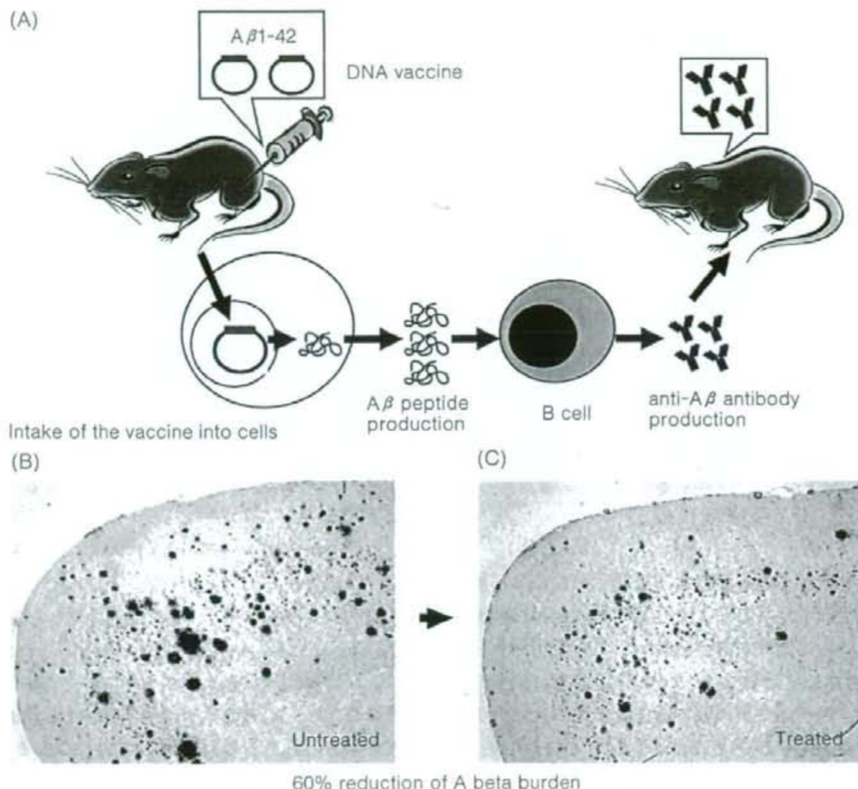

Fig. 1 Schematic representation of Aβ reduction by DNA vaccination

Plasmid vectors encoding Aβ 1-42 are injected intramuscularly to generate Aβ peptides and subsequent anti-Aβ antibodies. Aβ peptide production is maintained by muscle cells over a certain period (A). Amyloid plaques of various sizes were detected in the frontal cortex of untreated mice (B). Therapeutic administration of Aβ-Fc vaccines reduced cortical Aβ burden at 15 months (C).

チン単独で脳内Aβの減少を誘導することはできなかった[30]。

われわれの研究室では，これまでラットの実験的脳脊髄炎（EAE）などの自己免疫疾患モデルに対し非ウイルス性DNAワクチンを作製し，その効果を報告してきた[31]。そのデータの蓄積をもとに，哺乳類細胞発現ベクターにAβ-protein(1-42)および付属のシークエンスを挿入して3種類のアルツハイマー病非ウイルス性DNAワクチンを作製した。①Aβ 1-42のみを挿入したもの（K-Aβワクチン），②発現蛋白質の分泌を向上させるためにマウスIgκシグナルを付加したもの（IgL-Aβワクチン），③分泌した蛋白質の安定性を向上させるためにImmunoglobulinのFc領域を付加したもの（Aβ-Fcワクチン），の3種類である。3種類のベクターをHEK293Tにtransfectし，その細胞内にAβ1-42ペプチドが発現し，培養上清に分泌されているか否かをELISA法により検討した。IgL-Aβ，Aβ-Fcワクチンは培養上清中にAβ 1-42ペプチドが分泌されていた。Aβ1-42のみを挿入したK-AβワクチンはAβ 1-42ペプチドの分泌が認められなかった。その後，K-AβワクチンはAβ抑制効果が他の2つのワクチンに比べて低いことがわかったので，詳細な検討から除外した。

作製したDNAワクチンをアルツハイマー病のモデルマウス（APP23 Tg mouse）に2週間に1回，投与することにより治療を試みた。このモデルマウスは，スウェーデンの家族性アルツハイマー病家系にみられる遺伝子変異を持つアミロイド前駆蛋白が遺伝子導入されており，6カ月齢から老人斑が出現し，加齢とともに増加するこ

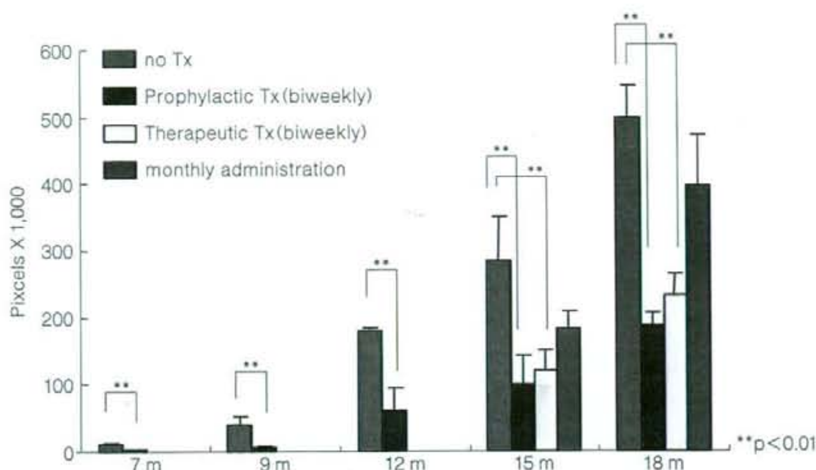

Fig. 2 Summary of quantitative analysis of Aβ burden in treated and untreated model mice

Blue, yellow, red and green bars represent the Aβ burden in untreated mice and in mice treated prophylactically treated (biweekly), therapeutically (biweekly) and monthly, respectively. Amyloid deposition was first detected in untreated mice at 7 months (7m) of age, and it was observed to rapidly increase after 15 months of age. Aβ deposition was significantly reduced in mice treated with DNA vaccine biweekly as compared with untreated control mice. Prophylactic administration of Fc-Aβ vaccine prevented Aβ deposition by 10-30% of that in untreated animals at 12 months of age and to 40-50% at 15 months. The effects of therapeutic administration were almost the same as those of prophylactic administration. Aβ reduction in mice treated monthly was lower than that in mice treated biweekly.

とがわかっている。ワクチン投与後，マウスの脳を免疫組織化学染色し，沈着したAβを画像解析した。Aβ沈着がまだ出現していない3～4カ月齢からワクチンを投与した予防的投与群（**Fig. 2** red bars）においては70～90%，既にAβ沈着が認められた12カ月齢から治療的にワクチンを投与した治療的投与群（**Fig. 2** yellow bars）においては50～60%のAβ沈着が，コントロール群(**Fig. 2** blue bars)に比較し有意に減少していた。ワクチンの効果は投与回数に依存する傾向があり，1カ月に1度投与した場合Aβの減少効果は減弱した（**Fig. 2** green bars)。DNAワクチン投与後の脳を免疫組織化学的に詳細に検索したが，Aβペプチド・ワクチンを投与したときにみられる，T細胞や炎症細胞の浸潤などの脳髄膜炎を思わせる所見はまったく認められなかった。さらにモデルマウスと同系で遺伝子操作を行っていないマウスにワクチンを投与し，そのリンパ節細胞からT細胞を分離しAβペプチドへの反応性を細胞増殖試験で解析したが，抗原反応性T細胞の活性化はまったく誘導されていなかった。これらの所見からDNAワクチンによる過剰な免疫反応はほとんどなく，Aβを減少する効果があると考えられた[32]。

V．アルツハイマー病に対する免疫療法の安全性

アルツハイマー病は数年から十数年の経過でゆっくり進行する。このため患者の治療を行う場合，長期に投与する必要性が高く，薬剤の安全性が強く求められる。能動免疫療法においては，脳炎などの過剰な免疫反応を惹起しないような薬剤を作製することが，今後のワクチン開発の課題になるであろう。また受動免疫療法では，抗体投与後に脳出血が起こる可能性があること，抗イディオタイプ抗体が産生され複数回の投与により効果が減弱する可能性があること，治療費が莫大になることなどに注意する必要がある。

DNAワクチンは，脳内の過剰な細胞免疫反応が誘導されにくく，能動免疫療法，受動免疫療法の欠点を克服できる可能性が高いが，遺伝子の導入に伴うベクターの安全性に留意する必要がある。遺伝子治療は，1990年に米国で先天的免疫不全であるADA欠損症の患者を対象

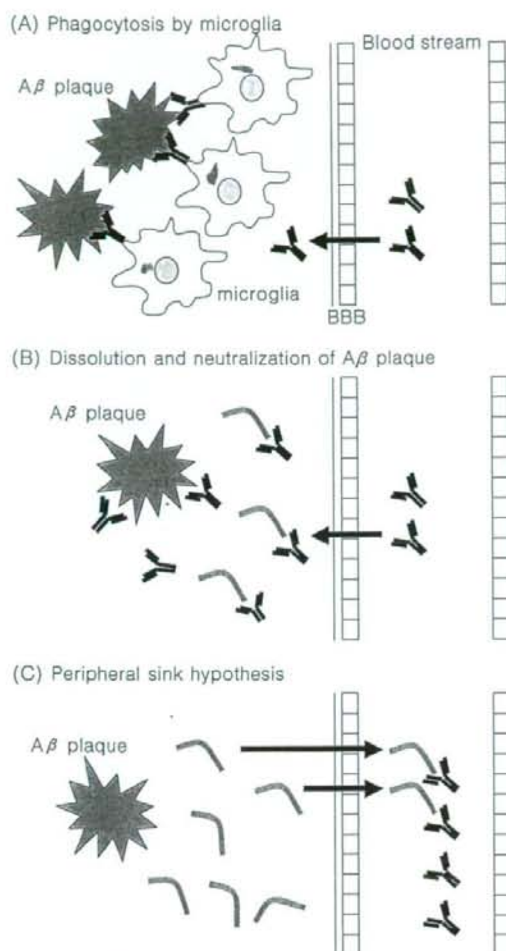

Fig. 3 Mechanisms of amyloid reduction with vaccine treatment

There are 3 hypotheses that can explain the amyloid reduction after vaccine therapy. (A) Phagocytosis by microglia: Anti-Aβ antibodies traverse the blood-brain barrier (BBB) and attach to Aβ deposits, which leads to Fc receptor-mediated phagocytosis by microglia. (B) Dissolution of Aβ plaques: antibodies bind to the N-terminus of Aβ deposits and dissolve amyloid fibrils. (C) The peripheral sink hypothesis: anti-Aβ antibodies in the circulation induce a net efflux of Aβ from the brain to plasma

にしてレトロウイルスベクターで実施されて以来，その有効性を拡大し，癌，HIVなどを対象に，世界で約4,000例が行われ，治療として確立した感があった。しかし1999年にペンシルバニア大学のWlisonらにより実施されていた先天性代謝疾患（OTC欠損症）の臨床試験で，大量のアデノウイルスを肝動脈から全身投与した18歳の男性患者が肝臓障害で死亡する事故が起こった。遺伝子治療で初の死亡例と報告されて以来，安全性に対する見直しが行われている[33]。その後，レトロウイルスベクターについても，フランスで1999年からX連鎖重症複合免疫不全症（X-SCID）に対して実施された遺伝子治療の患者11人に関して，2002年から2003年にかけて3人に有害事象（白血病の発症）が報告され，X-SCIDの遺伝子治療が一時凍結状態になった[34]。さらに，非病原性で安全性が高いと考えられていたAAVベクターに関しても，長期投与した場合のtumorigenecityが報告されている[35]。また，マウスに肝細胞癌を引き起こすという報告も認められる[36]。確率は極めて低いと考えられるものの，このような深刻な事態の出現は従来安全とされてきたウイルスベクターの安全性に関して，再度慎重な検討を行う必要性が出てきたことを示している。

これに対して，非ウイルス性ベクターは，特定の細胞に遺伝子を導入できないこと，導入効率が悪いことなどの欠点はあるものの，宿主細胞染色体へのintegrationは15万回に1回程度で，自然突然変異とほぼ同程度の無視できる割合であり[37]，安全性についてはほぼ確立されている。現時点ではアルツハイマー病のDNAワクチン療法のベクターとして非ウイルス性プラスミドベクターが最善であると思われる。

Ⅵ. DNAワクチンの作用機序

アルツハイマー病ワクチン療法におけるβアミロイド除去の機序として，現在以下の3つの説がある[6]。第1は，抗Aβ抗体が凝集したAβに結合し，Fc receptorを介してミクログリアに貪食されるために老人斑が除去されるという説である（**Fig. 3 A**）[38,39]。第2は抗Aβ抗体がAβのN末のアミノ酸を主に認識して結合し，凝集不溶化したAβを可溶化し，さらに分泌されたAβの凝集沈着を抗体が抑制することによりアミロイド沈着を減少させるという説である（**Fig. 3 B**）[40,41]。第3はAβに対する抗体は血液脳関門を越えず，末梢血末梢組織においてAβを減少させることにより脳組織から髄液を経由してAβを末梢血中に引き出すという，peripheral sink仮説である（**Fig. 3 C**）[15,42,43]。今回，筆者らのDNAワクチン投与の系で，これらの仮説が成り立つか否かを検証した。

まずDNAワクチン投与後のミクログリアの活性化の程度を知るために，ワクチン投与したマウスと対照群のマウスの脳切片をIba-1（microglia surface marker，茶色）と6F/3D（anti-Aβ 8-17，青）で二重染色し，病理

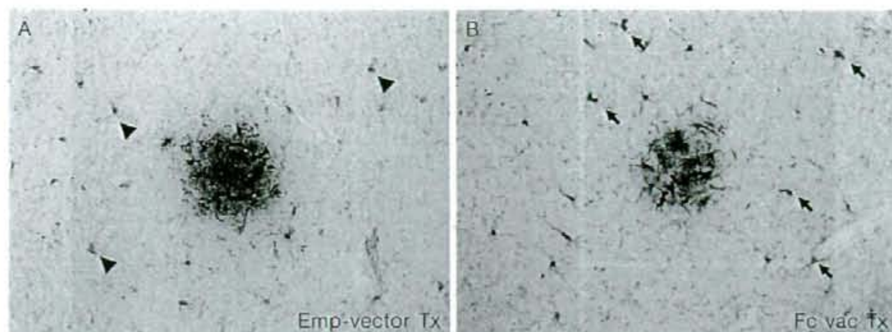

Fig. 4 Double staining with 6F/3D (amyloid plaques, blue) and Iba-1 (microglia, brown) in the brains of treated and untreated mice

Microglia with rich cytoplasms and processes with bulbous swellings are observed surrounding the plaques in the brains of untreated mice (A). After the treatment, more microglial cells infiltrated the amyloid plaques (B). In the remote brain regions of the non-treated APP23 mice, resting microglia were sparsely distributed (arrow heads in A). In the remote area of treated APP23 mice, microglial cells also increased in number and were present in the activated form (arrows in B).

学的解析を行った。この結果，野生型のB 6 マウスにおいては，小さな細胞質と細い突起を持つ休止型ミクログリアが脳全体に分布していた。未治療のモデルマウス(APP23)では，アミロイド斑周囲領域(periplaque area)に，大きな細胞質と太い突起を持つ活性化ミクログリアがアミロイド斑周囲に認められ，ミクログリアの突起はアミロイド斑に入り込んでいた。アミロイド斑から離れた領域(remote area)ではミクログリアは野生型B 6 マウスで観察されたように休止型であり，活性化像は認められなかった(**Fig. 4 A**)。これに対して治療群のモデルマウスでは，periplaque area のミクログリアはアミロイド斑の周囲で塊状となって有意に数を増しており($p<0.01$)，remote area でもミクログリアはその数を増して活性型に変化していた(**Fig. 4 B**)。ミクログリアの数の増加の割合は periplaque area よりも remote area で大きかった。

二重染色においてミクログリア内にアミロイドを認めることがしばしばあり，ミクログリアの貪食能が亢進している可能性が考えられた。それを定量的に分析するため，共焦点顕微鏡を用いてミクログリアの貪食能の変化を調べた。共焦点顕微鏡による観察では Cy3 でラベルされたミクログリア(赤)の中に，FITC ラベルされた Aβ 沈着(緑)が観察された(**Fig. 5**)。三次元解析をすることにより Aβ 沈着がミクログリア内にあることを確認した。アミロイドを貪食したミクログリア数は，ワクチン投与群で有意に増加していた($p<0.01$)。さらに，治療群でアミロイド斑表面に付着する抗体が増加することを免疫組織学的方法で確認した。

ミクログリアは，あるときは神経保護的に，あるときは神経損傷的に作用することが知られている。ワクチン投与後のミクログリアの増加がどちらの場合にあたるのかを，神経障害性サイトカインである TNF-α[44] を指標にして推測した。LPS 処理マウスおよび MOG-EAE−誘導マウスでは TNF-α が著明に増加していたものの，ワクチン投与後の増加は認められなかった。増加したミクログリアは神経保護的に作用しているものと考えられた。近年，oligomer として存在する可溶性 Aβ (soluble Aβ)に，シナプス障害等の神経細胞障害作用があることが報じられているが[45]，筆者らの系においてもワクチン投与により脳内の oligomer が減少することが western blotting 法により確かめられており，特に remote area の活性型ミクログリアの増加は，Aβ oligomer の除去に関与しているものと考えられた。

さらに，DNA ワクチンによって誘導された抗 Aβ 抗体が直接 Aβ 沈着に結合し，これを解離して除去する可能性[46]についても検討した。これは直接的評価が難しく，以下の手法でその程度を推測した。治療群および未治療群のマウス血清がアルツハイマー病モデルマウスのアミロイド斑と結合するか，否かを tissue amyloid plaque immunoreactivity (TAPIR)にて検討した。治療群の抗 Aβ 抗体は未治療群の抗体に比較して抗体価が高かったものの，Aβ に対する結合反応は著明ではなかった。抗体による Aβ の直接的解離，可溶化作用はさほど強くないと考えられた。次に，脳内から末梢血中に Aβ の

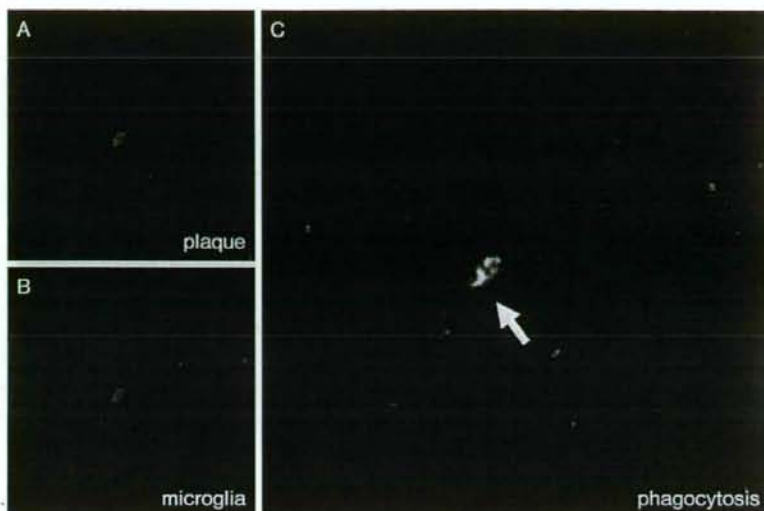

Fig. 5 Phagocytosis of Aβ deposits by activated microglia
Brain sections from treated APP23 mice were stained with 6F/3D (amyloid plaques, green) (A) and Iba-1 (microglia, red) (B) and observed with a confocal microscope. Single microglia present in remote brain region contain Aβ deposits (arrows in C).

引き抜きが起こっている可能性（peripheral sink hypothesis）を知るために，治療群および未治療群のマウスの血漿Aβを測定した。9カ月齢のマウスでは，治療群の一部で血漿Aβ量が高値であり，脳から血液へのAβの移行が亢進していると考えられた。しかし，15カ月齢では，治療群および未治療群で血漿Aβ値に変化はなかった。血管のアミロイド沈着が軽微な9カ月齢ではワクチン投与後，引き抜きが亢進している可能性がある。しかし15カ月齢では血管内のアミロイド沈着が進行し，引き抜きがほとんど起こらないことを示している。この機序はDNAワクチン療法では主要なルートではないと考えられた。

これらの事実から，DNAワクチン投与後のAβの除去はミクログリアによるAβの貪食によるところが大きく，抗Aβ抗体の直接作用や引き抜き作用の関与は存在しても弱いと考えられた。DNAワクチンはベクター内の遺伝子配列を変えることにより，簡単に再構成することができる。今後，さらに効果が高く副作用が少ないワクチンを開発発展する場合において有益である。

おわりに

コリンエステラーゼ阻害薬の塩酸ドネペジル（商品名アリセプト）は日本で唯一認可を受けたアルツハイマー病治療薬であり，症状の進行を遅らせることが知られている。しかし，効果は限定的で根治には至らないため，根本的な治療法の開発が社会的に極めて重要な課題であった。筆者らの作製したDNAワクチンがAβ沈着を有効に削減し，低価格で高い安全性を示したことが新聞，テレビなどで報道され，患者およびその家族からの多数の問い合わせが寄せられた。いかにアルツハイマー病が切実な問題であるか，ということを示していると言えよう。社会的反響を受け，現在筆者らの研究室では，プラスミド内に抗体産生を増加させるペプチドやミクログリアの貪食能を増進するペプチドを加え新たな数種類のワクチンを試作し，その効果を検討中である。さらに，創薬および臨床応用を具体的に視野に入れ，早期に医薬品製造管理基準（Good Manufacturing Practice: GMP）に対応した施設でヒト型DNAワクチンを製造しつつある。その後，医薬品の臨床試験の実施に関する基準（Good Clinical Practice: GCP）に沿い前臨床，および臨床試験を実施する予定にしている。

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ORIGINAL ARTICLE

Nonviral DNA Vaccination Augments Microglial Phagocytosis of β-Amyloid Deposits as a Major Clearance Pathway in an Alzheimer Disease Mouse Model

Yoshio Okura, MD, PhD, Kuniko Kohyama, MS, Il-Kwon Park, DVM, PhD, and Yoh Matsumoto, MD, PhD

Abstract

Immunotherapies markedly reduce β-amyloid (Aβ) burden and reverse behavioral impairment in mouse models of Alzheimer disease. We previously showed that new Aβ DNA vaccines reduced Aβ deposits in Alzheimer disease model mice without detectable side effects. Although they are effective, the mechanisms of Aβ reduction by the DNA vaccines remain to be elucidated. Here, we analyzed vaccinated and control Alzheimer disease model mice from 4 months to 15 months of age to assess which of several proposed mechanisms may underlie the beneficial effects of this vaccination. Immunohistochemical analysis revealed that activated microglial numbers increased significantly in the brains of vaccinated mice after DNA vaccination both around Aβ plaques and in areas remote from them. Microglia in treated mice phagocytosed Aβ debris more frequently than they did in untreated mice. Although microglia had an activated morphological phenotype, they did not produce significant amounts of tumor necrosis factor. Amyloid plaque immunoreactivity and Aβ concentrations in plasma increased slightly in vaccinated mice compared with controls at 9 but not at 15 months of age. Collectively, these data suggest that phagocytosis of Aβ deposits by microglia plays a central role in Aβ reduction after DNA vaccination.

Key Words: β-Amyloid, Alzheimer disease, DNA vaccine, Microglia.

INTRODUCTION

Alzheimer disease (AD) is the most common cause of age-related cognitive decline; it affects more than 12 million people worldwide (1). It is widely believed that accumulation of β-amyloid (Aβ) is the first event in the pathogenesis of AD and that it precedes tau phosphorylation, tangle formation, and neuron death (i.e. the amyloid cascade hypothesis) (2). Based on this hypothesis, Schenk et al (3) demonstrated that a vaccine composed of synthetic Aβ in complete Freund adjuvant induced high anti-Aβ antibody titers, leading to dramatic reductions of Aβ deposits in platelet-derived growth factor promoter–expressing amyloid precursor protein (PDAPP) transgenic mice (3). On the basis of these promising results, clinical trials with Aβ peptide (AN-1792) in conjunction with the T helper 1 adjuvant, QS-21, were initiated; however, the clinical trial was halted because some patients developed meningoencephalitis (4). Importantly, neuropathologic examination of treated patients showed apparent clearance of Aβ plaques from large areas of the neocortex (5, 6). Thus, it seems that vaccine therapy could be effective for AD if inflammatory/immune reactions are minimized.

We previously developed nonviral Aβ DNA vaccines with plasmid vectors and succeeded in reducing Aβ burden in APP23 mice without inducing side effects such as neuroinflammation (7). The mechanism of Aβ reduction after DNA vaccination, however, has not yet been elucidated. Three hypotheses explain how anti-Aβ antibodies reduce Aβ deposits in the brain (8, 9). The first is that anti-Aβ antibodies attached to Aβ plaques enhance Fc receptor–mediated phagocytosis of Aβ by microglia (10). The second mechanism is the direct effect of antibodies on Aβ, leading to the dissolution of amyloid fibrils or neutralization of Aβ oligomers (11, 12). Finally, the "peripheral sink hypothesis" postulates that anti-Aβ antibodies in the circulation results in a net efflux of Aβ from the brain into blood vessels (13, 14).

In this study, we examined whether these clearance mechanisms take place in our DNA vaccination system. We found that microglia were activated, increased in number, and phagocytosed Aβ deposits after vaccine administration. The results suggest that phagocytosis of Aβ deposits by activated microglia is a major clearance pathway of Aβ clearance after DNA vaccination and may provide important information for the development of effective new vaccines against AD.

MATERIALS AND METHODS

Animals

APP23 transgenic and wild-type B6 mice were used for analysis; and detailed information was provided in a previous

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report (7). Plasma was obtained from mice under deep inhalation anesthesia with ethyl ether via cardiac puncture with heparinized syringes before autopsy. Anesthetized mice were then killed. All procedures of animal experimentation were approved by the ethics committee of the Tokyo Metropolitan Institute for Neuroscience and performed in accordance with institutional guidelines.

Development and Administration of DNA Vaccines

We prepared Aβ DNA vaccines using a PTarget mammalian expression system (Promega, Tokyo, Japan) and injected them into APP23 mice on a weekly and then biweekly basis as used previously (15). Two DNA vaccines were used: immunoglobulin L (IgL)–Aβ vaccine, which possesses the Igκ signal sequence of mouse immunoglobulin to improve the secretion ability, and Aβ-Fc vaccine that has the Fc portion of human immunoglobulin at the 3′ end to maintain stability. APP23 mice received DNA vaccines (100 μg in 100 μL) regularly from 4 months of age, 2 months before amyloid plaque appearance, to the termination of experiments. Mice were killed at 9 and 15 months of age.

Immunohistochemistry

Mice were killed under deep anesthesia, and the brains were removed and immersion fixed in 4% paraformaldehyde. Paraffin-embedded sections were stained with monoclonal antibodies (mAbs) 6F/3D against Aβ8-17 (DAKO, Tokyo, Japan) and Iba-1 for microglia (WAKO, Tokyo, Japan). Sections were pretreated in formic acid for 3 minutes for 6F/3D staining and 0.1% trypsin for 10 minutes at 37°C for Iba-1 staining. After pretreatment, the sections were incubated in the primary mAbs followed by biotinylated horse anti-mouse immunoglobulin G (IgG) and horseradish peroxidase (HRP)–labeled Vectastain Elite ABC kit (Vector, Funakoshi, Tokyo, Japan). Horseradish peroxidase–binding sites were detected in 0.005% diaminobenzidine and 0.01% hydrogen peroxide. For confocal microscopic analysis, fluorescein isothiocyanate anti-mouse IgG and Cy-3 anti-rabbit IgG were used as secondary antibodies for 6F/3D and Iba-1 staining, respectively. The presence or absence of IgG depositions on Aβ plaques was determined by incubation of sections with biotinylated horse anti-mouse IgG followed by HRP-labeled Vectastain Elite ABC kit.

Quantitative Analysis of Aβ Burden and Microglia

β-Amyloid deposits were quantitated in the cerebral cortex and hippocampus according to the method used previously (15). All the procedures were performed by an individual blinded to the experimental conditions. The amyloid load was measured in 10 fields from the cingulate to retrosplenial cortex in the left hemisphere per mouse. Each field measured 600 × 400 μm and was randomly chosen. Analysis of the entire hippocampus was performed in a similar manner. β-Amyloid deposits that occupied the field were expressed as pixels using National Institutes of Health (NIH) image software.

After Iba-1 staining, the densities of microglia were determined by counting them in randomly selected 10 fields (600 × 400 μm each) from the cingulate to retrosplenial cortex in the left hemisphere of mice (n = 4–6 in each group). Microglia around the plaque (periplaque area) and those remote from the plaque (remote area) were counted separately and expressed as the mean ± SE per field. Using double-stained sections for Aβ and microglia, the densities of phagocytosing microglia were determined in a similar manner under confocal microscopy.

Western Blotting

Brain tissues were homogenized and sonicated in 10 volumes of Tris-buffered saline buffer in the presence of protease inhibitors. One milliliter of formic acid was added to 300 μg of homogenate in 10 μL. After a brief incubation, formic acid was vacuum dried with an acid-proof vacuum evaporator (miVac DNA, Scrum, Tokyo, Japan). After adding NuPAGE LDS sample buffer (Invitrogen, Tokyo, Japan), the samples were incubated at 70°C for 10 minutes and were run on NuPAGE 12% Bis-Tris gel (Invitrogen) (16). Before electrophoresis, protein concentration of each sample was determined, and the volume for loading was adjusted (equivalent to 40 μg); samples were then transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Tokyo, Japan). After blocking with 10% nonfat milk, the blots were incubated with anti-human Aβ 1–17 antibody (6E10; Cambridge, United Kingdom; 1:100) at 4°C overnight followed by incubation with Trueblot HRP-conjugated anti-mouse IgG (eBioscience, San Diego, CA; 1:1000) for 1 hour. The blots were developed by enhanced chemiluminescence reagents (Immunostar Kit Wako; WAKO) according to the manufacturer's instructions. The density of each band obtained by Western blot analysis was measured with a scanning laser densitometer (GS-700, Bio-Rad, Hercules, CA) and analyzed using the NIH image software.

Real-time Polymerase Chain Reaction

Total RNA was extracted from the indicated tissues using an RNAqueous Kit (Ambion), and complementary DNA was then synthesized by reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). SYBR Green real-time polymerase chain reactions (PCRs) were performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems) in a total volume of 25 μL using the SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). Each PCR was performed in duplicate using thermocycler conditions: Stage 1, 95°C for 10 minutes for 1 cycle and Stage 2, 95°C for 15 seconds and 58°C for 1 minute for 50 cycles. All primers were designed on an intron-exon junction to prevent coamplification of genomic DNA, and their sequences were shown in previous reports (17, 18). Relative quantification of messenger RNA (mRNA) was performed using the standard curve method. Glyceraldehyde-3-phosphate dehydrogenase was used as internal control. The absence of nonspecific amplification was confirmed by dissociation curve analysis.

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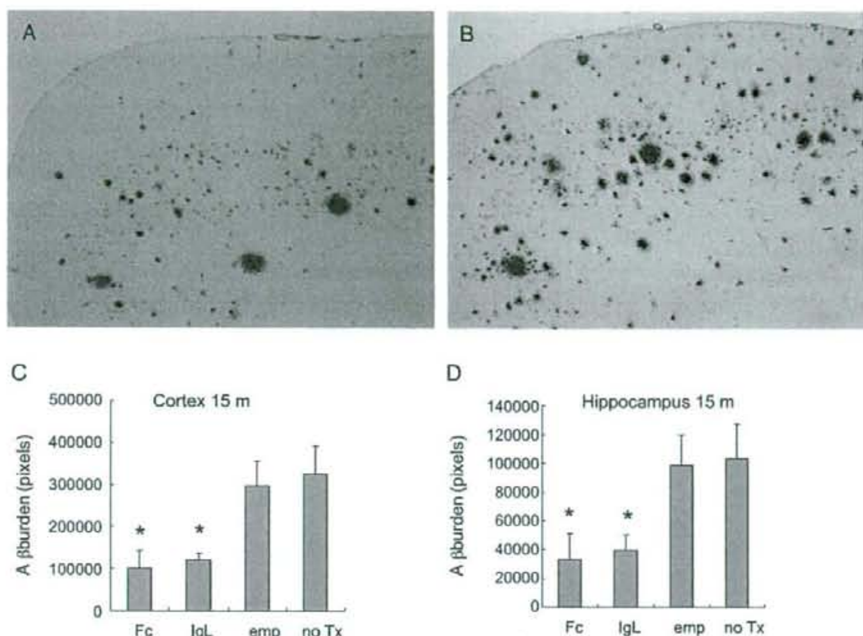

FIGURE 1. β-Amyloid (Aβ) reduction after DNA vaccination in mice at 15 months of age. There were fewer Aβ deposits in the frontal cortex of a mouse that had been treated with the Aβ-Fc vaccine **(A)** than in frontal cortex of a control mouse **(B)**. Semiquantitative analysis revealed that Aβ deposits were significantly reduced (* = $p < 0.01$) in the cortex of vaccinated mice (30.6% of untreated mice) **(C)**. β-Amyloid deposits in the hippocampus were also significantly reduced (* = $p < 0.01$) after vaccine treatment **(D)**. emp, empty vector; no Tx, untreated.

Tissue Amyloid Plaque Immunoreactivity Assay

Plasma to be tested were diluted to ×100, ×300, ×1,000, and ×3,000, and then applied to formic acid-pretreated APP23 brain sections, followed by incubation with biotinylated horse anti-mouse IgG and HRP-labeled Vectastain Elite ABC kit (Vector). The maximal dilution of plasma that gave positive staining was estimated as the amyloid plaque immunoreactivity titer.

Quantification of Tumor Necrosis Factor in the CNS Tissues and Plasma Aβ With ELISA

Brain tissue was homogenized in lysis buffer, and the supernatant was harvested after centrifugation. Each sample was adjusted to 10 mg/mL. The levels of brain tumor necrosis factor (TNF) and plasma Aβ were determined using the Mouse TNF Instant ELISA (Bender MedSystems, Vienna, Austria) and human Aβ (1–42) ELISA Kit (WAKO),

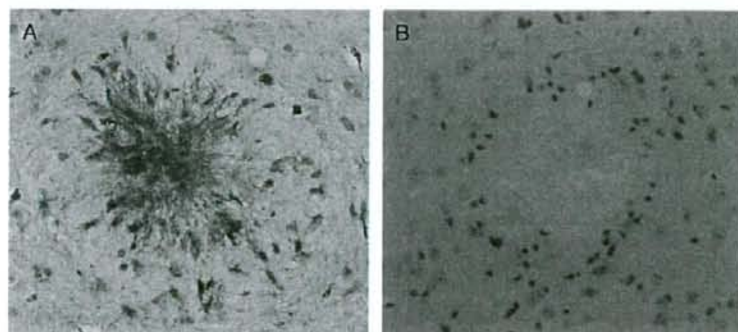

FIGURE 2. Immunoglobulin G (IgG) in the brains of DNA vaccine–treated and untreated APP23 mice at age 15 months. β-Amyloid (Aβ) plaques in the brain of an Aβ-Fc vaccine–treated mouse stained positively for IgG **(A)**, whereas a plaque in an untreated mouse was completely negative **(B)**. Immunohistochemistry with anti-mouse IgG. Original magnification: 200×.

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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

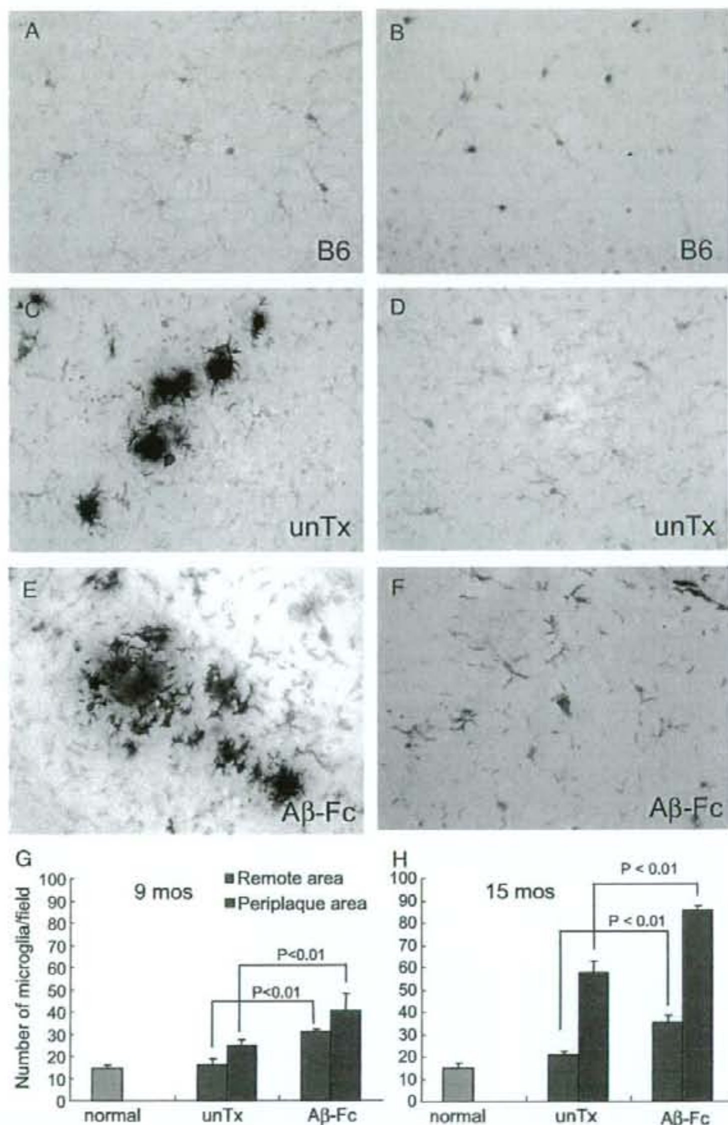

FIGURE 3. Double staining with monoclonal antibodies 6F3D against β-amyloid (Aβ) (blue) and Iba-1 against microglia (brown) of the brains of treated and untreated mice. In normal control B6 mice, ramified resting microglia were sparse in the cortex **(A)** and hippocampus **(B)**. Around plaques of untreated 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.

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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

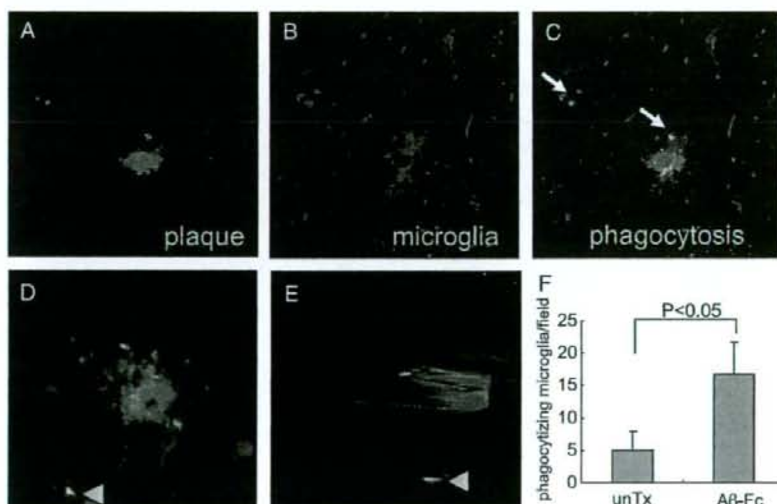

FIGURE 4. Phagocytosis of β-amyloid (Aβ) deposits by activated microglia in the cerebral cortex of mice at 15 months of age. Brain sections from treated **(A–E)** and untreated (not shown) APP23 mice were stained for Aβ with (6F3D, green) **(A)** and for microglia (Iba-1, red) **(B)** monoclonal antibodies 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.

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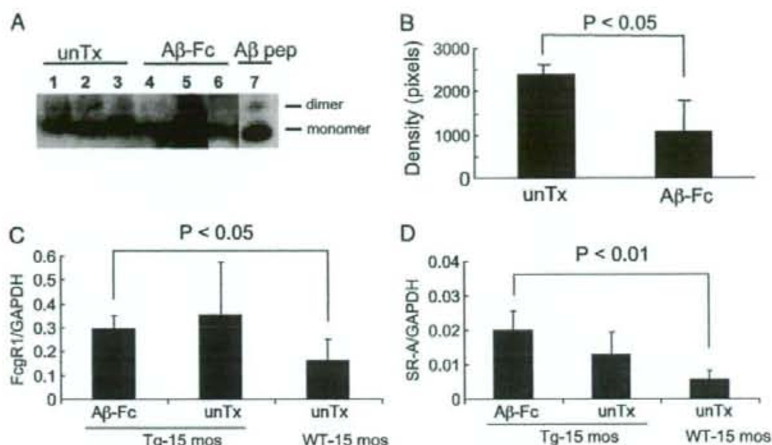

FIGURE 5. Western blot analysis **(A)** demonstrated that β-amyloid (Aβ) monomers plus dimers were reduced after DNA vaccination. Measurements of band densities revealed a statistically significant difference between vaccinated and control samples ($p < 0.05$). Lanes 1 to 5 and 7 were obtained from the 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 periplaque 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

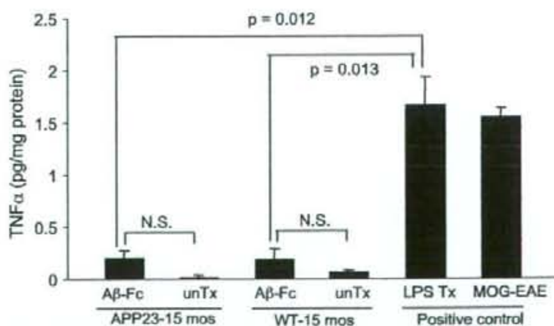

FIGURE 6. Tumor necrosis factor (TNF) levels in the brains of β-amyloid (Aβ)-Fc–treated and untreated B6 and APP23 mice detected by ELISA of CNS tissue homogenates. Large amounts of TNF were detected in the brains of positive control mice that had been given either an intraperitoneal injection of lipopolysaccharide 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.

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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

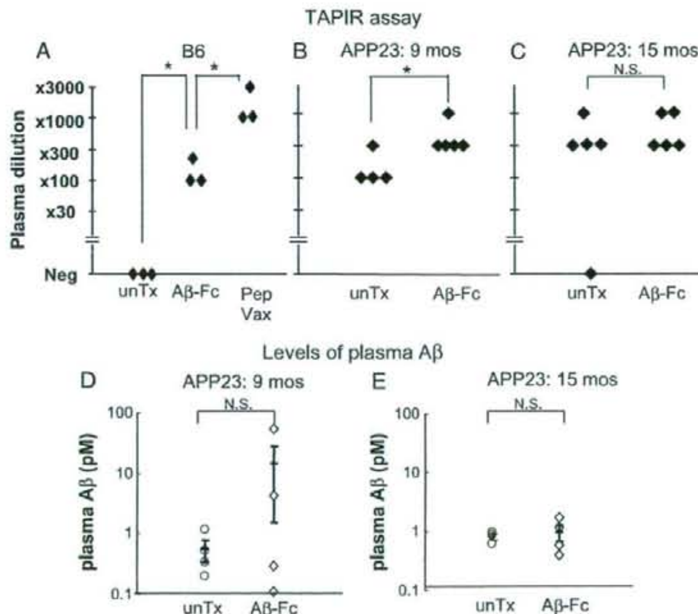

FIGURE 7. Tissue amyloid plaque immunoreactivity (TAPIR) assay and plasma β-amyloid (Aβ) levels in DNA vaccinated and untreated mice. **(A–C)** The binding of plasma from Aβ-immunized mice to Aβ plaques was determined using the TAPIR assay. **(A)** In plasma from untreated B6 mice, there was no Aβ-binding 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.

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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 nonimmunized 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

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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

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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 ($p <$

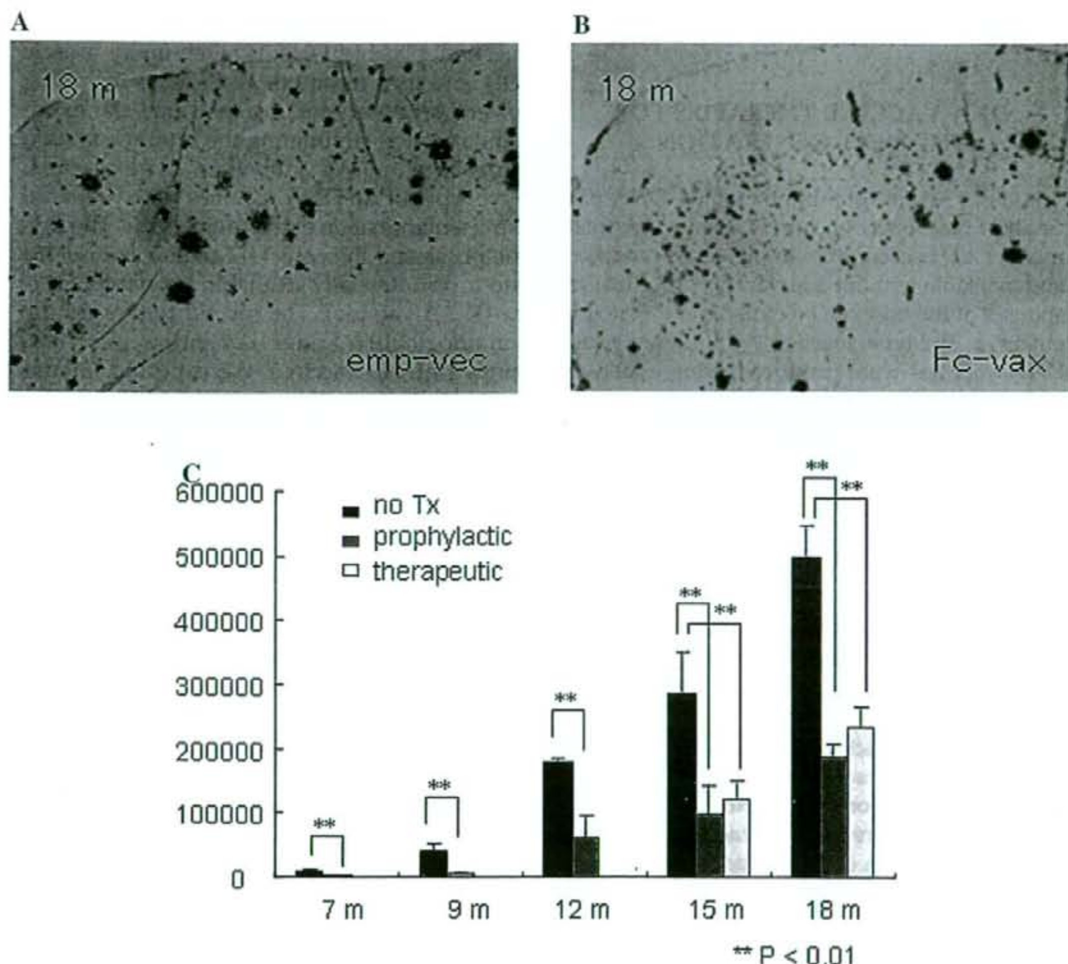

FIG. 1. Reduction of Aβ burden in APP23 mice after DNA vaccination. At 18 months of age, amyloid plaques with variable sizes were detected in the frontal cortex of untreated mice **(A)**. Therapeutic treatment with Fc-Aβ vaccines reduced cortical Aβ burden at 18 months **(B)**. **(C)** The overall quantitative 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.