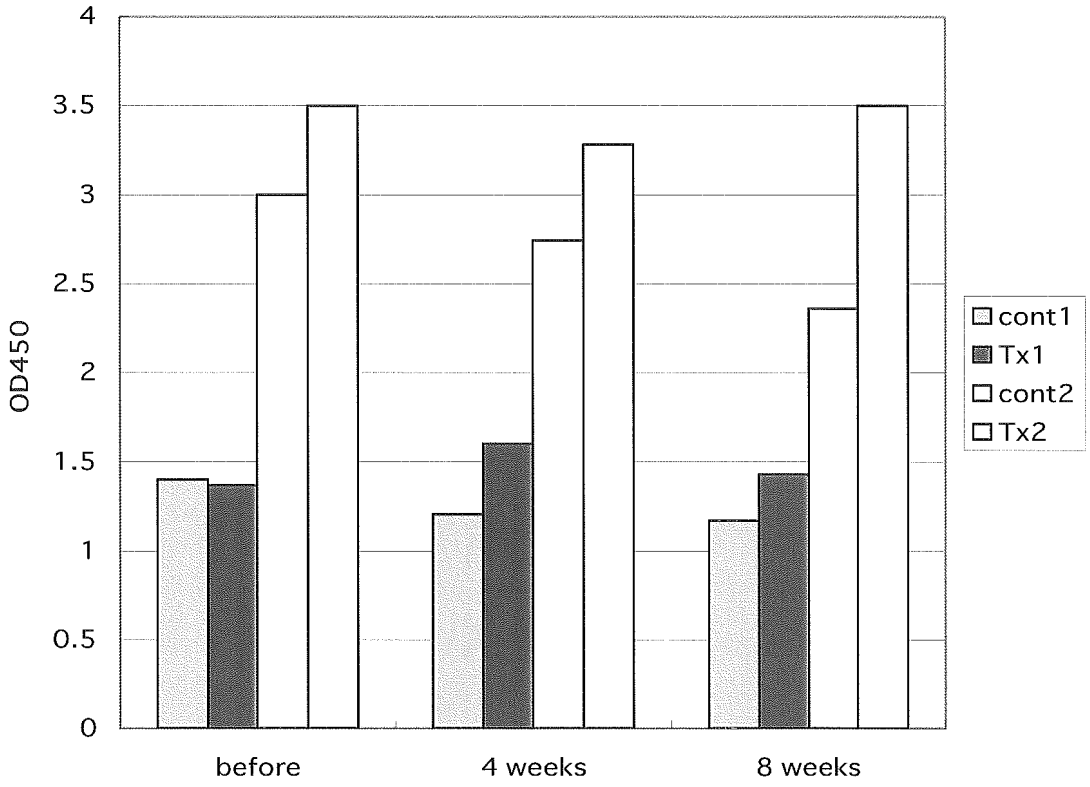
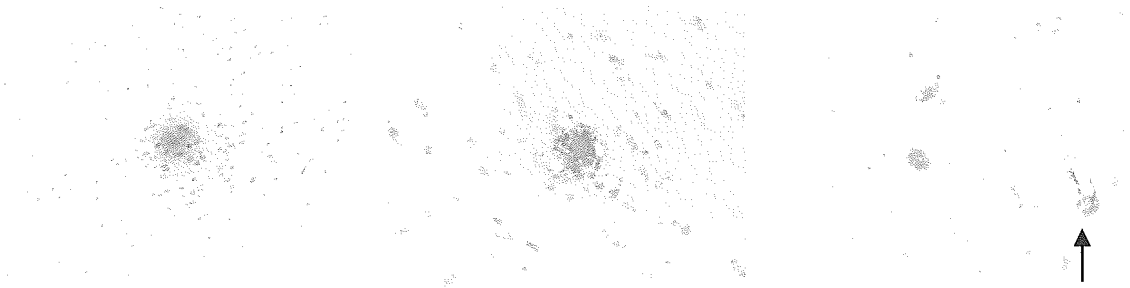
値を示し、観察期間中持続する傾向を示した。高抗体価を示す血清は、Aβペプチド以外の未同定の蛋白に交差反応している可能性も考えられる。

図１　アフリカミドリザル血清中の抗 Aβ抗体価の推移（1000 倍希釈）。



3.老人斑免疫組織染色

アフリカミドリザルの脳組織老人斑を抗 Aβ抗体(4G8)で染色した図２を示す。成熟型老人斑(mature senile plaques)とびまん型老人斑(diffuse senile plaques)を認める。小血管に沈着したアミロイドβ蛋白も認められる。



diffuse plaques　　mature plaques

図２　アフリカミドリザルの脳組織老人斑。成熟型老人斑(mature senile plaques)とびまん型老人斑(diffuse senile plaques)を認める。矢印は、血管に沈着したアミロイドβ蛋白。

アフリカミドリザルの前頭葉では、コントロール群および治療群とも老人斑の数は比較的少数であるが、コントロール群では神経細胞内 Aβ蛋白沈着が有意に増加していた（図３）。

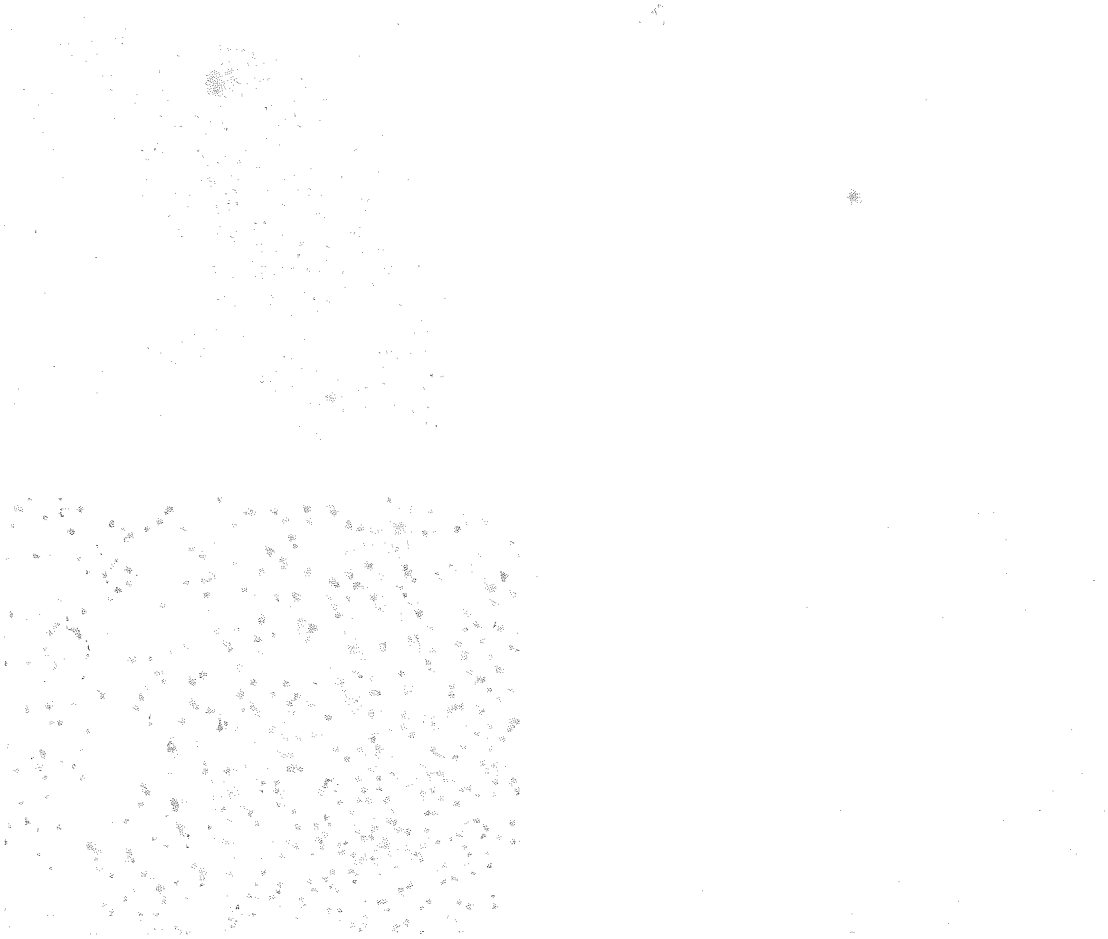


図３アフリカミドリザル前頭葉のアミロイドβ染色。

アフリカミドリザルの頭頂葉では、治療群 G-4 サルに老人斑がやや目立つが、コントロール群においても老人斑の形成や、神経細胞内 Aβ蛋白沈着が多く認められた（図４）。
治療群 G-5 サルでは、殆どアミロイドβ蛋白の沈着は認められない。

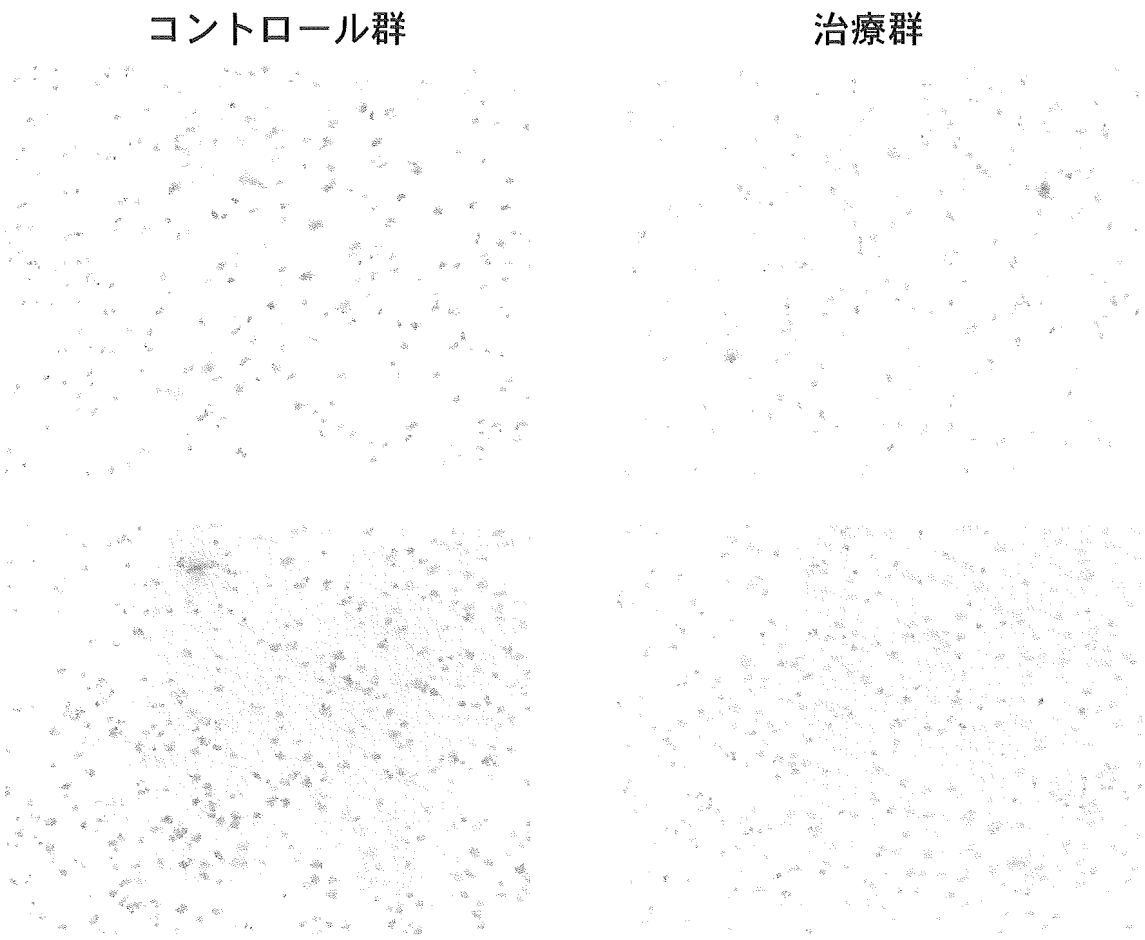


図４　アフリカミドリザル頭頂葉のアミロイドβ染色。

アフリカミドリザル側頭葉のアミロイドβ染色を示す（図５）。治療群 G-4 サルにアミロイドβ蛋白の沈着は軽微である。治療群 G-5 サルでは、殆どアミロイドβ蛋白の沈着は認められない。一方、コントロール群では、老人斑の形成や、神経細胞内 Aβ蛋白沈着が目立つ。

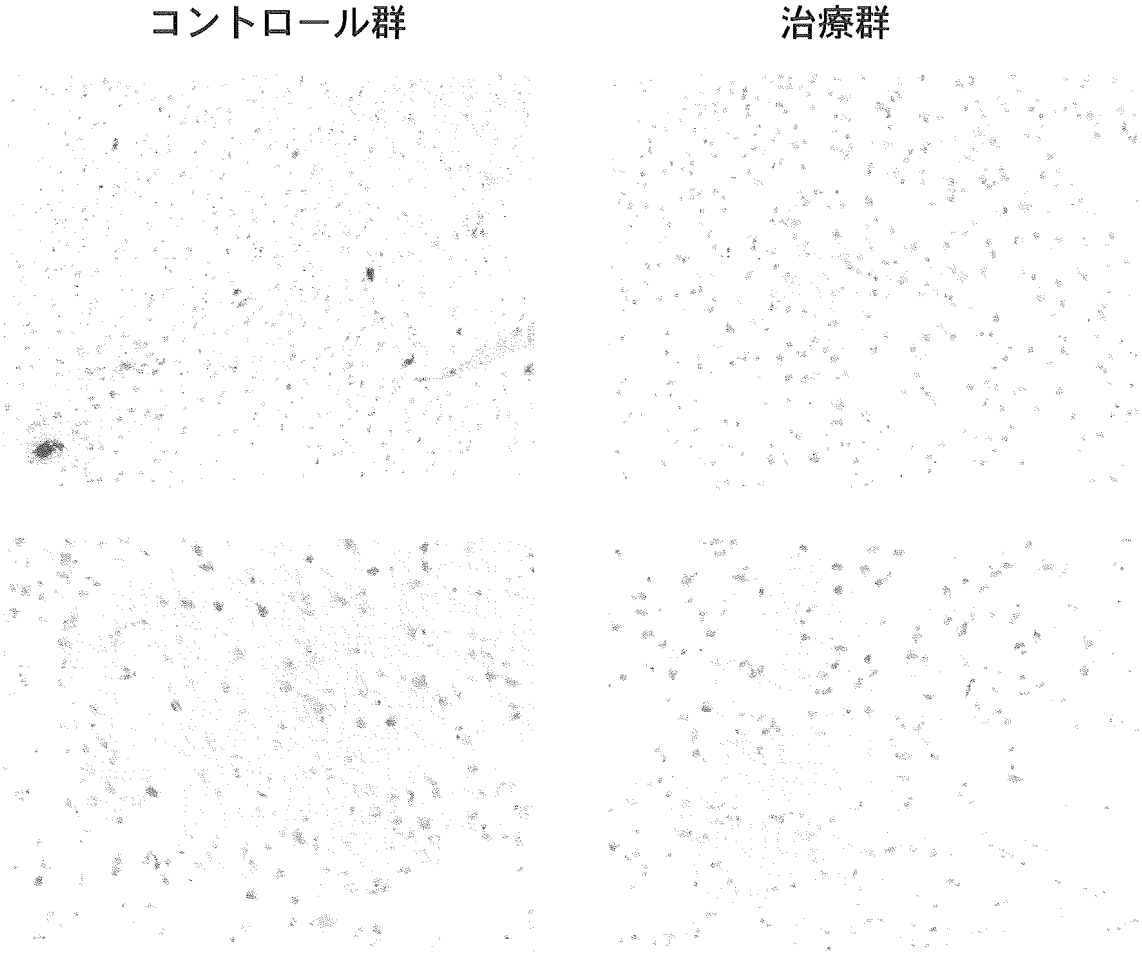


図５　アフリカミドリザル側頭葉のアミロイドβ染色。

アフリカミドリザル後頭葉では、コントロール群において老人斑の形成や、著明な神経細胞内 Aβ蛋白沈着が認められる。治療群では、小さな老人斑の形成を認めるのみで、神経細胞内 Aβ蛋白沈着の所見は認められない（図６）。

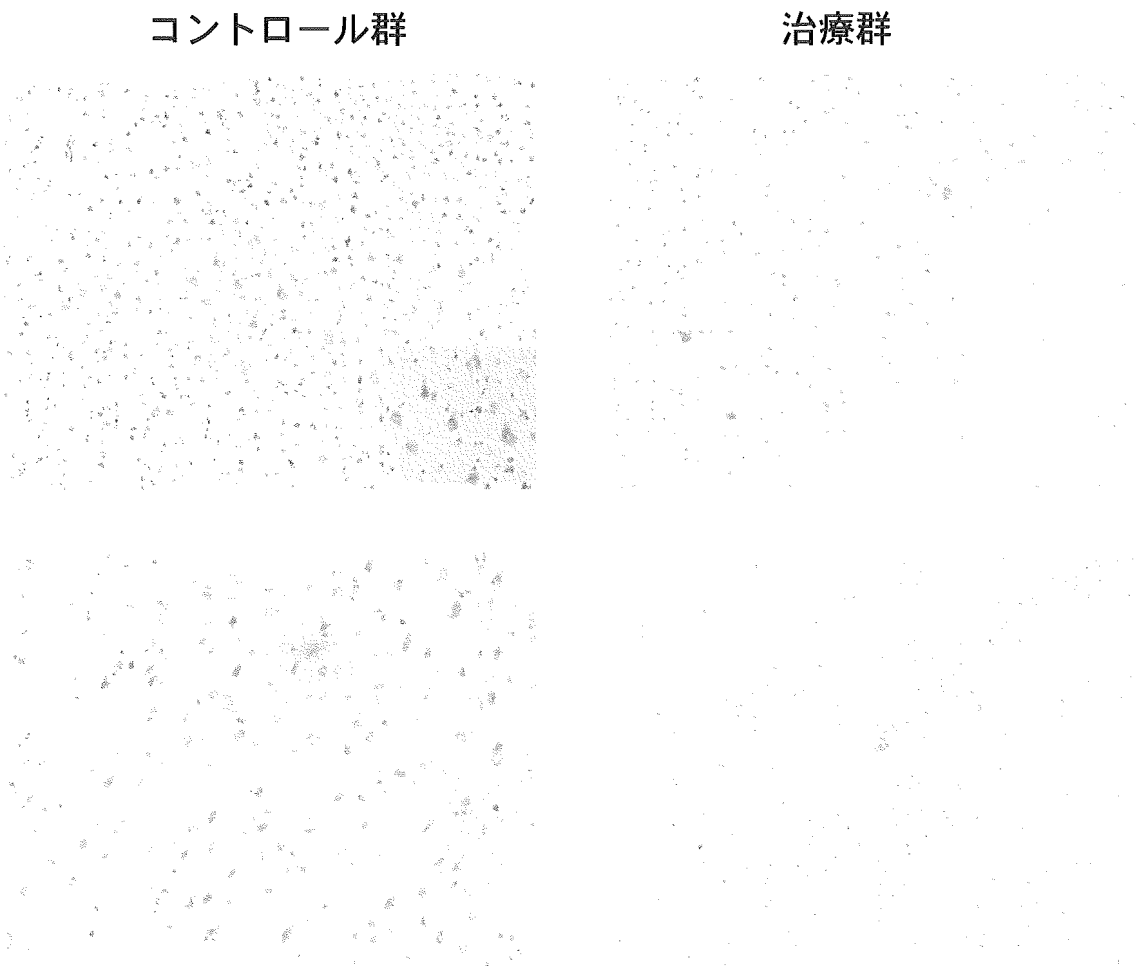


図６　アフリカミドリザル後頭葉のアミロイドβ染色。

アフリカミドリザル小脳においては（図７）、ヒトと同様に小脳プルキンエ細胞層にアミロイドβ蛋白が１列に沈着し、プルキンエ細胞層・顆粒層においては老人斑の形成が認められる。コントロール群に比べ、治療群では小脳プルキンエ細胞層のアミロイドβ蛋白沈着は少なく、顆粒層の老人斑も著明に減少していた。

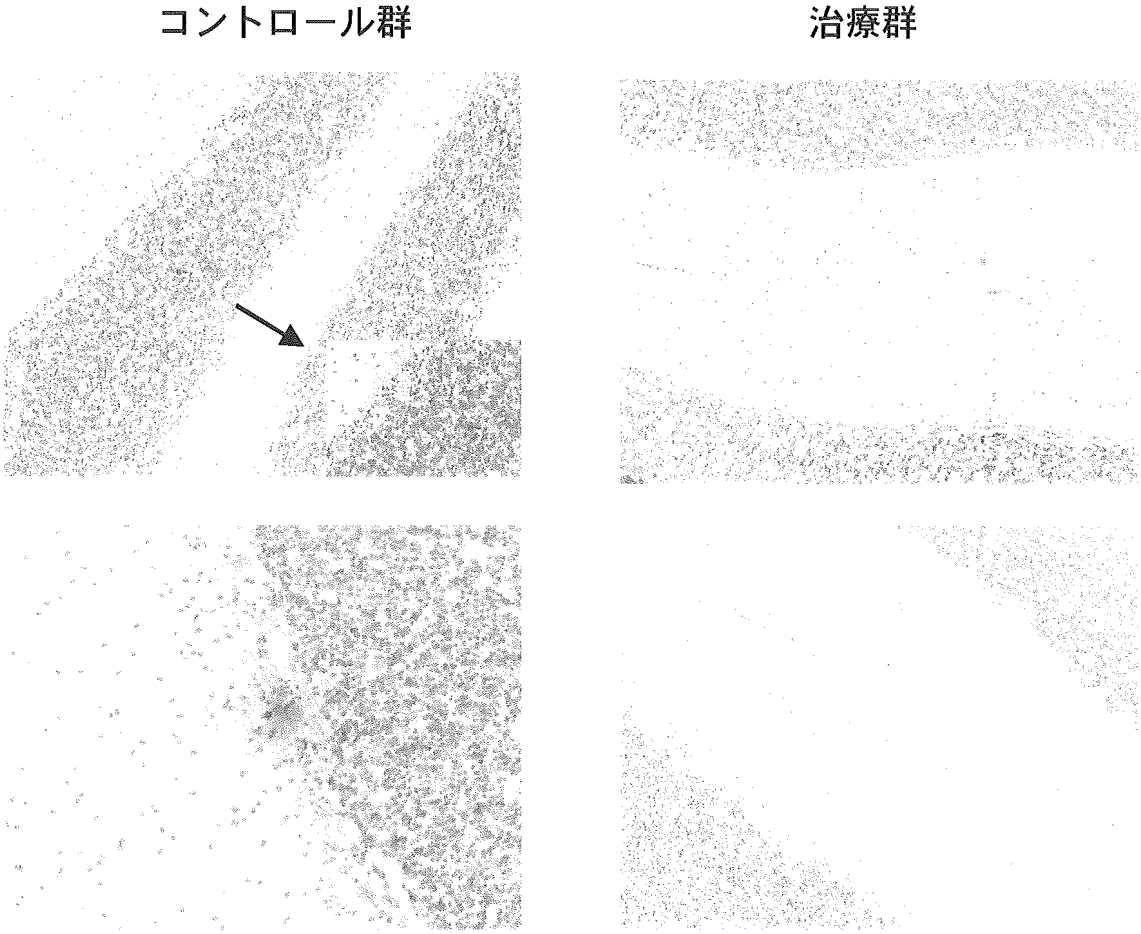


図７　アフリカミドリザル小脳のアミロイドβ染色。

D. 考察

神経性変性疾患（アルツハイマー病、パーキンソン病、脊髄小脳変性症、筋萎縮性側索硬化症等）の原因は、近年かなり明らかになってきたが、詳細な発症機序は未だに不明である。これらの疾患の治療法としては、明確な発症機序の解明後に開発される根治治療が理想であるが、例え発症機序が解明されたとしても、現段階の医学・分子生物学的技術では新たな治療法を開発出来ない可能性もある。アルツハイマー病の Aβワクチン療法は、免疫学的手法（抗体）を用いて自己蛋白である蓄積したアミロイドベータ蛋白を除去しようとする治療法であり、新しい治療ストラテジーの１つとして大変注目されている。

アルツハイマー病の病理学的所見として、神経細胞の萎縮・脱落、アミロイドβ(amyloid-β, Aβ) 蛋白の凝集・沈着による老人斑の形成、異常タウ蛋白からなる神経原繊維変化(neurofibrillary tangles: NFT)の 3 つが大きな特徴である。

我々は昨年度、アルツハイマー病の動物モデルである APP トランスジェニック(Tg2576)マウスに Aβを発現するアデノ随伴ウイルスベクターを経口投与し、13 ヶ月齢の APP トランスジェニックマウス脳組織を免疫染色で詳細に検索した結果、治療したマウス脳においてアミロイド沈着・老人斑形成がコントロールに比べ明らかに減少していることを報告した。

今回、さらにヒトに近い、老人斑形成が見られる老齢のアフリカミドリザルに経口ワクチンを投与し、短期間（３ヶ月間）での効果を解析した。

類人猿およびサルにおいても老人斑やアミロイドアンギオパチー(cerebral amyloid angiopathy; CAA)の出現については報告があるが、神経原線維変化については報告がない。サル類ではタウ蛋白の存在なしに多数の老人斑が認められる。これによりサル類での老人斑形成にはタウ蛋白の存在は必須でなく、むしろ apoE などの関与が推測されている。

カニクイザルの老人斑は、ヒトと同様に２つの型の老人斑、すなわち成熟型老人斑(mature senile plaques)とびまん型老人斑(diffuse senile plaques)に分類されている。カニクイザルでは、２０歳以上の動物に成熟型老人斑が認められている。出現初期の２０歳から３０歳以上の動物に至るまで老人斑の殆どが成熟型老人斑であり、びまん型老人斑から出現するヒトの傾向とは異なるとの報告がある。

総成熟型老人斑数は、加齢とともに増加する傾向があるが、個体差も存在する。成熟型老人斑の出現分布は、初期に外側溝に沿った上及び下側頭回皮質または扁桃核を中心に出現し、加齢に伴い前頭葉、頭頂葉、後頭葉など広範な大脳皮質に観察されると報告されている。

我々は、アフリカミドリザルの野生型で推定年齢２０歳以上の雌サル３頭と２３歳の雌サル１頭に経口ワクチンを投与した。

アデノ随伴ウイルスベクターは耐酸性があるが、我々が考案した方法は、腸管粘膜免疫を用いるため、胃組織より腸管粘膜免疫の発達した小腸を中心とする部位でのウイルスベクター感染を想定している。そのため単に経口投与した場合、胃組織への感染導入が多くなるため、分泌型Aβ発現アデノ随伴ウイルスベクター水溶液をゼラチン化し腸溶剤カプセルに詰めた薬剤の経口投与を行った。

アフリカミドリザル血清中の抗 Aβ抗体価は、投与前に比べ著しい増加はないが、４週後には上昇しており、８週には投与前の値と同等に下降した。

またサルの個体差もあるが、ELISAにて原因不明の抗 Aβ抗体価高値を示すサルも認められた。交差反応による可能性も考えられる。

カニクイザルでは、出現初期の２０歳から３０歳以上の動物に至るまで老人斑の殆どが成熟型老人斑であるとの報告と異なり、アフリカミドリザルでは、成熟型老人斑とびまん型老人斑の両方の型が認められた。今回使用したアフリカミドリザルは、年齢不詳のサルが大部分ではあるが、脳組織では老人斑の形成が全般的に比較的少量であった。

分泌型 Aβ発現アデノ随伴ウイルスベクターの経口ワクチン投与により、アフリカミドリザルの脳老人斑は減少傾向を示した。さらに著明な変化は、神経細胞内 Aβ蛋白に見られ、コントロール群では、神経細胞内 Aβ蛋白沈着の所見が多く認められたが、治療群では激減していた。さらに小脳プルキンエ細胞層のアミロイドβ蛋白沈着や、プルキンエ細胞層・顆粒層の老人斑の形成は、経口ワクチン投与により改善を示した。

E. 結論

我々が開発した分泌型 Aβ発現アデノ随伴ウイルスベクターの経口ワクチンは、老齢サルに投与後３ヶ月間の期間で、神経細胞内 Aβ蛋白沈着が激減し、小脳プルキンエ細胞層のアミロイドβ蛋白沈着や、プルキンエ細胞層・顆粒層の老人斑の減少などの改善を示した。

F. 健康危険情報

該当なし

G. 研究発表

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アルツハイマー病に対する経口ワクチン療法の開発

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アルツハイマー病に対する経口ワクチン療法の開発

原　英夫、Alon Monsonego、湯浅勝敏、足立香代、武田伸二、高橋慶吉、Howard L. Weiner、田平　武

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H. 知的所有権の取得状況

1. 特許取得

アルツハイマー病の治療のための組換えアデノ随伴ウイルスベクター；出願番号 2003-169714、平成 15 年 6 月 13 日, PTC 出願（発明者；原英夫、田平武）

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Development of a safe oral Aβ vaccine using recombinant adeno-associated virus vector for Alzheimer's disease

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Abstract. A new oral vaccine for Alzheimer's disease was developed using recombinant adeno-associated virus vector carrying Aβ cDNA (AAV/Aβ). Oral administration of the vaccine without adjuvant induced the expression and secretion of Aβ1–43 or Aβ1–21 in the epithelial cell layer of the intestine in amyloid precursor protein transgenic mice. Serum antibody levels were elevated for more than six months, while T cell proliferative responses to Aβ was not detected. Brain Aβ burden was significantly decreased compared to the control without inflammatory changes. This oral AAV/Aβ vaccine seems to be promising for prevention and treatment of Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is characterized by progressive loss of cognitive function due to amyloid β(Aβ) deposits in the central nervous system [1]. Immunization of amyloid Precursor protein (APP)-transgenic (Tg) mice with synthetic Aβ in complete Freund's adjuvant showed a marked decrease of amyloid burden in the brain [2]. Repetitive intranasal administration of the Aβ peptide and adjuvant [3] or passive transfer of Aβ antibodies [4] was also effective for reducing the amyloid deposits. Although Aβ is not an infectious agent, this treatment is now widely accepted as "vaccination". Since vaccinated mice showed an improvement of memory loss [5,6], clinical trials were performed in humans. The phase II trial of vaccine AN-1792 composed of synthetic Aβ1–42 and QS21 as adjuvant was halted because of complication, acute meningoencephalitis appeared in 18 of 298 (6%) patients [7], which was thought to be caused by autoimmune T cells reactive to Aβ. However, an autopsy case who died one year after the complication suggested effective clearance of β amyloid by vaccination [8], and patients who produced antibodies against senile plaque amyloid showed less cognitive decline than those who did not [9]. Therefore, Aβ vaccination seems to be a promising way to prevent the onset and progression of AD, if the T cell-mediated side effect is minimized. Here, we report a safe oral Aβ vaccine using the recombinant adeno-associated virus vector (AAV), which successfully reduced amyloid burden in APP transgenic

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mice, while T cell immune responses to Aβ were significantly suppressed.

2. Materials and methods

2.1. Mice

APP-Transgenic mice tg2576 [10] were obtained from Taconic (Germantown, NY). All animal experiments were performed in accordance with our institutional guidelines after obtaining the permission of the Laboratory Animal Ethical Committee.

2.2. Adeno-associated virus vector construction

Recombinant adeno-associated virus (AAV) vector for expression of Aβ1–43 was constructed using plasmid DNA pTRUF2 [11]. Primers were designed to amplify cytosolic Aβ1–43 from APP695. These primers amplify the entire Aβ sequence with an additional stop codon at the 3′ end to ensure translation. Cytosolic Aβ was amplified with the Aβ1–43 forward primer 5′-GAT GCA GAA TTC CGA CAT GAC TCA GGA-3′ and reverse primer 5′-GTC TTA AGT CGC TAT GAC AAC ACC GCC C-3′ (has a 3′ AflII site). Secreted Aβ was made by linking the APP signal peptide (SP) to the Aβ sequence. The SP adaptor was assembled by annealing the forward oligonucleotides and the reverse oligonucleotides (has single 3′ T residue) after the incubation at 90°C for 3 min. A ligation between SP adaptor (contains single 3′ T residue) and PCR-amplified Aβ coding sequence (has the 3′A-overhangs) was reamplified with the SP forward primer 5′-GGT CTA GAA TGC TGC CCG GTT TGG CAC-3′ (contains a 5′ XbaI site) and Aβ1–43 reverse primer. The PCR-amplified SP and Aβ sequence (3′ AflII/blunt) was further ligated to the nonfunctional "stuffer" sequences of pBR322 (PvuII-SalI fragment) to achieve the adequate genome size (4465 bp) for the efficient AAV packaging. The SP and Aβ nonfunctional "stuffer" sequences were cloned into the pTRUF2 vector through the XbaI/SalI restriction site.

2.3. Construction of SP- Aβ1–21 cDNA

SP-Aβ1–43 cDNA (XbaI-AflII/blunt) was ligated to pBluescript plasmid in the XbaI-SmaI site. Using this plasmid DNA as template, SP-Aβ1–21 was amplified by PCR with the 5′ primer: 5′-TGG CGG CCG CTC TAG AAT G-3′ and 3′ primer: 5′-CAC ATC TTA AGC AAA GAA CAC C-3′. Amplified SP-Aβ1–21 PCR product was digested with NotI and AflII (then, blunted) and cloned into pTRUF2 (NotI-SalI) with "stuffer" pBR322 PvuII-SalI DNA fragment.

2.4. Production of recombinant AAV

Human embryonic kidney (HEK) 293 cells were co-transfected with SP-Aβ pTRUF2 and plasmid pXX2 and pXX6 as described elsewhere [12]. Recombinant AAV titers were in the range of 1×10^{13} to 2×10^{13} viral genomes per ml. This study was approved by the Institutinal Recombinant DNA Experiment Safety Committee.

2.5. Western blot analysis of Aβ

To confirm the secretion of Aβ, the expression vector, SP-Aβ pTRUF2 was transfected into HEK293 cells. After 72 hrs incubation, cell lysate and conditioned medium were immunoprecipitated with monoclonal antibody 4G8 (Senetek, Napa, CA), samples were electrophoresed on a 16% Tris-Tricine gel and transferred onto nitrocellulose membrane. Monoclonal antibody 6E10 (Senetek, Napa, CA) was used to probe the blots. Bound antibody was visualized using horseradish peroxidase-conjugated anti-mouse IgG (at 1:10,000) and ECL+ detection kit (Amersham Pharmacia Biotech, Arlington Heights, IL).

2.6. Vaccination and tissue preparations

AAV/Aβ43 or AAV/Aβ21 was diluted with PBS to give 5×10^{11} genome in a final volume of 0.1 ml, and was administered once to tg 2576 mice using an orogastric tube in the treated group; control mice received once 0.1 ml of PBS. Since immunization with vector alone did not show any significant effect in our preliminary study, here we used PBS as control. Mice were randomized to four groups each; group A and D, treatment at age 15 weeks ($n = 4$); group B and E, treatment at age 30 weeks ($n = 4$); group C and F, treatment at age 45 weeks ($n = 4$); and control groups treated with PBS ($n = 6$) at each age, where groups A-C were treated with AAV/Aβ43 and groups D-F were treated with AAV/Aβ21. At the age of 12–13 months, all mice were anesthetized with Nembutal, exsanguinated and their brains were fixed in 4% paraformaldehyde with 0.1 M phosphate buffer, pH 7.6 for immunohistochemical analysis.

2.7. Immunohistochemistry

Immunohistochemistry was conducted as described before [13]. Standard methods were used for histological stains, including thioflavin S. The following primary antibodies were used: CD3, CD4, CD86, CD19 and CD11b (BD Biosciences Pharmingen, San Jose, CA; 1:50), Cy3-tagged anti-mouse GFAP (Sigma, Saint Louis, MS; 1:400), Iba-1 for microglia (kindly provided by Dr. U. Imai, National Institute of Neuroscience, NCNP, Tokyo), and 4G8 (Senetek, Napa, CA; 1:500) using a mouse on mouse (M.O.M) kit (Vector, Burlingame, CA) according to the manufacturer's instructions.

Quantitative analysis of amyloid burden was performed according to the method described [2] in three different brain regions: the hippocampus, frontal cortex, and parietal association cortex. Briefly, images were projected from an Olympus Vanox microscope onto a computer screen through a 3CCD Fujix Digital Camera. Images were captured and analyzed with an image analysis system (Mac Scope, Mitani Co.). The Aβ burden was expressed as % of area of Aβ deposits in the total tissue area examined.

2.8. ELISA

Anti-human Aβ antibody titers were quantified by a sandwich ELISA as described previously [14]. IgG subclasses were determined using the standard method.

2.9. PCR amplification of vector DNA

At 4 weeks after treatment and the end of the study, genomic DNA was extracted from heart, lung, spleen, liver, kidney and gut by standard methods. A 450-base pair (bp) product was amplified with DNA (200 ng) using primers CMV5′ (5′-AGT GAA CCG TCA GAT CGC-3′) and "stuffer" sequences of pBR322 (5′-CGG TAT CAG CTC ACT CAA-3′).

2.10. Cell proliferation assay

Spleen cells were tested *in vitro* for antigen-induced proliferation by Premix WST-1 Cell Proliferation Assay System (Takara, Kyoto) according to the manufacturer's instructions.

3. Results

To confirm the secretion of Aβ, we transfected HEK293 cells with SP-Aβ1-43pTRUF2 expression vector. An immunoprecipitation/western blot method revealed Aβ monomer in the cell lysate and Aβ oligomers in the conditioned medium (not shown). When AAV/Aβ was given in mice, Aβ expression was observed primarily in the lamina propria of the stomach and duodenum of the mice (Fig. 1(a)), and there was no increase of Aβ1–43 or Aβ1–21 in the serum (not shown). Transduction of AAV was confirmed by PCR in intestinal cells, but not in the liver, spleen, heart, lung and kidney 4, 11 and 21 weeks after treatment (Fig. 1(b)), suggesting an absence of widespread infection of the virus vector.

In the treated Tg2576 mice, IgG antibodies were detected in the serum at 4 weeks, and kept elevated for more than 6 months (Fig. 1(c)). The isotype of antibodies was mainly IgG1, and to a lesser amount IgG2b, but IgG2a was not detected and IgA was low. The immune sera from vaccinated mice stained the amyloid plaques in the brain (Fig. 2(g)). The proliferation response of spleen T cells against Aβ peptides was not detected in the vaccinated mice as well as in control mice (Fig. 1(d)).

Oral vaccination with AAV/Aβ43 or AAV/Aβ21 resulted in marked reduction of Aβ deposition in all treated groups compared to the control (Fig. 2(a–f)). Quantitative image analyses in three different regions of the brain showed a significant decrease of Aβ burden in all vaccinated mice compared to control mice examined at age 12-13-months-old (Fig. 2(m)).

HE staining of the brain sections of the treated mice showed no lymphocytic infiltration in either leptomeninges or cerebral white matter (Fig. 2(h)). We could not find any hemorrhagic lesion in the brain. Immunohistochemical studies did not reveal any cellular infiltration positive for CD3, CD4, CD86, CD19, and CD11b in brain sections. However, Iba-1+ activated microglia were more numerous in vaccinated mice (Fig. 2(i,j)). Microglia containing phagocytosed Aβ were not clear. In contrast, GFAP+ cells were less frequent in vaccinated mice (Fig. 2(k,l)).

4. Discussion

Safer Aβ vaccines are now being developed that produce antibodies against aggregated Aβ without Th1 type T cell activation, such as a vaccine using shorter

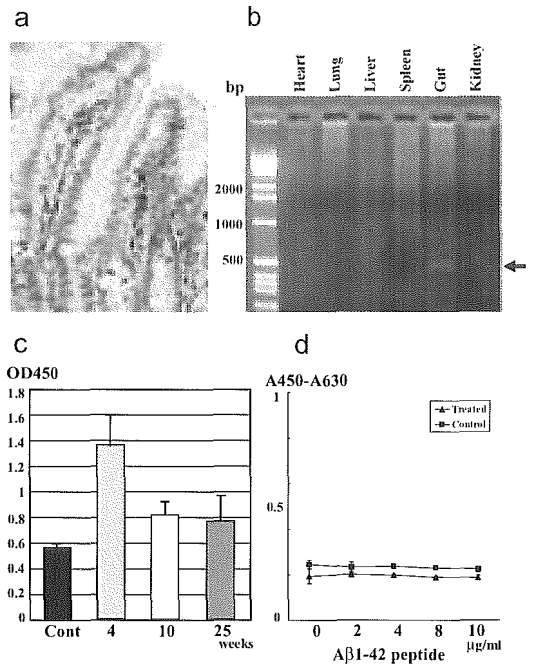

Fig. 1. AAV/Aβ expression and B- and T-cell immune responses. a, Aβ protein expression in lamina propria of the gut. The tissue was obtained from a mouse 6 months after vaccination, and sections were immunostained for Aβ with 4G8 antibody. b, Genomic DNA was purified from the liver, spleen, heart, lung, gut and kidney of a mouse immunized with AAV/Aβ43 at 30 wks of age and examined at 56 wks of age, the PCR product compatible with AAV is seen only in the gut (arrow). c, Serum anti-Aβ IgG antibodies were measured by ELISA at the dilution of 1:250 for Tg2576 mice ($n = 4$) treated with AAV/Aβ43 as well as for control mice ($n = 7$). As shown, Aβ antibodies were elevated for over 25 weeks. All data are the mean ± S.D. from four different experiments run in duplicate. d, Splenocytes isolated from control and vaccinated mice were incubated with Aβ1–42 peptide (0–10 μg/ml) for 3 days. As shown, T cell responses to Aβ were not detected. All data are the mean ± S.D. from four different experiments run in duplicate.

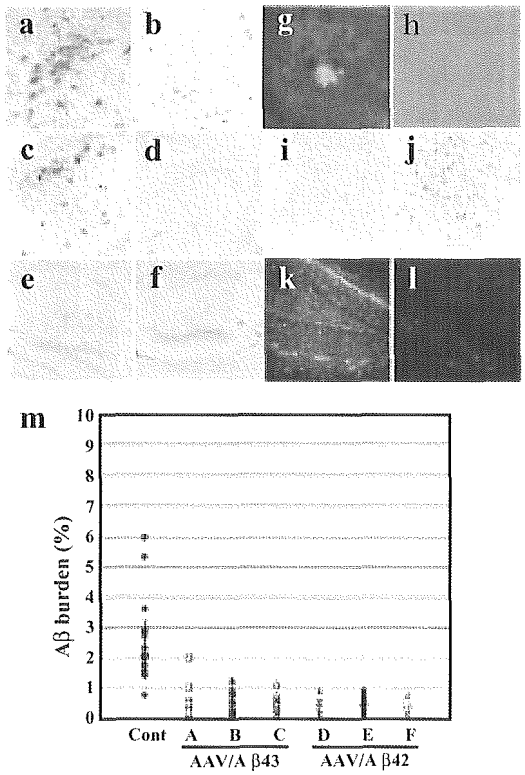

Fig. 2. Reduction of Aβ burden in Tg2576 mice after oral vaccination with AAV/Aβ. Brain sections of 12–13 months-old control (a, c) and vaccinated mice with AAV/Aβ43 (b) and AAV Aβ21 (d–f) were stained for Aβ deposits with 4G8. Numerous Aβ depositis in the frontal lobe cortex (a) and the hippocampus (c) were observed in control mice, but not in mice orally treated at 15 weeks of age (b–d), 30 weeks of age (e) and 45 weeks of age (f). Serum of vaccinated mice stained thioflavin S-positive amyloid plaques (g). HE staining showed no cellular infiltration in AAV/Aβ treated mice (h). Cortical areas had more numerous Iba-1+ microglial cells in the vaccinated mice (j) compared to control (i). Contrally, astrogliosis is more prominent in control (k) than vaccinated mice (l). m, Quantitative image analysis of Aβ burden in the three different regions of brain, hippocampus, frontal cortex and parietal association cortex. Aβ deposits were significantly reduced in the Tg2576 mice treated with either AAV/Aβ43 (group A-C) or AAV/Aβ21 (group D-F) at 15 weeks of age (group A, D), 30 weeks of age (group B, E) and 45 weeks of age (group C, F) compared to the control mice.

Aβ peptide lacking the C-terminal portion where most of the T cell epitope exist. However, there still exists a possibility that T cells respond to the N-terminal portion in certain individuals. Moreover, parenteral immunization requires adjuvant. Although an improved vaccine utilizes adjuvant which enhances Th2 responses, certain individuals still have a chance to get Th1-mediated encephalitis as in the case of measles, influenza and other virus vaccines. Passive transfer of Aβ antibodies is an alternative strategy. However, human monoclonal antibodies are not readily available, and *in vivo* production of neutralizing antibodies such as anti-idiotype antibodies must be considered. Our oral vaccine has an advantage in that it is efficient to induce antibodies without the use of adjuvant, and T cell responses are well suppressed. It is well known that the gut immune system suppresses Th1 responses and enhances Th2 responses [15]. Since anti-Aβ antibodies were continuously elevated for more than six months (Fig. 1(c)), it might be sufficient for patients to take the oral vaccine once or twice a year. In addition, AAV infected only

in epithelial cells of the upper gastro-intestinal tract without spreading over other organs including germ cells [16]. Adeno-associated viral DNA normally does not integrate into the cellular genome, instead it remains episomal. Since the turnover of epithelial cells of the GI tract is relatively quick, the recombinant AAV is eliminated along with the course of renewal of the epithelial cells, suggesting a lower risk in case of unexpected event.

The mechanism by which antibodies clear β amyloid from the brain tissue is still unknown. There are several hypotheses; First, Fc receptor-mediated uptake of Aβ-antibody complexes by local activated microglia [4]. Second, antibody-mediated disaggregation of amyloid fibrils [17]. Several reports indicated therapeutically active antibodies mainly recognized the residue 3–10 of Aβ peptide [18–20] and these antibodies inhibit Aβ fibrillogenesis and cytotoxicity [18]. Third, DeMattos et al. [21,22] hypothesized injected antibodies remove Aβ protein from the blood and then eventually pull out Aβ from the brain. In our vaccinated mice, microglias were activated, and sera from vaccinated mice showed inhibition of Aβ aggregation (unpublished data). Therefore, all three mechanisms seem to be likely. In conclusion, this oral vaccine would be safe and beneficial for AD.

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