ACTIVE IMMUNIZATION WITH NON-VIRAL DNA EPITOPE VACCINES
FOR ALZHEIMER DISEASE.
FUTURE STRATEGIES AND RESULTS.

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Abstract:

The Aβ peptide accumulation possesses fundamental role in the pathogenesis of Alzheimer disease (AD). The reduction effort of the brain amyloid burden through an antibody-medicated process, led the research to passive and active immunization practices aiming at the structural and functional rescue of the neurons in the AD patients brains. The use of the first peptidic vaccines gave beneficial results for the patients. However, their use has been stopped because of the undesirable aseptic meningoencephalitis appearance and the cerebral micro-hemorrhages intensification. Improved peptidic epitope vaccines are tested today in several clinical protocols so that the undesirable actions get limited. DNA epitope vaccines appear to assemble all the active immunization advantages without exhibiting undesirable actions, in the guinea-pigs, which they were tested in, up to date. These results remain to be proved in clinical trials so that these vaccines manage to constitute a safe and effective immune-therapeutical approach in AD.

1. **Introduction.**

The therapeutic treatment of Alzheimer disease (AD) was and also remains a big challenge for the world medical community. It is calculated that about 26 million people suffer from AD worldwide and the number is expected to get increased and reach the 35 millions the next 15 years [1].

The accumulation of both the amyloid peptides in the extracellular plaques and in the cerebrovascular depositions and the hyperphosphorylated or irregularly phosphorylated TAU protein in the intracellular neurofibrillary tangles, lead to neuroinflammation and dramatic loss of neurons in the cortex and in the hippocampus [2-3]. The inflammatory processes usually include effector mechanisms and innate immune response cells - activated microglial cells and astrocytes -, acute-inflammation phase proteins, chemokines, cytokines, complement components etc. [4]. In contrast with the intense activation of the local immune response, the systematic immune responses in AD are much less predominant.

It is clear that the interactions between the brain and the immune system are found to be in a continuous base [5]. In neuroinflammation conditions, inflammatory mediators from the C.N.S. can be delivered to the plasma and cause systematic immune responses, which, in turn, are able to influence the C.N.S. cells regardless of the local inflammatory reactions [6].

2. **Immune responses in AD.**

Researches in AD patients, describe altered lymphocytes subsets distribution in the blood, a general drop of the B and T lymphocytes and no change in the NK population [7].
These elements indicate weakening of the peripheral immunity function in the AD. It is important that at least some of the T cells are reactive to the Aβ peptide [8]. The activation of these cells by the Aβ was reduced in the AD patients [9]. An increased T cells reactivity was observed in the patients, after the use of a more immunogenic Aβ peptide. The cells role may be neuroprotective [10] via the neurotrophins secretion. However their intense activation may have neurotoxic effect and lead to the neuronal death [11].

The humoral immunity is regulated by soluble immune factors (acute-inflammation phase proteins, complement components, cytokines and other relative proteins). Most of them can be produced in the brain cells and also in the hemopoietic or other peripheral cells [12-13]. Their C.N.S. and plasma levels are in continuous communication and balance [14]. Aβ autoantibodies and autoreactive B lymphocytes are present in low levels in healthy individuals. It is important that autoantibodies against the toxic Aβ oligomers are found to be lower in AD patients in relation to the non-dementia control individuals [18-19]. On the contrary, the autoantibodies -which recognize Aβ monomers- quantity, did not manifest any difference in the two groups [15]. These natural autoantibodies are produced in absence of a concrete immunization and they target to the circulating products elimination, before they can extract a destructive response.

Studies in cytokines, chemokines, neurotrophic and growth factors and fractions of the complement in AD patients, have led to conclusions, which are frequently incompatible. Studies in genes level support that there is a total dysregulation of the gene expression in the mononuclear cells of the peripheral blood [16-17]. What is sure is that AD is accompanied by immune response changes and limited activation of the acquired immunity. Thus, the immunotherapeutical approaches in AD gain more and more ground in the past few years. Today, more than 10 clinical Aβ immunotherapy trials are under way in order to determine an effective antibody-mediated reduction of the brain amyloid burden, which is safe, so that we
achieve the structural and functional rescue of the neurons, the clinical stabilization of the patients and the inhibition or reduction of the dementia progress [20-21-22-23].

3. Active immunization in AD.

3.1 Peptidic vaccines in AD.

The significance of the active immunization in AD was imported by Schenck and his collaborators in 1999 [24], when he proved in transgenic AD mice that the vaccination with $A\beta_{42}$, which was formulated in complete Freund immunoadjuvant, extracted satisfactory titles of $A\beta$ antibodies that inhibited the amyloid plaques generation in young animals and they decreased $A\beta$ peptide from the depositions, which were found in the older laboratory animals. These results got confirmed by experiments to bigger mammal laboratory animals [25-26] and the preclinical trial with AD patients followed, which was interrupted when 6% of the vaccinated individuals manifested signs of aseptic meningoencephalitis [27]. This was attributed to the reactive T lymphocytes, which were specific for the T epitope of $A\beta$, and it was independent of the humoral immune response, since there was case of serious meningoencephalitis without detectable antibodies titles [28]. The Th1 pro-inflammatory response was connected with the immunoadjuvant presence or the reformulation of the vaccine [29-30]. This adverse outcome, including the fact of both the increased repercussion in the cerebral vascular $A\beta$ deposition [32] and the non-response of an important percentage of patients and especially of the elderly patients [33], led to the new strategies of active immunization essay [31]. This was because the autopsy findings in the vaccinated patients’ brains showed extensive regions of the cerebral cortex, which were free of amyloid plaques and, consequently, the vaccination was considered successful [34].

The understanding of the $A\beta$ peptide antigenic profile provided us with the potentiality to generate vaccines with altered peptide ligands. The $A\beta_{42}$ contains two T epitopes, one in
the carboxyterminal end (residues 29-42), one in the central region (residues 17-21) and a B epitope in the N-terminal end (residues 1-11) [35]. The elimination, replacement or the modification of the T epitopes, favour the humoral response and decrease the probability for a Th1 phenotype [36-37]. Nevertheless, the perforce of the immunoadjuvant utilization in the peptidic vaccines, does not exclude the possibility of the meningoencephalitis appearance.

3.2 DNA vaccines in AD.

The next step, for the active immunization in AD, was the DNA vaccination. It is simple, easily modified and it is obtainable without immunoadjuvant. The first DNA vaccines were based on the whole Aβ42 molecule. Their trial in the transgenic AD mice caused at the very best very low antibodies titles because of the immunoadjuvant non-utilization and the T lymphocytes immune tolerance towards Aβ, which occurs probably because of their long-term exposure to Aβ42 [9, 44-45-46]. Moreover, since this type of DNA vaccine contains the T epitopes of Aβ42, it cannot be considered safer in relation to the peptidic vaccine, in regard to the reactive autoimmune T cells production [8, 47]. The solution needed for the anti-Aβ B lymphocytes activation increase, which requires the necessary Th help for the Aβ antibodies production, with no autoimmune T cells production, seems to be provided by the DNA epitope vaccines. There are 2 types of DNA epitope vaccines: the emanating from virus and the plasmidic non-viral vaccines.

3.3 Viral DNA epitope vaccines in AD.

Initially, the DNA vaccines produced were using vectors emanating from the adenovirus (AAV) [38-39]. The results in the trasgenic AD mice models, were satisfactory. Just one vaccine administration caused extended and powerful production of Aβ antibodies, decreased the Aβ deposition and the astrocytosis in the brain and improved the memory [38]. However, their clinical application appears difficult, since a virus replication could not be
excluded and, additionally, the restrictions in the graduated increase of the AAV production [40], seriously limits the commercialization possibility and the use of the AAV vectors. On the contrary, the non-viral DNA vaccines [43] do not present any virus infection possibility [41-42], they can be massively produced and they have a low cost.

When the plasmids’ DNAs that code proteins are injected into the muscle, they are received by the muscular cells and they produce proteins in small quantity and for a relatively long time interval. These are released in the extracellular space and they induce the antibodies production [48-49].

3.4 Non-viral DNA epitope vaccines in AD.

DNA epitope vaccines, are genetically engineered, plasmids which include the B epitope sequence of Aβ, a promiscuous, foreign to Aβ, T cells epitope (PADRE) and the sequence of a molecular immunoadjuvant (e.g. the chemokine which is produced by the macrophages MDC/CCL22), something that causes powerful anti-Aβ antibodies responses via the recruitment of the anti-inflammatory CD4+ (Th2 -type) cells, which are specialized for the non-auto-antigen PADRE [50].

The manufacturing idea of these vaccines abuts on the results provided by the use of peptide epitope vaccines, which were constituted by the segment Aβ1-11 or Aβ1-15 with the T epitope PADRE [51]. Their utilization in the transgenic APP mice, gave us high Aβ antibodies titles without the reactive T cells production [52-53]. In regard to the problem of the obligatory use of a powerful conventional immunoadjuvant, something that may be inefficient, especially in elderly patients, but also in regard to practical issues, such as the difficulty of scaling up these vaccines in order to be clinically tested, the DNA epitope vaccines seem to be the answer.

Their use in the transgenic and wild-type mice induced high Aβ antibodies titles, as it
was expected. The response in the transgenic mice was considerably lower in relation to the wild-type mice one [54]. Supposing that there is a possibility for some antibodies to be bound to the Aβ peptide in the transgenic mice plasma but not in the wild type mice one, thereby resulting in the decreased antibodies titles, measurements of the Aβ antibodies have been conducted after the antigen-antibody complexes segregation. It appeared that the segregation did not increase the antibodies titles in the immune transgenic mice. Consequently, the difference in the antibodies concentrations after the vaccination in AD and wild type mice is not owed to the antibody binding to Aβ molecules, which circulate in the plasma. This difference is rather owed to a B cells tolerance to Aβ. The antibodies, which were produced, were IgG bound and they were naturally directed to the N-terminal end of Aβ (B epitope).

4. **Alzheimer disease and Aβ antibodies.**

4.1 **Aβ antibodies and amyloid pathology in AD.**

A crucial matter is the antibody transport beyond the blood-brain barrier (B.B.B.), since several of the proposed mechanisms of action (phagocytosis – plaques degradation), depend on the therapeutic concentrations presence in the brain [56-57].

Despite their high molecular mass, the IgG antibodies may pass through the B.B.B. in a percentage of 0.1-1% and they can bind to the β-amyloid fibrils, resulting in the very retarded percentages of IgG-Aβ interaction. Thus, in progress of time, small antibodies quantities which pass through the B.B.B., may be accumulated and decrease the cerebral concentrations. Alternatively, the peripheral mechanisms of action, as it is anticipated by the “sink” hypothesis, do not require the presence in the C.N.S. [55].

The antibodies effectiveness in the amyloid pathology of the brain was checked and confirmed by the plaques measurement in both the transgenic and control mice brains. On the whole, the results showed an effective reduction not only of the plaques amyloid burden but
also of the scattered one [58], in the brains of the vaccinated APP mice in relation to the not vaccinated. A critical query about the effectiveness of the vaccines, regards the differences that go for the preservative and the therapeutic vaccination. Thus, transgenic AD mice were vaccinated 5 months after the amyloid pathology appearance. The results exhibited a reduction of amyloid plaques in both the cortex and the hippocampus. However, the reduction was not as great as the one that occurred due to the preservative vaccination [59-60] (fig.1). Even if the preservative protocol appeared to be more effective than the therapeutic one, nevertheless, the effect of the vaccines, which were administered after the amyloid depositions appearance, is considered satisfactory. A potential problem of the immunotherapy could be the increase of the soluble toxic Aβ oligomers due to the insoluble Aβ reduction [61]. The examination of the Aβ oligomers reactivity toward the antibody showed a reduction of these forms in relation to the control mice. Consequently, the amyloid burden reduction regards both the fibrillar Aβ of the plaques and the toxic oligomers.

![Fig.1. Amyloid plaques depiction in the brain of the 18-month-old transgenic AD mice. Left: without vaccination, Right: after DNA vaccination. The clear reduction of amyloid plaques is distinguished in the immunized guinea-pigs.](image)

**Source:** Yoshio Okura and Yoh Matsumoto, “DNA Vaccine Therapy for Alzheimer’s Disease: Present Status and Future Direction”, REJUVENATION RESEARCH Volume 11, Number 2, 2008 DOI: 10.1089/rej.2007.0638.
4.2 Aβ antibodies and TAU formations.

May the amyloid pathology be ahead of the endogenous neurofibrillar formations - constituted by hyperphosphorylated or irregularly phosphorylated TAU protein - generation, however, the greatness of the dementia is related with the neurofibrillary tangles and not with the amyloid plaques [62]. The Aβ antibodies do not seem to affect the TAU formations. Measurements in the early, middle and late stages of the hyperphosphorylated TAU showed a reduction of the early forms of the TAU formations, something that does not appear to occur due to the Aβ antibodies and TAU protein direct reaction, but due to the Aβ pathology reduction that, as we know, accelerates the neurofibrillary tangles formation [63-64-65]. The improvement of the TAU pathology is owed in the amyloid burden reduction and concerns the early formations and not the already formed neurofibrillary tangles [66].

5. Mechanisms of Aβ antibodies action in AD.

5.1 Aβ antibodies and microglial activation.

Exploring the potential mechanisms of the amyloid burden reduction after the vaccination, the neuroglial activation and the lymphocytic infiltration was studied. Quantitative image analysis of the transgenic mice brain tissues after the vaccination, exhibited a reduction of both the astrocytosis and microglial activation, because of both the amyloid plaques and the neuroinflammation reduction. The microglia increase in the periplaque and the more distant regions, after the transgenic mice vaccination, appears to be in contrast with these observations, while the microglia even converted its form to ameboid type, exhibiting activation. More concretely, in the non-vaccinated transgenic mice, activated microglia was measured around the amyloid plaques. After the DNA vaccination, microglia was increased in both the periplaque regions and the distant, demonstrating explicit ameboid phenotype [67]. By labeling both the Aβ depositions and the microglia, the increased
phagocytosis, occurring after the vaccination, was confirmed, via the Fc receptor (fig.2). It is important that the activated microglia increase in the distant regions suggests the phagocytosis of the oligomers Aβ peptides, which are mainly responsible for the synaptotoxicity in AD (fig.3).

Microglia is considered to play a role either in neuroprotection or neurocatastrophic. The measurement of the levels of the TNF-α, which is a cytotoxic cytokine, was used as an indicator of the activated microglia nature characterization. In the non-transgenic wild-type mice, TNF-α was almost zero in the brain [68]. After the DNA vaccination, the levels were slightly increased but this increase was not statistically important in relation to the one which was caused after the vaccination in the transgenic mice models. This means that the amyloid burden reduction, occurring via the activated microglia Fc phagocytosis, appears to be an important Aβ reduction pathway and the nature of the activated microglia is neuroprotective. The hypothesis that the Aβ-IgG immune complexes probably bind to Fc receptors of the microglial cells, resulting in the release of inflammatory mediators and the pre-existing neuroinflammation deterioration in the AD brain, appears not to affect the vaccination with DNA epitope vaccines.

Fig.2  A) Labeled amyloid plaque (green color). B) Labeled microglial cell (red color). C) Characteristic picture of a microglial cell in the interior of which appears to be contained the amyloid plaque which has been phagocytosed and is presented in the picture as light-colored orange blot.

Fig. 3 A) Microglial cells around an amyloid plaque in the brain of a transgenic non-immunized AD mouse. C) After DNA vaccination microglia proliferation is appearing around the amyloid plaque and the characteristic microglia projection in the plaque. B) Microglial cells in a brain region of a transgenic non-immunized AD mouse far from amyloid plaque. The thin microglia distribution is characteristically distinguished. D) After DNA vaccination in the same region of the brain the microglial cells increase is distinguished, which are distributed more densely and exhibit an amoeboid phenotype.


Lymphocytic infiltrations and inflammatory reactions occurring due to the autoimmune T lymphocytes production were not observed as it was expected, neither in the transgenic nor in the wild type mice, even after long-lasting DNA vaccination. Study of brains of the vaccinated AD mice model by using monoclonal antibodies against both T lymphocytes (CD5) and macrophages (MAC-3), did not exhibit any autoimmune type inflammatory reaction. The anti-inflammatory Th2 cellular reaction was confirmed by measurements of both interferon-γ (IFN-γ) (Th1 phenotype) and IL-4 (Th2 phenotype), which showed that the cellular anti-PADRE immune response, providing the necessary help to B lymphocytes for antibodies production, was polarized to a Th2 phenotype. Moreover, in both
the vaccinated non-transgenic and the transgenic mice, the isotype of the produced IgG antibodies, which avails on the indirect indication of the Th2 cytokines contribution (IgG1) against Th1 cytokines (IgG2α), was consistent with the anti-inflammatory Th2-anti-PADRE cellular response [69].

![Diagram](image)

**Fig 4** Schematic representation of the antibodies action on Aβ monomers, oligomers and amyloid plaques. The mechanisms of: a) microglial activation and phagocytosis, b) the direct Aβ - detachment and removal from the amyloid plaques, c) the increased Aβ peptide efflux to the periphery according to the “peripheral sink” hypothesis, are distinguished.


**5.2 Aβ antibodies and amyloid Congophilic angiopathy.**

The amyloid plaques clearance, occurring by Aβ antibodies, may happen by the antibody binding on the target epitopes in the β-amyloid fibrils and, afterwards, by the direct segregation and immune complexes removal from the cerebral tissue [70-71]. The increased Aβ removal rate increases the aggravation probability of a pre-existing Congophilic amyloid angiopathy because of the deposition and the Aβ accumulation in the blood-vessels walls, a
matter which causes micro-haemorrhages [72]. In order to investigate whether Aβ antibodies separate and remove Aβ from the amyloid plaques, the immune-reactivity of the plaques was measured in vaccinated and non-vaccinated transgenic AD mice, using plasma, which was taken by the laboratory animal themselves. The immune-reactivity was found slightly increased in the immunized laboratory animals. The difference between the non-vaccinated and vaccinated mice was statistically non-important. These findings suggest that the direct segregation mechanism of Aβ from the amyloid plaques fibrils in the form of Aβ-IgG immune complexes, is not dominant in the amyloid burden clearance from AD brains. Moreover, the micro-haemorrhages detection in the neocortex, leptomeningeal, hippocampic and thalamic regions of the immunized mice brain, did not manifest any increase in relation to the non-vaccinated control models. DNA epitope vaccines do not cause micro-haemorrhages, since they do not intensify Congophilic amyloid angiopathy and of course they do not produce perivascular lymphocytic infiltrations by autoimmune reactive T-lymphocytes.

### 5.3 Peripheral sink hypothesis.

It is known that there is a balance between the Aβ\text{42} level in the periphery and its deposits in the brain. According to the “peripheral sink” hypothesis a reduction of the Aβ\text{42} level in the periphery, occurring because of the Aβ\text{42} binding to Aβ antibodies, will cause an increased peptide efflux from the brain to the periphery, so that the balance is maintained [55]. We know that proteins may leave from the C.N.S. and pass through the B.B.B. by active transport systems. It has been suggested that the perivascular drainage to the subarachnoid space and the CSF (cerebrospinal fluid) plays a role in the Aβ clearance in AD and in the cerebral amyloid angiopathy development. This efflux is also facilitated via the IgG binding ability on the Fc receptor (FcRn) in the endothelial cells albuminic side, which constitutes the B.B.B. As a result, an amyloid burden reduction of the brain emerges. Measurements of the Aβ\text{42} levels in the plasma of both vaccinated and non-vaccinated transgenic AD mice models,
showed a relatively high Aβ_{42} increase in the immunized laboratory animals in relation to the control laboratory animals [73]. This difference was decreased in the older animals’ next measurements. The results show that the hypothesis of the “peripheral sink” theory works after DNA vaccination, however it has a complementary character and Fc phagocytosis via the activated microglia, appears to be the most important pathway of the amyloid burden reduction in AD brains.

The antibodies binding on the Aβ peptide in the blood disturbs the balance between the Aβ deposits in the brain and its levels in the peripheral blood. This leads to an increased Aβ peptide efflux from the brain to the periphery and to the amyloid burden reduction in the central nervous system. The hypothesis, as we said above, is known as a “peripheral sink” mechanism [55] (fig.4).

**5.4 Memory improvement after DNA vaccination.**

It is known, through the study of the patients with familial AD, that a small reduction of the amyloid burden, transfers the disease symptoms several decades later. It was experimentally proved, that a 50% reduction of the amyloid burden restored the cognitive disturbance [74]. Using the DNA epitope vaccine in AD transgenic mice, it was found that both the short-term and long-term spatial memory in the vaccinated laboratory animals was improved in relation to the non-vaccinated transgenic laboratory control animals.

The current interest for the immune-therapy in AD is based on the amyloid cascade hypothesis and on the fact that the Aβ antibody may prevent the accumulation and it may increase the Aβ depositions clearance in the brains of the transgenic AD mice models. The presence of high antibodies titles was connected with the amyloid pathology reduction and the spatial learning improvement in the vaccinated transgenic mice. The cerebral inflammation appearance because of the auto-reactive T cells infiltration at the clinical trial of the peptidic
Aβ42 vaccine, led to new strategies of the active immunization.

6. **Conclusions and prospects.**

In the past few years, the non-viral DNA epitope vaccines technology gains more and more scientific interest, because of the advantages assembled. The facility of the production, the high stability and the genes modification potentiality in order to target an antigen with precision, by choosing the suitable anti-inflammatory immune response at the same time, gives a hopeful character to this active immunization type [75].

The beneficial results of the DNA epitope vaccine use in AD result from the Aβ42 peptide reduction of both the fibrillar and the toxic soluble oligomers. The attractive characteristic of the vaccine is, that it includes as a molecular immune-adjuvant, a mediatory substance of the inflammation, which polarizes the immune response to an anti-inflammatory Th2 phenotype. The amyloid burden reduction leads to less activated microglia and astrocytosis and to neuro-inflammation alleviation. The nature of the activated microglia is neuroprotective and the amyloid pathology reduction leads to the early TAU pathology clearance. The vaccination does not increase the cerebral micro-haemorrhages repercussion, it does not cause the reactive T cells production and it improves the behaviouristic disturbances.

In conclusion, the DNA epitope vaccines are a simple and efficient DNA vaccine strategy, which uses the B epitope of Aβ, a foreign promiscuous T epitope (PADRE) and a molecular immune-adjuvant. This vaccine is likely to be effective and safe in humans because: 1) it causes the specialized Aβ antibodies production in satisfactory titles without the autoreactive T lymphocytes production, 2) it uses a promiscuous T epitope which is effective in the human population, 3) it uses a molecular immune-adjuvant which activates the Th2 anti-inflammatory cells, specialized for the T epitope that is not expressed in the human brain.

The non-viral DNA epitope vaccines remain to be tested in bigger mammal-
laboratory animals, so that the effective humoral immunity without unfavourable side effects is confirmed, and that the vaccines manage to proceed in human clinical trials as a hopeful immune-therapy against AD.

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