clinical status of patients with cerebral demyelinating lesions accompanied by LESL, CSF pleocytosis or blindness. These patients should be classified as having NMO even if they have symptomatic cerebral lesions.

IFNB-1b treatment was discontinued in two NMO patients because of the skin ulcers. Patient 26 had severe skin ulcers at the injection sites requiring surgical repair. Patient 27 developed small skin ulcers and keloids within 1 month of IFNB-1b treatment. Then, IFNB-1b was halted before the skin ulcers became more severe.

4. Discussion

In the present study, we found that there were two phenotypes in the patient group showing symptomatic cerebral demyelination. One phenotype is characterized by the presence of the NMO-specific HLA allele, DPB1*0501, and severe optic-spinal demyelination represented by longitudinally extensive spinal cord lesion, blindness and CSF

pleocytosis. Importantly, patients carrying this phenotype showed a poor prognosis following IFNB-1b treatment. In another phenotype, patients did not show severe optic nerve and spinal cord demyelination and were successfully treated with IFNB-1b. We demonstrated that IFNB-1b treatment increases the frequency of severe optic nerve and spinal cord relapses or skin ulcer as side effects in the former group of MS. IFNB-1b treatment was also discontinued in two of our NMO patients because skin ulcers developed as side effects. We suppose that severe skin ulcer at the injection sites requiring surgical repair is a side effect common to both NMO and MS with severe optic-spinal demyelination. IFNB-1b should not be administered to patients with genetic and clinical characteristics mimicking NMO such as the HLA DPB1*0501 allele, longitudinally extensive spinal cord lesion, blindness and CSF pleocytosis, even if they have symptomatic cerebral lesions as typically seen in MS.

Three NMO patients (patients 18, 24 and 25) did not fulfill any of the three "supportive criteria" for NMO (Table 2). They should have been diagnosed as having MS and treated with IFNB-1b even if they did not demonstrate typical cerebral demyelination. IFNB-1b treatment has been reported to be effective for the Japanese MS even in those with lesions localized in the optic nerve and spinal cord [1]. This group should be distinguished from NMO and rather included to MS according to the effect of IFNB-1b treatment.

We agree with the revised criteria for NMO [7]. We consider that the presence or absence of symptomatic cerebral lesions cannot be the absolute criterion for diagnosing NMO and that the presence of longitudinally extensive spinal cord lesion is essential for classification. The question is whether these criteria are also applicable to Japanese demyelinating patients. Nakashima et al. reported that the frequency of NMO-IgG in Japanese NMO was only 63%, although 15% of Japanese MS had NMO-IgG [10]. We suspect that the reason for the low frequency of NMO-IgG in NMO and the high frequency in MS might be misdiagnosis. As discussed above, there are two types of borderline patients in Japan. One phenotype is characterized by cerebral demyelinating lesions accompanied by NMO-specific HLA allele, DPB1*0501, and severe optic-spinal demyelination represented by longitudinally extensive spinal cord lesion, blindness and CSF pleocytosis. Another is MS with lesions localized in the optic nerve and spinal cord. We consider that the former phenotype is NMO and the latter is MS. Diagnosis and the frequency of NMO-IgG should be reanalyzed to these Japanese borderline patients. Conversely, they reported that complete blindness in at least one eye, as well as longitudinally extensive spinal cord lesion, was a significant feature in NMO-IgG-positive patients [10]. However, the revised criteria for NMO do not address the issues of blindness and CSF pleocytosis [7]. We should continue to discuss the diagnostic criteria for NMO using HLA subtyping and the old "supportive criteria" for NMO, i.e., longitudinally extensive spinal cord lesion, blindness and CSF pleocytosis.

One limitation of our study is that the limited number of patients was treated with IFNB-1b. In Japan, it has only been several years since IFNB-1b was approved for use to date and the number of patients treated with IFNB-1b remains limited. We should closely monitor a larger numbers of patients to obtain firmer conclusions.

In the present study, we analyzed the clinical and genetic features of NMO, MS and borderline patients and examined the relationship between clinical and genetic phenotypes and the effects of IFNB-1b treatment. The outcomes demonstrated that IFNB-1b should not be administered to demyelinating patients with genetic and clinical characteristics mimicking NMO such as HLA DPB1*0501 allele, longitudinally extensive spinal cord lesion, blindness and CSF pleocytosis even if they demonstrate symptomatic cerebral lesions. These patients should be classified as having NMO even if they have symptomatic cerebral lesions.

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Nonviral Aβ DNA vaccine therapy against Alzheimer's disease: Long-term effects and safety

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It was recently demonstrated that amyloid β (Aβ) peptide vaccination was effective in reducing the Aβ 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-Aβ antibody therapy and other types of vaccination are now in trial. In this study, we have developed safe and effective nonviral Aβ DNA vaccines against Alzheimer's disease. We administered these vaccines to model (APP23) mice and evaluated Aβ burden reduction. Prophylactic treatments started before Aβ deposition reduced Aβ 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 Aβ deposition reduced Aβ burden to≈50% at the age of 18 months. Importantly, this therapy induced neither neuroinflammation nor T cell responses to Aβ peptide in both APP23 and wild-type B6 mice, even after long-term vaccination. Although it is reported that other anti-Aβ 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 β-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 β (Aβ) production and clearance (amyloid cascade hypothesis) (1, 2). On the basis of this hypothesis, Schenk *et al.* (3) developed an Aβ-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 Aβ-peptide and adjuvant (4) and the passive transfer of anti-Aβ antibodies were also effective in reducing amyloid deposits (5). Moreover, vaccinated mice showed an improvement in memory loss (6, 7). Thus, Aβ 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 Aβ1-42 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 Aβ 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 Aβ DNA Vaccines. We prepared three types of nonviral Aβ DNA vaccines using a mammalian expression vector, pTarget. The sequence of Aβ1-42 and additional sequences were inserted in the plasmid, as shown in Fig. 1*A*. The first one contains only the Aβ1-42 sequence with the Kozak sequence at the 5′ end (referred to as K-Aβ vaccine) (Fig. 1*A1*). To the second, the Igκ signal sequence of mouse Ig was added to improve the secretion ability (IgL-Aβ vaccine) (Fig. 1*A2*), and the third possesses the Fc portion of human Ig at the 3′ end to maintain stability (Aβ-Fc vaccine) (Fig. 1*A3*). Before *in vivo* administration, these DNA vaccines were transfected to HEK295T cells, and the secretion of Aβ1-42 peptide into the culture supernatant was assayed with Western blotting (Fig. 1*B*). The production of intracellular Aβ1-42 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-Aβ and Aβ-Fc vaccines contained translated proteins (4.5 and 35 kDa, respectively), whereas K-Aβ-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 Aβ DNA Vaccination. We used two types of regimens to examine the effect of Aβ 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. 1*C1*). The paraffin-embedded sections of the brain were stained immunohistochemically with 6F/3D against Aβ8-17, 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.

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

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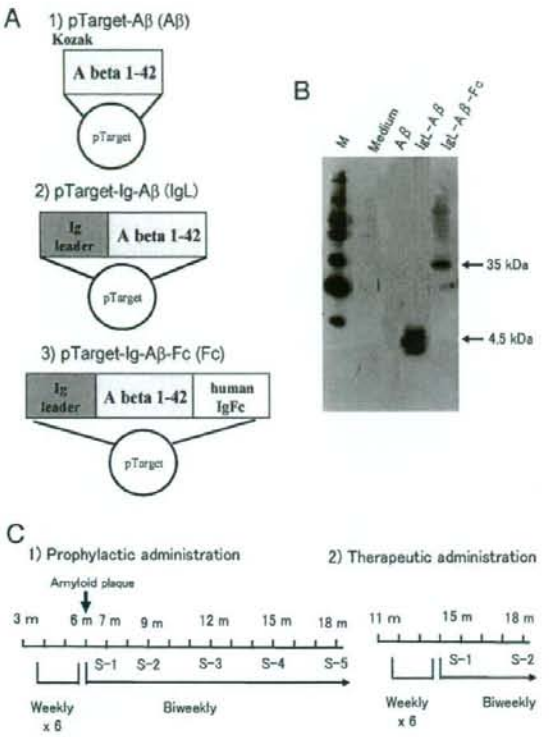

Fig. 1. Construction of DNA vaccines (*A*), *in vitro* characterization (*B*) and the treatment protocol (*C*). (*A*) Three DNA vaccines were produced by using a mammalian expression vector. DNA encoding the Aβ1-42 sequence was inserted in XhoI/KpnI site of the plasmid (K-Aβ vaccine) (*A1*). In the second 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 administrated 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. 2*B*). 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. 2*A*), 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. 2*E*). At 12 months of age, amyloid depositions in untreated mice were increased, and some of them became large (>50 μg) in the frontal cortices of the untreated mice (Fig. 4*C*, 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: Regimen	Weekly	Biweekly	Monthly	% Aβ burden (cortex): Fc	IgL	K-Aβ	Emp	No Tx	% Aβ burden (hippocampus): Fc	IgL	K-Aβ	Emp	No Tx	Inflammation: CD5	Mac3
1	1	Tg	15	4	7	3	Prophylactic	×6	×3		15.5	18.2	31.4	93.1	100	N.T.	N.T.	N.T.	N.T.	N.T.	Neg	Neg
	2	Tg	32	4	9	5	Prophylactic	×6	×8		12.7	13.3	28.1	103	100	N.T.	N.T.	N.T.	N.T.	N.T.	Neg	Neg
	3	Tg	17	4	12	8	Prophylactic	×6	×12		33.7	28.6	51.3	103	100	22	26	65.3	114	100	Neg	Neg
2	1	Tg	20	3	15	12	Prophylactic	×6	×24		30.6	37.2	N.T.	91.2	100	32.5	38.4	N.T.	96	100	Neg	Neg
	2	Tg	16	3	18	15	Prophylactic	×6	×29		38.5	46.2	N.T.	91.3	100	36.4	44.8	N.T.	99.5	100	Neg	Neg
3	1	Tg	23	12	15	3	Therapeutic	×6	×4		42.4	41.6	N.T.	98.1	100	40.3	43.5	N.T.	100	100	Neg	Neg
	2	Tg	19	12	18	6	Therapeutic	×6	×10		47	49.9	N.T.	96.7	100	38	46	N.T.	86.6	100	Neg	Neg
4	1	Tg	18	7	15	8	Therapeutic			×9	67.6	81.7	N.T.	90.6	100	69.4	73.9	N.T.	95.6	100	Neg	Neg
	2	Tg	16	7	18	11	Therapeutic			×12	79.6	88.1	N.T.	91.7	100	74.8	75.6	N.T.	92	100	Neg	Neg
5	1	B6	25	4	7	3	–	×6	×4		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	–	×6	×9		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	–	×6	×14		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.

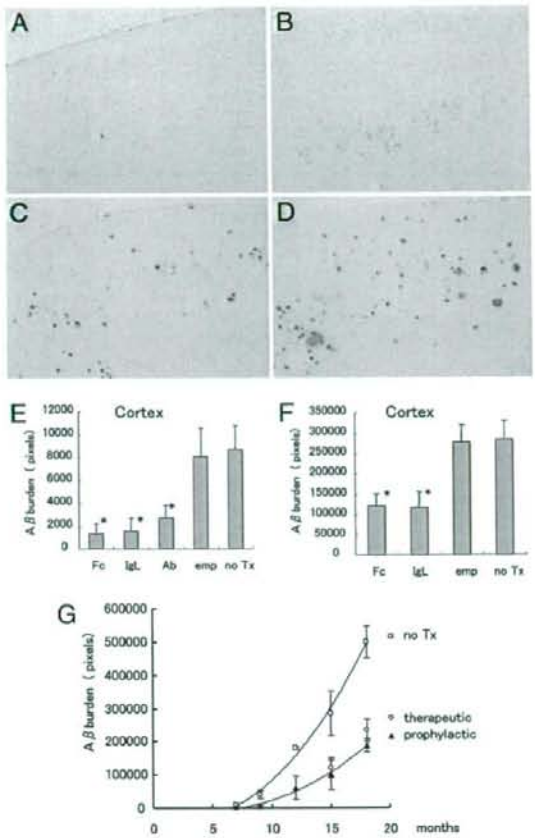

Fig. 2. Reduction of Aβ burden in APP23 mice after DNA vaccination. (*A*) In mice vaccinated with Aβ-Fc vaccine, amyloid plaques in the frontal cortex were reduced after 3 months of prophylactic treatment. (*B*) Immunohistochemical examinations revealed that granular amyloid depositions were detected in the frontal cortices of untreated mice at 7 months of age. (*C*) Amyloid plaques were reduced in mice treated with Aβ-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 Aβ burden at 7 months was significantly decreased ($P < 0.01$) after the prophylactic treatment with Aβ-Fc (15.5% of untreated controls), IgL-Aβ (18.2%), and Aβ vaccine (31.4%) compared with those found in untreated and empty-vector-vaccinated mice. (*F*) Therapeutic treatment with Aβ-Fc and IgL-Aβ vaccines significantly reduced ($P < 0.01$) cortical Aβ 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-Aβ vaccine prevented the Aβ 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, ×62 (*A* and *B*); ×24 (*C* and *D*).

Aβ depositions were significantly reduced ($P < 0.01$) to ≈30–50% of the untreated group (Fig. 4*D*) after Aβ-Fc (Fig. 4*A*) and IgL-Aβ (Fig. 4*B*) vaccine treatment. Aβ 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 Aβ-Fc vaccine was almost equal to IgL-Aβ vaccine. However, K-Aβ vaccine was less effective than the former two (Figs. 2*E* and 4*D*) 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. 2*G*, open squares) and hippocampus (data not shown). The prophylactic protocol, using Aβ-Fc vaccine, revealed that the final reduction rate of Aβ burden in the cerebral cortex at 18 months of age was ≈38.5% of untreated groups (Table 1, Exp. 2 and Fig. 2*G*, 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 Aβ 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. 2*F*) by Aβ-Fc and IgL-Aβ vaccination (Fig. 2*D*) compared with the controls (Fig. 2*C*). Aβ depositions in the hippocampus were also decreased ($P < 0.01$) (Table 1, Exp. 3). Although the therapeutic protocol (Fig. 2*G*, open circles) seemed to be less effective than the prophylactic one (Fig. 2*G* 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, Aβ DNA vaccines had sufficient effects, even if the vaccines were administrated after amyloid depositions appeared.

Recently, it was reported that the intracellular Aβ 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 Aβ depositions in the cortices of Aβ-Fc-vaccine-administered and control mice. Aβ-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 Aβ Antibody Titer After Vaccine Administration. The titers of serum anti-Aβ antibodies after the prophylactic treatment (Table 1, Exps. 1 and 2) were determined by ELISA. The levels of anti-Aβ antibodies were significantly increased (**, $P < 0.01$; *, $P < 0.05$) 2- to 4-fold compared with the untreated and empty-vector-vaccinated mice (Fig. 3*A*). 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 Aβ in the sera of aged mice. In contrast, the anti-Aβ 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-Aβ titers (Fig. 3*B*). 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 Aβ burden was observed at 7 months ($r = -0.642$) (Fig. 3*B*, 7 months) and 9 months ($r = -0.38965$) (Fig. 3*B*, 9 months) of age by the CORREL function. The difference became less clear at a later stage (Fig. 3*B*, 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 Aβ peptide/CFA. Three weeks after the first injection, lymphocytes were isolated and cultured with Aβ peptide (0–10 μM) for 3

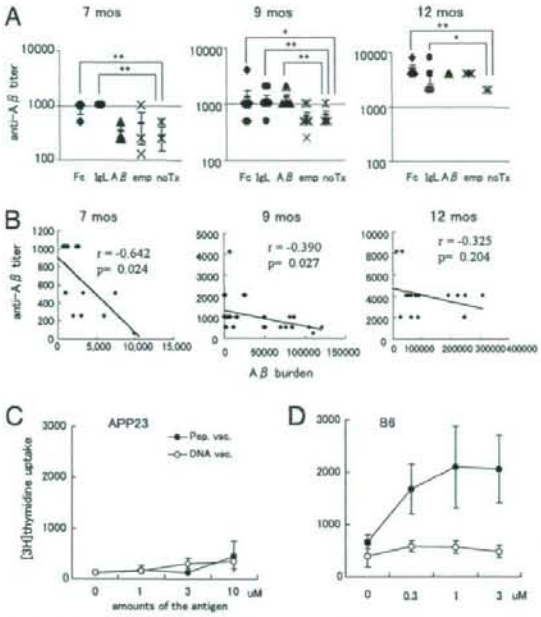

Fig. 3. B and T cell responses of mice treated with Aβ DNA vaccines. (*A* and *B*) Titration of anti-Aβ antibodies in vaccinated APP23 mice. The anti-Aβ antibody titer in treated mice was significantly increased (**, $P < 0.01$; *, $P < 0.05$) ≈2- to 4-fold compared with the untreated group. The titer 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-Aβ antibody titer and the reduction of amyloid depositions at 7 months of age (CORREL function $r = -0.642$, $t0 = 2.648 > t10 = 2.228$) (*B*). At 9 months of age, a significant correlation was present, but the difference was less marked compared with that at 7 months ($r = -0.38965$, $t0 = 3.10 > t22 = 2.086$). A significant difference was not noted at 12 months of age ($r = -0.325$, $t0 = 1.3309 < t17 = 2.110$). (*C* and *D*) T cell responses in APP23 (*C*) and B6 (*D*) mice after immunization with Aβ peptide/CFA or DNA vaccination. Lymphocytes isolated from two strains were incubated with Aβ peptide (0–10 μM) 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 Aβ1-42 (*C*). In contrast, Aβ peptide immunization, but not DNA vaccination, induced a significant T cell response in B6 mice (*D*). All of the data are the mean values ± 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 Aβ peptide after both Aβ DNA vaccine and Aβ peptide administration (Fig. 3*C*). In sharp contrast, T cells from B6 mice responded significantly to Aβ peptide after immunization with Aβ peptide, but not after Aβ DNA vaccination (Fig. 3*D*). Notably, Aβ DNA vaccination did not induce T cell proliferation in either APP or B6 mice after three injections (Fig. 3*C*) 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 Aβ1-42, probably because of long-term exposure to a high level of Aβ1-42.

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, pathological 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. 7*A*, which is published as supporting information on the PNAS web site) and spleen (Fig. 7*B*), 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. 7 *C* and *D*) or B6 (Fig. 7 *E* and *F*) mice.

Discussion

In this study, we developed nonviral Aβ DNA vaccines against Alzheimer's disease and demonstrated satisfactory effects in the Aβ reduction in model mice. With the prophylactic and therapeutic protocols, treatment with both IgL-Aβ and Aβ-Fc reduced Aβ burden in the cerebral cortex to ≈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 Aβ burden. Because it was demonstrated in the other experimental setting that 50% reduction of Aβ 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 Aβ immunotherapy reduces not only extracellular Aβ plaques but also intracellular Aβ 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-Aβ antibodies was also detected after DNA vaccination. However, the antibody elevation was mild to moderate (≈2- to 4-fold) compared with that found in mice that had received Aβ 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-Aβ 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 Aβ between the Alzheimer model and wild-type mice. As clearly demonstrated here, there was no Th1 cell response to Aβ peptide in APP23 mice after Aβ peptide/CFA injection, whereas, in B6 mice, the same immunization protocol induced a significant T cell response against Aβ peptide (Fig. 3*D*). These findings strongly suggest that autoreactive Th1 cells in model mice are in a state of immune tolerance because of a high Aβ 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-Aβ antibodies. Similar findings were reported by Qu *et al.* (24), with gene-gun administration of Aβ 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 Aβ 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-γ 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, Aβ DNA vaccines were developed by using virus vectors (33, 34). Although, these vaccines effectively decreased Aβ 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 Aβ DNA vaccines are highly effective and safe in reducing the Aβ 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. Aβ plaques have most of the characteristics of human Alzheimer's disease plaques, including fibrillary Aβ 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 Aβ DNA vaccines using a pTarget mammalian expression system (Promega, Tokyo) (Fig. 1*A*). The DNA fragment, encoding Aβ1-42, was made to anneal two oligonucleotides covering the entire Aβ1-42 sequence. The Kozak sequence was inserted at the 5′ end of the Aβ1-42 sequence (referred to as K-Aβ vaccine). In the second vaccine, the signal sequence of mouse Igκ was added to the 5′ end of the Aβ1-42 sequence to improve the secretory efficiency of Aβ peptide (IgL-Aβ vaccine). The third vaccine was made by adding the Fc portion of human immunoglobulins to the 3′ end of the Aβ1-42 sequence to stabilize the secreted protein (Aβ-Fc vaccine). To prevent unwanted disulfide bonds, three cystines 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 Aβ 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-Aβ, IgL-Aβ, or Aβ-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 Aβ1–17 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 (VectorLaboratories) 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 μg) and bupivacaine (0.25 mg) in 100 μl 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 μm) were stained immunohistochemically with mAb (6F/3D) against Aβ8–17 (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 Aβ Burden. Aβ 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 cingulated to retrosplenial cortex in the left hemispheres of the mice (600 × 400 μm each), chosen randomly. Analysis in the hippocampus was performed on the entire hippocampus in a similar manner. Aβ depositions that occupied the field were expressed as pixels by using the NIH IMAGE software.

ELISA. Microtiter plates were coated with 2 μg/ml human Aβ1-40 (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 Aβ, which was determined by dividing the highest OD_{450} 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 [^{3}H]thymidine. Aβ1-42 peptide (50 μg) emulsified with CFA (twice) and Fc-Aβ 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 (2×10^5 cells per well) were cultured with 0.3–10 μM Aβ1-42 peptide for 3 days and subsequently pulsed for 18 h with 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (Amersham Pharmacia Biotech). Incorporation of [^{3}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 Aβ burden was estimated by the CORREL function.

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