Invited Review for the 90th Anniversary

Immunization Therapy for Alzheimer Disease: A Comprehensive Review of Active Immunization Strategies

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Based on the amyloid cascade hypothesis, various strategies targeting amyloid β protein (Aβ) have been invented for prevention and treatment of Alzheimer disease (AD). Active and passive immunizations with Aβ and Aβ antibodies successfully reduced AD pathology and improved cognitive functions in an AD mouse model. However, active immunization with AN-1792, a mixture of Aβ1-42 peptide and adjuvant QS21 induced autoimmune encephalitis in humans. Surprisingly, although AN-1792 cleared senile plaque amyloid, it showed no benefit in humans. It is speculated that AN-1792 failed in deleting more toxic forms of Aβ such as oligomers and intracellular Aβ, suggesting that newly developing vaccines should delete these toxic molecules. Since T cell epitopes exist mainly in the C-terminal portion of Aβ, vaccines using shorter N-terminal peptides are under development. In addition, since T helper 1 (Th1) immune responses activate encephalitogenic T cells and induce continuous inflammation in the central nervous system, vaccines inducing Th2 immune responses seem to be more promising. These are N-terminal short Aβ peptides with Th2 adjuvant or Th2-stimulating molecules, DNA vaccines, recombinant viral vector vaccines, recombinant vegetables and others. Improvement of vaccines will be also achieved by the administration method, because Th2 immune responses are mainly induced by mucosal or trans-cutaneous immunizations. Here I review recent progress in active immunization strategies for AD.

Keywords: Alzheimer/vaccine/immunotherapy/autoimmune/T helper 1/T helper 2


The elderly population defined as 65 years old or older is now a little over 22% of the whole population in Japan (from the report of Ministry of Health, Welfare and Labor, Japan), and a recent report says that 11% of the elderly people suffer from dementia (Wada-Isoe et al. 2009). A variety of diseases cause dementia in the elderly, and approximately 55% of dementia patients are caused by Alzheimer disease (AD) including mixed types (Matsui et al. 2009). The quality of life of AD patients has been slightly but significantly improved by the use of Donepezil® and other acetylcholine esterase inhibitors or Memantin®, an antagonist of NMDA receptor, we are far from prevention and cure of this devastating disease.

Neuropathological findings of AD are characterized macroscopically by diffuse brain atrophy, and microscopically by senile plaques (SPs), neurofibrillary tangles (NFTs) and neuronal loss in the brain. SPs are formed initially by extracellular deposits of β amyloid, and at a later stage SPs are accompanied by microglia and astrocyte responses with inflammatory and neuritic changes. β amyloid is a glycosylated fibrillar protein mainly composed of aggregated amyloid β protein (Aβ) which derives from amyloid precursor protein (APP). A large body of knowledge regarding to the pathological mechanism established the amyloid cascade hypothesis (Hardy and Selkoe, 2002), which is illustrated in Fig. 1. Based on the hypothesis, novel preventive and therapeutic strategies targeting the amyloid cascade are being established. Among, immuno-therapies targeting Aβ are expected to be highly promising (Weiner and Frenkel, 2006). Here I review active immunization (vaccination) strategies for prevention and treatment of AD.

Amyloid β is a key molecule in the pathological mechanism of AD

β amyloid is composed mainly of aggregated Aβ which derives from its precursor protein APP by the cleavage with β-secretase and γ-secretase. Aβ is a small peptide composed mainly of 40 amino acids, Aβ40 and 42 amino acids, Aβ42. The latter has additional two hydrophobic amino acids in its carboxyl terminal, and thus it is easily aggregated.

SPs are seen in non-demented elderly, otherwise SPs

Received November 30, 2009; revision accepted for publication January 7, 2010. doi:10.1620/tjem.220.95
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are specific for AD in dementing disorders. Although Aβ monomer is not toxic, aggregated forms, particularly oligomers are toxic to synapses and neurons (Noguchi et al. 2009). Thus, it is widely accepted that AD is one of so-called conformational diseases. NFTs are mainly composed of phosphorylated tau and deposited in neural processes and neurons. It is speculated that NFTs contribute partly to degeneration of neurons and neuronal processes (Gómez-Isla et al. 1997). However, amyloid deposition appears about 10 years prior to NFT formation in human brains (Wisniewski et al. 1985). Injection of Aβ into the brain of APP transgenic (tg) mice (Götz et al. 2001) and APP tg × Tau tg mice shows acceleration of phosphorylated tau deposition (Ribé et al. 2005).

Thus, tau pathology exists in the downstream of Aβ. Moreover, mutations of familial AD genes such as APP, presenilin 1 (PS1) and presenilin 2 (PS2) enhance amyloid formation by enhancing production of total Aβ or Aβ42. Genetic risk factor gene apolipoprotein E (ApoE) ε4 also enhances amyloid formation. Thus, all these findings suggest that Aβ has the central role in the pathological mechanism of AD (Tabira, 2004). Indeed, reduction of Aβ pathology by vaccination induced reduction of tau pathology (Wilcock et al. 2009).

**Invention of vaccination therapy for AD**

The vaccination therapy for AD was invented by Dale Schenk and his colleagues in 1999. They immunized young APP tg mice with Aβ with adjuvant and found that amyloid deposits were significantly reduced in the immunized mice when examined at a later time. They also found that immunization of APP tg mice with Aβ at the age when amyloid deposits had started in the brain reduced amyloid plaques and prevented further amyloid deposition (Schenk et al. 1999). The similar effect was obtained by injection of certain monoclonal and polyclonal antibodies to Aβ (passive immunization), and immunized mice showed improvement of cognitive functions (Bard et al. 2000; Janus et al. 2000; Morgan et al. 2000).

**Clinical trial of Aβ vaccination in AD patients**

The phase I clinical trial of AN-1792 vaccine composed of synthetic Aβ1-42 and adjuvant QS21 was started in April, 2000 using moderately severe AD patients in UK. Since it was found safe, the phase IIa clinical trial was started in September, 2001 using mild to moderate AD in
Europe and USA. However, 6% of the patients developed subacute meningencephalitis after one to three intramuscular injections of the vaccine, the trial was halted in February 2002 (Orgogozo et al. 2003). It is speculated that appearance of the adverse effect is due to an addition of Polysorbate-80, a detergent to enhance exposure of the antigenic epitope in the phase II study.

**Subacute meningencephalitis induced by AN-1792**

Patients who developed subacute meningencephalitis after injection of AN-1792 vaccine showed slight to moderate increase of lymphocytes and total protein in the cerebrospinal fluid, but no specific pathogens were identified. MRI scans showed diffuse and multifocal high intensity lesions in the white matter, gray matter and meninges in T2 weighted and FLAIR images. This condition mimics so called post-vaccinal or post-infectious encephalitis.

\( \alpha \beta \) is a self antigen, and most of self-reactive T cells are deleted in the thymus during an embryonic developmental period. However, T cells reactive to \( \alpha \beta \) are not the case, and they exist in peripheral blood under the control of regulatory T cells in humans. The frequency of \( \alpha \beta \)-reactive T cells in the peripheral blood is higher in the elderly than the youth, and it is slightly more in AD patients (Monsonego et al. 2003). Immunization of wild type mice with \( \alpha \beta \) and Freund complete adjuvant (CFA) hardly induced encephalitis in mice and rats, because most of the tested animals were tolerant to \( \alpha \beta \). However, immunization of T helper 1 (Th1)-prone APP tg mice crossed with interferon-\( \gamma \) (IFN-\( \gamma \)) tg mice could reproduce similar encephalitis (Monsonego et al. 2006). Thus, the meningencephalitis induced by AN-1792 is probably mediated by an autoimmune mechanism. This is compatible with that QS21 is an adjuvant that strongly activates Th1 T cells.

**Autopsy cases who had received AN-1792 vaccine**

Although AN-1792 trial was halted, it gave us extremely important information. It was our surprise to see that an autopsy case who had received a total 5 immunizations of AN-1792 and died of pulmonary embolism 1 year after the last immunization showed reduced numbers of SPs and infiltration of phagocytes containing \( \beta \) amyloid, suggesting that \( \alpha \beta \) immunization cleared amyloid deposits in the patient (Nicoll et al. 2003). It is also of interest to note that plaque-associated dystrophic neurites containing phosphorylated tau also disappeared. However, intra-neuronal NFTs and vascular amyloid were not cleared. Since the patient had developed meningencephalitis, the brain showed remnant inflammatory foci where CD4+ T cells were infiltrated.

Similar autopsy cases were also reported by others (Ferrer et al. 2004; Masliah et al. 2005). The case reported by Masliah et al. did not have encephalitis, suggesting that amyloid plaques can be cleared in the absence of encephalitis. However, none of the cases showed improvement of cognitive functions in spite of significant plaque clearance.

**Clinical course of vaccinated patients**

Although the trial was halted, the clinical follow-up was continued. Hock et al. at University of Zurich reported results of one year follow-up after the cessation of the phase II trial (Hock et al. 2003). The patients who had developed antibodies reactive to SPs showed significantly slower decline of cognitive functions assessed by mini-mental state examination (MMSE) and disability assessment scale of dementia (DAD) than those who did not have developed such antibodies. The antibodies were designated as tissue amyloid plaque immuno-reactive (TAPIR) antibodies, which were examined immuno-histochemically by staining of AD brain using patients’ sera.

Gilman et al. reported a follow-up study of all patients who joined the AN-1792 trial (Gilman et al. 2005). Antibody responders who had developed high titers of antibodies examined by enzyme-linked immuno-sorbent assay (ELISA) showed slight but significant better scores in a certain memory functions of the neuropsychological test battery (NTB), but there was no difference in MMSE and Alzheimer disease assessment scale (ADAS-cog), and the responders showed a significant reduction in CSF tau levels. The responders were only 20% of patients who received AN-1792 vaccine. This is because the vaccine was given only 1 to three times, and immune functions are deteriorated in the elderly. Vellas et al. also reported that antibody responders tended to show significantly better scores in DAD \((p = 0.015)\) in their 4.7-year follow-up study in France (Vellas et al. 2009).

Recently, results of a 6-year follow-up study were reported from UK (Holmes et al. 2008). Although the trial was started with 80 cases, only 26 cases (20 vaccine, 6 placebo) agreed and completed the follow-up study. Therefore, we must be careful in interpretation of the results, but there was no difference in the rate and time course of becoming severe AD (MMSE scores below 10) and the survival rate between vaccine and placebo groups. Autopsy was done in 9 patients during the follow-up period and 8 cases were diagnosed as AD. Six of the 8 cases showed moderate or outstanding clearance of SPs, yet all became severe AD and their MMSE scores were 0 before death. Thus, it is definitely clear that SPs have little or no contribution to cognitive dysfunctions in AD. It is highly possible that AN-1792 cleared SP amyloid, but might not have cleared toxic \( \alpha \beta \) oligomers or intracellular \( \alpha \beta \) (Tabira et al. 2002). It might be also said that \( \alpha \beta \) vaccine may be too late, once a progressive mechanism has started in AD. Further, we must consider that AN-1792 activated \( \alpha \beta \)-reactive Th1 T cells. As some patients developed autoimmune encephalitis by activation of \( \alpha \beta \)-reactive Th1 T cells, it is highly probable that \( \alpha \beta \)-reactive Th1 T cells were continuously activated and entered into the brain for immune surveillance, where they contributed to continuous inflammatory processes in the central nervous system (CNS) tissues.
**Development of future Aβ vaccines**

From above findings, Aβ vaccines of the next generation require following conditions.

a. It should not induce autoimmune encephalitis, in other words, vaccines activate Th2 T cells rather than Th1 T cells.

b. It is useful for prevention, if given at an early stage.

c. It modifies the disease course and hopefully improves cognitive functions, if given after the disease progression mechanism has started.

d. It is efficient in the elderly whose immune functions are deteriorated.

e. It is not painful and has a good compliance.

f. It is inexpensive.

It is now well known that Th1 T cells are activated by interleukin (IL)-12, produce pro-inflammatory cytokines such as IFN-γ and tumor necrosis factor (TNF)-α, and help cellular immunity. Th1 T cells are involved in the effector mechanism of autoimmune encephalitis. On the other hand, Th2 T cells are activated by IL-4, produce IL-4 and IL-10, and help humoral immunity. Th1 T cells and Th2 T cells regulate each other. Therefore, vaccines that activate Th2 T cells would help antibody production and suppress autoimmune encephalitis (Fig. 2). T helper cells also influence on the immunoglobulin (Ig) class switch. Th1 T cells enhance IgG2a antibodies, while Th2 T cells enhance IgG1 and IgG2b antibodies.

Here I summarize active immunization strategies for AD (Table 1 to 4) and briefly explain those.

**Peptide vaccines**

A short N-terminal Aβ peptide with a nonencephalitogenic T cell epitope of carrier protein: Since T cell epitopes exist in the C-terminal portion of Aβ (Aβ16-30) in mice (Monsonego et al. 2001) and humans (Monsonego et al. 2003), N-terminal peptide of Aβ is relatively safe. To get good antibody responses to a short N-terminal peptide of Aβ, Aβ1-15 was conjugated with a T cell epitope of bovine serum albumin (BSAT) and injected in animals subcutaneously with CFA (Monsonego et al. 2001).

It is possible to elicit better antibody responses if multiple antigenic peptides are used. To this end lysine cores are used; a two-lysine core has two arms and a three-lysine core has 4 arms. For instance, Aβ1-5 peptide was conjugated to the 3 arms of a three-lysine core and ovalbumin T cell epitope (OVAT) to the remaining arm, and injected intraperitoneally in mice with CFA (Bard et al. 2003).

To enhance Th2 immune responses, Aβ1-15 is conjugated to the 3 arms of a lysine core and pan HLA DR-binding peptide (PADRE) to the remaining arm, and injected subcutaneously in mice with Alum, a Th2 adjuvant (Agadjanyan et al. 2005). Alternatively, repeated subcutaneous injections of Aβ1-28 conjugated with Mannan elicited Th2 immune responses predominantly (Ghochikyan et al. 2005).

![Fig. 2. Subtypes of T Helper Cells.](image)

T helper (Th) cells are largely divided to Th1 and Th2 cells. Th1 cells are activated by interleukin (IL)-12, produce pro-inflammatory cytokines such as interferon-γ and tumor necrosis factor-α, and help cellular immunity. Th1 T cells per se become effector cells in certain autoimmune diseases. Recently, it is suggested that autoimmune encephalitis is mediated by Th17 cells in mice, but it is uncertain yet in humans. On the other hand, Th2 cells are activated by IL-4, produce cytokines such as IL-4 and IL-10, and help humoral immunity. Since the gut immune system is markedly shifted to Th2, Th2-type antibodies are easily elevated and Th1-mediated autoimmune encephalitis is suppressed, because Th1 and Th2 cells suppress each other.
Table 1. Aβ Peptide and Non-Aβ Peptide Vaccine.

2) 3 × (Aβ1-15)-BSAT + CFA I.P. (Bard et al. 2003)
7) Aβ1-15-Parmitoyl liposome I.P. (Muhs et al. 2007)
8) Aβ1-42-Chorela Toxin Transcutaneous (Nikolic et al. 2007)
10) Aβ affitope + Alum S.C. (Schneeberger A et al. 2009)
11) Copolymer1 (Glatiramer Acetate) S.C. with adjuvant (Butovsky et al. 2006)

Table 2. DNA Vaccine.

1) Plasmid-Aβ1-42-MHC class II-targeting sequence I.D. (Qu et al. 2004)
3) Plasmid-Aβ1-42 I.M. (Okura et al. 2006)
4) Plasmid-CCL22-3 × (Aβ1-11)-PADRE I.D. (Movsesyan et al. 2008)

Table 3. Recombinant Vegetable, Bacteria and Phage.

1) Recombinant potato expressing 5 × (Aβ1-42) Protein Extract + CT Oral (Youm et al. 2005)
3) Recombinant Salmonella Oral (Boutajangout et al. 2009)
4) Recombinant phage expressing Aβ3-6 (EFRH) in pIII or pVIII I.V. or I.P. (Frenkel et al. 2000; Lavie et al. 2004)

Table 4. Recombinant Viral Vectors.

1) AAV-ΔAβ1-43 or 1-21 Oral (Hara et al. 2004; Mouri et al. 2007)
2) AAV-ΔAβ1-42 – CTB Oral (Zhang et al. 2003)
3) SeV-ΔAβ1-43 – IL-10 Nasal (Hara et al. in preparation)
4) pCA-PEDI-11 × (Aβ1-16) boosted with rAV- PEDI*-11 × (Aβ1-16) Nasal (Kim et al. 2007a, 2007b)
5) pHSV-ΔAβ1-42-IL-4 S.C. (Frazer et al. 2008)

AAV, adeno-associated virus vector; AV, adenovirus vector; CTB, cholera toxin B; pCA, a plasmid; pHSV, herpes simplex virus amplicon; SeV, Sendai virus vector; PEDI, receptor binding domain of Pseudomonas exotoxin A.

of SPs, soluble and insoluble Aβ titers of anti-Aβ injected intravenously in APP23 mice, they produced high 1-15-PDGFR fusion protein. When 1 × 10^6 particles were injected intravenously in APP23 mice, they produced high titers of anti-Aβ antibodies, and the subtypes were IgG1 and IgG2b. The immunized mice showed significant reduction of SPs, soluble and insoluble Aβ (Bach et al. 2009). Each virus-like particle expresses several thousands epitopes of Aβ1-15 and the injected particles were calculated as containing 150 ng of Aβ1-15. However, it was not reported whether it reduced Aβ oligomers and improved cognitive functions.

**Human trials of N-terminal peptide vaccine:** ACC-001, an N-terminal Aβ peptide vaccine conjugated with diphtheria toxin and adjuvant QS21 is now under the phase II clinical trial in the world including Japan by Wyeth. The precise construct of the vaccine is unknown. Since the encephalitogenic epitope mainly exists in the C-terminal portion of Aβ, the N-terminal peptide may be safe. However, the encephalitogenic epitope was examined in Caucasians, and nothing is known in Orientals. Humans are hybrid and the encephalitogenic epitope could exist in the N-terminal portion. Also considered is that QS21 is the Th1 adjuvant, which may induce Th1 responses to the peptide and induce continuous inflammatory responses in the CNS.

Recently, a new technology to express non-self structures of self antigens, named “affitope” vaccine was invented (Schneeberger et al. 2009). This is easy to break immune barrier and elevates antibodies to N-terminal Aβ peptide. A human trial of this vaccine is planned by GlaxoSmithKline.

**Non-Aβ peptide and adjuvant vaccine**

Copolymer1 (Cop1), Glatiramer Acetate® is now used for treatment of multiple sclerosis (MS). Autoimmune encephalitogenic T cells that recognize myelin antigens are involved in MS. Cop1 is a mixture of randomly polymerized peptides composed of Glutamic acid, Lysine, Alanine and Tyrosine, and activates regulatory T cells reactive to myelin antigen-reactive and encephalitogenic T cells. It is interesting to note that APP tg mice immunized with Cop1 and adjuvant showed significant reduction of SPs in association with an increase of Th2 T cells (Butovsky et al. 2006). It is speculated that Cop1-activated-Th2 T cells in the brain activated IGF1-producing CD11c+ microglia cells, which in turn cleared amyloid deposits. Cop1 is proven safe in humans, so it seems to be easier to go to the human trial. However, tolerability may be the problem, because it must be injected subcutaneously every day for MS patients.

Intra-nasal administration of Cop1 with an adjuvant Protollin (IVX-908) effectively reduced amyloid burden in APP tg mice without inducing inflammatory change (Frenkel et al. 2005). Protollin is a non-covalent formulation of outer membrane proteins (proteosomes) of Neisseria meningitidis and LPS from Shigella flexneri. Nasal vaccine is not painful, but regulatory T cells against encephalitogenic T cells may be mainly activated in the white matter where amyloid pathology is very few. Since this vaccine does not use Aβ, it is uncertain how long such nonspecific vaccines continue to work.

**DNA vaccine**

Qu et al. made a gene construct using an expression plasmid containing cDNA of monomer or dimer of Aβ1-42...
under the control of SP72, a synthetic mammalian cell-specific promoter, an $\alpha$-antitrypsin leader sequence and an MHC class II-targeting sequence. Gold particles coated with the DNA were given intra-dermally in the ear of mice by a helium-driven gene gun 3 times. Consequently, they could observe a good immune response to A$\beta$ without activation of cytotoxic T cells (Qu et al. 2004). Later they changed the leader sequence to that of Adenovirus E3, and immunized APPsw × PS1$\Delta$E9 mice 15 times similarly using a gene gun. They showed a significant elevation of Th2 type antibodies and reduction of A$\beta$ burden (Qu et al. 2007). Recently, they made a vaccine mixed with an activator plasmid carrying a GAL4 activator sequence under the control of CMV promoter and a responder plasmid carrying a GAL4 upstream activating system. 3-tandem repeat cDNAs of A$\beta$1-42, the E3 leader sequence and the MHC class II endosomal targeting sequence. They compared immune responses in wild type mice immunized with the DNA vaccine and those with A$\beta$1-42 peptide vaccine mixed with adjuvant Quil A. The antibody responses in the DNA-vaccinated mice showed a significantly higher ratio of IgG1/IgG2a than those in peptide-vaccinated mice (Lambracht-Washington et al. 2009). Thus, DNA vaccine seems to induce Th2 dominant immune responses. However, the IgG1/IgG2a ratio was about 10 in the DNA-vaccinated mice, the IgG2a response was not nil.

A similar DNA vaccine was made using an expression plasmid pTarget carrying an Ig$\alpha$ signal sequence and A$\beta$1-42 or A$\beta$1-42 with a fusion gene of Ig Fc (Okura et al. 2006). APP tg mice received this vaccine repeatedly into the muscle, and they showed a significant reduction of A$\beta$ burden without T cell activation. However, antibody subtypes and cognitive functions were not examined.

Movsesyan et al. made a pCMV DNA vaccine carrying an IP10 signal sequence, a gene encoding CCL22 chemokine, 3 repeat of A$\beta$1-11 and PADRE gene (Movsesyan et al. 2008). This vaccine was given 3 times into the shaved abdominal skin of mutant APP tg × mutant PS1 tg × mutant tau tg (3X-Tg-AD) mice using a gene gun. The use of CCL22 further shifted the immune response to Th2 and produced IgG1 and IgG2b antibodies. It is good to know that SPs were significantly reduced and cognitive functions were improved. In addition, insoluble A$\beta$40, A$\beta$42 and soluble A$\beta$ oligomers (3-mers and 6-mers) were significantly reduced, but tau pathology was unchanged.

Since post-mitotic muscle cells are poor for antigen presentation, DaSilva et al. made a DNA vaccine carrying wild type A$\beta$1-42 and a mutant caspase gene. The expression of the mutant caspase was not enough to induce apoptosis of muscle cells but enough to induce apoptosisomes, which enhanced antigen presentation. TgCRND8 mice which carry APPsw and APP$\Delta$INDANA mutations were repeatedly injected with the vaccine intramuscularly. They showed Th2 type antibody responses and reduction of amyloid plaques and insoluble A$\beta$42. Cerebral amyloid angiopathy and soluble A$\beta$ oligomers tended to show reduction, but not significant (DaSilva et al. 2009).

Recombinant vegetable, recombinant bacteria, recombinant phage

Recombinant potato expressing 5 tandem repeats of A$\beta$1-42 was made, and Tg2576 mice were fed with 25 mg of the protein extract, which contained 0.9 $\mu$g/mg of A$\beta$, and adjuvant cholera toxin B once a week for 3 weeks. The result showed elevated anti-A$\beta$ antibodies and reduction of senile plaques (Youm et al. 2005). This suggested that in a certain condition orally given food extracts with a certain adjuvant can break immune tolerance. However, it is unknown whether taking recombinant food instead of food extract and an adjuvant is enough to induce the immune response. In addition, how much recombinant potato is required to be eaten, and how cholera toxin B is mixed with the potato before eating?

Since we eat cooked potato, antigenicity of A$\beta$ may be inactivated by heat. Therefore, they produced recombinant tomato for eating without cooking. Mice just fed with the soluble extract of recombinant tomato did not develop any anti-A$\beta$ antibodies. However, mice injected with a small amount of A$\beta$ after feeding with transgenic tomato developed high titers of anti-A$\beta$ antibodies, suggesting that mice acquired immune conditioning by eating A$\beta$-containing tomato (Youm et al. 2008). Similar effect may be obtained by eating potato infected with virus which has high homology with A$\beta$ (Friedland et al. 2008).

APP tg mice fed with non-pathogenic recombinant Salmonella expressing A$\beta$ showed reduction of A$\beta$ burden (Boutajangout et al. 2009). In this case, secreted A$\beta$ from the bacteria may not be immunogenic. Instead, A$\beta$-expressing Salmonella phagocytosed by M-cells or dendritic cells seems to be immunogenic. If this is the case, yogurt containing recombinant Lactobacillus expressing A$\beta$ may be useful.

Frenkel et al. made a recombinant filamentous phage expressing 10 copies of A$\beta$1-36 (EFHR) in protein III (pIII) or 300 copies in major coat protein VIII (pVIII). APP tg mice inoculated and repeatedly boosted intra-peritoneally or nasally with 10$^{10-11}$ of the recombinant phage produced antibodies to A$\beta$, and showed significant reduction of SPs and improvement of the Morris water maze test (Frenkel et al. 2000; Lavie et al. 2004). The recombinant phage with high copy numbers of A$\beta$ was much more efficient. The antibodies inhibited A$\beta$ aggregation and A$\beta$-induced neurotoxicity. It is unknown whether the antibodies react with 3-pylo-glutaminyl A$\beta$ which is the major form of aggregated A$\beta$ in AD brain (Harigaya et al. 2000). Phage is a virus for E. coli, exists ubiquitously on the earth and in our body, and is said non-toxic to humans.

Recombinant viral vectors

Recombinant Adeno-associated Virus vector (AAV) carrying A$\beta$1-43 or A$\beta$1-21: We developed recombinant AAV carrying an APP signal sequence and A$\beta$1-43 or...
Aβ1-21 cDNA (Hara et al. 2004). We used Aβ1-43 in order to differentiate it from native Aβ, because majority of native Aβ is composed of Aβ40 and Aβ42. Tg2576 mice were given orally once with $5 \times 10^{11}$ viral genome of the recombinant AAV using a naso-gastric tube at the age of 15, 30 or 45 weeks old, and their brains were examined at 56 weeks old. The vaccinated mice showed elevated anti-Aβ antibodies for over 46 weeks and the subtypes were IgG1 and IgG2b. IgA antibodies were low and IgG2a antibodies were not detected. SPs were significantly reduced in all vaccinated mice compared with PBS controls, and Aβ1-43 was not increased in the brain. The antibody titers were much lower in mice vaccinated with Aβ1-21 vaccine, but the effect was the same. Splenic T cells did not proliferate when stimulated with Aβ1-42 peptide. There was no infiltration of T cells and B cells in the brain, and Iba-1$^+$ activated microglia were increased and GFAP$^+$ astrocytes were decreased. When serum cytokines were examined using a set of over 30 conventional cytokine microbeads and Luminex (Millipore), only transforming growth factor β-1 (TGF β-1) was significantly reduced (Hara and Tabira, 2006).

The recombinant AAV-Aβ1-43 vaccine was given similarly in Tg-2576 mice at the age of 10 months and cognitive functions were examined at 13 months. The vaccinated mice showed significant improvement in the Y-maze test, Morris water maze test, novel object recognition test and conditioned fear learning test. After the cognitive function tests, all the brains were examined. Reduction of SPs was confirmed, and SDS-soluble and formic acid (FA)-soluble Aβ40 and Aβ42 were also significantly reduced in the vaccinated mice. They also showed significant reduction of soluble 9-mers and 12-mers (Mouri et al. 2007).

Cynomolgus monkeys over 20 years old were given orally with $1 \times 10^{13}$ viral genome of AAV-Aβ1-43 vaccine twice in a three months interval and the brain was examined 6 months after the first vaccination. Senile plaques were significantly reduced and there was no inflammation in the brain. There were no significant abnormalities in the laboratory tests including complete blood counts and serum chemistry, and pathological examinations of the systemic organs showed no particular changes (manuscript in preparation).

The intestine is the biggest and the most efficient immune organ in humans (Fig. 3). Therefore, immune responses were easily obtained even in the elderly. As a matter of fact, one or two oral administrations of the vaccine were enough in mice and monkeys. In addition, since the gut immune system is shifted to Th2, Th1-mediated autoimmune encephalitis is supposed to be suppressed. Further, since gut epithelial cells are renewed in a few days, majority of the transfected genes are deleted quickly. AAV is a safe vector and transfected genes are retained in the epitope.

**Fig. 3. Oral Vaccine using Recombinant Adeno-associated Virus.**

Recombinant adenovirus vector (AAV) carrying Aβ1-43 or 1-21 with an APP signal sequence was made. When this was given orally, the gut immune system responded well and produced Th2 type antibodies efficiently. It cleared senile plaque amyloid, reduced insoluble Aβ and soluble Aβ oligomers, and improved cognitive functions without inducing encephalitis or other adverse effects.
some. However, a certain amount of the transfected genes may be retained for a while, if infected in M-cells, dendritic cells and stem cells of the gut epithelium. Thus, our vaccine fulfills most of the requirement for the next generation of Ab vaccine. In order to do a clinical trial of this vaccine, we need a company or a facility to provide an enough amount of the vaccine at the GMP level.

Recombinant AAV-Ab1-42-CTB: A similar vaccine was reported in China (Zhang et al. 2003). However, their construct contains Ab1-42 and cholera toxin B (CTB) as an adjuvant. They did not see antibody responses if CTB is not included. Our vaccine carries Ab1-43 only and does not require adjuvant.

Ab1-43 - IL-10 in SeV: We have also developed recombinant Sendai virus vector (SeV) carrying Ab1-43 and IL-10. Sendai virus induces common cold-like symptoms in murines, but it is not pathogenic in humans. Nasal administration of this vaccine induced significant reduction of senile plaques without inducing inflammatory changes in the brain and improved cognitive functions (manuscript in preparation).

pCA-PEDI-II x (Ab1-16) boosted with recombinant Adenovirus carrying PEDI-II x (Ab1-16): Kim et al. immunized APP tg x PS1 tg mice intra-nasally twice with a plasmid DNA vaccine containing 11 tandem repeats of Ab 1-16 cDNA and the receptor binding domain of Pseudomonas exotoxin A (PEDI), and then the mice were boosted nasally every 3 weeks for 10 months with recombinant Adenovirus containing the same gene construct (Kim et al. 2007a, 2007b). The mice produced high titers of IgG1 and IgG2b antibodies to Ab and SPs were significantly reduced. Splenocytes from the vaccinated mice produced a significant amount of IL-10, when stimulated with Aβ. However, IgG2a antibodies were also produced, probably because PEDI contains a lot of CpG motif. CpG binds to a Toll-like receptor and activates the innate immune system, which in turn activates the acquired immune system. Th1 immune responses are induced by CpG in a certain condition.

pHSV14, Ab1-42-cmv-IL-4: Frazer et al. produced a recombinant Herpes simplex virus (HSV) ampiclon carrying Ab1-42 gene under the control of HSV immediate early promoter (IE) and IL-4 gene under the control of Cytomegalovirus (CMV) immediate early promoter. 3X-Tg-AD mice were inoculated subcutaneously with 1 x 10^6 transduction units of the vaccine and were boosted with the same vaccine 1 month and 6 months after. They described that the vaccinated mice developed antibodies to Ab42 and showed a significant reduction of SPs and phosphorylated tau, and improvement in the Barnes maze test (Frazer et al. 2008). They described that the antibody pattern was a Th2 type. However, antibody subtypes were IgG1, IgG2a, IgG2b and IgG3, and no specific pattern was shown.

Miscellaneous

Adjuvant alone: Nasal administration of Protollin, a proteosome-based adjuvant activated brain microglia and reduced SPs (Frenkel et al. 2005).

Juzen-taiho-to, an herbal medicine: Plaque amyloid is suggested to be phagocytosed mainly by bone marrow-derived microglia/macrophages (Simard et al. 2006). Brain derived microglia and bone marrow derived macrophages were activated in vitro and in vivo by Juzen-taiho-to, an herbal medicine and enhanced phagocytosis of Ab amyloid (Liu et al. 2008). When Tg-2576 mice were treated with Juzen-taiho-to in drinking water, amyloid burden was significantly reduced (Hara et al. 2010). This is not a vaccine, but it seems to have an vaccine-like effect in activating bone marrow-derived phagocytes in the brain.

Memapsin 2: Tg2576 mice were immunized with memapsin 2 (β-secretase) and CFA, then with the antigen and IFA, and later with the antigen alone. The actively immunized mice and mice treated with anti-memapsin 2 antibodies showed reduced memapsin 2 activity, reduced SPs and plasma Ab, and improvement of cognitive functions (Chang et al. 2007).

APP beta-cleaving site: Rakover et al. immunized mice with APP cleaving site peptide and obtained antibodies that inhibit β-secretase cleavage. Tg2576 repeatedly injected with the antibodies showed reduction of brain inflammation, reduction of brain hemorrhage, and improvement of cognitive functions without changing brain Ab (Rakover et al. 2007).

HA-KLH: Homocysteic acid (HA) is a neurotoxin binding to the NMDA receptor. Animals fed with vitamin B6 deficient food showed cognitive dysfunctions in association with an increase of intracellular Ab. Active and passive immunization targeting HA improved cognitive functions in Vitamin B6 deficient animals and 3X-Tg-AD mice (Hasegawa et al. 2010).

In conclusion, there are numerous ideas and strategies in immunotherapy for AD. Some of them have been proven safe and effective in animals, and will be applied for AD patients in near future. As we learned from the study of AN-1792, only the result in a human trial can tell the efficacy and safety, unless good monkey models are established.

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