

Alzheimer's disease vaccines: promises and pitfalls

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Feasibility of immunotherapy for Alzheimer's disease has been postulated, and numerous strategies of active and passive immunization have been applied in animals and humans. This article summarizes accumulated knowledge in clinical trials and animal experiments, and foresees the future perspective. Clinical trials have clearly shown that clearance of senile plaque amyloid is insufficient for improving clinical symptoms and signs, suggesting a possibility that in addition to plaque amyloid, amyloid β oligomers and intracellular amyloid β should be targeted. Furthermore, avoidance of autoimmune encephalitis and adverse inflammation must be considered. Since the exact targeting molecule is unknown at present, polyclonal immunization strategies with activation of T helper type 2 T cells seem to be promising. Also prevention rather than treatment will be successful until we establish measures to cope with the progression mechanism of Alzheimer's disease.

Keywords: amyloid • AN1792 • DNA vaccine • immunotherapy • monoclonal antibody
• mucosal immunity • viral vector

Alzheimer's disease (AD) is characterized by insidious onset and slow progression of memory disturbance and other cognitive functions. Pathological hallmarks are amyloid β (A β) deposits, neurofibrillary tangles, loss of synapses and neurodegeneration. Although it has long been accepted that AD is a neurodegenerative disorder, recent findings in experimental treatment in model animals and clinical trials in patients suggest that immunotherapy or immune-mediated prevention may be feasible. Although there are already many reviews on this topic [1–4] including my own [5], it is important to revisit the previous clinical trials, review the procedure and results carefully, and give perspectives for future development of new immune-mediated prevention and treatment. Here I will try to summarize what we learned from the clinical trials and animal experiments, and give suggestion to the future direction.

AN1792 & AN1792-PS-80 vaccine trials

■ Phase I study

A randomized, double-blind, multiple-dose Phase I study of AN1792 was started in April 2000 at four clinical centers in the UK to investigate its safety, tolerability and immunogenicity, in which mild-to-moderate AD patients were enrolled [6]. The vaccine was composed of a combination of prefibrilized synthetic A β_{1-42} (50 or 225 μ g) and adjuvant QS21 (50 or 100 μ g). Patients aged 85 years or younger were divided into four groups; each group consisted of 16 patients who received AN1792 and four patients who received QS21 as placebo control. When the vaccine was given intramuscularly at 0, 4, 12 and 24 weeks, 21.6% showed elevated IgG antibodies to A β by ELISA. This Phase I study clearly showed safety and tolerability. However, immunogenicity was found low, probably because most of humans are tolerant to the self antigen A β and immune functions deteriorate due to aging.

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After confirming the safety, an extension study was undertaken. In this study, a modified vaccine was used to enhance immunogenicity, which contained $A\beta_{1-42}$, QS21 and 0.4% polysorbate 80 (here designated as AN1792-PS-80). PS-80, also named Tween 80, is an anionic surfactant or detergent that stabilizes aqueous formulations of medications as well as increases antigen availability. AN1792-PS-80 was given at 36, 48, 60 and 72 weeks, and the study was ended in June 2002. Adverse events were not significantly different between the vaccine and placebo groups except for more frequent injection site pain in the vaccine group. In fact, addition of PS-80 increased the antibody responder rate to 56.9%, and immunization with 225 μg $A\beta_{1-42}$ + 50 μg QS21 + 0.4%PS-80 elicited antibody responses in 86.7% [6]. Therefore, AN1792-PS-80 was applied to the Phase IIa study. It is of note that one participant had developed meningoencephalitis during the extended study of the Phase I, which appeared 6 weeks after the first injection of AN1792-PS-80. The patient died of pulmonary embolism 1 year after the last immunization. Autopsy showed for the first time that amyloid deposits can be cleared by $A\beta$ immunization in humans [7].

A long-term follow-up study after the extended study was commenced in June 2003. At this point, 20 patients had already died, and only 36 patients joined the study with informed consent. The total 6-year follow-up study ended in September 2006 [8]. The results will be discussed later.

■ Phase IIa study

A randomized, multicenter, placebo-controlled, double-blind Phase IIa study was started in September, 2001 at 28 clinical centers in the EU and USA to see its safety, tolerability and a pilot efficacy [9]. Patients aged 50–85 with mild-to-moderate AD (Mini-Mental State Exam [MMSE] scores 15–26) were enrolled in this study; 300 cases received AN1792-PS-80 and 72 cases received placebo (physiological saline this time instead of QS21). A total of six injections at 0, 1, 3, 6, 9 and 12 month was planned. However, since subacute meningoencephalitis was developed in 6% of the patients who received AN1792-PS-80 vaccine as an adverse event, the clinical trial was halted in January 2002 [10]. Until this point, 274 (91%) patients had received two injections, two patients one injection, and 24 patients three injections [9]. In this Phase IIa study, only 59 (19.7%) of the 300 patients were antibody responders (anti- $A\beta$ IgG titers of $\geq 1:2200$ after the first immunization), probably because the study was suspended early. Frequencies of adverse events were not different between the vaccine and placebo groups, although severe adverse events were more frequent in the vaccine group, particularly in the responder group.

■ Clinical efficacy in the Phase I & IIa studies

Although the clinical efficacy was not the primary end point in these trials, evaluation of cognitive functions was undertaken throughout the study. In the Phase I study, all patients declined during the study period, and scores of AD Assessment Scale-Cognitive Subscale (ADAS-cog), MMSE and Clinical Global Impression of Changes (CGI-C) were not significantly different between the vaccination group and control group. However, scores of Disability Assessment of Dementia (DAD) were significantly better in the vaccinated group ($p < 0.002$ at week 84, $p < 0.001$ at the final visit) [6]. Here, the fact must be considered that controls received QS21.

At the end of the Phase IIa study, all cases were subjected for evaluation. Again there was no significant difference between the treated group and placebo group in ADAS-cog, DAD, clinical dementia rating, MMSE and CGI-C. However, some memory assessment in Neuropsychological Test Battery was slightly but significantly better ($p = 0.047$) in the vaccine group compared with the placebo controls [9]. CSF tau was significantly reduced from the baseline in the antibody responder patients ($n = 11$, -204 ± 57 pg/ml) compared with non-responders ($n = 10$, $+42 \pm 52$ pg/ml) ($p < 0.001$), but the level of CSF $A\beta_{42}$ was not significantly altered [9]. However, in other small cohort, plasma $A\beta_{42}$ tended to be higher in tissue amyloid plaque immuno-reactive (TAPIR) antibody responders [11] (see later).

Volumetric studies of MRI showed that ELISA antibody responders had greater brain volume decrease (3.12 ± 1.98 vs $2.04 \pm 1.74\%$, $p < 0.007$) and greater ventricular enlargement (1.10 ± 0.75 vs $0.48 \pm 0.40\%$, $p < 0.001$) than placebo patients, but the change of the hippocampal volume was not statistically significant [12]. It is unclear at present whether this is due to amyloid removal and associated cerebral fluid shift or enhanced brain atrophy in the vaccinated patients. Since cognitive decline was not significantly increased in the antibody responders, it does not seem at least to be due to enhanced loss of neurites and neurons. Since amyloid itself is voluminous and immature amyloid contains more water [13], the former seems to be the likely mechanism.

A 4.6-year follow-up study was continued in the Phase IIa multicenter study. A total of 159 patients were enrolled in this study; 25 ELISA antibody responders, 104 nonresponders and 30 placebo patients. The responders showed still elevated levels of anti- $A\beta$ titers, although the titer declined. In the responders, scores of MMSE, ADAS-cog, Neuropsychological Test Battery and Clinical Dementia Rating Scale Sum of Boxes were not different, but those of DAD ($p = 0.015$) and Dependence Scale ($p = 0.033$) were slightly but significantly better compared with the placebo [14]. Thus, patients' activity of daily living seems to be slightly

improved, but cognitive functions deteriorated. The MRI follow-up study showed no more difference between responders and placebo patients during the follow-up period, but in responders a significant ventricular enlargement from the baseline at the entry of Phase IIa was confirmed even 4.6 years after. This seems that no further enhanced loss of the brain volume was observed in responders, but the brain volume loss did not return during the follow-up period.

In a cohort in Zurich, those patients who developed TAPIR antibodies showed a significantly slower decline of cognitive functions assessed by ADAS-cog and DAD during 1-year follow-up period after cessation of vaccination [11]. Here, TAPIR antibodies were defined as antibodies reactive to senile plaques in the brain of amyloid precursor protein (APP) transgenic (tg) mice by immunohistochemistry, which were absorbed by aggregated $A\beta_{1-42}$ but not by monomeric $A\beta$, suggesting that the antibodies seem to recognize the conformational structure of aggregated $A\beta$ or $A\beta$ oligomers. Therefore, the antibodies seem different from those examined by ELISA, which recognized N-terminal peptide of $A\beta$ ($A\beta_{1-8}$) [15]. However, the result of further follow-up has not been reported yet.

■ A 6-year follow-up of Phase I cases

A long-term follow-up of Phase I cases started in June 2003 and completed in September 2006 [16]. In this study, 36 patients (30 treated, six placebo) were enrolled at four clinical centers in the UK. Although the dose of $A\beta$ was not high in all, ELISA antibody responders were higher (58.8%) than those enrolled in the Phase IIa study. The antibody titers were higher in treated patients, however, their cognitive functions declined equally as in placebo patients, and the ratio of severe AD (MMSE <10), the duration to severe AD, and the survival ratio were not significantly different. Approximately 50% of patients died during the 6-year follow-up period in both treated and placebo patients. Although it seems that AN1792 and AN1792-PS-80 vaccine did not alter the disease course, it must be noted that not all patients were enrolled in this follow-up study. Particularly, placebo cases were too few to compare. In addition, placebo cases received adjuvant QS21, a purified natural saponin isolated from the soapbark tree *Quillaja saponaria* Molina. It is well known that systemic inflammation alters disease course of AD [17]. Therefore, it is highly possible that QS21 affected the disease course. Intranasal administration of adjuvant alone affected AD pathology (see later). It should also be noted that even an herbal medicine with an immune activating effect reduces AD pathology in mice [18]. Therefore, the effect of the vaccine might have been offset by QS21, and it appears that QS21 may not be suitable as a placebo.

During the follow-up period, nine autopsy cases were added [16]. Pathologically one case was diagnosed as progressive supranuclear palsy, and the remaining eight cases were compatible with AD. Among the eight cases, senile plaque amyloid was cleared very extensively in two cases and intermediately in four cases, yet the MMSE score in these patients was 0, which was obtained when the patients' consciousness was still alert before death, suggesting that those patients were at the end stage of AD. Thus, it is obvious that removal of senile plaque amyloid has nothing to do with cognitive functions. Alternatively, removal of senile plaque amyloid made a good effect, but it was offset by a certain bad effect caused by the vaccination such as increase of inflammation and tau pathology.

Meningoencephalitis associated with AN1792 vaccination

One of 64 cases (1.6%) in the Phase I extended study developed meningoencephalitis [7]. Of 298 patients who received AN1792-PS-80 vaccine in the Phase IIa study, 18 developed subacute meningoencephalitis, but none in placebo saline controls [10]. It was independent of the ELISA antibody titers. Most of the cases (16 out of 18) developed meningoencephalitis after two immunizations, one after three immunizations and one after one immunization. The latency period from the last injection to onset of symptoms was 5–71 days (median 40) with two outliers of 156 and 168 days. Presented manifestations were confusion, headache, lethargy and other variable symptoms and signs suggesting meningitis and encephalitis. In addition, some patients showed manifestations due to large white matter lesions. The disease course was mostly monophasic, but four patients experienced relapses. CSF cells were increased to 15–130 cells/ μ l (71–100% lymphocytes) in all 17 cases examined. Total protein was 33–310 mg/dl, and IgG was markedly elevated in three out of four cases tested. MRI scan showed variable high intensity signals in the meninges, cortex and white matter of the cerebrum and cerebellum in T2 and fluid attenuated inversion recovery (FLAIR) images with or without gadolinium enhancement. The meningoencephalitis was aseptic, in other words no specific bacteria or virus was found. Most of the cases were treated with corticosteroid and two patients were put on plasmapheresis, and 12 cases recovered to or close to their baseline status, whereas six cases had persistent disabling.

Plaque & tangle pathology in autopsy cases

The first autopsy case was reported from the UK, who was enrolled in the Phase I study [7]. At 6 weeks after the last immunization with AN1792-PS-80 ($A\beta_{1-42}$ = 250 μ g), a 72-year-old woman with MMSE score 26 at the baseline developed acute meningoencephalitis. MRI

showed extensive bilateral alterations in the cerebral white matter and enhancement on the brain surface. Although the patient was put on steroids, the patient remained unchanged and died of pulmonary embolism 12 months after the last immunization.

Postmortem examination showed brain atrophy with focal white matter softening. A β plaques were observed in the medial frontal cortex, where the distribution pattern was patchy. In these areas, moth-eaten plaques and plaques of dense cores without surrounding amyloid were frequently observed. A β plaques were almost none in other cerebral cortices, while numerous plaques were present in basal ganglia and cerebellum. In the plaque-free areas, A β -immuno-reactive CD68⁺ HLA-DR plus microglia/macrophages were scattered. These findings suggest that plaque amyloid was disrupted by certain immune mechanisms and A β was phagocytosed by activated microglia/macrophages. The moth-eaten plaques are thought to be partially disrupted plaques, and the core alone plaques are due to removal of surrounding amyloid of classical plaques.

There was an infiltrate of CD3⁺, CD4⁺, CD45RO⁺ T lymphocytes in the leptomeninges, and in perivascular spaces and parenchyma of the cerebral cortex. CD8⁺ T cells were few, and B cells were absent. The white matter lesion was a rarefaction with reduced myelin staining and numerous lipid-laden macrophages without T-cell infiltrations. This could be caused by a small vessel disease due to enhanced amyloid angiopathy as suggested [19]. It could also be secondary to damage of aquaporin 4 and edema, which was observed in acute multiple sclerosis (MS)-like lesions [20].

In the second autopsy case, a 76-year-old man with MMSE score 18 at the baseline was reported from Spain, who was enrolled in the Phase IIa study [21]. Six months after the second injection with AN1792-PS-80, the patient experienced progressive speech and gait disturbances without fever. Cranial MRI taken 3 months after onset showed bilateral white matter lesions without contrast enhancement. CSF demonstrated 60 cells/ μ l with aseptic results. The patient was put on steroids, and MRI showed some improvement. However, the patient deteriorated clinically and showed agitation and confusion. Since the patient developed endocarditis and bronchopneumonia, steroid was withdrawn and antibiotics were given. However, the patient further deteriorated with reactivation signs in MRI, and he died 4 months later.

Autopsy findings were similar to those in the case one. In addition, multinucleated giant cells engulfing A β and multiple cortical hemorrhages were present. The giant cells are said nonspecific and not uncommon in aged individuals. Regarding the inflammatory infiltrates in this case, the majority of infiltrated cells were CD8⁺ T cells, less often CD4⁺ T cells and B cells,

and CD16⁺, CD57⁺ natural killer cells were absent. The inflammation was observed mainly in the entorhinal cortex, hippocampus and amygdala in association with collapsed plaques and multinucleated giant cells. Collapsed plaques were devoid of phospho-tau-positive dystrophic neurites, although neurofibrillary tangles and neuropil threads were maintained as in the case one.

The third autopsy case was a 71-year-old man with a 10-year history of moderate-to-severe AD [22]. The patient received three injections of AN1792-PS-80 at 0, 4 and 12 week without noticeable adverse events, and died of failure to thrive. The ELISA anti-A β titer was 1:2771 at 6 months after the first injection. In this case, A β plaques were absent in frontal cortex with scattered A β laden CD68⁺ microglia/macrophages, but A β plaques remained in other areas. Tau pathology was consistent with the Braak stage VI. This case suggested A β plaque removal in absence of encephalitis. However, it is of note that there were minimal but significant cell infiltrates in the leptomeningeal vessels, which consisted of CD20⁺ B cells and CD3⁺ T cells, while multinucleated giant cells were absent.

In the Phase I and its extended studies, a total of ten autopsy cases were reported so far, including the case one mentioned above. One case was found progressive supranuclear palsy which is characterized by vertical gaze palsy, gait disturbance, parkinsonism and subcortical dementia.

Three autopsy cases were added in University of California San Diego [23]. A 78-year-old male with a total of 5-year disease duration died 36 months after one immunization with AN-1792-PS-80. Postmortem diagnosis of this case was Lewy body variant of AD with the Braak stage III. The second case was an 86-year-old male at death, who also received one immunization with AN1792-PS-80 and died 60 months after. The diagnosis was AD with the Braak stage IV. The third case received one AN1792-PS-80 immunization and died 48 months after. The diagnosis was hippocampal sclerosis with minimal A β deposits. Thus, so far two non-AD and one AD variant cases were included in the trials, suggesting that establishment of clinical diagnostic criteria for AD is necessary. By adding PET imaging and CSF biomarkers, revised NINCDS-ADR criteria may give more accurate diagnosis of AD [24].

The autopsy cases in the Phase I study were systematically reviewed [19]. Immunized AD cases showed significantly reduced A β ₄₂ load in the neocortex and hippocampal formation ($p < 0.001$), suggesting successful removal of A β plaques. Quantitation of phospho-tau and dystrophic neuritis were also significantly reduced in immunized AD cases compared with non-immunized AD. This suggests that on removal of A β plaques, phospho-tau is also removed and remodeling

of neuronal processes takes place in human brains even at the age of 80 years. However, phospho-tau positive neurons were not significantly different, suggesting a failure in removal of neurofibrillary tangles. There is no evidence suggesting that the immunization enhanced tangle formation.

Vascular pathology after active

A β_{42} immunotherapy

Eight autopsy cases including the above mentioned first case were carefully studied and compared with age matched unimmunized archival cases of AD in UK [8,25]. All cases were enrolled in the Phase I study of AN1792 and in the extended study of AN1792-PS-80. In association with reduction of A β plaques, there was a significant increase of vessels with A β_{42} as well as A β_{40} in the cerebral cortex and meninges. There was also a significant increase of microvascular lesions including microhemorrhages without cellular infiltrates. They speculate that plaque A β is solubilized by A β antibodies, allowing A β to diffuse to the perivascular space and deposited in the vasculature. Since long standing cases tended to show A β -laden vessels less frequently, they speculate that once increased deposits of vascular A β after immunization is also slowly cleared [19]. Immunized patients deteriorated equally in comparison to placebo controls, therefore increased vascular deposits of A β and microhemorrhages do not seem to affect the clinical course.

Chemical findings after active

A β_{42} immunotherapy

Biochemical analysis was performed using the frozen brain material of the two autopsy cases; case one [21] and case two [22]. Total A β in the water-soluble fraction of gray matter was high in both cases (61.1 and 13.5 $\mu\text{g/g}$, respectively) compared with un-immunized seven AD cases (3.9 $\mu\text{g/g}$) [26]. Both A β_{40} and A β_{42} were high in case one, but only A β_{40} was high in case two. The water-soluble A β seems to be derived from disrupted plaque amyloid by immunization. This is compatible with the increased immunoreactivity of A β in the vasculature of immunized cases [25].

It is interesting to see both A β_{40} and A β_{42} dimers in both water-soluble and sodium dodecyl sulfate (SDS)-insoluble fractions [26]. The western blot showed that A β dimers were much higher than A β monomers. In addition, there were several other oligomers with a molecular weight up to 30 kDa in the SDS-insoluble fraction. Unfortunately there was no information whether these oligomers were reduced or increased compared with un-immunized AD controls. Nonetheless, it is obvious that certain A β oligomers still remain in AN1792-PS-80-immunized cases, although plaque amyloid was significantly removed.

The chemical findings were confirmed using the five autopsy cases obtained in UK and additional case in the USA [23]. In addition, it was shown that TNF- α was significantly elevated in the gray matter of immunized patients (34 pg/mg protein) compared with un-immunized AD (14 pg/mg protein, $p < 0.0001$) [23]. This suggests that brain inflammation was significantly increased in vaccinated patients even though A β plaques were significantly removed. However, it should be again in mind that the un-immunized AD controls received physiological saline.

Mechanism of meningoencephalitis

Although self-reactive T cells are deleted in the thymus during an embryonic developmental period, A β -reactive T cells persist in the peripheral lymphoid organs under the control of regulatory T cells in humans. In fact A β -reactive T cells exist in the peripheral blood, and the frequency is higher in the elderly than the youth, and it is slightly higher in AD patients than aged non-AD individuals [27]. There is a report describing that immunization of wild type C57BL/6 mice with A β_{1-42} and complete Freund's adjuvant (CFA) followed by pertussis toxin induced inflammatory lesions in the brain with production of a high amount of T helper 1 (Th1) cytokines [28]. Although this study was hardly repeated by us and others, immunization of APP tg mice crossed with a tg strain of mouse IFN- γ with the H-2^s background under the control of myelin basic protein promoter could reproduce similar encephalitis [29]. It is interesting to see that in mice immunized with proteolipid protein, inflammatory cells infiltrated mainly in the spinal cord and cerebellum, while in mice immunized with A β , T cells infiltrated mainly in the hippocampus [30]. Thus, it is highly probable that the meningoencephalitis induced by AN-1792 is mediated by the autoimmune mechanism against A β , because QS21 strongly activates Th1 T cells. Here we must aware that absence of T-cell infiltrations in AD model mice should be carefully explained, because most of these animals are tolerant unless they are genetically manipulated to enhance Th1 immune responses.

It is well established that Th1 T cells are activated by IL-12, which produce pro-inflammatory cytokines such as IFN- γ and TNF- α . Th1 T cells help cellular immunity and are involved in the effector mechanism of autoimmune encephalitis. On the other hand, Th2 T cells are activated by IL-4, which produce IL-4 and IL-10, and help humoral immunity. Th1 T cells and Th2 T cells regulate each other. Recently Th17 T cells are also known to be involved in autoimmune encephalitis. However, it has not been examined whether Th17 T cells are involved in the vaccine-induced encephalitis.

As 6% of patients showed clinically overt meningoencephalitis, it is highly probable that Th1 T cells were activated subclinically in the remaining patients. Peripherally activated Th1 T cells migrate into the brain, where re-activated T cells induce

inflammatory processes by activation of microglia and astrocytes. Therefore, in order to avoid Th1 T-cell-mediated autoimmune adverse events, vaccines predominantly activating Th2 T cells would be strongly recommended (Figure 1).

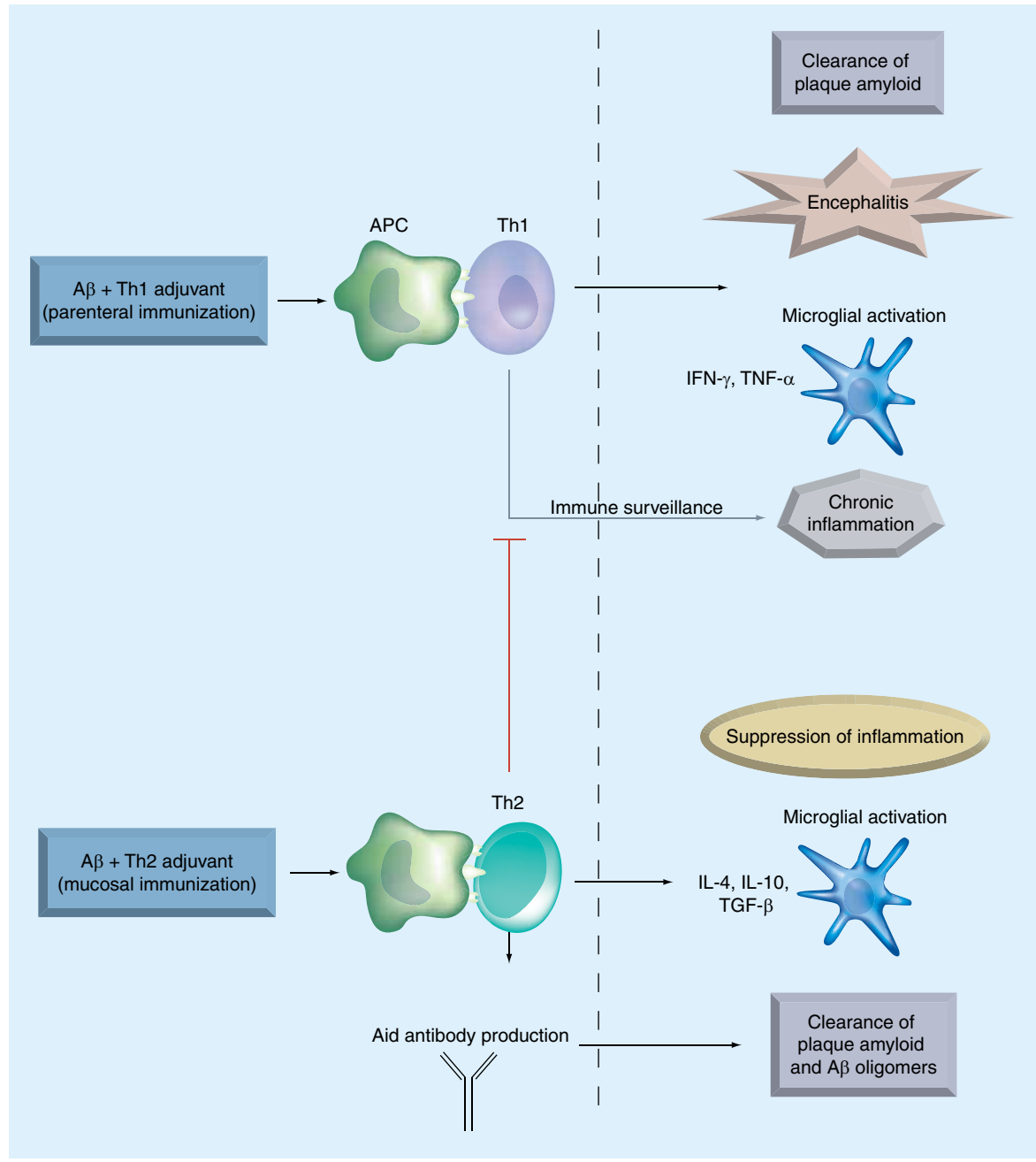


Figure 1. T cell-mediated mechanisms of Aβ immunization. Parenteral immunization of Aβ with Th1 adjuvant activates Aβ-specific Th1 T cells, which induce autoimmune encephalitis on further activation in the CNS. Th1 T cells are speculated to induce chronic inflammation in the CNS as a consequence of immune surveillance; this still needs to be proven in animals and humans. Mucosal immunization of Aβ with or without Th2 adjuvant activates Th2 T cells, which help antibody production and suppress detrimental inflammation in the CNS. Consequently, plaque amyloid and Aβ oligomers are cleared by microglia.

Experience gained from the AN1792 vaccine trial

The most important finding is that A β plaques can be cleared by immune mechanisms. Since A β plaques are most characteristic and outstanding in AD, we have long thought that plaque amyloid is pathogenetic in AD. However, results of AN1792 trial have made us speculate that fibrillar amyloid deposits may not be pathogenetic or have little effect in the pathological mechanism of AD. Although there exists a possibility that the removal of amyloid deposits was incomplete and the removal of amyloid deposit was largely variable among patients, it would be suggested that in addition to fibrillar amyloid, A β oligomers, intracellular A β , phospho-tau and inflammation should be targeted for treatment. Indeed, AN1792 vaccine succeeded in clearing β amyloid, but significant amount of A β oligomers remained in the brain. Although there exists a possibility that solubilization of A β deposit formed more toxic oligomers, this is less likely because responders were at least clinically not worse. In addition, intraneuronal phospho-tau was not reduced, and continuous high production of TNF- α was observed. Thus, for developing future immunotherapies these points should be addressed.

For the lack of treatment effects of AN1792 in both Phase I extended study and Phase II study, one might argue that the cognitive measures were not sensitive to detect the subtle changes. Since ADAS-cog and other measures worked well for development of allopathic drugs such as donepezyl, galantamine and others, they should work well for disease modifying drugs. However, since it will take longer durations for clinical trials of disease modifying drugs, more sensitive measures are definitely worth to be established.

In addition, it is also speculated that amyloid deposits may trigger a certain progressive mechanism. Therefore, vaccination may be required before the progressive mechanism starts. In both Phase I and Phase II studies, mild-to-moderate AD patients were enrolled. Even patients with MMSE 26 were included, suggesting that even mild AD may be too late. In this sense, prevention rather than treatment may work better, or treatment should be given at the prodromal stage of AD.

A β oligomers and intracellular A β

The growing body of knowledge suggests the particular importance of A β oligomers in the pathological mechanism in AD [31]. There are a variety of A β oligomers: low molecular oligomers such as dimers and trimers [32], middle size molecules such as 56 kDa 12-mers (A β *56) and A β -derived diffusible ligands [33,34], a higher molecular size oligomers such as 30–150-mers (amylopherooids) [35] and

protofibrils [36]. However, it is still unknown which oligomer is responsible in AD, one of them or all of them? If all of them are involved, all of them should be deleted. Since a common structure may not exist in all of the oligomers, polyclonal immune responses are required to delete all of them.

Significance of intraneuronal A β is also suggested [37]. A β is produced in the endosome of presynaptic terminals, and secreted extracellularly [38]. However, in certain conditions A β seems to accumulate intracellularly, which is degraded in the proteasome. In fact, proteasome activation reduces intracellular A β [39]. In APP mutant neurons, the ubiquitin-proteasome system is altered and intracellular A β accumulates in multivesicular bodies [40]. Although the mechanism is unknown yet, A β immunization reduces intracellular A β in mice [41,42]. Thus intracellular A β can be targeted by immunotherapy. The mechanism how antibodies clear intracellular A β is unclear. Since certain viruses are neutralized by antibodies intracellularly [43,44]. In a certain paraneoplastic syndrome, human antibodies against an intracellular molecule was successfully transferred to rats [45]. Thus, intracellular immune surveillance by antibodies does occur.

A β pathology is an upstream event to tau pathology

A β pathology appears 10 years prior to tau pathology [46]. Intracerebral injection of A β fibrils facilitated tau pathology in tau tg mice [47], and tau pathology was significantly facilitated in tau tg mice crossed with APP tg mice [48]. These observations suggest that A β pathology is the upstream event of tau pathology. In animals, A β immunotherapy lead to clearance of early, but not late, hyperphosphorylated tau aggregates [41]. In humans, A β immunization reduced aggregated tau in neuronal processes but not in the cell bodies [19]. Therefore, it may be possible to prevent or to delay tau pathology if A β immunization is given at an earlier stage.

Development of future A β vaccines

Taking into account the above findings, A β vaccines of the next generation should feature the following properties:

- They should be safe;
- They should avoid autoimmune encephalitis. In other words, vaccines activate Th2 T cells rather than Th1 T cells, and suppress adverse inflammation;
- They should not cause vasculitis, microhemorrhages and vasogenic edema;

- They should clear not only plaque amyloid but also other toxic molecules such as A β oligomers, intracellular A β and phospho-tau;
- They should be useful for prevention, if given at an earlier stage;
- They should modify the disease course and hopefully improves cognitive functions, if given after the disease progression mechanism has started;
- They should be efficient in the elderly whose immune functions are deteriorated due to aging;
- They should not be painful and has a good compliance;
- They should be inexpensive.

Passive immunization

■ Antibodies on clinical trial

Passive immunization reduced A β burden and improved cognitive functions in animal models of AD [49,50]. To avoid T cell-mediated meningoencephalitis, clinical trials of monoclonal antibodies to A β and human polyclonal antibodies are underway (Table 1).

Bapineuzumab is the humanized mouse monoclonal antibody 3D6, which has the antigenic epitope in the N-terminus of A β (A β_{1-5}) and recognizes A β fibrils preferentially. It is known that it reduces A β plaques in an animal model of AD [49], but it is unknown whether it reduces A β oligomers. Bapineuzumab (0.15, 0.5, 1 or 2 mg/kg) was infused six-times with a 13-week

interval in 124 clinically probable AD patients with MMSE 16–26, and compared with 110 placebo-controlled patients at 72 weeks. Although overall clinical efficacy was not confirmed in the primary efficacy analysis, exploratory analyses showed potential difference in ADAS-cog and DAD in patients without carrying apolipoprotein E (ApoE) $\epsilon 4$ [51]. However, cognitive functions declined continuously during the observation period. A noticeable adverse event was vasogenic edema which was common in ApoE $\epsilon 4$ carriers with high dose bapineuzumab. CSF A β_{42} and total tau were not significantly different. Brain volume loss was significantly less in treated ApoE $\epsilon 4$ noncarriers. Pittsburgh compound B/PET showed reduction of [11 C] Pittsburgh compound B in the treated patients' brain, while it was increased in placebo controls [52]. This is now in the Phase III study with lower doses.

Solanezumab is the humanized mouse monoclonal antibody m266, which recognizes the central portion of A β (A β_{13-28}). Since m266 recognizes A β monomer preferably and not A β fibrils, administration of m266 in APP tg mice reversed memory deficits without reducing brain amyloid burden [53]. From the observation that m266 sequesters peripheral A β and reduces brain A β burden, the sink hypothesis was postulated [54]. However, recent observation in mice shows that intracerebrally injected radioisotope-labeled A β did appear in the peripheral blood in the absence of m266 antibody. However, m266 antibody was injected, A β did not come out of the brain. They speculate that peripherally administered m266 enters into the brain, where it stabilizes A β monomer and presumably prevents A β oligomer formation [55]. Whatever the mechanism is, it

Table 1. Passive immunizations for Alzheimer's disease under clinical trials.

Name	Company	Type of antibody	Antigenic epitope	Target	Injection	Phase
AAB-001 (Bapineuzumab)	Elan/Johnson & Johnson/Pfizer	Humanized mouse monoclonal, IgG1	N-terminus A β_{1-5}	Amyloid	Intravenous (subcutaneous)	III (I)
LY2062430 (Solanezumab)	Eli Lilly	Humanized mouse monoclonal, IgG1	Central part A β_{13-28}	Soluble A β	Intravenous	III
γ -gard	Baxter Bioscience	Human polyclonal immunoglobulin	?	?	Intravenous	III
Octagam	Octapharma	Human polyclonal immunoglobulin				II
PF-4360365 (Ponezumab)	Pfizer	Humanized mouse monoclonal, IgG2	C-terminus A β_{33-40}	Amyloid	Intravenous	II
R1450 (Gantenerumab)	Roche/ Chugai	Human monoclonal, IgG1	N-terminus and central part	Conformation	Intravenous	I
BAN2401	Eisai/ Bioarctic	Humanized mouse monoclonal	?	Protofibril	Intravenous	I
GSK93376A	GlaxoSmithKline				Intravenous	I

A β : Amyloid β .

has an advantage in that it does not increase microhemorrhages because of poor recognition of A β fibrils [56]. Indeed, 19 patients were well tolerated to a single dose of solanezumab (0.5, 1.5, 4 and 10 mg/kg) without significant adverse events including vasogenic edema, and substantial dose-dependent increases of plasma A β_{40} and A β_{42} with a peak at 500–1000 h after infusion were observed [57]. Although clinical efficacy is unknown at present, this is now in the Phase III study.

Ponezumab is a humanized mouse monoclonal antibody 2H6 that recognizes C-terminus of A β (A β_{33-40}). In an animal model of AD, deglycosylated 2H6 reduced parenchymal amyloid and improved cognitive deficits with minimal microhemorrhages, while original 2H6 increased microhemorrhages significantly [58]. Ponezumab is now in Phase II study.

Gantenerumab is a human IgG1 monoclonal antibody which recognizes conformation of A β aggregate, but precise information is not available yet. Gammagard is human intravenous immunoglobulin (IVIG) containing polyclonal antibodies from healthy human donors. In a pilot study, IVIG 0.4 g/kg was given on three consecutive days every 4 weeks over 6 months in five AD patients, and the result showed significant decrease of CSF A β and improvement of ADS-cog [59]. Further, an open-label dose-ranging study was conducted in eight mild AD patients, involving 6 months IVIG, 3 months washout, and 9 months extension of IVIG. IVIG induced reduction of A β in CSF, increase of A β in plasma and improvement of MMSE scores by 2.5 points [60]. These pilot studies suggest that there exist natural antibodies against A β in healthy individuals, which are beneficial for reducing the pathological mechanism in AD. Indeed, humans who had received at least one IVIG reduced risk of AD and related disorders: 2.6% of 847 versus 4.6% of 84,700 untreated individuals ($p = 0.02$) [61]. Decoration of senile plaques with natural antibodies was demonstrated, suggesting that natural antibodies enter into the brain [62,63]. A possible mechanism is that natural antibodies catalyze hydrolysis of A β and disrupt plaque amyloid [64]. In addition, IVIG reduces complements and reduces inflammation (Figure 2).

■ Antibodies at preclinical stage

There are many antibodies at the preclinical stage. The most expecting antibodies are those against A β oligomers. Again it is unknown which oligomers are responsible for AD. Therefore, we must wait for results of clinical trials. We established a TAPIR-like monoclonal antibody, which recognized conformation of A β , stained the peripheral part of senile plaques preferentially and tended to show reduction of A β *56 in AD mice [65].

A β peptides are cleaved by β -site APP cleaving enzyme-1 between amino acids 2–3 and 10–11 of the peptide, and the terminal glutamate of the N-truncated A β peptide is modified to pyro-glutamate by glutaminyl cyclase. The 3pE-A β is the major fraction of plaque amyloid in AD [66]. Recently, antibodies against N-terminally truncated A β peptides starting with pyro-glutamate were developed and tested in an AD mouse model [67,68]. Since 3pE-A β is also the source of A β oligomers, such antibodies may also work well.

■ Pros and cons of antibody therapy

There are several advantages and disadvantages of antibody therapies.

Advantages:

- When a serious adverse event appears, antibody elimination from the body is relatively easy by stopping administration;
- The dose can be controlled;
- T cell-mediated autoimmune encephalitis can be avoided.

Disadvantages:

- Since target molecules are unknown, limitations exist in the monoclonal antibody. For instance, an A β dimer-specific monoclonal antibody may not work, if other molecules are responsible for AD. Although certain monoclonal antibodies recognize different A β molecules, polyclonal antibodies may be better until the target molecule is identified;
- Since only a small amount of injected antibodies can penetrate into the brain, a large amount of antibody is required;
- Microhemorrhage, vasogenic edema and vasculitis may occur. These adverse events are also seen in patients with active immunization. However, the incidence is higher in passive immunization;
- Neutralizing antibodies and anti-idiotypic antibodies may appear;
- Repeated intravenous injections or a relatively large amount of subcutaneous injections are required;
- Prophylactic use is limited;
- Expensive.

Therefore, active immunization, if it is safe, is still worth developing.

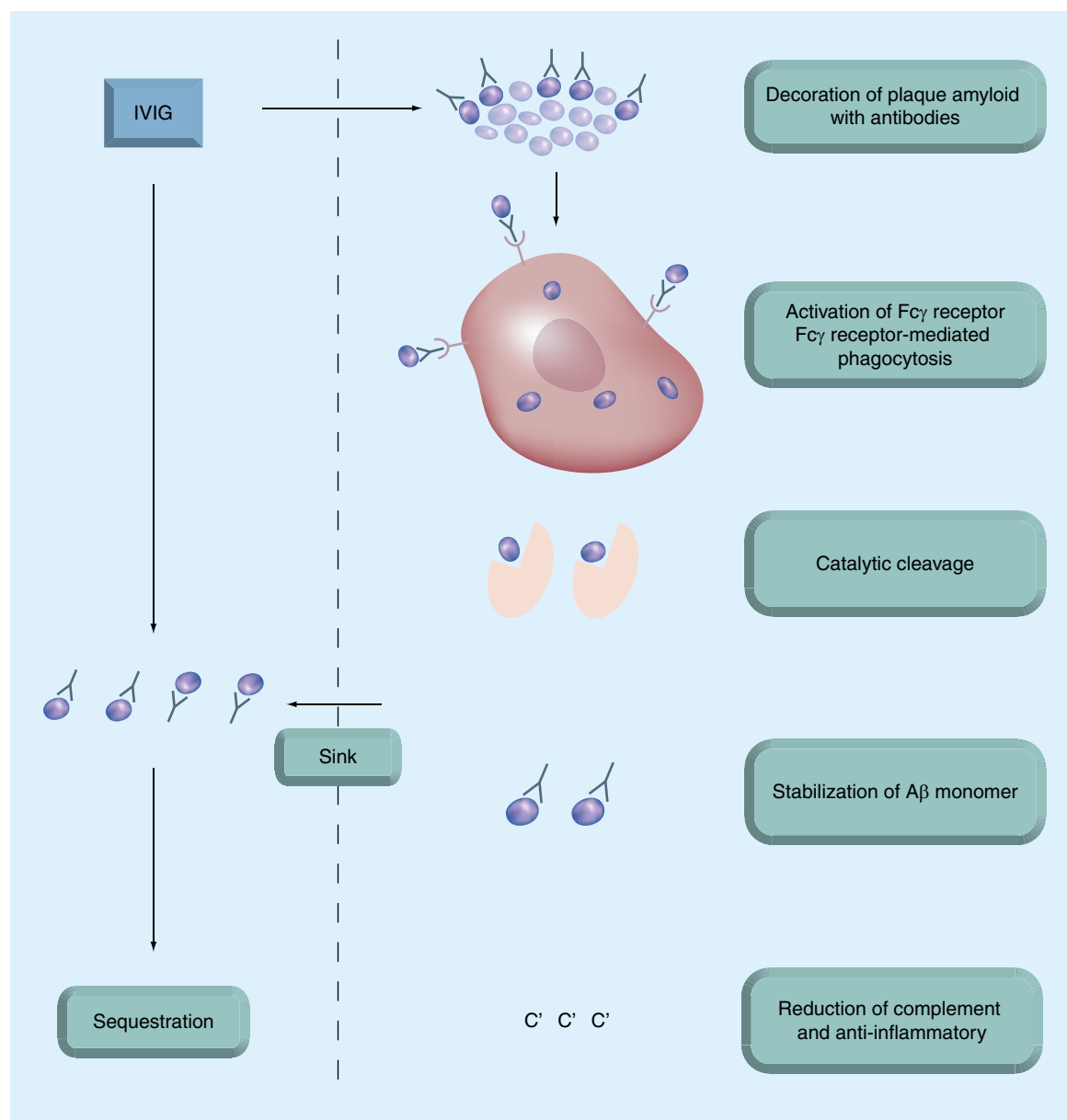


Figure 2. The possible mechanism of intravenous immunoglobulin. When a high dose of intravenous immunoglobulin is injected, natural antibodies to A β species penetrate into the brain and decorate plaque amyloid [62], resulting in Fc γ receptor-mediated phagocytosis of fibrillar A β and A β oligomers. Some natural antibodies stabilize A β monomers and prevent oligomer formation [55], and some cleave A β by catalytic enzyme activities [64]. A peripheral sink hypothesis may also work [54]. Intravenous immunoglobulin has effect for reducing complement and anti-inflammatory effect [122].

Active immunization

■ A β N-terminus vaccine

The most important point exists in that A β peptide vaccine should avoid autoimmune encephalitis which is mediated by Th1 T cells. T cells including Th1 T cells are activated by presenting the antigen with the major histocompatibility complex (MHC) class II molecule by antigen presenting cells. The extent of T cell activation

by A β differs significantly among mouse strains and humans bearing different MHC class II molecules. The dominant T-cell epitope in SJL (H-2^s) and C57BL/6 (H-2^b) mice was mapped to A β _{10–24} and A β _{16–30}, respectively [29]. T-cell epitopes detected by analysis of A β -specific T-cell lines were mapped to A β _{16–30}, A β _{18–32}, and A β _{28–42} in humans [27]. It was also mapped to A β _{25–42} in DR B1*1501 tg mice (= DR15 or DRw2) [69]. Thus,

N-terminal peptide seems to be relatively safe. However, T-cell responses against A β ₁₋₁₆ were observed in A β ₁₋₄₂-immunized HLA-DR3, HLA-DR4 and HLA-DQ8 tg mice [70]. Thus, it cannot be said that the N-terminus peptide is entirely safe.

There are a number of approaches using N-terminus peptide in animals (Table 2).

A short N-terminus A β peptide conjugated with a nonencephalitogenic T-cell epitope of carrier protein Prototype vaccines utilize a single or triple repeat of A β ₁₋₁₅ peptide conjugated with a T-cell epitope such as bovine serum albumin, injected in animals subcutaneously or intraperitoneally with CFA [71,72]. To enhance Th2 immune responses, A β ₁₋₁₅ is conjugated to the three arms of a lysine core and the promiscuous nonself T-cell epitope, pan HLA DR-binding peptide (PADRE) to the remaining arm, and injected subcutaneously in mice with Alum, a Th2 adjuvant [73]. Their second generation vaccine using A β ₁₋₁₁ and PADRE eliminated amyloid deposits, but not soluble A β and A β oligomers [74]. This is because the vaccine produced antibodies to fibrillar A β , not to soluble A β and A β oligomers.

Alternatively, repeated subcutaneous injections of A β ₁₋₂₈ conjugated with Mannan elicited Th2 immune responses predominantly [75]. Mannan is a molecular adjuvant that enhances uptake of antigens by dendritic cells and production of IgG1, IL-10 and IL-4. Their later report says that it increased microhemorrhages in APPs mice [76].

Mucosal immunization also induces Th2 dominant immune responses. Weiner and colleagues immunized APP tg mice with A β ₁₋₄₂ nasally and showed efficient reduction of amyloid burden with an increase of microhemorrhages [77]. Maier M and coworkers made constructs such as A β ₁₋₁₅-K-K-A β ₁₋₁₅, RGD-A β ₁₋₁₅-K-K-A β ₁₋₁₅, T1-A β ₁₋₁₅, and T1-RGD-K-K-A β ₁₋₁₅. Here, K-K is a lysine core which binds two peptides, RGD is a cell attachment motif which enhances antigen uptake and presentation by antigen presenting cells in the mucous membrane, and T1 is a T-cell epitope of HIV IIIB gp120. Mice were immunized intranasally with the vaccine and *E. coli* heat-labile enterotoxin [LT(R192G)] as an adjuvant [78]. It must be cautious that intra-nasally administered A β and adjuvant enter into the brain through the olfactory nerve, which may have some effects in the CNS.

ACC001 is a vaccine composed of an A β ₁₋₆ peptide conjugated with a diphtheria toxin T-cell epitope, Diphtheria toxin CRM₁₉₇ (synthetic *Corynebacterium diphtheriae*) [201,202], and adjuvant QS21, which is now on the Phase II trial (Table 3). As mentioned above, QS21 is an adjuvant that activates Th1 T cells, and A β N-terminus is not entirely safe. However, meningoencephalitis has not been reported so far.

Table 2. Peptide vaccines in animals.

Antigen	T-cell help	Adjuvant	Injection	Ref.
A β ₁₋₁₅	BSAT	CFA	Subcutaneous	[71]
3×A β ₁₋₁₅	BSAT	CFA	Intraperitoneal	[72]
3×A β ₁₋₁₅	PADRE	Alum	Subcutaneous	[73]
A β ₁₋₁₅		Mannan	Subcutaneous	[75,76]
A β ₁₋₄₂			Nasal	[77]
2×A β ₁₋₁₅ RGD-2×A β ₁₋₁₅ T1-RGD-A β ₁₋₁₅	T1	EHLE	Nasal	[78]
Parmitoyl A β ₁₋₁₅		Liposome	Intraperitoneal	[80]
A β ₁₋₄₂		CTX	Transcutaneous	[81]
MLV-A β ₁₋₁₅ -PDGFR	PDGFR	MLV?	Intravenous	[82]
SDPM1		Adjuvant?	Subcutaneous	[84,85]
Cop 1		Adjuvant	Subcutaneous	[87]

A β : Amyloid β ; BSAT: Bovine serum albumin T-cell epitope; CFA: Complete Freund's adjuvant; Cop: Copolymer; CTX: Cholera toxin; EHLE: *E. coli* heat-labile enterotoxin; MLV: Murine leukemia virus; PADRE: Pan-HLA-DR binding peptide; RGD: Arginine, glycine, aspartic acid motif; SDPM1: Mimotope of A β tetramer.

UBITh[®]1 contains A β ₁₋₁₄ conjugated with CpG oligonucleotide and a synthetic T-cell epitope of measles virus F protein, while a synthetic T-cell epitope of hepatitis B surface antigen is used for UBITh[®]2. Intramuscular injections of these vaccines with an adjuvant aluminum salt elicited Th2 immune responses in guinea pigs, mice and monkeys [79]. This vaccine is now on a clinical trial as UB311 (Table 3). The problem of CpG motif will be discussed later.

A β peptide/liposome vaccine

A liposome vaccine was made by C16 parmitoylation at both end of A β ₁₋₁₅ peptide, and repeated intra-peritoneal inoculations of the vaccine without adjuvant elicited IgG1 and IgG2b antibodies (=Th2 type antibodies) recognizing β sheet structures of A β [80]. The reasons why it induced Th2 type immune responses and why it induced antibodies against β sheet structures are unknown. A liposome vaccine by adding polyethylene glycol (PEG) in both ends of A β ₁₋₁₅ induced antibodies recognizing an α helix of A β .

Transcutaneous vaccine using A β ₁₋₄₂ peptide & cholera toxin

The precursor cells of CD14⁺ Langerhans cells in the epidermis are antigen presenting cells that induce Th2 immune responses predominantly. In order to activate these cells, a solution containing A β ₁₋₄₂ peptide and cholera toxin was applied repeatedly on the carefully shaved skin in mice for 2 h every 1–2 weeks [81].

Table 3. Peptide vaccines under clinical trials.

Name	Company	Antigen	T-cell epitope/adjuvant	Injection	Phase	Ref.
ACC001	Pfizer	A β ₁₋₇	Diphtheria toxin/QS21	Intramuscular	II	
UB311	United Biomedical	A β ₁₋₁₄ /CpG ODN	Synthetic viral T-cell epitope/ aluminum salt	Intramuscular	I	[79]
CAD106	Novartis	A β ₁₋₆	Q β bacteriophage	Intramuscular	II	[83]
AD01, AD02	GlaxoSmithKline/Affiris	Affitope*	None	Subcutaneous	I	[88]
V950	Merck	A β N-terminus	Iscomatrix	Intramuscular	I	

*Affitope: Synthetic peptide of mimotope, a mimic of epitope.
A β : Amyloid β ; CpG ODN: Oligonucleotide containing CpG motif.

This procedure successfully activated Th2 immune responses. To improve the drug delivery, a transcutaneous patch vaccine is being developed using a sticky tape containing small needles with a length of less than the depth of the epidermis and an antigen/adjuvant complex [NAKAGAWA *SET AL.* PERS. COMM.].

Virus-like particles expressing A β ₁₋₁₅ peptide & PDGF receptor protein

Plasmid pHIT60 containing *MLV gag* and *pol* genes and plasmid pDisplay containing A β ₁₋₁₅, PDGF receptor and an immunoglobulin κ signal sequence were co-transfected in HEK-293 T cells, which produced virus-like particles expressing A β ₁₋₁₅-PDGFR fusion protein. When 1×10^{10} particles were injected intravenously in APP23 mice, they produced high titers of anti-A β IgG1 and IgG2b antibodies. The immunized mice showed significant reduction of plaque amyloid and soluble as well as insoluble A β [82]. Each virus-like particle expresses several thousands epitopes of A β ₁₋₁₅ and the injected particles were calculated as containing 150 ng of A β ₁₋₁₅. However, it has not been reported whether it reduces A β oligomers and improves cognitive functions.

CAD106 is a virus particle-like vaccine using bacteriophage Q β and A β ₁₋₆ peptide, which does not need an adjuvant [83]. This is presently being studied in a clinical trial (Table 3).

■ Mimotope & mimotope-like peptide vaccine

A peptide that binds to aggregated A β ₁₋₄₀ but not to monomeric A β ₁₋₄₀ was obtained by screening a phage peptide library. SDPM1, the peptide with cysteine residue at both end bound aggregated A β ₁₋₄₀ with high affinity. Interestingly, antibodies to SDPM1 recognized A β ₁₋₄₀ and A β ₁₋₄₂ tetramers, and reduced A β burden in APPswPSEN1 tg mice [84,85]. Thus, SDPM1 turned out to be a mimotope-like peptide of A β tetramer.

'Mimotope' (mimic of epitope) is a peptide obtained from phage libraries by using an antibody as a mold, which has similar but different amino acid sequences from the original peptide. Since the mimotope is not

self, it breaks immune tolerance easily and elevated antibodies recognize antigens as the original antibody [86]. AD01 and AD02 are such mimotope vaccines, which are now in a clinical trial as Affitope (Table 3).

■ Non-A β peptide & adjuvant vaccine

Glatiramer acetate (Copolymer 1[®]) is now used for treatment of MS, in which autoimmune encephalitogenic T cells against myelin antigens are supposed to be pathogenic. Glatiramer acetate 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 glatiramer acetate and T cell adjuvant showed significant reduction of plaque amyloid in association with an increase of Th2 T cells [87]. It is speculated that glatiramer acetate-activated-Th2 T cells activate IGF1-producing CD11c⁺ microglia cells in the brain, which in turn clear amyloid deposits. Glatiramer acetate is proven safe in humans, so it seems to be easier to go to a human trial. However, tolerability may be a problem, because it must be injected subcutaneously every day for MS. Moreover, it is uncertain how long such a nonspecific strategy can keep affecting the specific pathological mechanism of AD.

To overcome the compliance problem, intranasal administration of glatiramer acetate with an adjuvant Protollin[™] (IVX-908) was invented, which effectively reduced amyloid burden in APP tg mice without inducing inflammatory change [88]. Protollin is an adjuvant composed of noncovalent formulation of outer membrane proteins (proteosomes) of *Neisseria meningitidis* and lipopolysaccharide from *Shigella flexneri*. Again, since this vaccine does not use A β , it is uncertain how long such a nonspecific vaccine continues to work.

■ DNA vaccine

A gene construct was made using an expression plasmid containing cDNA of monomer or dimer of A β ₁₋₄₂ under the control of SP72, a synthetic mammalian

cell-specific promoter, an α -antitrypsin leader sequence and an MHC class II-targeting sequence. Intradermal injections of gold particles coated with the construct were made into the ear of mice by a helium-driven gene gun three-times. Consequently, good immune responses to A β without activation of cytotoxic T cells were observed [89]. Later they changed the leader sequence to that of Adenovirus E3, and immunized APP^{sw} \times PS1 Δ E9 mice 15-times similarly. A β burden was significantly reduced in association with elevation of Th2 type antibodies [90]. 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, three-tandem repeat cDNAs of A β 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 β ₁₋₄₂ peptide vaccine mixed with adjuvant Quil A. The antibody responses in mice given the DNA vaccine showed a significantly higher ratio of IgG1/IgG2a than those in peptide-vaccinated mice (Table 4) [91]. Thus, intradermal injections of DNA vaccine seem to induce Th2 dominant immune responses.

A similar DNA vaccine was made using an expression plasmid pTarget carrying an Ig κ signal sequence and A β ₁₋₄₂ alone or A β ₁₋₄₂ with a fusion gene of Ig Fc. APP tg mice given the vaccine repeatedly into the muscle showed a significant reduction of A β burden without T-cell activation. However, antibody subtypes and cognitive functions were not examined [92]. Recently they reported the effect and safety of the vaccine in rhesus monkeys [93].

Movsesyan *et al.* made a pCMV DNA vaccine carrying an IP10 signal sequence, a gene encoding CCL22 chemokine, three repeat of A β ₁₋₁₁ and PADRE gene. This vaccine was given three-times into the shaved abdominal skin of mutant APP tg \times mutant PS1 tg \times 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.

Plaque amyloid was significantly reduced and cognitive functions were improved. In addition, insoluble A β ₄₀, A β ₄₂ and soluble A β oligomers (3- and 6-mers) were significantly reduced, but tau pathology was unchanged [94].

Since postmitotic muscle cells are poor for antigen presentation, a DNA vaccine carrying wild type A β ₁₋₄₂ and a mutant caspase gene was made. Expression of the mutant caspase is not enough to induce apoptosis of muscle cells but enough to induce apoptosomes, which enhances antigen presentation. TgCRND8 mice which carry APP^{sw} and APP^{INDIANA} mutations were repeatedly injected with the vaccine intramuscularly. They showed Th2 type antibody responses and reduction of amyloid plaques and insoluble A β ₄₂. Cerebral amyloid angiopathy and soluble A β oligomers tended to show reduction, but not significant [95].

■ Recombinant viral vectors

Recombinant adeno-associated virus vector carrying A β ₁₋₄₃ or A β ₁₋₂₁
We have developed recombinant adeno-associated virus vector (rAAV) carrying an APP signal sequence and A β ₁₋₄₃ or A β ₁₋₂₁ cDNA (Table 5) [96]. We used A β ₁₋₄₃ in order to differentiate it from native A β , because majority of native A β is composed of A β ₄₀ and A β ₄₂. Tg2576 mice were given orally once with 5×10^{11} viral genome of the rAAV using a naso-gastric tube at the age of 15-, 30- or 45-weeks, and their brains were examined at 56-weeks. The vaccinated mice showed elevated anti-A β antibodies for over 46 weeks and the subtypes were IgG1 and IgG2b. IgA antibodies were low and IgG2a antibodies were not detected. Plaque amyloid was significantly reduced in all vaccinated mice compared with phosphate buffered saline controls, and A β ₁₋₄₃ was not detected in the brain. The antibody titers were much lower in mice vaccinated with A β ₁₋₂₁ vaccine, but the effect was the same. Splenic T cells did not proliferate when stimulated with A β ₁₋₄₂ 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

Table 4. DNA vaccines.

DNA construct	Injection	Ref.
Plasmid-A β ₁₋₄₂ -MHC II TS	Intradermal	[89,90]
Plasmid-GAL4 activator + plasmid-GAL4 responder-3 \times A β ₁₋₄₂ -MHC II TS	Intradermal	[91]
Plasmid-A β ₁₋₄₂	Intramuscular	[92,93]
Plasmid-CCL22-3 \times A β ₁₋₁₁ -PADRE	Intradermal	[94]
Plasmid- A β ₁₋₄₂ -mutant caspase	Intramuscular	[95]

A β : Amyloid β ; CCL22: CCL22 chemokine; GAL4: β galactosidase 4; MHC II TS: Major histocompatibility complex class II targeting sequence; PADRE: pan-HLA-DR binding peptide.

Table 5. Recombinant viral vector vaccines.

Vector	Construct	Immunization	Ref.
AAV	A β ₁₋₄₃ or ₁₋₂₁	Oral	[96,100]
AAV	A β ₁₋₄₂ -CTB	Oral	[101]
SeV	A β ₁₋₄₃ -IL-10	Nasal	[HARA ET AL. UNPUBLISHED DATA]
AV	pCA-PEDI-11 \times A β ₁₋₆	Nasal	[102,103]
pHSV	A β ₁₋₄₂ -IL-4	Subcutaneous	[104]

A β : Amyloid β ; AAV: Adeno-associated virus; AV: Adenovirus; CTB: Cholera toxin B; PEDI: Receptor binding domain of *Pseudomonas* exotoxin A; pHSV: Herpes simplex virus amplicon; SeV: Sendai virus.

cytokines were examined using a set of over 30 conventional cytokine microbeads and Luminex[®] (Millipore), only TGF β -1 was significantly reduced [97]. TGF- β 1 enhances vascular amyloid deposits [98]. Reduction of TGF- β 1 is beneficial for autoimmune encephalitis [99]. Therefore, reduction of TGF- β 1 is an advantage of this vaccine.

The rAAV-A β ₁₋₄₃ vaccine was given similarly in Tg2576 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. Significant reduction of plaque amyloid was confirmed, and SDS-soluble and formic acid-soluble A β ₄₀ and A β ₄₂ were also significantly reduced in the vaccinated mice. They showed significant reduction of soluble oligomers, particularly of 9- and 12-mers [100].

The gut is the largest and the most efficient immune organ. Therefore, immune responses were easily obtained even in the elderly. As a matter of fact, one oral administrations of the vaccine was enough in mice. In addition, since the gut immune system is shifted to Th2, Th1-mediated autoimmune encephalitis is suppressed. Further, since gut epithelial cells are renewed in a few days, majority of the transfected genes are deleted quickly. The AAV vector is safe and transfected genes are retained in the episome. 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. This vaccine is also good for prevention. Thus, our vaccine fulfills most of the requirements for the next generation of A β vaccine. However, this is a gene therapy and regulation is high.

rAAV-A β ₁₋₄₂-cholera toxin B

A similar vaccine was reported from China [101]. However, their construct contains A β ₁₋₄₂ and cholera toxin B (CTB) as an adjuvant. In their construct, CTB was required to induce antibody responses, while we could see a good immune response to A β without CTB.

CTB is commonly used to elicit immune responses to orally given antigens. According to their construct, secreted A β with CTB in the gut lumen is also immunogenic. In this sense, they need not use AAV vector. It should be enough to give recombinant A β and CTB in enteric-dissolving coated capsules. This would be the same as food vaccine (see below). In addition, since they use A β ₁₋₄₂, vaccine-derived A β cannot be differentiated from native one.

A β ₁₋₄₃ - IL-10 in recombinant Sendai virus vector

We have also developed recombinant Sendai virus vector (rSeV) carrying A β ₁₋₄₃ and IL-10. Nasal administration of this vaccine induced significant reduction of senile plaques without inducing inflammatory changes in the brain and improved cognitive functions (manuscript submitted). Sendai virus induces common cold-like symptoms in murines, but it is not pathogenic in humans. Since it is an RNA virus, transgenes are not incorporated in nuclear DNA. A clinical trial using rSeV for arteriosclerosis obliterans is ongoing in Japan without any serious side effect, and rSeV/HIV vaccine will be tested in humans.

pCA-*Pseudomonas* exotoxin A-11 \times A β ₁₋₁₆ boosted with recombinant adenovirus carrying *Pseudomonas* exotoxin A-11 \times A β ₁₋₁₆

A plasmid DNA vaccine containing 11 tandem repeats of A β 1-16 cDNA and the receptor binding domain of *Pseudomonas* exotoxin A (PEDI) was given intranasally into APP tg \times PS1 tg mice twice, and then the mice were boosted nasally every 3 weeks for 10 months with rAV containing the same gene construct. The mice produced high titers of IgG1 and IgG2b antibodies to A β and plaque amyloid was reduced significantly. Splenocytes from the vaccinated mice produced a significant amount of IL-10, when stimulated with A β [102,103].

pHSV_{IE} A β ₁₋₄₂-CMV-IL-4

A recombinant herpes simplex virus amplicon was made, which carries A β ₁₋₄₂ gene under the control of herpes simplex virus immediate early promoter and *IL-4*

gene under the control of Cytomegalovirus immediate early promoter. 3X-Tg-AD mice were inoculated subcutaneously with 1×10^6 transduction units of the vaccine and were boosted with the same vaccine after 1 and 6 months. They described that the vaccinated mice developed antibodies to A β 42 and showed a significant reduction of plaque amyloid and phospho-tau, and improvement in the Barnes maze test [104]. However, antibody subtypes were IgG1, IgG2a, IgG2b and IgG3, and no specific pattern was shown.

■ Recombinant vegetable, recombinant bacteria & recombinant phage

Recombinant vegetables

Recombinant potato expressing five tandem repeats of A β ₁₋₄₂ was made, and Tg2576 mice were fed with 25 mg of the protein extract (= 22.5 μ g A β) and cholera toxin B once weekly for 3 weeks. The results showed elevated anti-A β antibodies and reduction of plaque amyloid [105]. Since we eat cooked potato, antigenicity of A β may be inactivated by heating. Therefore, recombinant tomato were produced. Mice fed with the recombinant tomato and adjuvant did not develop any anti-A β antibodies. However, mice injected with a small amount of A β peptide after feeding with tg tomato developed high titers of anti-A β antibodies, suggesting that mice acquired immune conditioning by eating A β -containing tomato (Table 6) [106].

Recombinant green pepper expressing A β was made using tobacco mosaic virus, and extract of the leaf was given orally to APP tg mice with cholera toxin B. Antibody to A β was elevated and amyloid burden was reduced [107].

Thus, in a certain condition orally given food extracts with an adequate adjuvant can break immune tolerance. However, taking recombinant food without an adjuvant is not enough to induce immune responses. When an adjuvant is added, immune responses to other molecules in food may also be elicited. In addition, it is difficult to determine the dose of recombinant food, and it is questioned how cholera toxin B is mixed with the recombinant food before eating? There are a number of natural products that have molecular mimicry to A β . One example is potato virus Y, which has high homology to A β , and antibodies to potato virus Y bind to A β [108]. Since potato virus Y is widely infected in potato and tomato, eating these infected vegetables may be useful. However, plant virus may not be able to infect in human tissues, the effect would be the same as food.

Recombinant bacteria

APP tg mice orally gavaged with recombinant *Salmonella typhimurium* expressing 4 copies of A β ₁₋₃₀ and tetanus toxin showed reduction of A β burden [109]. In this case, secreted A β from the bacteria may not be immunogenic. Instead, A β -expressing *Salmonella* phagocytosed by M-cells or dendritic cells seems to be immunogenic. Although the bacteria was attenuated, many animals died after vaccination. If this kind of strategies work, nonpathogenic bacteria such as *Lactobacillus* expressing A β in yogurt may be useful.

APP tg mice repeatedly inoculated intraperitoneally or nasally with 10^{10-11} of a recombinant filamentous phage expressing 10 copies of A β ₃₋₆ (EFRH) in protein III (pIII) or 300 copies in major coat protein VIII (pVIII) produced antibodies to A β , and showed significant reduction of plaque amyloid and improvement of the Morris water maze test [110,111]. The recombinant phage with high copy numbers of A β was much more efficient. The antibodies inhibited A β aggregation and A β -induced neurotoxicity. It is unknown whether the antibodies react with 3-pylo-glutaminy A β which is the major form of plaque amyloid in AD brain. Phage is a virus for *Escherichia coli*, exists ubiquitously on the earth and in the human body, and is said nontoxic to humans.

■ Miscellaneous

Adjuvant alone

Nasal administration of Protollin alone, a proteosome-based adjuvant activated brain microglia and reduced plaque amyloid [88]. This means nasally given adjuvant enters into the brain and gives a significant effect in the nervous system. However, activated microglia are not only good ones, but bad ones would be activated as well.

Juzen-taiho-to, an herbal medicine

Plaque amyloid is suggested to be phagocytosed mainly by bone marrow-derived microglia/macrophages [112]. Brain-derived microglia and bone marrow derived macrophages were activated *in vitro* and *in vivo* by Juzen-taiho-to, an herbal medicine and enhanced

Table 6. Recombinant vegetables, bacteria and phages.

Species	Construct	Immunogen	Route	Ref.
Potato	5×A β ₁₋₄₂	Protein extract + CT	Oral	[105]
Tomato	5×A β ₁₋₄₂	Soluble extract + CT	Oral	[106] [†]
Green pepper		Leaf extract + CT	Oral	[107]
Salmonella		Whole bacteria	Oral	[109]
Phage	pIII-A β ₃₋₆ pVIII-A β ₃₋₆	Whole bacteriophage	Intravenous, Intraperitoneal	[110,111]

[†]No immune response by oral soluble extract + CT. Booster with A β peptide required.
A β : Amyloid β ; CT: Cholera toxin.

phagocytosis of β -amyloid without enhancing nitric oxide production [113]. When Tg-2576 mice were treated with Juzen-taiho-to in drinking water, amyloid burden was significantly reduced [18]. This is not a vaccine, but it seems to have a vaccine-like effect in activating bone marrow-derived phagocytes in the brain.

Unmethylated DNA CpG motif

Unmethylated DNA CpG motifs activated microglia through Toll-like receptor 9 and attenuated AD pathology *in vitro* without inducing inflammatory cytokines and nitric oxide [114]. However, it must be given into the cerebral ventricles to have *in vivo* effect. Otherwise, it must be injected repeatedly intraperitoneally [115]. Further, CpG oligonucleotide is an adjuvant that activates Th1 T cells and induces autoimmune encephalitis in the presence of encephalitogenic autoantigens [116]. CpG induced antigen-specific proliferation of Th17 cells to the same extent as that by CFA, although encephalitogenic activity was much weaker than CFA [117]. Thus, safety must be examined carefully.

Memapsin 2

Tg2576 mice were immunized with memapsin 2 (β -secretase) and CFA, then with the antigen and incomplete Freund adjuvant, and later with the antigen alone. Both active immunization and passive immunization showed reduced memapsin 2 activity, reduction of plaque amyloid and plasma A β , and improvement of cognitive functions [118].

APP β -cleaving site

Immunization of mice with an APP cleaving site peptide produced β -secretase inhibiting antibodies. Tg2576 repeatedly injected with the antibodies showed reduction of brain inflammation, reduction of brain hemorrhage, and improvement of cognitive functions without changing brain A β [119].

Homocysteic acid-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 HA and intracellular A β . Active

and passive immunization targeting HA improved cognitive functions in Vitamin B6 deficient animals and 3X-Tg-AD mice [120].

Peptide vaccine using M-cell specific antibody

A peptide vaccine using an M-cell specific monoclonal antibody and cholera toxin was developed [121]. M-cells exist in the epithelial layer of the small intestine and take up bacterial flora and other food materials for immune surveillance. Oral administration of an M-cell-specific antibody conjugated with a peptide and an adjuvant efficiently activates gut immune system to the peptide. However, this technique has not been applied to A β , and a human M-cell specific monoclonal antibody has not been established yet.

Future perspective

As reviewed above, numerous strategies for immune-mediated prevention and treatment of AD have been established. It would be highly possible that some of these will be successful and applied in humans in the near future. Initially, polyclonal antibodies or safe active immunizations would be used. Once a specific targeting molecule is found, more specific approach such as monoclonal antibodies or more specifically targeted active immunizations will be established. Together with other strategies such as secretase inhibitors, inhibitors of A β aggregation, activators of A β degrading enzymes and others, prevention of AD or at least a method for significantly delaying onset of AD would be established.

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Executive summary

- Immunological strategies for treatment and prevention of Alzheimer's disease would be highly feasible.
- Initially, polyclonal antibodies or safe active immunization strategies would be established.
- Once targeting molecules were identified, more specific strategies, such as monoclonal antibodies or more specific active immunization would be used.
- Since A β was found in 1984 by Glenner *et al.*, our understand of Alzheimer's disease is rapidly advanced in the last quarter of century. It may not be able to conquer this devastating disease for decades, but at least some medicines to delay the disease onset and progression would be available in the near future as in other disorders, such as multiple sclerosis and diabetes mellitus.

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