respectively. For positive controls in the TNF assay, 2 types of brain and spinal cord tissue samples were used. For the first type, C57Bl6 mice were given an intraperitoneal injection of 100 μg lipopolysaccharide, and brain tissue was harvested 1 hour later and subjected to ELISA. Second, C57Bl6 mice were immunized twice on Days 0 and 7 with 300 μg of recombinant rat myelin oligodendrocyte protein (MOG) emulsified with complete Freund adjuvant. On Days

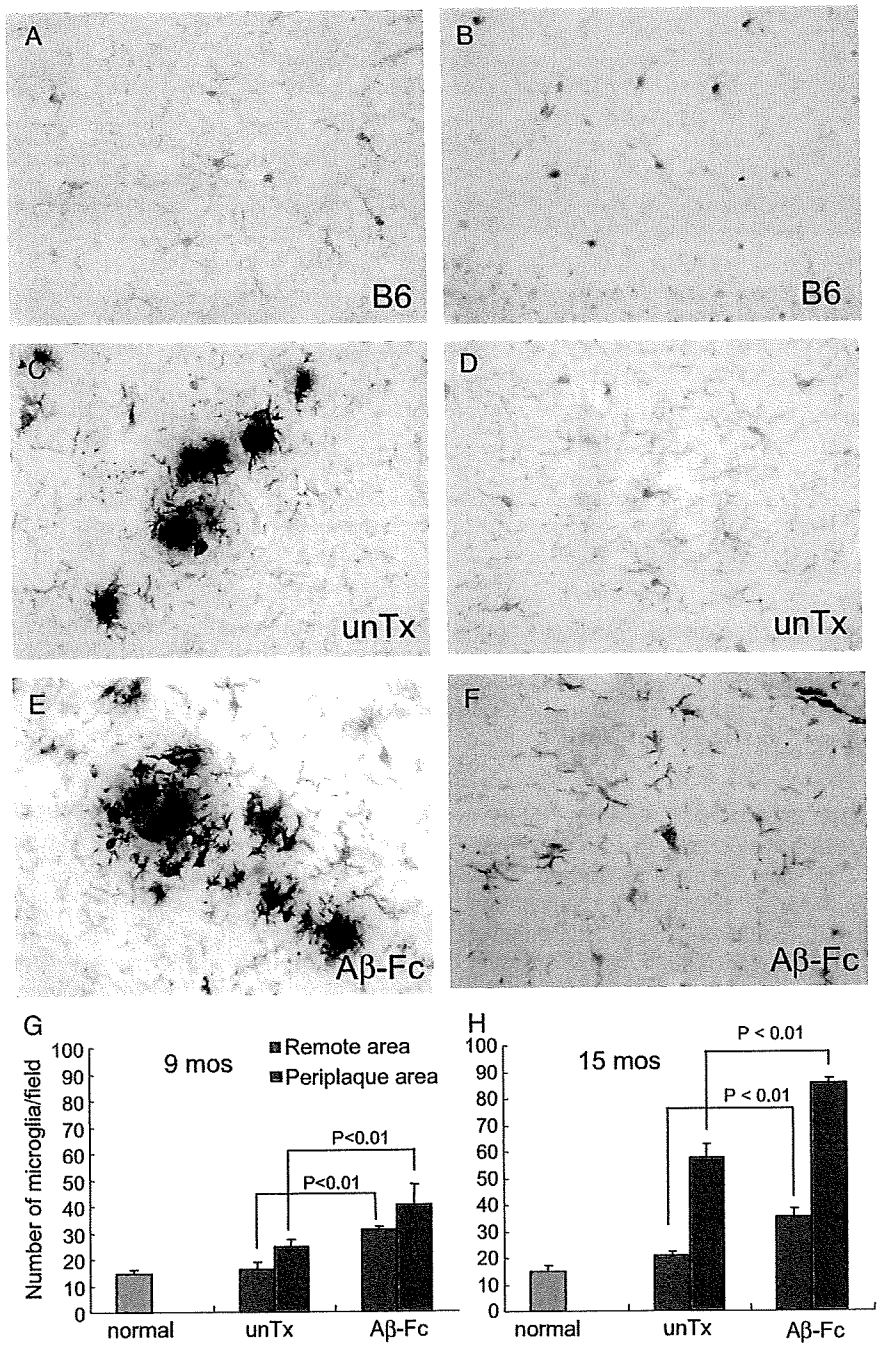

FIGURE 3. Double staining with monoclonal antibodies 6F3D against β-amyloid (Aβ) (blue) and Iba-1 against microglia (brown) of the brains of treated and untreated mice. In normal control B6 mice, ramified resting microglia were sparse in the cortex **(A)** and hippocampus **(B)**. Around plaques of untreated APP23 mice, there were microglia with abundant cytoplasm and processes that had bulbous swellings **(C)**. In areas remote from the plaques in nontreated APP23 mice, resting microglia were sparsely distributed as in control B6 mice **(D)**. In vaccinated mice, more microglia infiltrated amyloid plaques **(E)**. In the area remote from plaques in treated APP23 mice, microglia were more numerous and showed an activated phenotype **(F)**. Semiquantitative analysis was performed at 9 months **(G)** and 15 months **(H)** by counting microglial cell number in 10 fields (3–4 mice per group). Normal, normal B6 mice; unTx, untreated APP23 mice; Aβ-Fc, vaccinated mice.

0 and 2, the mice received intraperitoneal injection of pertussis toxin (300 ng). When they showed complete hind leg paralysis on Day 20 (i.e. clinical experimental autoimmune encephalomyelitis [EAE]), lumbar spinal cords were removed and subject to ELISA analysis.

Statistical Analysis

Student *t*-test or the Mann-Whitney U test was used for the statistical analysis. Values of $p < 0.05$ were considered significant.

RESULTS

DNA Vaccination Reduces Aβ Burden in the Brains of AD Model Mice

We prepared nonviral Aβ DNA vaccines and injected them (100 μg each) into APP23 mice beginning at 4 months of age on a weekly and then biweekly basis (7). At 15 months of age, Aβ deposits were considerably reduced (Figs. 1A, B). Semiquantitative analysis revealed that Aβ deposits in treated mice were reduced to approximately one third of those in nonvaccinated control mice in both the cerebral cortex and hippocampus (Figs. 1C, D).

IgG Deposits Were Detected on Aβ Plaques in the Brains of DNA-Vaccinated, But Not Control Mice

To determine the possible mechanisms of Aβ reduction after DNA vaccination, it was essential to know whether the anti-Aβ antibodies raised by vaccination reach the brain and decorate Aβ plaques. We previously found that the DNA vaccination protocol resulted in a mild but significant induction of anti-Aβ antibodies in plasma in vaccinated mice (7). We performed immunohistochemistry using anti-mouse IgG antibodies to identify IgG on Aβ plaques. Plaques in the brains of treated mice were stained positively for IgG (Fig. 2A), whereas those in untreated mice were completely negative (Fig. 2B). Some cells with morphological features of microglia were also positive for IgG (Fig. 2A). Interestingly, Aβ plaques in empty vector–administered mice were also negative for IgG (data not shown). Thus, antibody binding to the Aβ plaques may occur in the brains of vaccinated mice in vivo.

Microglial Activation and Phagocytosis Induced by DNA Vaccination

We assessed phagocytosis of Aβ deposits by microglia after DNA vaccination. Brain sections from treated and control (i.e. untreated APP23 and wild-type) mice were double-stained with Iba-1 and 6F/3D mAbs. We previously determined that DNA vaccination did not elicit neuroinflammation in either AD model or wild-type mice (7). Because in the present study, Iba-1–positive cells in the CNS showed typical features of resident microglia, they likely were microglia and not macrophages. In untreated B6 mice, ramified microglia with small cytoplasm and fine processes were sparsely distributed throughout the cerebral cortex

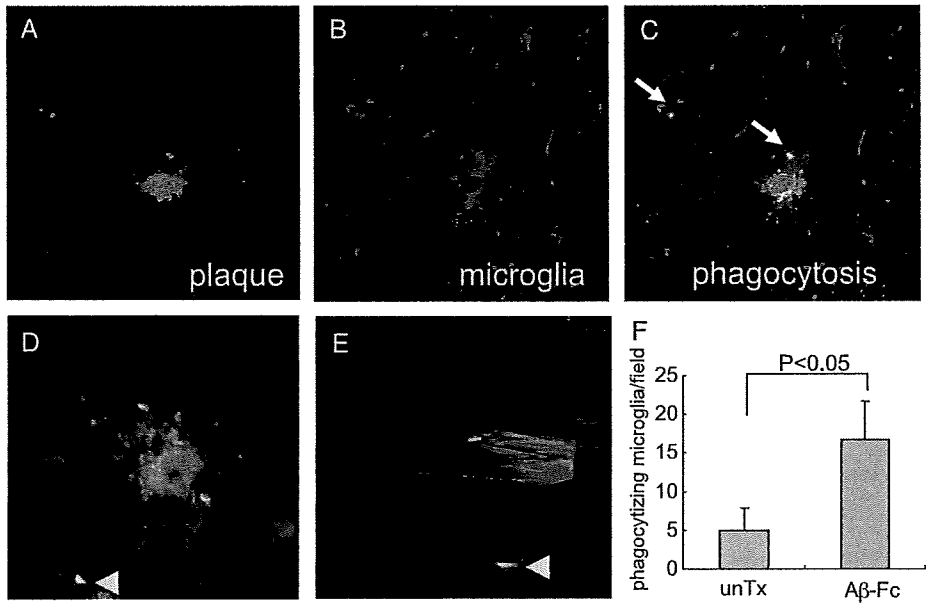

FIGURE 4. Phagocytosis of β-amyloid (Aβ) deposits by activated microglia in the cerebral cortex of mice at 15 months of age. Brain sections from treated **(A–E)** and untreated (not shown) APP23 mice were stained for Aβ with (6F3D, green) **(A)** and for microglia (Iba-1, red) **(B)** monoclonal antibodies and observed with a confocal microscope. Some microglia surrounding the amyloid plaque contained Aβ deposits (C, arrows). Microglia (red) in areas away from Aβ plaques had Aβ staining (**D**, arrowhead) within the cytoplasm. Using 3-dimensional reconstruction, a different plane of the view was shown in (**E**). The Aβ deposit ingested by a microglial cell was indicated by an arrowhead in (**E**). Semiquantitative analysis revealed that the number of phagocytosed Aβ deposits increased approximately 2.5-fold in treated compared with untreated mice ([**F**] $p < 0.05$). Phagocytosed particles in 10 fields from 4 treated and 4 control mice (total, 40 fields in each group) were counted and compared.

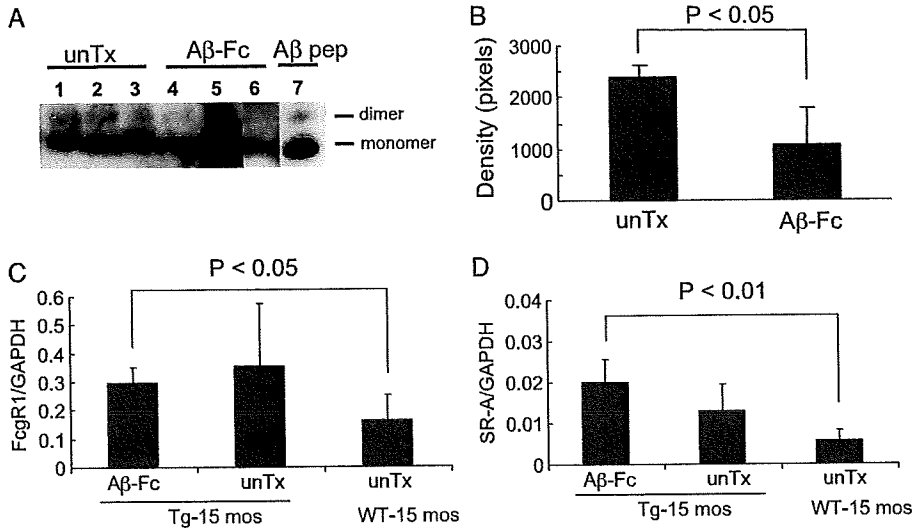

FIGURE 5. Western blot analysis **(A)** demonstrated that β-amyloid (Aβ) monomers plus dimers were reduced after DNA vaccination. Measurements of band densities revealed a statistically significant difference between vaccinated and control samples ($p < 0.05$). Lanes 1 to 5 and 7 were obtained from the same blot. Although Lane 6 was from a different blot, it was confirmed that the densities of the standard synthetic peptide in 2 blots were identical. unTx, untreated APP23 mice; Aβ-Fc, vaccinated mice; Aβ-pep, synthetic Aβ peptide positive control. **(C, D)** Real-time polymerase chain reaction analysis of messenger RNA (mRNA) levels of phagocytosis-related receptors, Fcγ receptor 1 (FcγR1) and scavenger receptor A (SR-A). Messenger RNA for these receptors was significantly greater in vaccinated APP mice than in untreated wild-type mice. Receptor mRNAs were also upregulated in untreated APP mice, consistent with the observations that microglia were activated and increased in untreated APP mice (Fig. 3). There were no significant differences between vaccinated and untreated APP mice or between untreated APP and wild-type mice in **(C)** and **(D)**. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Fig. 3A) and hippocampus (Fig. 3B). In untreated APP23 transgenic mice, activated ameboid microglia were seen around amyloid plaques (periplaque area); their processes were present deep within the plaques (Fig. 3C). In areas away from the plaques (remote area), ramified microglia were similar to those in wild-type mice (Fig. 3D). After DNA vaccination, microglia in the periplaque area seem to be increased in number and were clustered around the plaques (Fig. 3E). The major difference between vaccinated and nonvaccinated AD mice, however, was the morphological change of microglia in the remote areas. In vaccinated mice, microglia had more ameboid forms with long processes (Fig. 3F). To analyze the increase of microglia in a semi-quantitative manner, microglia were counted in both peri-plaque and remote areas in brain sections from normal, untreated, and treated mice. At 9 months of age, there were significantly more microglia in both areas in treated compared with untreated AD mice ($p < 0.01$); in treated mice, microglia were more numerous in periplaque areas (40.3 ± 7.9 in each 600 × 400 μm field) compared with remote areas (30.9 ± 2.7 in each field; Fig. 3G). At 15 months of age, immunostained microglia were also more numerous, particularly in periplaque areas in AD mice, with patterns similar to those at 9 months of age (Fig. 3H).

In double-stained brain sections, small Aβ deposits seemed to be located inside microglia. This was confirmed by confocal microscopy. In periplaque area, Cy3-labeled microglia (red) (Fig. 4B) enclosed fluorescein isothiocyanate–labeled Aβ deposits (green) (Fig. 4A). The merged image indicates small Aβ deposits within microglia (arrows in Fig. 4C). Ingestion of Aβ deposits was confirmed by 3-dimensional reconstruction view. Localization of Aβ deposits within the cytoplasm of microglia was demonstrated by the

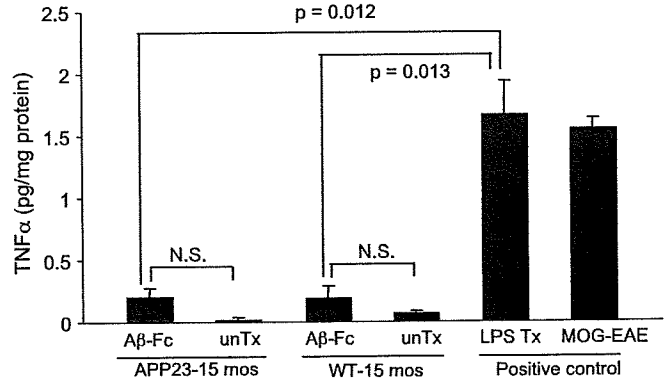

FIGURE 6. Tumor necrosis factor (TNF) levels in the brains of β-amyloid (Aβ)-Fc–treated and untreated B6 and APP23 mice detected by ELISA of CNS tissue homogenates. Large amounts of TNF were detected in the brains of positive control mice that had been given either an intraperitoneal injection of lipopolysaccharide or myelin oligodendrocyte protein (MOG)–induced experimental autoimmune encephalomyelitis ([EAE] spinal cord sample). Tumor necrosis factor in the brains of untreated B6 and APP23 mice was almost undetectable. p values are indicated. Additional p values for Aβ-Fc APP23 versus MOG-EAE and Aβ-Fc wild-type versus MOG-EAE are $p = 0.0001$ and $p = 0003$, respectively. Aβ-Fc, vaccinated mice; unTx, untreated APP23 mice.

different plane of the z axis view (Figs. 4D, E). Semiquantitative analysis revealed that the numbers of microglia phagocytosing Aβ deposits were significantly increased in vaccine-treated compared with untreated mice ($p < 0.05$; Fig. 4F). Phagocytosis in remote areas was interpreted as indicating clearance of invisible small Aβ aggregates such as Aβ oligomers by activated microglia.

To confirm this, we performed Western blot analysis. As shown in Figures 5A and B, Aβ aggregates were reduced by DNA vaccination compared with untreated controls. Thus, Aβ phagocytosis away from amyloid plaques and Aβ oligomer reduction after DNA vaccination may be beneficial for cognitive decline in AD patients because Aβ oligomers show toxic effects on neurons in AD brains (19, 20). We also quantitated mRNA levels of phagocytosis-related receptors, Fcγ receptor 1 and scavenger receptor A, by real-time PCR. As shown in Figures 5C and D, mRNA for these receptors was significantly upregulated in vaccinated APP mice compared with untreated wild-type mice. However, receptor mRNA was also upregulated in untreated APP mice. This finding was consistent with morphological observations that microglia were activated and increased in untreated APP mice (Fig. 3).

TNF Did Not Increase Significantly in the Brain After DNA Vaccination

To determine whether activated microglia in AD mice are neurotoxic or neuroprotective, we measured the TNF levels with ELISA. Tumor necrosis factor is a proinflammatory cytokine and is regarded as a biomarker of risk for the development of meningoencephalitis (21). Large amounts of TNF were detected in the brains of LPS-treated mice and in the spinal cords of mice with MOG-induced EAE, but levels of TNF in the brain of vaccinated and control B6 and APP23 mice assayed in the same manner were very low (Fig. 6). Thus, activated microglia in the brains of DNA vaccinated AD model mice did not produce large amounts of TNF and seem to be nonneurotoxic.

Direct Effects of Anti-Aβ Antibodies on Aβ Plaques as Suggested by Amyloid Plaque Immunoreactivity Assay

The second hypothesis to explain the mechanism of Aβ reduction is the direct effect of anti-Aβ antibodies on Aβ deposits, leading to the dissolution of amyloid fibrils or neutralization of Aβ oligomers (22). Because it was difficult

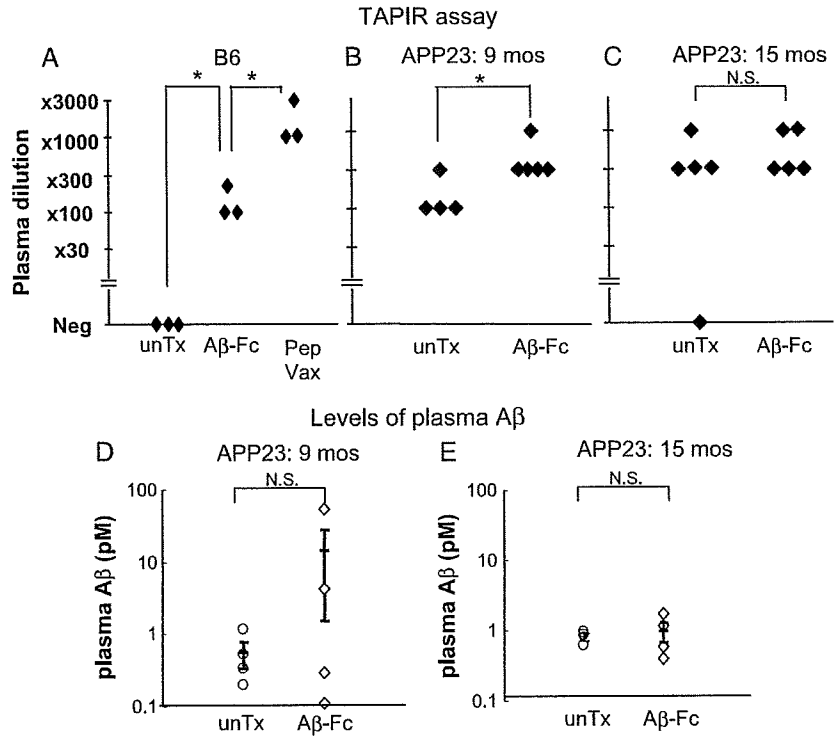

FIGURE 7. Tissue amyloid plaque immunoreactivity (TAPIR) assay and plasma β-amyloid (Aβ) levels in DNA vaccinated and untreated mice. **(A–C)** The binding of plasma from Aβ-immunized mice to Aβ plaques was determined using the TAPIR assay. **(A)** In plasma from untreated B6 mice, there was no Aβ-binding activity (unTx). By contrast, plasma from Aβ peptide–immunized B6 mice (Pep Vax) showed high titers, and plasma from DNA-vaccinated mice (DNA Vax) showed intermediate values. Asterisks indicate $p < 0.05$. **(B)** Plasma from untreated APP23 mice at 9 months of age showed intermediate binding, which was significantly different from the treated group ($p < 0.05$); differences were not significant (N.S.) at 15 months of age **(C)**. **(D, E)** At 9 months of age, plasma Aβ levels were slightly increased in some mice after DNA vaccine therapy **(D)**, but at 15 months of age, plasma Aβ levels in treated mice were almost the same as those of untreated mice **(E)**. No differences between treated and untreated groups at either age were significant (N.S.). unTx, untreated APP23 mice; Aβ-Fc, vaccinated mice.

to estimate the direct effects in vivo, we measured Aβ-binding activities of plasma from treated and untreated mice using a tissue amyloid plaque immunoreactivity assay on sections from APP23 mice. First, we determined the plaque-binding ability of plasma taken from nonimmunized and immunized B6 mice (Fig. 7A). Plasma samples from non-immunized mice did not show detectable levels of amyloid plaque immunoreactivity activities, whereas samples from Aβ peptide–immunized mice showed significantly higher levels. Plasma from DNA vaccine–injected mice showed intermediate levels (Fig. 7A). The binding activity of plasma from vaccinated APP23 mice was significantly higher than that from untreated APP23 mice at 9, but not at 15, months of age (Figs. 7B, C). It should be noted that amyloid plaque immunoreactivity activities of plasma of untreated APP23 mice were elevated, especially at 15 months. This may correspond to elevation of the plasma antibody titer of untreated model mice as previously reported (7). The Aβ plaques were, however, negative for IgG in these mice (Fig. 2B). Collectively, these findings indicate that the binding activities of anti-Aβ antibodies to Aβ were augmented by DNA vaccination at early stages of the disease, but that the direct effects of antibodies are not as strong at later stages.

Plasma Aβ Levels in Vaccinated and Untreated Mice

We next measured the levels of plasma Aβ peptide to evaluate the so-called sink effect by blood-circulating anti-Aβ antibodies. At 9 months of age, plasma Aβ was slightly elevated in some cases after vaccine treatment (Fig. 7D). At 15 months of age, the levels of plasma Aβ in the treated group were almost the same as those in the untreated group (Fig. 7E). These findings suggest that Aβ efflux from the brain to blood (i.e. "peripheral sink") is present in some treated mice at an early stage, but does not seem to be the major route of Aβ reduction after DNA vaccination.

DISCUSSION

Immunotherapies against AD are effective not only in the mouse model (3, 23), but also in human clinical trials (5); however, the mechanisms by which raised or transferred anti-Aβ antibodies reduce Aβ deposition in the brain remain to be elucidated. We examined 3 possible Aβ reduction mechanisms to determine the major route of Aβ clearance in our DNA vaccination system and found that DNA vaccination enhances the phagocytosis of Aβ deposits by microglia. Because Aβ plaques in the brains of vaccinated, but not of empty vector–administered and untreated mice, were positive for IgG, an IgG-mediated immune-mediated mechanism such as Fc-mediated phagocytosis of Aβ by microglia may take place after DNA vaccination. Although it has been reported that Aβ reduction by activated microglia after glatiramer acetate treatment was achieved without the involvement of anti-Aβ antibodies (24, 25), we believe that the antibodies play an essential role in microglial activation in our DNA vaccination system. This was because plasmid DNAs containing the CpG motif without the Aβ sequence (i.e. empty vector) were not effective in Aβ reduction (Fig. 1). Increase of plaque-binding properties of plasma in vaccinated mice at 9 months of age also suggested the presence of anti-Aβ antibodies on Aβ plaques. There was, however, no significant difference in this activity between the treated and untreated groups at 15 months of age. Sink effects of plasma anti-Aβ antibodies may be present at the early stage in some treated mice but become unclear at later stages. There are at least 2 explanations for these results. First, anti-Aβ antibodies in plasma were only mildly elevated after DNA vaccination (7). Second, cerebral amyloid angiopathy may progress, especially in the late stage, and interfere with the perivascular drainage pathway of Aβ (26). Thus, microglial activation and their subsequent enhanced phagocytosis of Aβ deposits is a major Aβ clearance pathway in DNA vaccine therapy. Importantly, DNA vaccination reduced not only visible Aβ deposits, but also Aβ oligomers (Fig. 5). Thus, the findings obtained in this study provide useful information for the development of new and more effective DNA vaccines against AD.

There have been some controversies with regard to the role of microglia in AD pathogenesis. Previously, microglia were thought to be harmful and toxic to neurons in the AD brain because there were sustained inflammatory responses, including complement activation (27). β-Amyloid plaques and interferon-γ–activated microglia have synergistic effects on neuronal degeneration, which may have a role in the pathogenesis of aging and AD (28). Upon activation, microglia are known to secrete a wide variety of molecules involved in inflammation, many of which are potentially neurotoxic (29). It has been shown, however, that microglia react with Aβ plaques and phagocytose Aβ deposits under various conditions (30–33). Furthermore, activated microglia may play a protective role in the brain through the secretion of neurotrophic factors and cytokines (34). In the present study, we demonstrated that DNA vaccination induced microglial activation and augmentation of phagocytosis but did not induce large amounts of TNF production in the brains of vaccinated APP23 mice. We therefore speculate that only microglia attached to Aβ plaques may secrete TNF locally, which does not influence the level of TNF detected by ELISA, and this is less likely because microglial activation was diffuse in both periplaque and remote areas. These findings suggest that microglia after DNA vaccination may in part be neuroprotective in AD.

Increasing evidence suggests that microglia do not constitute a single uniform cell population, but rather a family of cells with diverse phenotypes—some that are beneficial and others that are destructive (35). Proper regulation of inflammatory responses to injury will arrest degeneration and promote regrowth, whereas inappropriate regulation will lead to ongoing degeneration (36). Microglial differentiation, neuroprotective or neurotoxic, might be determined by the strength of the stimulus.

The success of vaccine therapies depends on how to control microglial function to obtain beneficial effects in the AD brain. From this standpoint, DNA vaccination has advantages over other immunotherapies. The constructs of DNA vaccines can be easily manipulated by adding appropriate additional sequences to control microglial functions. Moreover, DNA vaccines may be safer because their half-life

within the body is shorter than those of others (37). If adverse side effects occur, they can be easily controlled by stopping further administration of the vaccine. Therefore, DNA vaccination may be a promising therapy for AD in the near future.

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DNA Vaccine Therapy for Alzheimer's Disease: Present Status and Future Direction

Yoshio Okura and Yoh Matsumoto

ABSTRACT

Alzheimer's disease is the most common cause of dementia characterized by progressive neurodegeneration. Based on the amyloid cascade hypothesis, a vaccine therapy for Alzheimer's disease (AD) was developed as a curative treatment. In 1999, the amyloid beta (Aβ) reduction in AD model transgenic mice with active vaccination with Aβ peptide was first reported. Although the clinical trials of active vaccination for AD patients were halted due to the development of meningoencephalitis in some patients, from the analysis of the clinical and pathological findings of treated patients, the vaccine therapy is thought to be effective. Based on such information, the vaccines for clinical application of human AD have been improved to control excessive immune reaction. Recently, we have developed non-viral DNA vaccines and obtained substantial Aβ reduction in transgenic mice without side effects. DNA vaccines have many advantages over conventional active or passive immunization. In this article, we review conventional vaccine therapies and further explain our non-viral DNA vaccine therapy. Finally, we show some data regarding the mechanisms of Aβ reduction after administration of DNA vaccines. DNA vaccination may open up new avenues of vaccine therapy for AD.

INTRODUCTION

ALZHEIMER'S DISEASE (AD) is the most common cause of age-related cognitive decline, affecting more than 12 million people worldwide.[1] The disease is characterized by progressive memory impairment and cognitive decline, altered behavior, and language deficit. Later in the disease process, patients show global amnesia, slowing of motor function, and finally death, within 9 years after diagnosis. Therefore, development of therapies against AD is extremely important from the medical, social, and economic perspective. Recently, it is generally believed that accumulation of amyloid beta (Aβ) is the first event in the pathogenesis of AD. In other words, Aβ deposition is an upstream event of tau phosphorylation, tangle formation, and neuronal death (amyloid cascade hypothesis).[2] Therefore, already deposited or depositing Aβ should be the first target of immunotherapy of AD. Recently, several immunotherapies have been developed as a curative treatment of AD by targeting the underlying cause (Table 1).

ANTI-Aβ IMMUNOTHERAPY AND HUMAN CLINICAL TRIALS

In 1999, Schenk and colleagues demonstrated that monthly inoculation with synthetic

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TABLE 1. DEVELOPMENT OF Aβ VACCINE THERAPY FOR ALZHEIMER'S DISEASE

Year	*Author*	*Event*	*Ref*
1991	Hardy	Amyloid cascade hypothesis	2
1997	Solomon	Disaggregation of amyloid fibril by anti-Aβ antibody	30
1999	Schenck	Reduction of Aβ by active immunization on mice Tg model	3
2000	Janus	Reduction of behavioral impairment by active immunization	5
2000	Morgan	Prevention of memory loss by active immunization	4
2000	Bard	Passive immunization	12
2001	DeMattos	Peripheral sink hypothesis	31
2002	Pfeife	Cerebral hemorrhage after passive immunotherapy	13
2003	Orgogozo	Interruption of clinical trial due to the development of meningocephalitis	6
2003	Nicoll	Neuropathology of autopsy case after human clinical trial	7
2003	Ghochikyan	Generation of antibodies after DNA vaccination on B6 mice	24
2003	Zhang	Effectiveness of AAV DNA vaccine on Tg mice model	18
2004	Schulz	Combined DNA vaccine therapy with low-dose Aβ peptide	25
2005	Kim	Effectiveness of adenoviral DNA vaccine on Tg model mice	20
2006	Okura	Long-term effectiveness and safety of non-viral Aβ DNA vaccine therapy	26

Aβ in complete Freund adjuvant (CFA) could lead to high anti-Aβ antibody titers and dramatic reductions of Aβ deposition in PDAPP transgenic mice.[3] The vaccine was able to slow or reverse amyloid deposit formation, even if administrated after Aβ deposition occurred. Subsequent studies demonstrated that clearance of Aβ deposits following immunization protected APP-transgenic (Tg) mice from developing memory deficits.[4,5] Based on the promising results using model mice, clinical trials were started for AD patients. However, the phase II-A study was halted in 2002 because 18 patients developed meningoencephalitis.[6] It was suggested that vaccination with Aβ peptide in a Th-1 type adjuvant may induce T cell responses against Aβ, which in turn resulted in the development of meningoencephalitis. Later, autopsy of an AD patient revealed apparent clearance of Aβ plaques from large areas of the neocortex, as well as a decrease in plaque-associated astrocytes and neuritic dystrophy.[7] Collectively, vaccine therapy is potentially effective for human AD if excessive immune reactions are minimized to avoid unwanted neuroinflammation. To control harmful T cell responses, other vaccination approaches using different routes, adjuvants, and immunogens have been developed after the trial. In addition, nasal vaccination with proteosome-based adjuvant plus glatiramer acetate cleared Aβ plaques in AD mouse model.[8] A short Aβ immunogen (Aβ1-15) that contains antibody epitopes but lacks T cell reactive sites that reside in full-length Aβ 1-42 induced the production of Aβ-specific antibodies in the absence of Aβ-specific cellular immune responses in wild-type mice[9] and significantly reduced Aβ plaque in APP transgenic mice.[10] After further improvement of peptide vaccines, there are two phase I clinical trials of active immunization with minimum side effects using ACC-001 (Elan, Dublin, Ireland and Wyeth, Philadelphia, PA), which contains Aβ 1-7 derivatives, and CAD106 (Novartis, Basel, Switzerland), which consists of an Aβ fragment coupled to a carrier.[11] There are still some possibilities of meningoencephalitis in active immunization because adjuvants are necessary for peptide vaccination.

Passive transfer of anti-Aβ antibodies is an alternative strategy that is also as effective as active immunization in the mouse model of AD. Peripheral administration of antibodies against Aβ peptide was sufficient to reduce amyloid burden. Despite relatively modest serum levels, passively administrated antibodies were able to enter the central nervous system, decorate plaques, and induce clearance of pre-existing Aβ deposits.[12] Passive immunization is more acceptable than active vaccination because it does not need adjuvants and does not elicit hazardous cellular responses found in the clinical trial of active immunization. In addition, the dose can be controlled easily. However, this approach requires caution when conducted in human trials. Long-term adoptive transfer of N-terminal specific anti-Aβ antibodies reduced amyloid loads, but induced a

number of microhemorrhages in old APP mice.[13] *In vivo* production of neutralizing antibodies, such as anti-idiotype antibodies, must be considered. Furthermore, a serious disadvantage of passive immunization is the cost of monoclonal antibodies; many patients cannot afford such expensive medical costs. Although there are still many problems, passive administration of Aβ-specific humanized monoclonal antibodies is currently in a phase II clinical trial (Elan and Wyeth) for AD patients.[11]

DNA VACCINE THERAPIES FOR THE NEXT GENERATION

Among several immunotherapies, DNA vaccination may open up a new avenue for treatment of AD because it is simple, easily modified, and available without adjuvant.[14,15] Immune responses of the host can be easily manipulated to obtain a Th2-type reaction.[14,16,17] Initially, Aβ DNA vaccines were produced using adeno-associated virus (AAV) vectors[18,19] or adenovirus vector.[20] A single administration of the AAV vaccine induced a prolonged and strong production of Aβ-specific serum IgG in model mice and resulted in improved ability of memory and cognition and decreased Aβ depositions and plaque-associated astrocytosis in the brain.[18] Much higher titers of antibodies against Aβ were obtained when an adenovirus vector encoding GM-CSF was co-administered with the vector encoding 11 tandem repeats of Aβ 1-6.[20] However, limitations to scale up the AAV vector production severely restrict the commercialization and use of AAV vectors.[21] Moreover, a viral replication could not be completely excluded when adenovirus vectors are used for vaccines. Thus, the clinical application of DNA vaccines with viral vectors seems to be difficult at present.

From this standpoint, we and others have focused on plasmid vectors. DNA vaccines with plasmid vectors have many advantages over those with virus vectors because the vaccines can be mass-produced at a low cost[14,15] and have no possibility of viral infection or transformation.[22,23] Ghochikyan et al. developed an Aβ 1-42 DNA vaccine with Th-2 cytokine sequence (IL-4) and confirmed the generation of anti-Aβ antibodies after vaccination in wild-type B6 mice.[24] Schulz et al. developed an Aβ DNA vaccine with a secretary signal, tissue-type plasminogen activator (tPA). With simultaneous use of low-dose Aβ peptide, DNA vaccine therapy reduces amyloid plaque in mice model.[25] However, significant reductions of Aβ deposition were not obtained only with these DNA vaccines.

Recently, we have been interested in the benefit of the plasmid vector and have developed non-viral DNA vaccines.[26] After intramuscular injection, plasmid DNA is taken up by muscle cells, and then recombinant proteins including Aβ are produced and secreted into the extracellular space, stimulating the immune system to produce anti-Aβ antibodies. In our system, three types of Aβ DNA vaccines were prepared using a mammalian expression vector. The first one possessed the core 1-42 sequence inserted into a commercially available expression vector (K-Aβ vaccine). The second possessed the immunoglobulin leader sequence at the N-terminus (IgL-Aβ vaccine). We expected this sequence to increase the secretion of the Aβ peptide. Furthermore, we added the human immunoglobulin Fc portion to the third vaccine to stabilize the vaccine product (Fc-Aβ vaccine). The sequences inserted into the plasmid were important for the Aβ secretory property of the vaccines. It was clearly demonstrated that supernatants of cultured cells that had been transfected with IgL-Aβ and Fc-Aβ vaccines contained translated proteins, whereas K-Aβ-transfected cells did not secrete the peptide into the extracellular space. Consistent with this finding, K-Aβ vaccine was less effective in Aβ reduction than the former two and was not used in subsequent experiments.

We first administrated the vaccines at 3–4 months of age before the appearance of amyloid deposition using prophylactic administration. AD model mice received 6 weekly and, subsequently, biweekly injections of the vaccines. At 7 months of age, granular amyloid depositions were recognized in the frontal cortex in the control groups. In sharp contrast, cortical Aβ depositions in mice treated with Fc-Aβ or IgL-Aβ were significantly reduced ($p < 0.01$). At 12 months of age, amyloid depositions in untreated mice were increased and some of them became large (>50 μg) in the frontal cor-

tex of the untreated mice. Untreated model mice showed an age-dependent increase of amyloid plaques in the cerebral cortex and hippocampus. The prophylactic protocol, using Fc-Aβ vaccine, revealed that the final reduction rate of Aβ burden in the cerebral cortex at 18 months of age was approximately 39% of untreated groups (Fig. 1C). Aβ depositions in the hippocampus were also decreased equally.

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, 5 months after the appearance of Aβ deposition, and the brains were examined at 15 and 18 months (therapeutic administration). In therapeutic treatments, amyloid plaques in the cortex were significantly decreased ($p <$

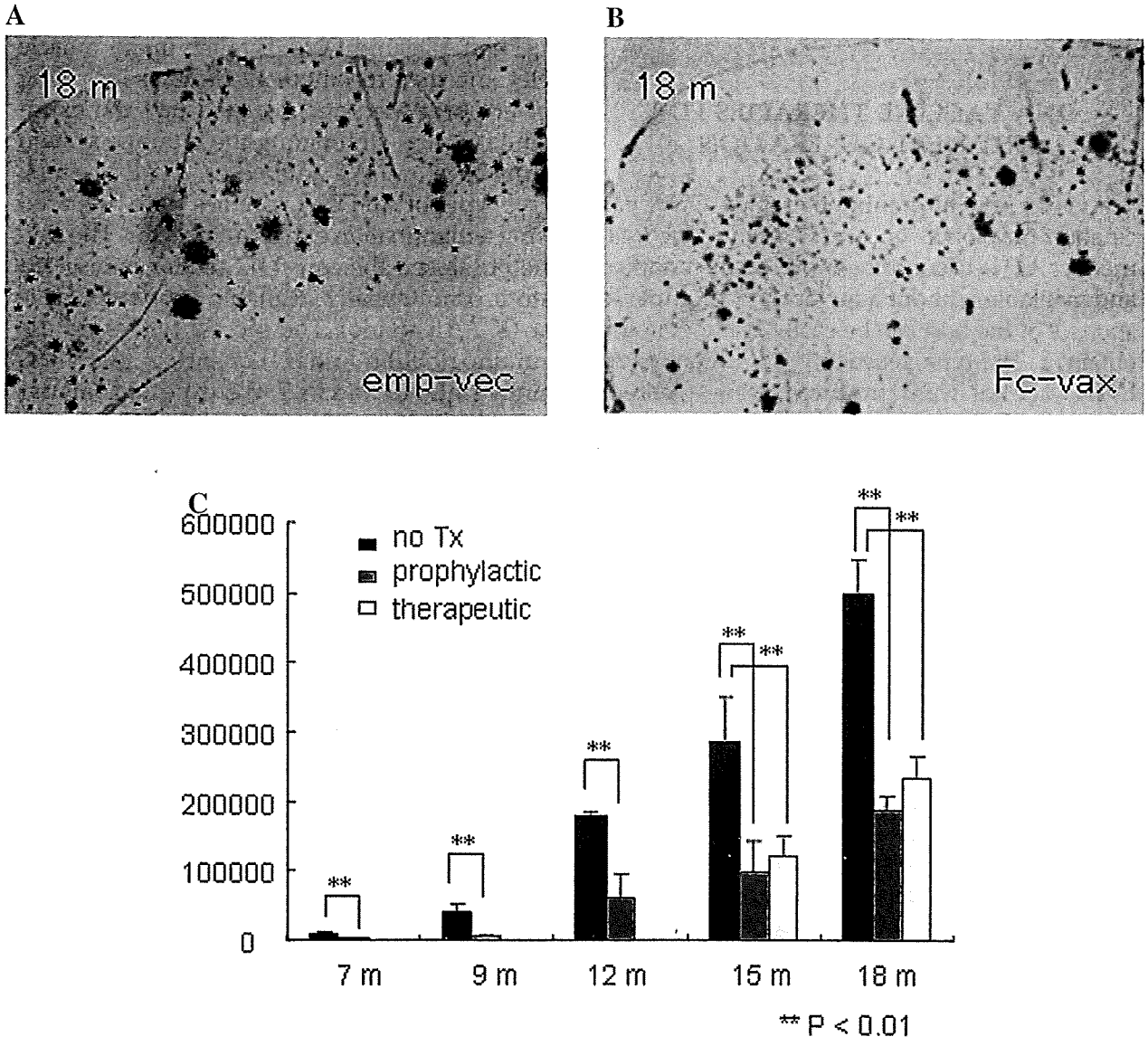

FIG. 1. Reduction of Aβ burden in APP23 mice after DNA vaccination. At 18 months of age, amyloid plaques with variable sizes were detected in the frontal cortex of untreated mice **(A)**. Therapeutic treatment with Fc-Aβ vaccines reduced cortical Aβ burden at 18 months **(B)**. **(C)** The overall quantitative analysis. The blue, yellow, and red bars indicate Aβ burden of untreated, prophylactically treated, and therapeutically treated mice, respectively. DNA vaccination reduced Aβ deposition to one third to a half of controls. The amyloid deposition was first detected in untreated mice at 7 months of age and rapidly increased after 15 months of age. 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. The effects of therapeutic administration were almost the same as those of prophylactic administration.

0.01) by Fc-Aβ (Fig. 1A) and IgL-Aβ vaccinations compared with the controls (Fig. 1B). Aβ depositions in the hippocampus were also decreased. Although the therapeutic protocol seemed to be less effective than the prophylactic one (Fig. 1C), the difference was not statistically significant. Thus, Aβ DNA vaccines had sufficient effect even if the vaccines were administrated after amyloid depositions appeared. We also confirmed that the level of anti-Aβ antibodies in plasma of model mice significantly increased after DNA vaccination. The levels of increase in our system were mild compared with those reported in active immunization.[3] The safety of our vaccines has been established as well as the effects. T cell activation and proliferation, as measured by [^{3}H]-thymidine incorporation of T cells from vaccinated mice, was negative in both wild-type B6 and model mice. Pathological examinations using monoclonal antibodies, CD5 (anti-T cell) and Mac-3 (anti-macrophage), demonstrated no inflammatory lesion in the brain after long-term treatments.

As mentioned above, there are two types of DNA vaccines, viral and non-viral DNA vaccines. We believe that non-viral DNA vaccines are superior to viral DNA vaccines for several reasons. Non-viral DNA vaccines can be prepared in large amounts with standard technology. They are safe because they lack a viral component. One can make the vaccines at a low cost. When considering clinical use, AD patients receive vaccines for the span of their life,

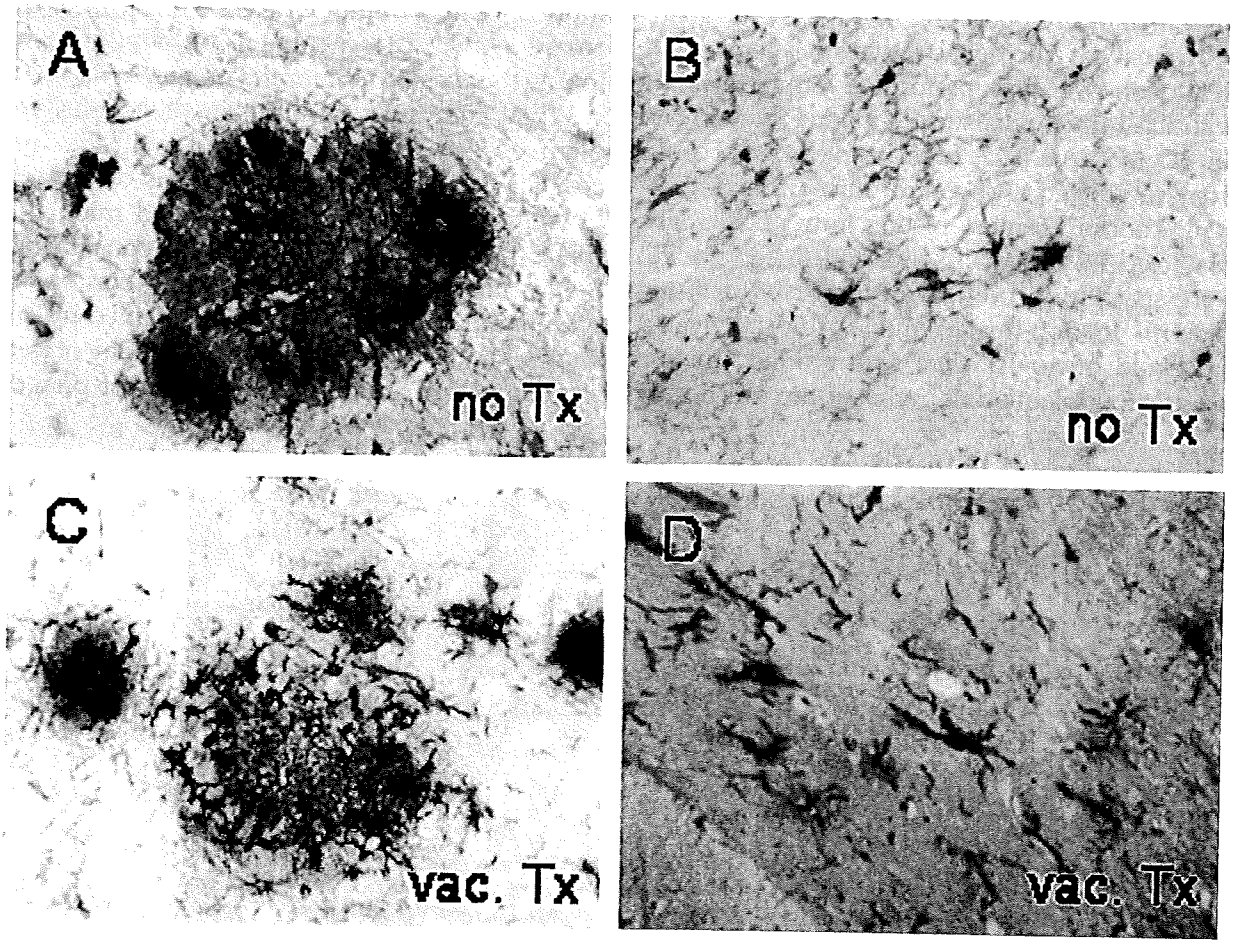

FIG. 2. Double staining with 6F3D (amyloid plaque, blue) and Iba-1 (microglia surface marker, blown) in the brains of treated and untreated mice. Around plaques of untreated mice, there are microglia with rich cytoplasms and processes that show bulbous swellings **(A)**. After the treatment, more microglial cells infiltrated the amyloid plaques **(C)**. In the remote area of non-treated APP23 mice, resting microglia were sparsely distributed **(B)**. In the remote area of treated APP23 mice, microglial cells also increased in number and demonstrate the activated form **(D)**.

and such advantages are quite important factors for the choice of their treatment.

MECHANISMS OF AMYLOID REDUCTION WITH VACCINE THERAPIES

Although Aβ DNA vaccination has significant effects on Aβ reduction in the mouse model, the mechanisms of Aβ clearance remain unclear. There are three hypotheses to explain how anti-Aβ antibodies reduce Aβ deposits in the brain. One possible mechanism is augmentation of Fc-mediated phagocytosis by microglia.[27,28] The second mechanism is direct dissociation by anti-Aβ antibodies.[29,30] The third mechanism is augmentation of Aβ efflux from the brain to the blood circulation.[31,32] We examined whether these mechanisms are operating in our system.

To analyze microglial activation after vaccine treatment, we first performed double-staining with Iba-1 (microglia surface marker, blown) and 6F/3D (anti-Aβ 8-17, blue) using brain sections from vaccinated and control mice. In non-transgenic control B6 mice, resting form of microglia characterized by small soma and fine projection were sparsely distributed in the brain (data not shown). In non-treated model mice, activated amoeboid microglia were seen around amyloid plaques (periplaque area), whose processes deeply entered into the plaques (Fig. 2A). In the area remote from plaques (remote area), the retesting form of microglia was observed, as was seen in wild-type mice (Fig. 2B). After DNA vaccination, microglia in periplaque area increased in number and clustered around plaques (Fig. 2C). The significant change after the vaccination was the morphological change of microglia in the remote area. Microglia increased in number and switched their form to the amoeboid type, which have long and stringy cytoplasmic processes all over the brain.

To quantitatively analyze the increase of microglia after the vaccination, the number of the microglia was counted both in periplaque and remote areas. At 9 months of age, microglia were increased in number significantly ($p < 0.01$) in both areas after the vaccination. The number of microglia in the periplaque area was larger than that in the remote areas. At 15 months of age, microglia were significantly increased ($p < 0.01$) in number, in similar fashion as that seen in 9 months of age.

While analyzing double-stained sections, we often observed small Aβ deposits inside microglial cells. This phenomenon was confirmed by confocal microscopy. Cy3-labeled microglia (Fig. 3B) enclosed FITC-labeled Aβ deposits (Fig. 3A). A merged image indicates ingestion of Aβ within microglia (Fig. 3C). We confirmed by 3D analysis that this was not a simple overlay of two structures (not shown). The number of phago-

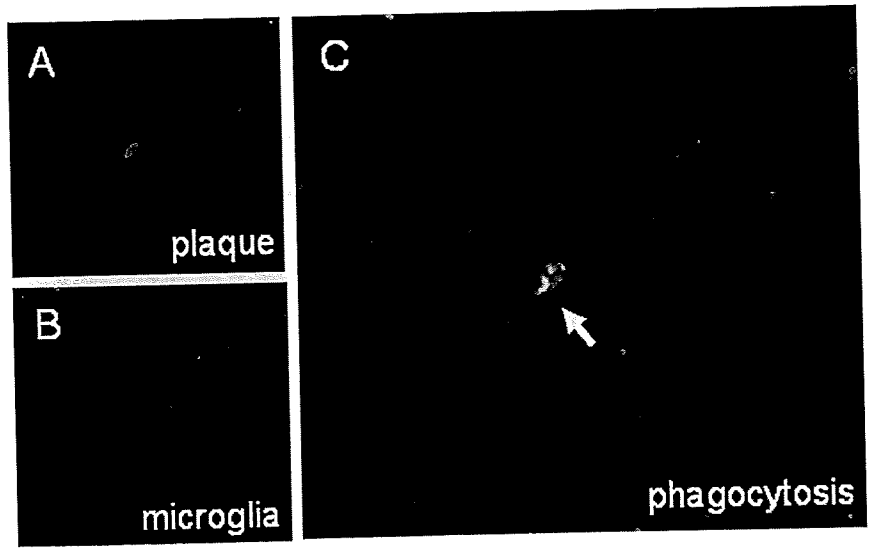

FIG. 3. Phagocytosis of Aβ deposits by activated microglia. Brain sections from treated and untreated APP23 mice were stained with 6F3D (amyloid plaque, green) **(A)** and Iba-1 (microglia, red) **(B)** and observed with a confocal microscope. Solely existed microglia in remote area contain Aβ deposits (C, arrows).

cytosing microglia was significantly increased ($p < 0.01$) after vaccine treatment. The increase of activated microglia in remote areas suggests that clearance of invisible small Aβ aggregates is performed by activated microglia. If this is the case, it is greatly beneficial for AD patients because Aβ oligomers are responsible for AD pathology[33,34] and synaptotoxicity.[35]

We also examined that direct dissociation by anti-Aβ antibodies[36] was operating in our treatment system. It is very difficult to evaluate *in vivo* direct effects of anti-Aβ antibodies on Aβ dissociation. We determined the levels of anti-Aβ antibodies with high affinity by tissue amyloid plaque immunoreactivity (TAPIR) assay using plasma taken from treated and untreated mice. Although the titer from treated mice was slightly higher than that of plasma from untreated mice, the difference was not statistically significant. Thus, direct dissociation of Aβ deposits by the antibodies is not so marked in this system although it may be present.

We next examined the sink effects of anti-Aβ antibodies by measuring the levels of Aβ in plasma taken from treated and untreated mice. At 9 months of age, some treated mice showed relatively high levels of plasma Aβ, suggesting that Aβ migration from the brain to the blood is upregulated. However, there was not significant difference at 15 months of age. These findings suggest that the Aβ efflux from the brain to blood (peripheral sink) is not the major route of Aβ reduction after DNA vaccination.

Microglia are thought to play a role in either neuroprotection or neurodamage. We examined the nature of activated microglia in our DNA vaccine therapy. The levels of tumor necrosis factor-alpha (TNF-α), one of cytotoxic cytokines,[37] were determined using brain homogenates of treated and untreated mice. A large quantity of TNF-α was detected in the brain and spinal cord from the positive control, i.e., LPS-treated and MOG-EAE-induced mice. Without DNA vaccine therapy, TNF-α in the brain of wild-type or model mice was nearly zero. After DNA vaccination, the levels of TNF-α in these mice seemed to be slightly increased; however, they were not significantly different from control mice. These findings suggest that activated microglia detected after DNA vaccination may be neuroprotective.

Taken together, in DNA vaccine therapy, Fc-mediated phagocytosis of Aβ deposits by activated microglia is a major route of Aβ reduction. Direct Aβ dissociation and sink effects may be weak in this situation. This information is very important for improvement of DNA vaccines. DNA vaccines are easily reconstructed by adding or changing the sequence in the plasmid vector. We expect that more effective vaccines will be developed and applied for human AD in the near future.

CONCLUSION

Vaccine therapy for AD is a promising strategy if excessive immunoreactions are controlled. We have developed non-viral DNA vaccines for AD that were effective in the animal model without side effects. In our system, Fc-mediated phagocytosis of Aβ depositions by activated microglia is a major route of Aβ reduction. The accumulation of such data helps to develop more effective, safer vaccines that may be clinically applied in the near future.

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LOOKING AHEAD

Although interrupted, the phase II clinical trial of AN-1792 provides further support for Aβ immunotherapy of Alzheimer's disease. Alternative vaccine therapies, such as nonviral DNA vaccines, are being investigated to reduce excessive immune reactions of the host brain.

Anti-Aβ Vaccination as a Promising Therapy for Alzheimer's Disease

by Yoshio Okura and Yoh Matsumoto

Summary

Alzheimer's disease is the most common cause of dementia characterized by progressive neurodegeneration. Recently, a vaccine therapy for Alzheimer's disease was developed as a curative treatment. Although clinical trials of active vaccination for Alzheimer's disease were halted due to the development of meningoencephalitis in some patients, the clinical and pathological findings of treated patients suggest that the vaccine therapy is effective. Hence, newly designed vaccines are being invented to control excessive T-cell immune reactions after the human clinical trial. In this article, we will review conventional vaccine therapies and newly developed vaccine therapies, mainly DNA vaccines, for possible clinical application in the near future.

Alzheimer's disease, first reported by Alzheimer 100 years ago, is most common cause of age-related cognitive decline, affecting more than 12 million people worldwide.[1] The disease is characterized in its earlier stage by progressive memory impairment and cognitive decline, altered behavior and language deficit. Later, patients develop global amnesia and slowing of motor function, and finally die typically within 9 years after diagnosis. Current drug therapies such as donepezil hydrochloride slow cognitive decline; however, the effect is limited. Recently, it is generally believed that accumulation of amyloid beta (Aβ) is the first event in the pathogenesis of Alzheimer's disease. In other words, Aβ deposition is an upstream event of tau phosphorylation, tangle formation and neuronal death (amyloid cascade hypothesis).[2] Based on this hypothesis, vaccine therapy has been developed for curative treatment of Alzheimer disease by targeting the underlying cause.

Antiamyloid immunotherapy

Schenk et al. for the first time demonstrated the effect of Aβ vaccines. Monthly inoculation with synthetic Aβ in complete Freund's adjuvant (CFA) could lead to high anti-Aβ antibody titers (Fig. 1A), and dramatic reductions of amyloid deposition in PDAPP transgenic mice.[3] The vaccine was able to slow or reverse amyloid deposit formation, even if administrated after amyloid deposition occurred. Neuritic plaques and astrocytic reactions were also decreased by the vaccination. They speculated that acceleration of Fc receptor-mediated microglial phagocytosis plays a major role in plaque reduction. Subsequent studies demonstrated that clearance of Aβ depositions following immunization

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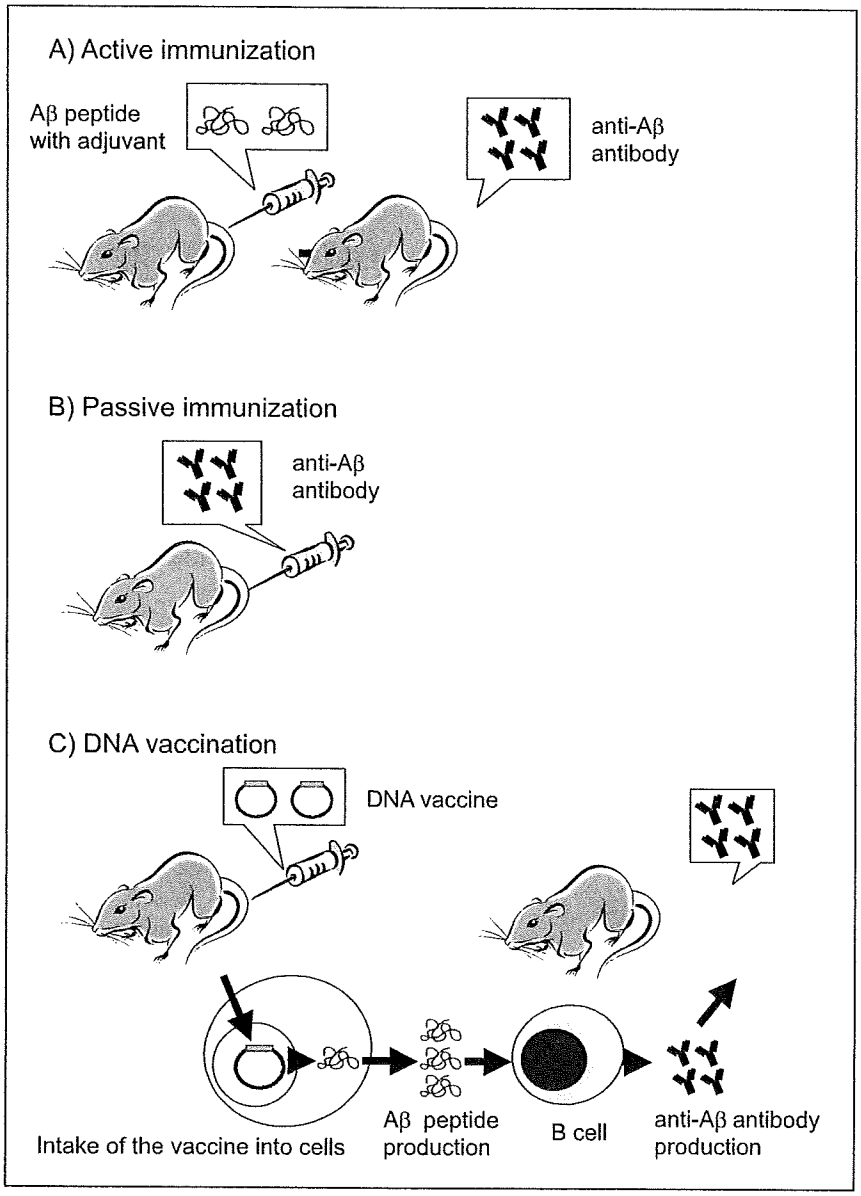

Fig. 1. Types of vaccine therapies for Alzheimer's disease. A) Active immunization: $A\beta_{1-42}$ peptides are administered with an adjuvant to induce anti-Aβ antibodies. B) Passive immunization: anti-Aβ antibodies are administrated directly. C) DNA vaccination: plasmid vectors encoding $A\beta_{1-42}$ gene are injected to generate Aβ peptides and subsequent anti-Aβ antibodies. Aβ peptide production is continued for a certain period.

protected APP-Tg mice from developing memory deficits.[4,5] Clearance of Aβ depositions and improvement of memory were also observed after passive administration of antibodies against Aβ (Fig. 1B).[6,7]

Mechanisms of amyloid reduction with vaccine therapies

Although Aβ peptide vaccination has effects on Aβ reduction in the mouse model, the mechanisms of Aβ clearance remain unclear. There are three hypotheses to explain how anti-Aβ antibodies reduce Aβ depositions in the brain (Fig. 2). One possible mechanism is that anti-Aβ antibodies enhance Fc receptor-mediated phagocytosis of Aβ by microglial cells (Fig. 2A). Following peripheral administration of anti-Aβ antibodies, activated microglia were found surrounding the plaques.[8] The culture of microglial cells with anti-Aβ antibodies on brain slices from Tg mice induced the clearance of Aβ.[9]

The second mechanism is a direct effect of antibodies on Aβ leading to dissolution of amyloid fibrils or neutralization of Aβ oligomers (Fig. 2B). Direct injection of $F(ab')_2$ antibodies into the brain equally mediated a decrease in Aβ.[10] Antibodies raised against the N-terminal region (1-28) of the Aβ peptide binded to *in vitro*-formed Aβ assemblies, leading to disaggregation and increased solubility of Aβ fibrils.[11]

The third mechanism, the peripheral sink hypothesis, postulates that administration of anti-Aβ antibodies to the circulation results in a net efflux of Aβ from the brain to the plasma (Fig. 2C).[7] Rapid improvement in cognition was observed in animals after intravenous injection of antibodies and increased plasma concentrations of Aβ.[12] Injection of an agent that has high affinity for Aβ (gelsolin or GM1) reduced the level of Aβ in the brain.[13]

A clinical trial of amyloid vaccination

Based on the promising results obtained using transgenic mice, clinical trials with $A\beta_{42}$ (AN-1792) in conjunction with the T helper (Th) 1 adjuvant QS-21 were initiated. The phase I studies using single or multiple doses of the vaccine demonstrated good immunological responses and tolerability to the vaccine. However, a phase IIa study performed in 375 patients at several sites was halted because meningoencephalitis developed in 18 patients.[14] It was suggested that vaccination with the Aβ peptide vaccine in a Th1 type adjuvant induced T-cell responses against Aβ. However, the autopsy case showed apparent clearance of Aβ plaques from large areas of the neocortex as well as a decrease in plaque-associated astrocytes and neuritic dystrophy.[15] Thus, the clinical trial clearly demonstrated benefits of vaccine therapy. Taken together, the results indicate that the vaccine therapy is potentially effective for human

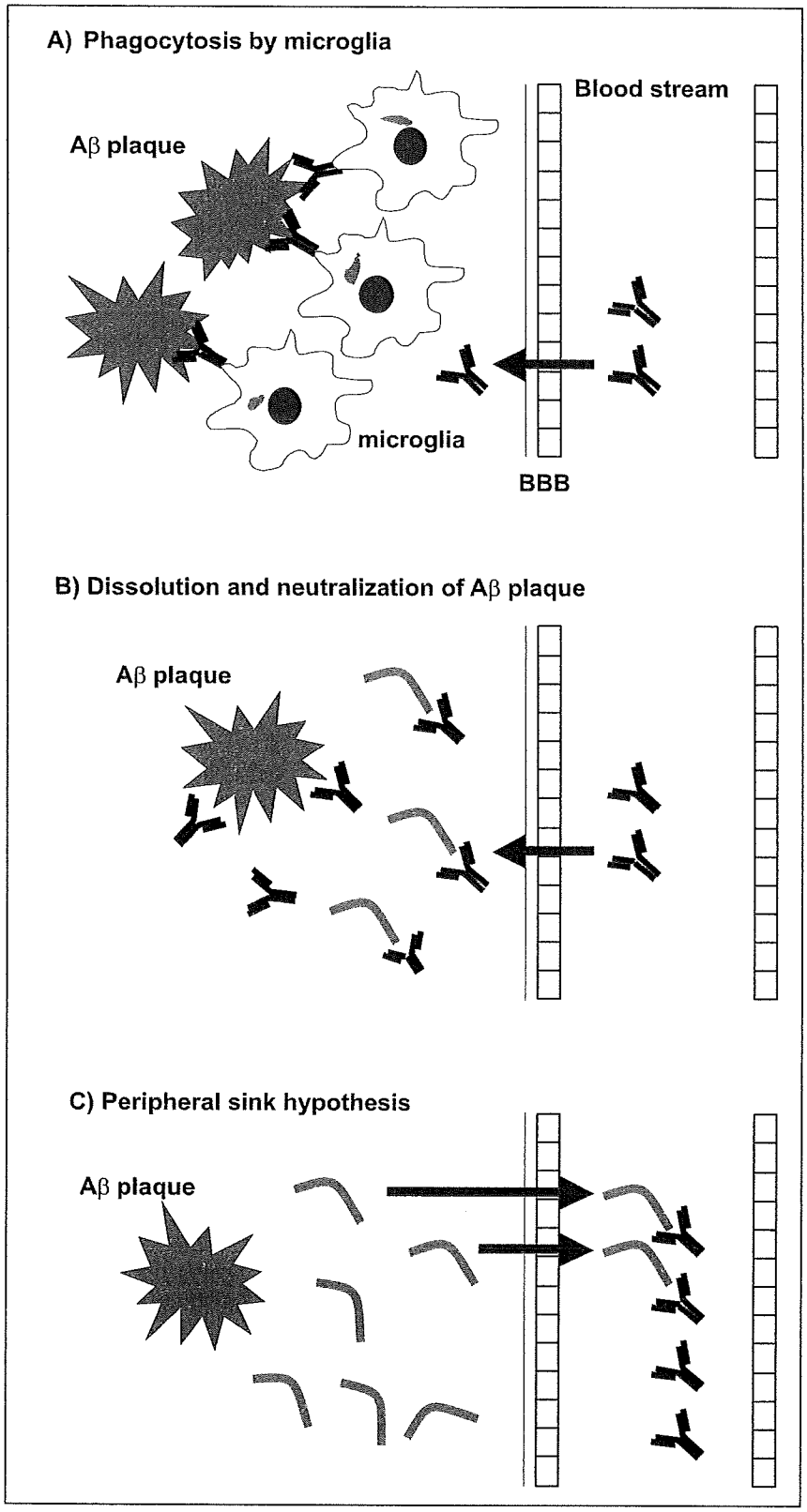

Fig. 2. Mechanisms of amyloid reduction with a vaccine treatment. A) Phagocytosis by microglia: anti-Aβ antibodies traverse blood–brain barrier (BBB) and attach to Aβ deposits, which leads to Fc receptor-mediated phagocytosis by microglia. B) Dissolution and neutralization of Aβ plaque: antibodies bind N-terminal end of Aβ depositions and dissolve amyloid fibrils or neutralize Aβ oligomers. C) The peripheral sink hypothesis: anti-Aβ antibodies in the circulation induce a net efflux of Aβ from the brain to the plasma.

Alzheimer's disease if excessive immune reactions are minimized to avoid unwanted neuroinflammation.

After further improvement of peptide vaccines, there are two phase I clinical trials of active immunization with minimum side effects using ACC-001 (Elan and Wyeth), which contains Aβ 1-7 derivatives, and CAD-106 (Novartis), which consists of an Aβ fragment coupled to a carrier.[16] However, there is still the possibility of meningoencephalitis in active immunization because adjuvants are necessary for peptide vaccination.

Passive immunization

Passive transfer of anti-Aβ antibodies is an alternative strategy (Fig. 1B), which is as effective as active immunization in the mouse model of Alzheimer's disease. Peripheral administration of antibodies against Aβ peptide was sufficient to reduce amyloid burden. Despite relatively modest serum levels, the passively administered antibodies were able to enter the central nervous system, decorate plaques and induce clearance of preexisting amyloid.[6] Direct injection of antibodies into the brain induced rapid parenchymal Aβ clearance.[10] Passive immunization is more acceptable than active vaccination because it does not need adjuvant injection and does not elicit the hazardous cellular responses observed in the clinical trial of active immunization. Moreover, the dose can be controlled easily. However, this approach will require caution in the conduct of human trials. Long-term adoptive transfer of the antibodies in old APP transgenic mice reduced amyloid loads, but doubled the number of microhemorrhages in 27-month-old APP mice treated for 5 months with an N-terminal specific anti-Aβ monoclonal antibody.[17] Moreover, after passive immunization, *in vivo* production of neutrizing antibodies such as anti-idiotype antibodies must be considered. A serious disadvantage of passive immunization is the cost of monoclonal antibodies. An enormous number of patients cannot afford the expensive medical costs.

Although there are some problems to be solved, passive administration of an Aβ-specific humanized monoclonal antibody (bapineuzumab; Elan and Wyeth) is currently in a phase II clinical trial in patients with Alzheimer's disease.[16]

Development of new vaccine therapies for the next generation (DNA vaccines)

Among alternative vaccine therapies, DNA vaccination may open up a new avenue for the treatment of Alzheimer's disease because it is simple, easily modified and can be used without adjuvant (Fig. 1C).[18,19] The immune responses of the host induced by DNA vaccination are generally Th2 type.[18,20,21]

Initially, Aβ DNA vaccines were developed using adeno-associated virus (AAV) vectors[22,23] or adenovirus vector.[24] A single administration of the AAV vaccine induced a prolonged and strong production of Aβ-specific serum IgG in Tg mice and resulted in improved ability of memory and cognition, decreased Aβ depositions in the brain, and a resultant decrease in plaque-associated astrocytosis.[22] Much higher titers of antibodies against Aβ were obtained when an adenovirus vector encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) was co-administered with the vector encoding 11 tandem repeats of $A\beta_{1-6}$.[24] However, the ability to scale up the AAV vector production severely restricts the commercialization and use of AAV vectors.[25] Moreover, a viral replication could not be completely excluded when the adenovirus vector is used for vaccines. Thus, the clinical application of DNA vaccines with virus vectors seems to be difficult at present.

We and others have focused on plasmid vectors. DNA vaccines with plasmid vectors have many advantages over those with virus vectors because the vaccines can be mass produced at a low cost[18,19] and have no possibility of viral infection or transformation.[26,27] Ghochikyan et al. developed an $A\beta_{1-42}$ DNA vaccine with Th2 cytokine sequence (IL-4) and confirmed the generation of anti-Aβ antibodies after vaccination in wild-type B6 mice.[28] Schulz et al. developed and Aβ DNA vaccine with a secretary signal, tissue-type plasminogen activator (tPA). With simultaneous use of low dose Aβ peptide, DNA vaccine therapy reduces amyloid plaque in a mouse model.[29] However, significant reductions of Aβ deposition were not obtained with these DNA vaccines alone.

We also focused on the benefit of the plasmid vector and prepared three types of Aβ DNA vaccines using a mammalian expression vector.[30] The sequence of $A\beta_{1-42}$ and additional sequences were inserted in the plasmid, as shown in Figure 3A. The first

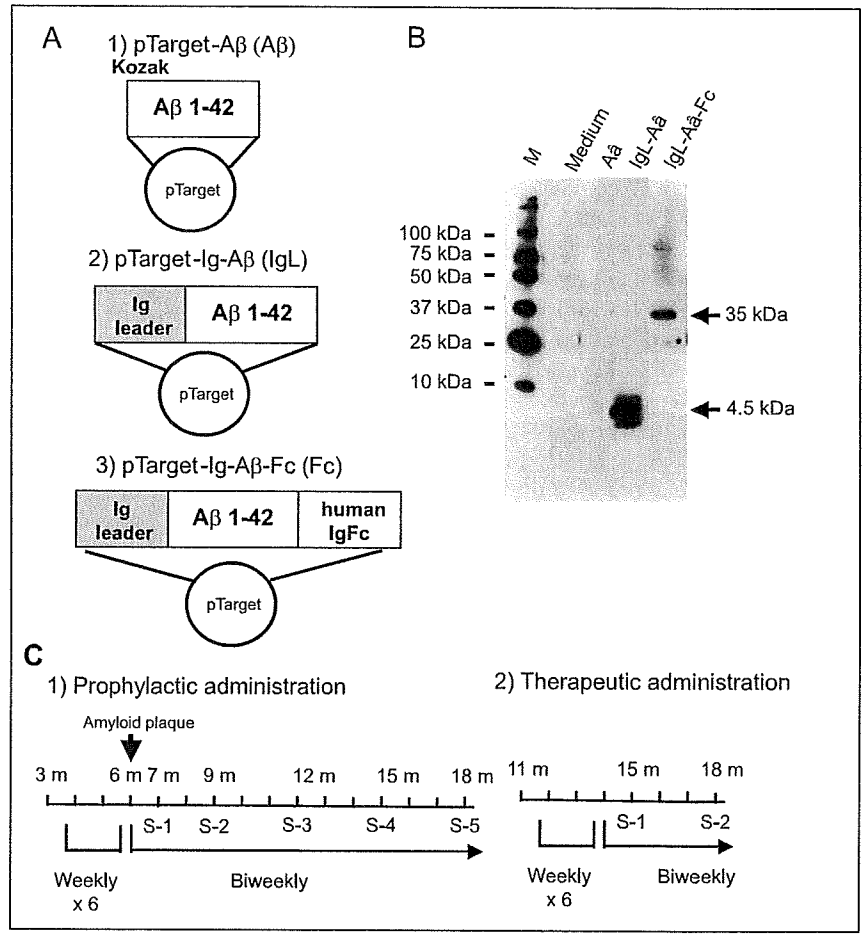

Fig. 3. Construction of DNA vaccines (A), *in vitro* characterization (B) and the treatment protocol (C). A) Three DNA vaccines were produced using a mammalian expression vector. DNA encoding the $A\beta_{1-42}$ sequence was inserted in XhoI/KpnI site of the plasmid (K-Aβ vaccine) (A-1). In the second vaccine, the signal sequence of mouse Igκ is added to the 5' end to improve the secretive efficiency (IgL-Aβ vaccine) (A-2). The third vaccine possesses the Fc portion of human immunoglobulins to improve the stability of the secreted protein (Aβ-Fc vaccine) (A-3). 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 from 3-4 months of age before the appearance of amyloid depositions. The mixture of one of the vaccines (100 mcg) 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 (C-1). For therapeutic treatment, the vaccines were administered to APP23 mice from 12 months of age, after the appearance of amyloid plaques. Samplings were performed at 15 and 18 months of age (C-2). (Reproduced from Okura, Y., Miyakoshi, A., Kohyama, K. et al. *Nonviral Abeta DNA vaccine therapy against Alzheimer's disease: Long-term effects and safety.* Proc Natl Acad Sci U S A 2006, 103: 9619–24. © 2006 National Academy of Sciences, U.S.A.)

one contains only the $A\beta_{1-42}$ sequence with the Kozak sequence at the 5' end (referred to as K-Aβ vaccine) (Fig. 3A-1). To the second, the Igκ signal sequence of murine immunoglobulin was added to improve the secretion ability (IgL-Aβ vaccine) (Fig. 3A-2), and the third possesses the Fc portion of human immunoglobulin at the 3' end to maintain stability (Fc-Aβ vaccine) (Fig. 3A-3). Before *in vivo* administration, these DNA vaccines were transfected to HEK295T cells and the secretion of $A\beta_{1-42}$ peptide into the culture supernatant was assayed with Western blotting (Fig. 3B). 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 were 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 and that this event is critical for the effects of DNA vaccines.

We employed two types of regimens, prophylactic and therapeutic, to examine the effect of Aβ DNA vaccination. For the prophylactic protocol, vaccine administration was started from 3 to 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. 3C-1). At 7 months of age, granular amyloid depositions were recognized in the frontal cortex in the control groups (empty vector-administrated and untreated mice) (Fig. 4B). At this stage, Aβ plaques were not detected in the hippocampus. In sharp contrast, cortical Aβ depositions in mice treated with Aβ-Fc (Fig. 4A), IgL-Aβ and Aβ vaccines were significantly reduced ($p < 0.01$). The Aβ burden was reduced to approximately 15–30% that of the untreated groups (Fig. 4E). At 12 months of age, amyloid depositions in untreated mice were increased and some of them became large (> 50 μg) in the frontal cortex of the untreated mice (Fig. 4D). Aβ depositions in the hippocampus were also equally decreased ($p < 0.01$). It was shown that the suppressive effect of Aβ-Fc vaccine was almost equal to that of IgL-Aβ vaccine. However, K-Aβ vaccine was less effective than the former two (Fig. 4E and 4F) and was not used in subsequent experiments. At 15 and 18 months of age, the plaques in untreated groups were rapidly increased. Untreated APP23 mice showed an age-dependent increase of amyloid plaques in the cerebral cortex (Fig. 5, open square) and hippocampus. The

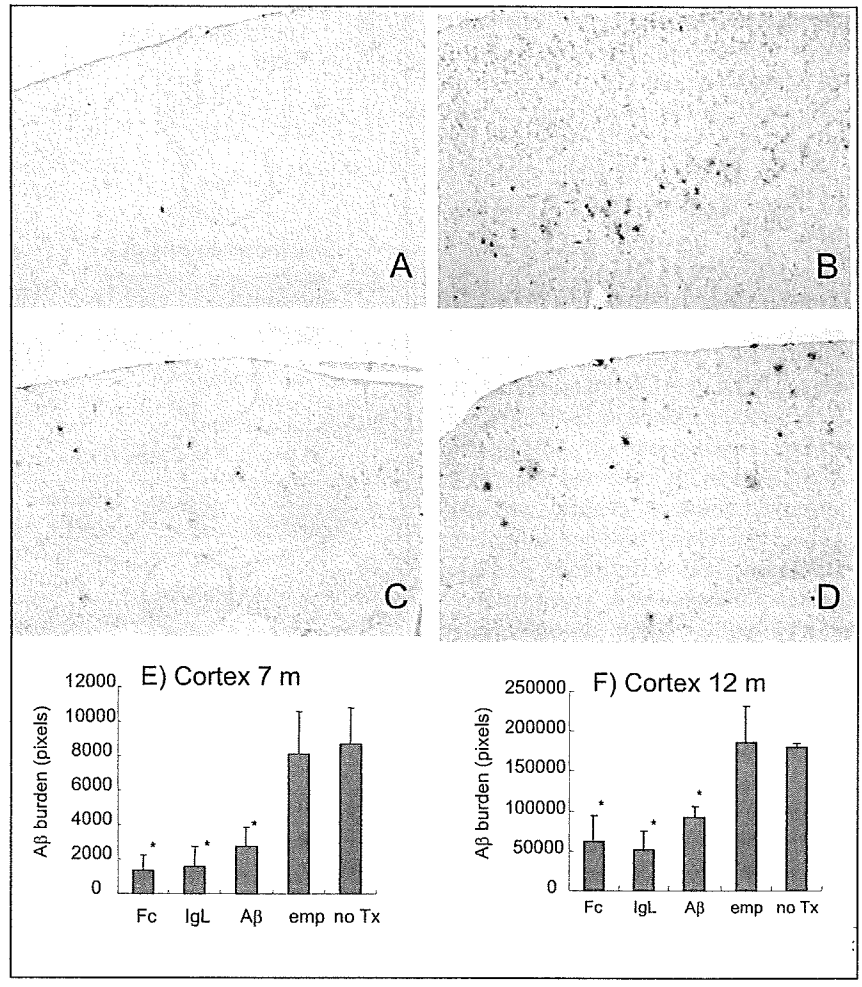

Fig. 4. Reduction of Aβ burden in APP23 mice at 7 months after DNA vaccination starting from 4 months (A, B and E). Immunohistochemical examinations revealed that granular amyloid depositions were detected in the frontal cortex of untreated mice at 7 months of age (B). In mice vaccinated with Aβ-Fc vaccine, amyloid plaques in the frontal cortex were reduced (A). 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%) than those found in untreated and empty vector-vaccinated mice (E). Reduction of Aβ burden in APP23 mice at 12 months after DNA vaccination starting from 4 months (C, D and F). Many Aβ deposits were observed in the frontal cortex of control mice (D), but were significantly reduced after treatment with Aβ-Fc (C) vaccines. Quantitative image analysis of Aβ burden in the cortex at 12 months of age revealed that Aβ deposits were significantly reduced (*$p < 0.01$) in mice with prophylactic treatment with Aβ-Fc (33.7% of untreated mice), IgL- Aβ- (28.6%) and K-Aβ (51.3 %) vaccines (F). K-Aβ vaccine was less effective than the former two. Magnification A-B ×62, C-D ×24. (Reproduced from Okura, Y., Miyakoshi, A., Kohyama, K. et al. *Nonviral Aβ DNA vaccine therapy against Alzheimer's disease: Long-term effects and safety.* Proc Natl Acad Sci U S A 2006, 103: 9619–24. © 2006 National Academy of Sciences, U.S.A.)

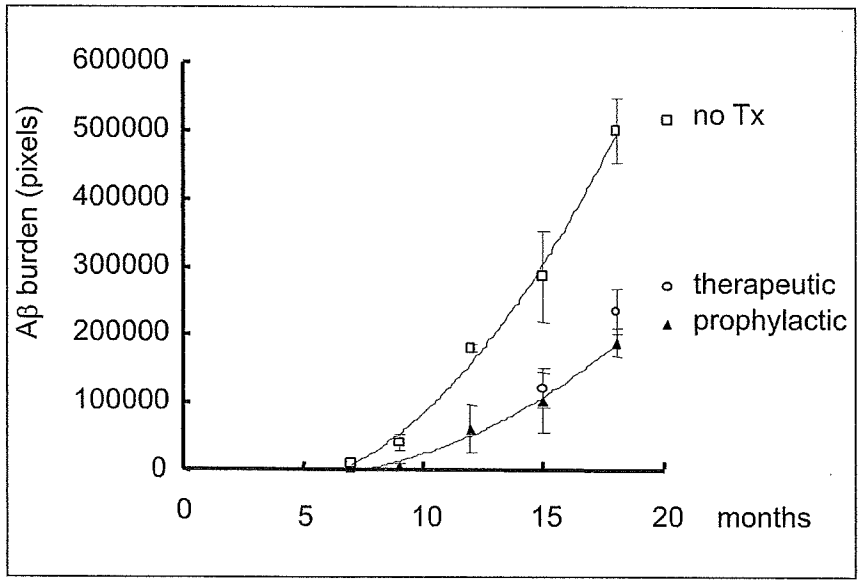

Fig. 5. The overall quantitative analysis. The amyloid deposition was first detected in untreated mice at 7 months of age and rapidly increased after 15 months of age (open squares). Prophylactic administration of Fc-Aβ vaccine prevented the Aβ deposition to 10–30% of that in untreated animals before 12 months of age and to 40–50% after 15 months (closed triangles). The effects of therapeutic administration (open circles) were almost the same as those of prophylactic administration (closed triangles).

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 approximately 38.5% that of untreated groups (Fig. 5, closed triangles). These results demonstrated that two of three vaccines produced in this study were effective in the 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 and 18 months (Fig. 3C-1). In therapeutic treatments, amyloid plaques in the cortex were significantly decreased ($p < 0.01$) by Aβ-Fc and IgL-Aβ vaccination (Fig. 6A) compared with the controls (Fig. 6B). Aβ depositions in the hippocampus were also decreased ($p < 0.01$) (Fig. 6D). Although the therapeutic protocol (Fig. 5, open circle) seemed to be less effective than the prophylactic one (Fig. 5, closed triangle), the difference was not significant. 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.[31] Therefore, we counted the number of neurons containing intracellular Aβ depositions in the cortex 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 (59.5%, $p < 0.05$) treatments at 15 months of age (Fig. 7).

The titers of plasma anti-Aβ antibodies after the treatment were determined by ELISA. The levels of anti-Aβ antibodies were significantly increased compared with the untreated

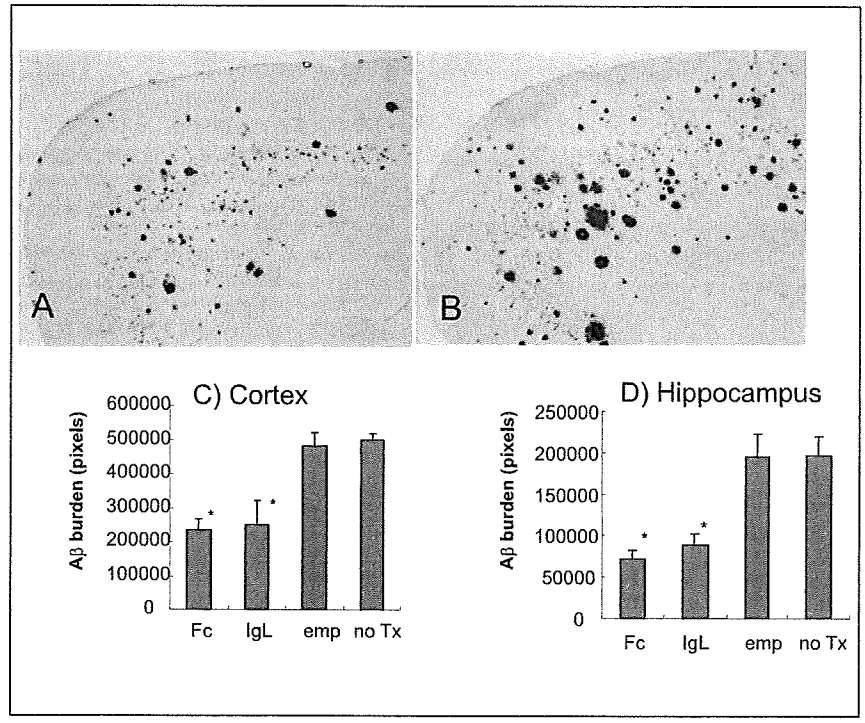

Fig. 6. Aβ burden reduction at 18 months of age after therapeutic treatment starting from 12 months. While large Aβ deposits (> 100 μm) were observed in the frontal cortex of control mice at 18 months of age (B), significant reduction was observed after 6-month therapeutic administration of the IgL-Aβ vaccine (A). Quantitative image analysis of Aβ burden in the cortex at 18 months of age revealed that Aβ deposits were significantly reduced (*$p < 0.01$) in mice with therapeutic treatment of Aβ-Fc (47.0% of untreated mice) and IgL-Aβ (49.9%) vaccines. Aβ deposits in the hippocampus were also significantly reduced (*$p < 0.01$) after Aβ-Fc (38.0% of the control) and IgL-Aβ (46.0%) vaccine treatment (D). Magnification A and B, ×24.