いては、「動物の愛護及び管理に関する法律」「厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針」及び東京都神経科学総合研究所動物実験倫理委員会が定める動物実験指針を遵守して行なった。

また、本研究では遺伝子操作によりDNAワクチンを作製し、それを遺伝子改変マウス、及びサルに投与した。研究目的での遺伝子操作はすでに当研究所の研究倫理委員会、遺伝子組換実験委員会での審査を受け承認されており、動物実験については動物実験倫理委員会の審査を受けている。

C．研究結果

まず初めに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)、及び 6F/3D (anti-Aβ 8-17)で二重染色し、病理学的に検索した。Wild typeのB6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。未治療のアルツハイマーモデルマウス(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wild type B6マウスで観察されたように休止型であり、活性化像認められなかった。これに対して、治療群のアルツハイマーモデルマウスでは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(P<0.01)おり、remote areaでも、ミクログリアはその数を増し、活性型に変化していた。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ために、蛍光染色を行い、共焦点顕微鏡を用いてミクログリアの貪食能の変化を検索した。共焦点顕微鏡による観察ではミクログリアの中に、Aβ沈着が観察された。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)。

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おり、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を指標にして推測した。LPS処理マウス及び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)にも、シナプス障害等の神経細胞障害作用が認められることが報じられているが、我々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

ものと考えられる。

さらに我々は、DNA ワクチンの投与後、体内で誘導された抗 Aβ 抗体が直接 Aβ に反応し、解離、可溶化を引き起こし沈着した Aβ を除去する可能性について検討した。これは直接的評価が難しく、治療群及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反応性を持つか否かを tissue amyloid plaque immunoreactivity (TAPIR)にて検討し、間接的に推測した。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及び未治療群のマウスの血清 Aβ を測定した。9ヶ月齢のマウスでは治療群の一部で血清 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ヶ月齢では治療群及び未治療群で血清 Aβ 値に変化はなかった。血管にアミロイドーシスが進行していない 9 ヶ月齢ではワクチン投与後、引抜が亢進している可能性があるものの、15 ヶ月齢では血管のアミロイドーシスが進行し、引抜がほとんど起こらないことを示している。Peripheral sink 仮説は DNA ワクチン治療後の主要なルートではないと考えられた。

D．考察

ワクチン投与により活性化したミクログリアは A ベータ沈着周囲に付着し、沈着物の貪食に関与する事が示された。さらに、活性化したミクログリアは目に見えない Aβ 沈着物を貪食している事を明らかにした。本研究で明らかになったワクチンの作用機序は新型ワクチンを開発する上で有用な情報を提供すると考えられる。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いと考えられ、抗 Aβ 抗体の直接作用や peripheral sink 仮説の関与は少ないと考えられた。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ことから、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。

E．結論

DNA ワクチン療法における Aβ 蓄積の削減はワクチン投与によって活性化したミクログリアによる貪食が主体である。

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G. 知的財産権の出願・登録状況

新型ワクチンの出願を準備中。

厚生労働科学研究費補助金こころの健康科学　研究事業

分担研究報告書

DNA ワクチン投与動物の行動解析に関する研究

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研究要旨

DNA ワクチンによる治療群と対照群に Morris 水迷路試験を行い、ワクチン投与群で学習能力が改善する傾向が認められた。

A．研究目的

アルツハイマー病は認知障害（記憶障害、見当識障害、学習の障害、注意の障害、空間認知機能、問題解決能力の障害など）を主症状として中年以降に多発し、世界中で 1200 万人を超える患者が存在すると考えられている。症状は、数年の経過を経て徐々に進行し、発症後数年から十数年で寝たきりになり死に至る。その根治療法の開発は急務である。

近年、種々のアルツハイマー病のモデルマウスが開発され、候補薬剤のスクリーニングは容易になった。治療効果の判定は組織学的に Aβ を検出し、治療によって Aβ 蓄積がどれだけ減少するかを定量解析する方法と行動実験で明らかとなった認知障害がどれだけ改善するかを分析する方法の 2 種がある。

本研究は DNA ワクチン投与群と対照群の行動実験を以下の方法で行い、治療による認知障害の改善を明らかにすることを目的としている。

B．研究方法

定法のごとく Morris 水迷路試験を行う。直径 1 メーターの円形プールを用い、隠れたプラットフォームにたどり着く時間を計測する。一日 3 回のトライアルを 4 日間行い、その改善時間で認知障害の有無を判定する。すべての被検動物の行動はビデオ撮影し、PhenoScan (Primetech. Corp.) 分析装置で解析する。

(倫理面への配慮)

動物の飼養および実験上の処置については、「動物の愛護及び管理に関する法律」

「厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針」及び東京都神経科学総合研究所動物実験倫理委員会が定める動物実験指針を遵守して行なう。

また、本研究では遺伝子操作によりDNAワクチンを作製し、それを遺伝子改変マウス、及びサルに投与する。研究目的での遺伝子操作はすでに当研究所の研究倫理委員会、遺伝子組換実験委員会での審査を受け承認されており、動物実験については動物実験倫理委員会の審査を受けている。

C．研究結果

予備実験ではワクチン投与群で学習能力が改善する傾向が認められた。しかし、研究期間が短く(産休のため平成19年4月より8月まで)、十分な個体数の検索ができなかった。

D．考察

文献的にはモデルマウスのAβ沈着を半減すると認知障害はほとんど認められなくなると言う。今回の検索ではそれほどはっきりした結果は出なかったが、DNA ワクチン療法はAβ 沈着の削減だけでなく、認知障害も改善することが期待できる。

E．結論

DNA ワクチン療法によるアルツハイマー病モデルマウスの認知障害の改善は本法によって評価可能である。

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G．知的財産権の出願・登録状況

新型ワクチンの出願を準備中。

厚生労働科学研究費補助金こころの健康科学　研究事業

分担研究報告書

DNA ワクチンの作製に関する研究

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研究要旨

DNA ワクチンによる Aβ 蓄積の削減メカニズムの解析から、脳内ミクログリアの活性化が重要であることが明らかとなった。その効果を増強することができると考えられる 2 種の新型ワクチンを作製して、現在その効果を検定中である。

A．研究目的

作用機序の解析から DNA ワクチン療法における Aβ 蓄積の削減はワクチン投与によって活性化したミクログリアによる貪食が主体である事が明らかとなった。この事実はミクログリアの活性化をより強く刺激する構造を DNA ワクチンに付加すれば、さらにワクチンの効果を増強することが期待できる。

本研究では以下の方法により 2 種の新型ワクチンを作製し、その効果を検討しつつある。

B．研究方法

マウス脾臓由来の cDNA より、X 配列或いは Y 配列のコード領域をはさむプライマーを用いて各遺伝子を増幅し、クローニングした。作製済みの pTarget-IgL (Ig leader)-Aβ1-42-Fc (IgG の Fc 領域)の Fc 遺伝子を制限酵素切断によって切り出し、この部分に同じ制限酵素サイト（及びスペーサー配列）を付加した X 配列遺伝子をライゲーションして、Aβ1-42 と X 配列をタンデムに発現する pTarget-IgL-Aβ1-42-X 配列 (以降 Aβ-X 配列) を作製した。同様の手法で、以前作製した pIRES2-IgL-Aβ1-42-EGFP を用い、pIRES2- IgL-Aβ1-42- Y 配列(以降 Aβ- Y 配列)を作製した。Y 配列はホモダイマーを形成して活性を発現するサイトカインであるため、IRES (mRNA 内部のリボソーム結合サイト)を保有する pIRES ベクターの使用によって、Aβ と Y 配列を独立に発現させることを目的とした。

2 種の新型ワクチンによる目的蛋白発現を検討するため、各ワクチンプラスミドをトランスフェクション試薬により HEK293 細胞に導入し、48 時間後に培養上清及び細胞を回収して、ウェスタンブロッティングを行った。培養上清は 10 倍濃縮、細胞は蟻酸処理後に電気泳動用サンプルバッファーに溶解し、市販の 12%アクリルアミド Bis-Tris ゲル及び MES ランニングバッファーを用いて低分子蛋白(約 2 kDa 以上)のバンドを分離し、メンブレンにトランスファーした。5% スキムミルクでブロッキング後、抗ヒト Aβ1-16 抗体 (6E10, x100)で一晩、HRP 標識抗マウス IgG (x1000)で 1 時間反応し、ケミルミ試薬によってシグナルを検出した。

(倫理面への配慮)

動物の飼養および実験上の処置については、「動物の愛護及び管理に関する法律」「厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針」及び東京都神経科学総合研究所動物実験倫理委員会が定める動物実験指針を遵守して行なった。

また、本研究では遺伝子操作により DNAワクチンを作製し、それを遺伝子改変マウス、及びサルに投与した。研究目的での遺伝子操作はすでに当研究所の研究倫理委員会、遺伝子組換実験委員会での審査を受け承認されており、動物実験については動物実験倫理委員会の審査を受けている。

C．研究結果

ウェスタンブロッティングの結果、Aβ-X 配列は、培養上清、細胞ともに、Aβ1-42 と X 配列をタンデムに繋げた分子量である約 20 kDa の位置にバンドを検出した。Aβ1-42 単独 (約 3.5 kDa) のバンドは検出されなかった。

一方、Aβ- Y 配列は、抗 Aβ 抗体により Aβ1-42 単独のバンドが検出されることを期待したが、ここまでの実験結果では培養上清、細胞ともバンド自体が検出されず、Aβ 発現量は本検索による検出感度以下であることが示唆された。

D．考察

今回開発した 2 種の新型ワクチンのうち X 配列を持つものは DNA ワクチンとしての特性を持ち、アルツハイマー病の治療薬として有効であることが期待できる。今後、第 3 の入れるを持つワクチンの開発も計画中である。

E．結論

DNA ワクチン療法の発症機序に基づく新型ワクチン開発はより有効なワクチンを見いだす上で重要である。

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G. 知的財産権の出願・登録状況

新型ワクチンの出願を準備中。

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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IV. 研究成果の刊行物・別刷

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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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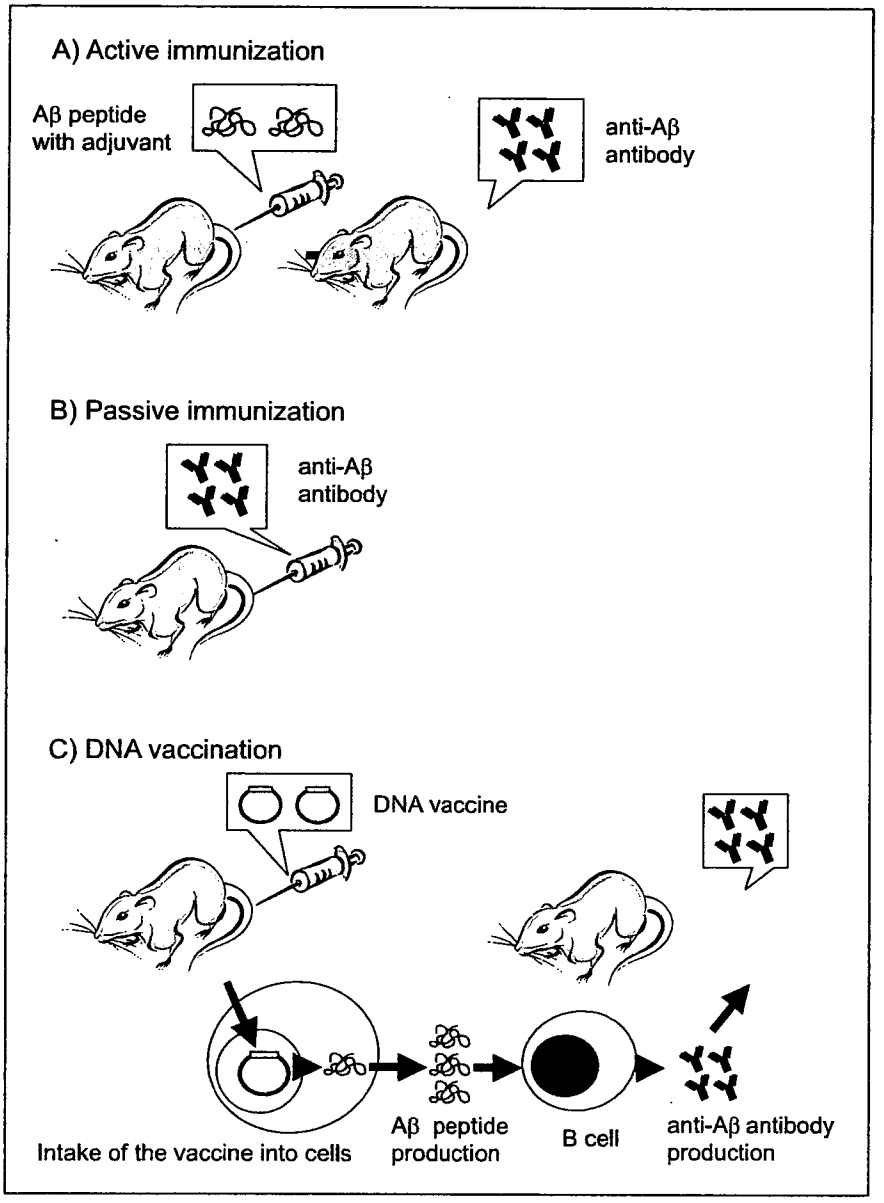

Fig. 1. Types of vaccine therapies for Alzheimer's disease. A) Active immunization: $A\beta_{1-42}$ peptides are administered with an adjuvant to induce anti-Aβ antibodies. B) Passive immunization: anti-Aβ antibodies are administrated directly. C) DNA vaccination: plasmid vectors encoding $A\beta_{1-42}$ 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

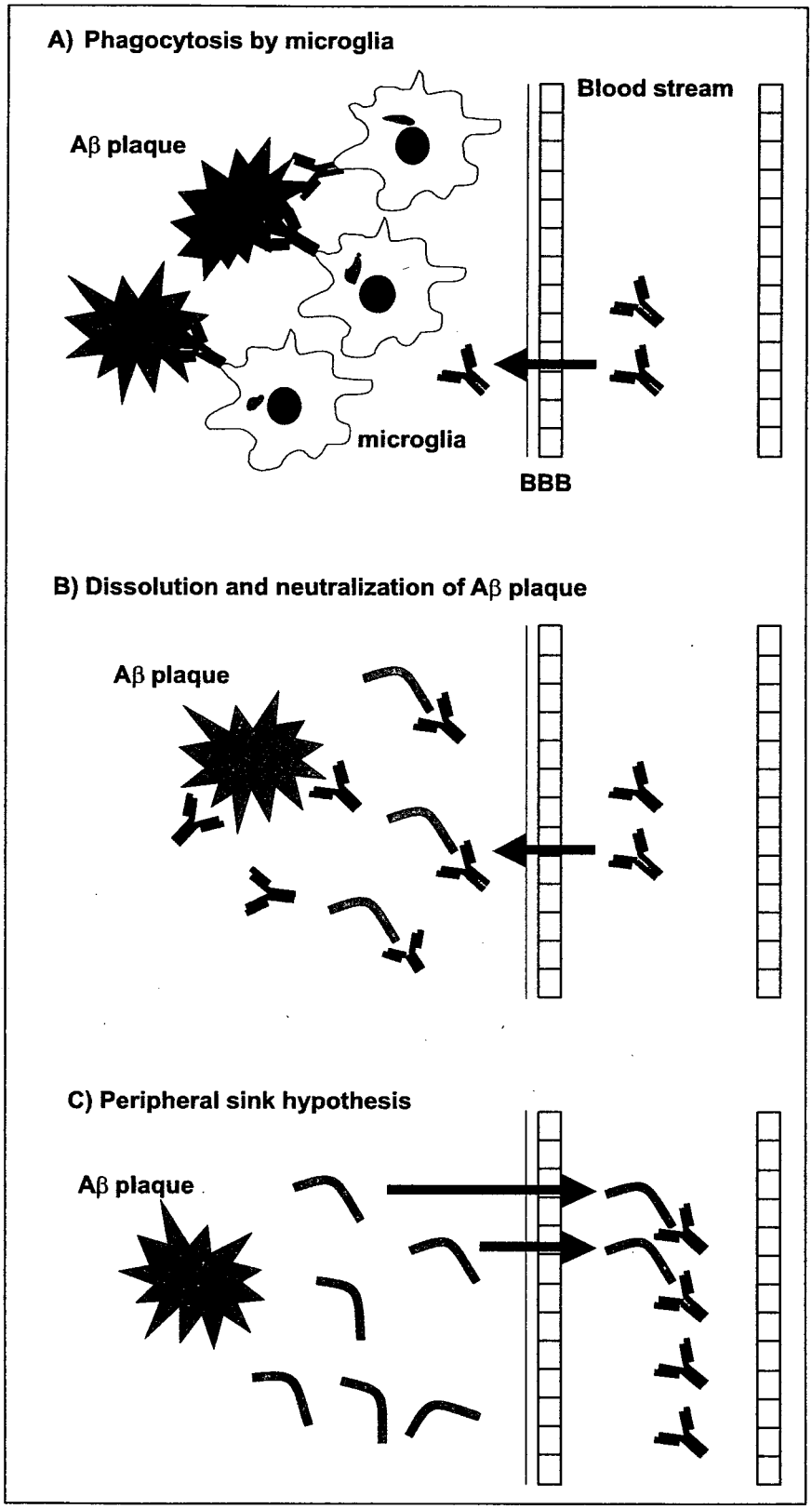

Fig. 2. Mechanisms of amyloid reduction with a vaccine treatment. A) Phagocytosis by microglia: anti-Aβ antibodies traverse blood–brain barrier (BBB) and attach to Aβ deposits, which leads to Fc receptor-mediated phagocytosis by microglia. B) Dissolution and neutralization of Aβ plaque: antibodies bind 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

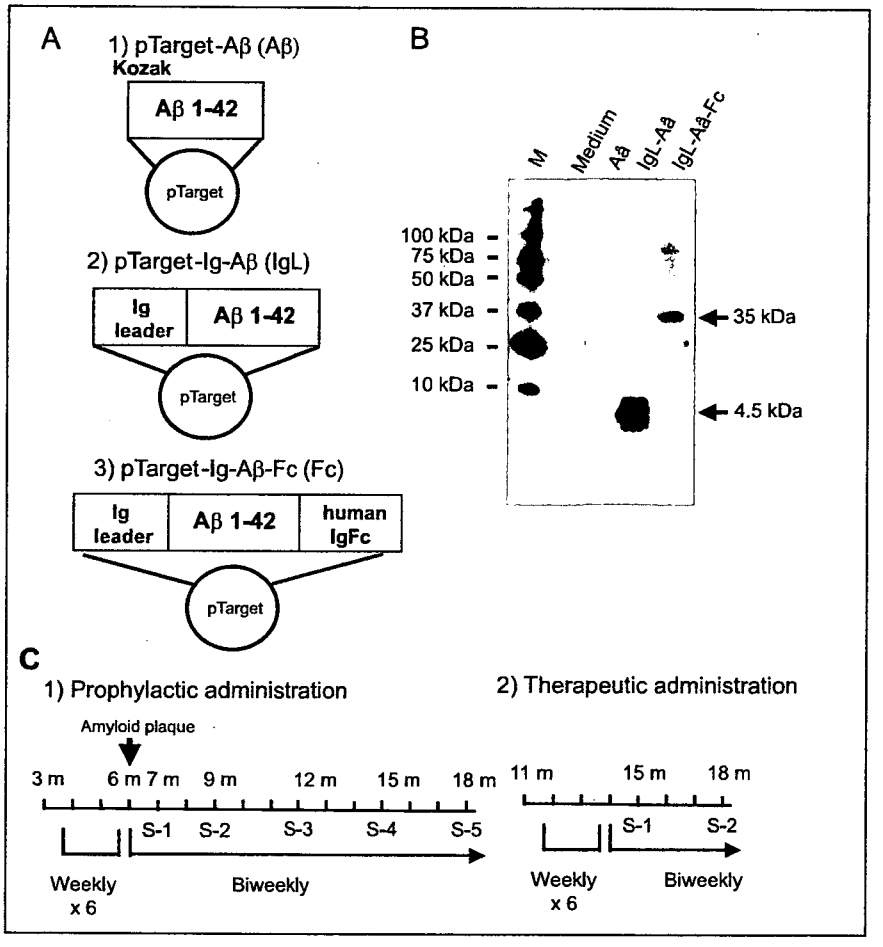

Fig. 3. Construction of DNA vaccines (A), *in vitro* characterization (B) and the treatment protocol (C). A) Three DNA vaccines were produced using a mammalian expression vector. DNA encoding the $A\beta_{1-42}$ sequence was inserted in XhoI/KpnI site of the plasmid (K-Aβ vaccine) (A-1). In the second 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

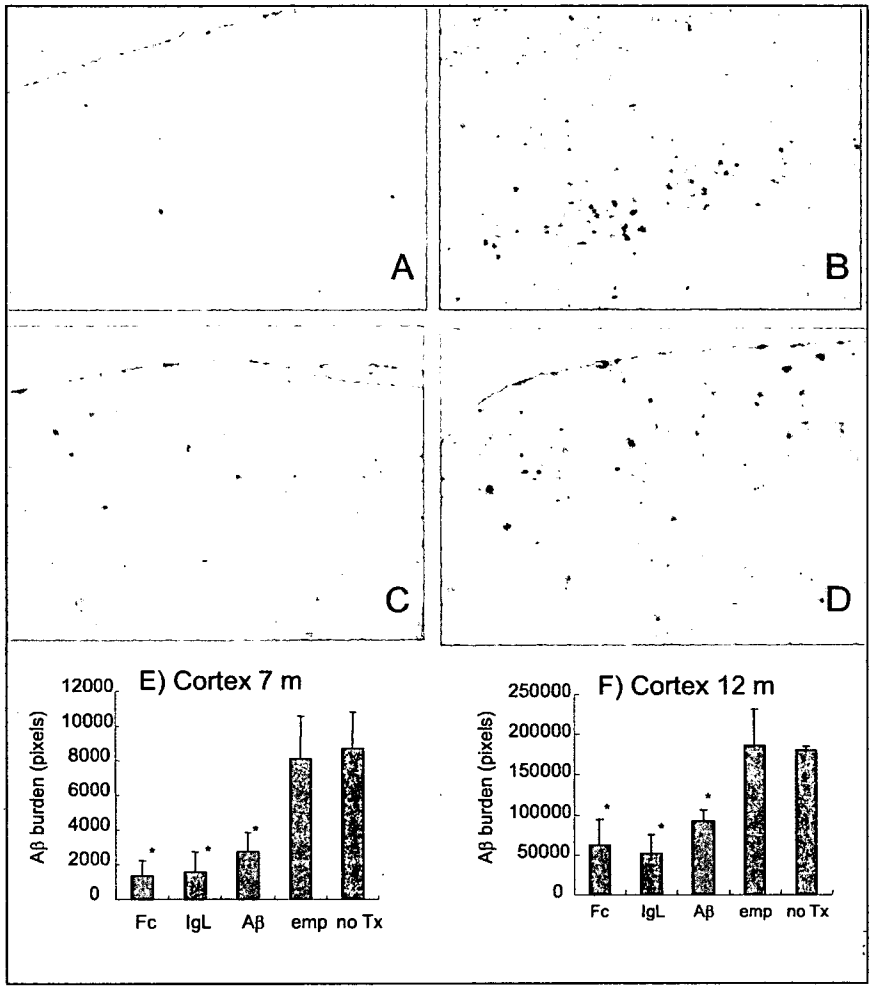

Fig. 4. Reduction of Aβ burden in APP23 mice at 7 months after DNA vaccination starting from 4 months (A, B and E). Immunohistochemical examinations revealed that granular amyloid depositions were detected in the frontal cortex of untreated mice at 7 months of age (B). In mice vaccinated with Aβ-Fc vaccine, 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.)

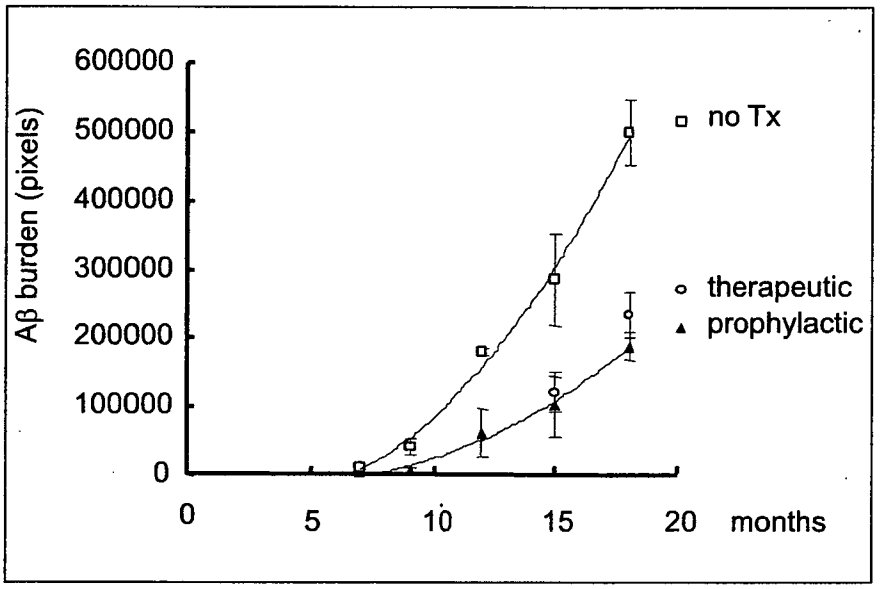

Fig. 5. The overall quantitative analysis. The amyloid deposition was first detected in untreated mice at 7 months of age and rapidly increased after 15 months of age (open squares). Prophylactic administration of Fc-Aβ vaccine prevented the Aβ deposition to 10–30% of that in untreated animals before 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

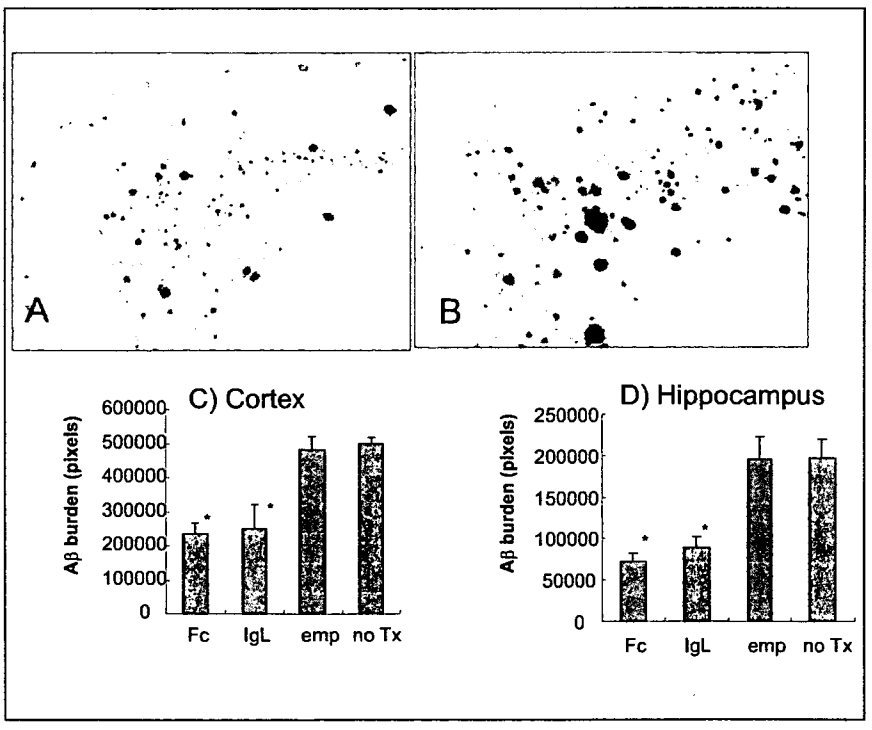

Fig. 6. Aβ burden reduction at 18 months of age after therapeutic treatment starting from 12 months. While large Aβ deposits (> 100 μm) were observed in the frontal cortex of control mice at 18 months of age (B), significant reduction was observed after 6-month therapeutic administration of the IgL-Aβ 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.

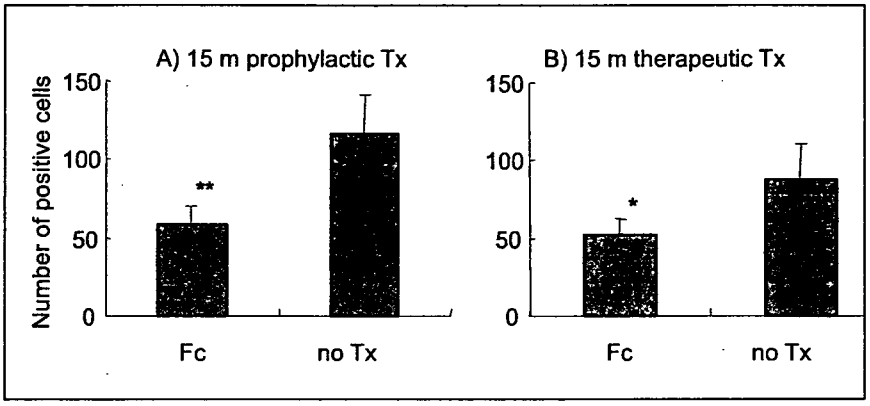

Fig. 7. Quantitative analysis of intracellular Aβ depositions after the prophylactic (A) and therapeutic (B) treatment with Aβ-Fc vaccine at 15 months of age. Microphotographs of the cerebral cortex (8 fields/mouse) were taken and neurons containing Aβ depositions were counted in a blinded manner. The numbers of positive neurons in vaccinated mice were significantly reduced compared with those in untreated mice ($*p < 0.05$; $**p < 0.01$).

and empty vector-vaccinated mice. Double staining with 6F/3D (anti-Aβ) and Iba-1 (antimicroglia) demonstrated the increase of phagocytizing microglia in the cerebral cortex of vaccine-treated Tg mice (unpublished data). It suggests that Aβ phagocytosis by activated microglia is a major pathway of Aβ clearance during nonviral DNA vaccine therapy.

The safety of our vaccines has been established as well as the effects. T-cell activation and proliferation, [^{3}H]-thymidine incorporation of T cells from vaccinated mice was negative in both wild-type B6 and APP23 Tg mice strain. 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 (data not shown). Thus, our nonviral Aβ DNA vaccines are highly effective and safe and promising as vaccine therapy against human Alzheimer's disease.

Conclusions

Although interrupted, the phase II clinical trial of AN-1792 provides further support for Aβ immunotherapy of Alzheimer's disease. Alternative vaccine therapies have been investigated and developed to reduce excessive immune reactions of the host brain. As discussed in this article, nonviral DNA vaccines are being introduced as a promising therapy against human Alzheimer's disease.

Acknowledgements

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