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Abeta DNA Vaccination for Alzheimer's Disease: Focus on Disease Prevention

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Abstract

Pre-clinical and clinical data suggest that the development of a safe and effective anti-amyloid-beta (A β) immunotherapy for Alzheimer's disease (AD) will require therapeutic levels of anti-A β antibodies, while avoiding proinflammatory adjuvants and autoreactive T cells which may increase the incidence of adverse events in the elderly population targeted to receive immunotherapy. The first active immunization clinical trial with AN1792 in AD patients was halted when a subset of patients developed aseptic meningoencephalitis. The first passive immunotherapy trial with bapineuzumab, a humanized monoclonal antibody against the end terminus of A β , also encountered some dose-dependent adverse events during the Phase II portion of the study, vasogenic edema in 12 cases, which were significantly over represented in ApoE4 carriers. The proposed remedy is to treat future patients with lower doses, particularly in the ApoE4 carriers. Currently there are at least five ongoing anti-A β immunotherapy clinical trials. Three of the clinical trials use humanized monoclonal antibodies, which are expensive and require repeated dosing to maintain therapeutic levels of the antibodies in the patient. However, in the event of an adverse response to the passive therapy antibody delivery can simply be halted, which may provide a resolution to the problem. Because at this point we cannot readily identify individuals in the preclinical or prodromal stages of AD pathogenesis, passive immunotherapy is reserved for those that already have clinical symptoms. Unfortunately those individuals have by that point accumulated substantial neuropathology in affected regions of the brain. Moreover, if A β pathology drives tau pathology as reported in several transgenic animal models, and once established if tau pathology can become self propagating, then early intervention with anti-A β immunotherapy may be critical for favorable clinical outcomes. On the other hand, active immunization has several significant advantages, including lower cost and the typical immunization protocol should be much less intrusive to the patient relative to passive therapy. However in the advent of A β -antibody immune complex-induced adverse events the patients will have to receive immuno-suppressive therapy for an extended period until the anti-A β antibody levels drop naturally as the effect of the vaccine decays over time. Obviously, improvements in vaccine design are needed to improve both the safety, as well as the efficacy of anti-A β immunotherapy. The focus of this review is on the advantages of DNA vaccination for anti-A β immunotherapy, and the major hurdles, such as immunosenescence, selection of appropriate molecular adjuvants, universal T cell epitopes, and possibly a polyepitope design based on utilizing existing memory T cells in the general population that were generated in response to childhood or seasonal vaccines, as well as various infections. Ultimately, we believe that the further refinement of our AD DNA epitope vaccines, possibly combined with a prime boost

regime will facilitate translation to human clinical trials in either very early AD, or preferably in preclinical stage individuals identified by validated AD biomarkers.

Keywords

Immunotherapy; AN1792; polysorbate-80; bapineuzumab; immuno-conjugate; immunosenescence; thymic involution; PADRE; molecular adjuvant; macrophage-derived chemokine; T cell polyepitope; prime boost; electroporation

AMYLOID-BETA AS A THERAPEUTIC TARGET IN ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common form of dementia in the elderly and is characterized clinically by an insidious onset and progressive cognitive decline that impacts memory, language, judgment, and orientation to time and space. It is estimated that there are currently about 18 million people worldwide with AD. This number is projected to nearly double by 2025 to 34 million. The neuropathological features of the disease include neurofibrillary tangles, deposition of amyloid- β (A β) in senile plaques, and neuronal cell loss in affected regions of the central nervous system (CNS) [1]. These pathological changes result in a profound loss of synapses over the course of the disease, thereby contributing to a progressive reduction in the functional capacity of the patient. A critical aim in developing therapeutic interventions for AD was the identification of suitable targets. The A β peptide is cleaved from the amyloid precursor protein (APP) by β - and γ -secretases [2–4] and is thought to play a central role in the onset and progression of AD, which has led to the amyloid cascade hypothesis [5–7]. Accordingly, many therapies for AD are aimed at reducing the level of A β in the brain and/or blocking assembly of the peptide into pathological forms that disrupt cognitive function [8, 9]. Currently, there are no effective treatments for AD. Therefore new therapeutic approaches for treating AD are essential. Anti-A β immunotherapy represents a potentially powerful strategy for reducing pathological forms of A β in the brains of AD patients [10–20].

THE POTENTIAL OF IMMUNOTHERAPY TO REDUCE A β IN THE CNS OF APP-TRANSGENIC MICE

In 1999, Schenk and colleagues reported that a vaccine formulation that contained fibrillar A β_{42} as the immunogen and Freund's adjuvant system when injected into APP-transgenic (Tg) mice prevented the deposition of amyloid plaques in the brain, as well as development of dystrophic neurites and astrogliosis. Other researchers have reproduced and extended the original findings to include studies showing that active A β immunization with different peptide immunogens [21–27], adjuvants [28–30], and routes of administration [30, 31] can enhance production of therapeutic anti-A β antibodies, and that anti-A β antibodies can attenuate the behavioral deficits that develop in APP-Tg mice as they accumulate A β with aging [32, 33]. Studies of peripheral (passive immunization) administration of anti-A β antibodies showed the presence of anti-A β antibodies in the brain [33, 34] and reduction of cerebral A β load if given before robust plaque deposition. Passive immunization improves behavior in APP-Tg mice, even in very old APP-Tg mice with extensive cerebral A β loads [35–38]. These results clearly demonstrated that anti-A β -specific antibodies are sufficient for clearing amyloid deposits from the brains of APP-Tg mice. The only adverse response to passive immunization observed in APP-Tg mice was the occurrence of microhemorrhages within the cerebral vasculature of very old APP-Tg mice injected weekly with high doses of anti-A β monoclonal antibody [39]. Interestingly, the sites of anti-A β antibody-induced microhemorrhages co-localized with cerebral vascular deposits of A β [38–41]. More

recently, microhemorrhages have been reported in very old actively immunized APP-Tg mice as well [27, 42].

INSIGHTS FROM THE FIRST IMMUNOTHERAPY CLINICAL TRIAL IN AD PATIENTS (AN1792)

Based on the impressive results in APP-Tg mice, and the lack of adverse autoimmune-type reactions to AN1792 in several large animal models, Elan Inc. and Wyeth Inc. began a clinical trial on AD patients with their AN1792 vaccine, which consisted of fibrillar A β ₄₂ as the immunogen, and QS21, a strongly Th1-polarizing adjuvant [28]. Although results from the Phase I trial showed good tolerability of the vaccine, the overall response to the AN1792 vaccine in this elderly cohort was rather poor. For the phase IIa portion of the trial the AN1792 vaccine was reformulated to include polysorbate-80. Additional injections with the same AN1792, plus polysorbate-80, increased the number of antibody responders up to 58.8%. Ultimately, the phase IIa portion of the AN1792 immunotherapy vaccine trial was halted when a subset (6%) of individuals immunized with the AN1792 vaccine developed adverse events (aseptic meningoencephalitis) in the CNS [43–50]. Importantly, only vaccinated participants (n=18) developed meningoencephalitis, whereas none of the control patients (n=72) injected with placebo developed adverse events [44]. Although 13 of these 18 patients were antibody responders, the remaining five patients did not show significant titers of antibodies and did develop meningoencephalitis. Postmortem examination of the brains from two vaccinated AD patients with neuroinflammation [43, 45] showed an infiltration of T cells in the leptomeninges, densest in the areas with amyloid angiopathy. There was also sparse T cell involvement in the cerebral cortex, perivascular spaces and within the parenchyma. A third case report on an immunized AD patient that did not clinical symptoms of encephalitis also reported some lymphocytic infiltration in the leptomeninges [46]. These results suggest that anti-A β -specific T cells may induce significant side effects in AD patients vaccinated with full-length A β ₄₂. In fact, the low to moderate titers of anti-A β antibodies generated in a subset of immunized AN1792 patients were capable of reducing parenchymal amyloid pathology [43, 45, 46, 49–51].

However, analysis of total brain A β , as measured by immunoassay, revealed that total soluble amyloid levels were significantly elevated in vaccinated patient brains compared with non-immunized AD cases [49]. Finally, researchers from Wyeth Research have attempted to identify a possible causative factor in the development of the AN1792-induced meningoencephalitis [52]. They took peripheral blood mononuclear cells obtained from patients who received original AN1792, as well as patients that received the re-formulated vaccine, and re-stimulated the cells *in vitro* with A β . Interestingly, the A β -induced T-cell responses from patients' cells in an earlier multiple dose study (original vaccine) were anti-inflammatory Th2 biased, whereas those from the Phase II (+ polysorbate 80) were biased toward a proinflammatory Th1 response. They speculated that the addition of polysorbate 80 to the AN1792 vaccine formulation used in Phase IIa (Study 201) was the most likely culprit in the development of the adverse events that caused the cessation of the AN1792 clinical trial [52]. Although polysorbate 80 is used ubiquitously as solubilizing agent in many commercial available products and medicines and was generally considered to be inert, recent reports have found that Polysorbate 80 can cause severe nonimmunologic anaphylactoid reactions [53, 54]. Finally, an upsurge in the incidence of antibody-mediated pure red cell aplasia (PRCA) among patients taking one particular formulation of recombinant human erythropoietin (epoetin-alpha, marketed as Eprex(R)/Erypo(R); Johnson & Johnson) in Europe caused widespread concern. The PRCA upsurge coincided with removal of human serum albumin from epoetin-alpha in 1998 and its replacement with glycine and polysorbate 80. Although they mentioned that the “immunogenic potential of

this particular product may have been enhanced by the way the product was stored, handled and administered, it should be noted that the subcutaneous route of administration does not confer immunogenicity per se” [55]. The possible role of micelle (polysorbate 80 plus epoetin-alpha) formation in the PRCA upsurge with Eprex is currently being investigated [55]. Considering all of the information provided above, the reformulation of the AN1792 vaccine to include polysorbate 80 was probably the determining factor in triggering the adverse events that caused the clinical trial to fail.

Additional problems that plagued the AN1792 trial were the low number of positive responders (i.e., reach trial target antibody titer) and the generally low titers. In all fairness, many of the patients in the clinical trial did not receive the full course of immunizations proposed in the AN1792 protocol. Moreover, based on results from the recent passive immunotherapy clinical trial (AAB-001. Therapeutic Applications: Mild to moderate Alzheimer disease) using bapineuzumab, a humanized monoclonal antibody against the end terminus of A β , higher titers may increase the risk of adverse events [56]. During the Phase II study with bapineuzumab 12 cases of vasogenic edema were reported. The incidence of vasogenic edema was dose-dependent (8 of the 12 cases were reported in the highest dose group), and ApoE4 carriers were over-represented (10 out of 12 cases) (Alzheimer’s Research Forum: DRUGS IN CLINICAL TRIALS. October 22, 2009).

Another concern regarding the AN1792 clinical trial was the selection of QS21 as the adjuvant. While QS21 is a powerful adjuvant, the risk of adverse events due to immunization with strong Th1-type adjuvant (QS21) in an elderly population in which there is already substantial parenchymal and cerebrovascular inflammation needs to be considered. In particular, the meningeal and cortical blood vessels in many AD patients have activated perivascular macrophages and astrocytes, which will become further activated by strong peripheral inflammatory events [57–60]. There is even evidence that systemic inflammation that produces elevated levels of interleukin-1 β in the CNS can contribute to cognitive decline in AD [61].

However, not all data from the AN1792 clinical trial were negative. A recent report on a long-term, 4.6 years after initiation of the AN1792 study, followed of patients immunized with AN1792. Patients originally defined as positive responders in the phase IIa study maintained low but detectable levels of anti-A β antibodies, and demonstrated significantly reduced functional decline compared with placebo-treated patients. Brain volume loss in antibody responders was not significantly different from placebo-treated patients approximately 3.6 years after the end of the clinical trial. The author’s interpreted these results as support for the hypothesis that anti-A β immunotherapy may have long-term functional benefits in AD patients [62].

Obviously, improvements in vaccine design are needed to improve both the safety as well as the efficacy of anti-A β immunotherapy. The remainder of this review will focus on the advantages of DNA vaccination for anti-A β immunotherapy, and the major hurdles, such as immunosenescence, selection of appropriate adjuvants and T cell epitopes, that need to be overcome for active immunization approaches to move forward in the clinic to treat AD patients.

IMMUNOSENESCENCE IN THE ELDERLY PRESENTS PROBLEMS FOR ANTI-A β IMMUNOTHERAPY

A potentially major obstacle for developing an active vaccination approach for AD patients is immunosenescence in the elderly that contributes to the poor outcome of vaccination and increased susceptibility of the elderly to infectious disease [63–65]. Currently, defects in T

cells appear to be the primary cause of the reduced vaccine efficacy in the elderly. During normal aging there is a decline in the number of naïve T cells, cells capable of responding to new antigens, in favor of T cells with a memory phenotype [63]. A major contributor to this age-related phenomenon is thymic involution, which retards T cell development and inhibits migration of naïve T cells to the periphery [66]. The lack of a sufficient T helper cell activation in the elderly in response to active immunization typically results in diminished B cell proliferation and low titers of antibody against the B cell epitope in the vaccine immunogen. In fact immunosenescence was probably a significant factor in the low number of responders and relatively low anti-A β antibody titers in the elderly patients that received the AN1792 vaccine, and may also have been a factor in the selection of QS21 as the adjuvant in an attempt to overcome the effects of immunosenescence in the patient cohort recruited for that clinical trial.

FOCUSING ON DISEASE PREVENTION BY USING EARLY INTERVENTION MAY IMPROVE IMMUNOTHERAPY EFFICACY AND REDUCE THE INCIDENCE OF ADVERSE EVENTS

One possible solution to enhance the anti-A β response to active immunization would be to immunize at an earlier age before immunosenescence becomes a significant problem. However, for this to occur active anti-A β immunization protocols will have to show excellent safety profiles. Moreover, if anti-A β immunotherapy does show some clinical benefit, as suggested in both the long-term follow-up of the AN1792 trial and the more recent passive bapineuzumab clinical trial in mild cognitive impairment or mild to moderate AD patients, and the recent reports on putative brain imaging methodologies, cerebrospinal fluid and blood biochemical biomarkers for AD become validated then selection of individuals to receive anti-A β immunization can improve the risk-to-reward profile of the patient population. Significant therapeutic benefits of early immunization may include attenuating A β deposition, thereby possibly attenuating tau hyperphosphorylation before tau pathology becomes self-propagating in the CNS. A second potential benefit of early immunization, based on immunotherapy studies in young versus old APP-Tg mice, may be a reduced incidence of adverse events in the cerebral vasculature. Alternatively, boosting the health of the thymus *via* caloric restriction, interleukin-7, growth hormone, or keratinocyte growth factor (also known as FGF7) have been proposed as possible therapies to rejuvenate the thymus thus increasing the population of naïve T cells, which may improve T cell response to new antigens in the elderly. This approach may at least partially reduce the need for powerful adjuvants to induce an adequate immune response in the elderly [66, 67].

Another possible strategy to counteract immunosenescence is to recruit previously generated memory T cells produced during childhood vaccination or prior exposure to human pathogens. Thus the majority of people already possess a broad panel of specific memory T cells. Vaccination of these people with an epitope vaccine composed of a therapeutic B cell epitope of A β peptide and Th cell epitopes from conventional vaccines may induce the pre-existing memory T cells in the elderly to expand rapidly and differentiate into effector T cells, which may lead to a more robust anti-A β antibody response. Tetanus toxin (TT), hepatitis B virus (HBV), and influenza virus are examples of such conventional vaccines where there is widespread T helper memory in the human population, and some of the T helper epitopes in these pathogens are recognized by multiple MHC class II haplotypes, in some cases approaching universal epitope status. The value of these universal/promiscuous T helper epitopes is that almost everyone should have memory CD4⁺ T cells specific for epitopes in TT, HBV, and conserved epitopes in influenza. Thus, a booster injection with an epitope vaccine containing these conventional Th epitopes along with a strong Th2 molecular adjuvant should be enough for rapid activation of pre-existing anti-TT, anti-HBV

and anti-influenza memory Th cells, hopefully resulting in induction of potent anti-A β antibody production in the elderly.

THE ADJUVANT PROBLEM FOR ANTI-A β IMMUNOTHERAPY IN ELDERLY AD PATIENTS

The issue of selecting an appropriate adjuvant to amplify the immune response to produce therapeutic levels of anti-A β antibodies in the elderly without triggering unacceptable adverse events is complicated by several factors. The first is that currently alum is the only approved adjuvant for human vaccines, and while alum is known to be good at inducing antibodies, it is not considered a very potent adjuvant, which may restrict its usefulness in an elderly population [68]. Interestingly, we found alum to be quite an effective adjuvant for inducing therapeutic levels of anti-A β antibodies, as measured by a reduction amyloid plaque load in all of the elderly canines that were immunized with fibrillar A β bound to alum [69]. Importantly, adjuvants can control the type of immune response generated against A β -containing immunogens to alter the response toward either a Th1 (proinflammatory) [52] or Th2 (anti-inflammatory) [28, 30, 70–74], which may be important in avoiding adverse events caused by elevated levels of various cytokines and chemokines, as well as the isotype of the antibodies that are generated in response to the vaccine immunogen. The isotype of the antibody determines the type of effector functions, such as activation of complement or Fc-receptor mediated activation of macrophages, that can be induced when the antibody binds its' target antigen and forms an immune complex.

Although researchers are making excellent progress in understanding how adjuvants work to amplify the immune response to vaccine immunogens, and many companies have developed adjuvants with commercial and therapeutic potential, getting new adjuvants through U.S. Food and Drug Administration approval process has proved to be quite difficult. Therefore clinical trials often include both an experimental immunogen, as well as experimental adjuvant in the vaccine formulation. For example, Elan and Wyeth selected QS21 as the adjuvant for the AN1792 clinical trial, and QS21 was also selected for Elan, Johnson & Johnson, and Wyeth's current active immunization clinical trial (ACC-001), a novel A β immuno-conjugate that draws on conjugate technology that Wyeth has used previously. An A β fragment is attached to a carrier protein intended to help induce an antibody response against A β . In the fall of 2005, Elan and Wyeth began Phase I dosing patients with the active A β immunotherapeutic conjugate, and are currently enrolling patients for Phase II (Alzheimer's Research Forum: DRUGS IN CLINICAL TRIALS. Dec 10, 2008).

ADVANTAGES OF DNA VACCINES OVER CONVENTIONAL PEPTIDE/ PROTEIN VACCINES

DNA-based vaccination utilizes direct injection of plasmid DNA encoding genes for protein or peptide antigens. The facilitated uptake of the DNA by cells that then express the antigen, which when encountered by cells of the immune system provokes an immune response against expressed antigen [75–77]. DNA vaccination is currently being used for the development of vaccines against a variety of pathogens, as well as for human diseases including cancer, autoimmune disorders, and AD. A unique property of DNA-based vaccination is the ability to induce prolonged, endogenous antigen synthesis and processing within the immunized host own cells. DNA immunization has been shown to generate protective humoral and cellular immune responses against viral, tumor, and foreign antigens [78–85]. Among many significant advantages of DNA immunization are less complicated technologies of production, high stability, the capability to modify genes encoding the desired antigen/s, the ability to make changes in the cellular localization of an antigen by

means of adding or removing signal sequences or transmembrane domains; and the ability to target the desired type of immune response to enhance the therapeutic potential of the immune response. The immune response to DNA immunization can be enhanced by using molecular adjuvants (immune modulators), such as cytokines in conjunction with the immunogen, which can direct the T helper cell toward the desired pathway.

DESIGNING EPITOPE VACCINES COMPOSED OF THE N TERMINAL REGION OF A β ₄₂ AND FOREIGN PROMISCUOUS T CELL EPITOPE

Our first DNA construct to incorporate a molecular adjuvant into the design of the anti-A β DNA epitope vaccine contained interleukin-4 (IL4). We generated a gene encoding human A β ₄₂ fused with murine IL4 (pA β ₄₂-IL4). Immunization of B6SJLF1 mice with this construct (gene gun bombardment) generated predominantly IgG1 and IgG2b anti-A β ₄₂ antibodies [86]. Both isotypes of anti-A β antibodies bound to A β ₁₋₁₅ epitope in an ELISA and were capable of binding to amyloid plaques in brain tissue from AD cases [86]. Thus, we demonstrated that DNA immunization is capable of inducing Th2-type therapeutically potent anti-A β ₄₂ antibodies in wild type mice. The pA β ₄₂-IL4 prototype vaccine allowed us to break tolerance and induce primarily Th2-type IgG1 antibodies specific to the self-A β ₄₂ antigen in APP-Tg2576 mice. DNA vaccination, however, induced significantly lower anti-A β antibody production in APP-Tg2576 mice [87] compared with wild-type animals [86]. These results were not totally unexpected, because previously we and others reported that immune responses to A β in APP-Tg2576 mice were significantly impaired even when protein immunizations with fA β ₄₂ was used [22, 88].

Other groups have also attempted to generate anti-A β antibodies using DNA vaccines encoding the A β ₄₂ peptide [89, 90]. Only pA β ₄₂ mixed with aggregated A β ₄₂ peptide slightly increased antibody production compared with that after immunization with vector mixed with the same peptide. Another group demonstrated that pA β ₄₂ is capable of inducing T cell proliferation in mice of different immune haplotypes as well as HLA Class II transgenic mice; however, they did not report on the generation of anti-A β antibodies [90]. The last group was somewhat more successful inducing in one out of three wild-type mice significant titer of anti-A β antibodies after immunization with a plasmid encoding an A β ₄₂ dimer gene. In addition, only one APP-Tg mouse out of three induced very low titer of antibodies specific to A β ₁₋₁₆ after vaccination with mixture of pA β ₄₂ and pA β ₁₋₁₆ [89].

In order to avoid the generation of autoimmune anti-A β T cell responses in actively immunized patients we generated a prototype epitope vaccine consisting of the self B cell antigenic determinant of A β ₄₂ (A β ₁₋₁₅) and the non-self T cell antigenic determinant PADRE (PADRE-A β ₁₋₁₅) [23, 87]. We used the N-terminal region of A β ₄₂ since it represents the major B cell epitope in mice [28, 91–99] and humans [100]. In addition, this region of A β ₄₂ does not possess T cell antigenic determinants mapped in mice [22, 28] or humans [101]. Thus, we designed the replacement of the self-T cell epitope of A β ₄₂ with a foreign T cell epitope while keeping the self-B cell epitope to avoid autoreactive anti-A β /APP T cell responses. We selected PADRE, a synthetic, non-natural Pan HLA DR-binding epitope, as our candidate for a foreign T cell epitope because of the potency of this molecule in generating strong T helper (Th) cell responses [102–106]. In our original experiments to test the anti-A β epitope vaccine concept we utilized multiple antigen peptides (MAPs) containing PADRE-A β ₁₋₁₅ epitope vaccine. MAPs provide multiple copies of immunogen attached to the core matrix and significantly enhance antibody responses [107, 108]. Immunization of BALB/c mice with the A β ₁₋₁₅-PADRE-MAP epitope vaccine produced high titers of anti-A β IgG1 antibodies, but not antibodies specific to PADRE or MAP peptides. On the contrary, splenocytes from immunized mice showed robust T cell stimulation *in vitro* in response to PADRE, but not autoreactive anti-A β T cells [23, 87].

More recently we demonstrated that 2 copies of A β ₁₁ fused with PADRE and MAP is even more immunogenic, because it induced very high titers of therapeutically relevant anti-A β ₁₁ antibodies in two different strains of APP-Tg mice. Thus, our epitope vaccine activates non-self antigen-specific T lymphocytes, which initiates and directs the formation of therapeutically potent anti-A β antibodies. Taken together, the data discussed above suggest that our prototype AD vaccine composed of N terminal region of A β ₄₂ and a foreign T cell epitope may be safe for use in humans, because it is capable of inducing anti-A β humoral immune responses without generation of autoreactive (anti-A β) T cell-mediated cellular immune responses, or production of high levels of proinflammatory cytokines.

SECOND-GENERATION DNA EPIOTOPE VACCINES INCORPORATING NOVEL MOLECULAR ADJUVANTS FOR ANTI-A β IMMUNOTHERAPY

More recently we have made additional improvements in the design of the anti-A β DNA epitope vaccine design. Our prototype second generation DNA epitope vaccine encoding 3 copies of A β ₁₋₁₁ fused with foreign Th epitope, PADRE plus the a potent Th2-promoting molecular adjuvant, human macrophage derived chemokine (MDC). The attractive feature of a vaccine including MDC/CCL22 as the molecular adjuvant is that it is controlled by the expression of Th2-type chemokine that plays a critical role in the antigen-induced recruitment of Th2 cells *via* chemotaxis, and activation of CCR4-expressing Th2-type CD4⁺ T cells followed by B-cell activation [109, 110]. These features of MDC have been associated with its' superb efficiency to induce humoral and Th2 responses without detectable CD8⁺ T cell responses when used as fusion proteins with weakly immunogenic antigens [109]. Typically, approaches that target various endocytic cell surface receptors are known to increase the efficiency of antigen presentation between 100- to 10,000-fold [111]. In concordance with our previous reports on the mechanism of chemokine-based vaccines [112, 113], we believe that our DNA epitope vaccine was efficiently delivered and internalized into endo/lysosomal compartments of target CCR4⁺ antigen-presenting cells (APCs). In fact, only a few μ g of the DNA epitope vaccine were required for induction of the strong anti-PADRE Th2 polarized responses and high levels of anti-A β antibody in wild-type and 3xTg-AD mice. Vaccination initiated in young 3xTg-AD mice without pre-existing AD-like pathology was considered therapeutic based on the attenuated cognitive dysfunction in 18 $\pm$ 0.5 month-old animals. This cognitive improvement was correlated with reduction of amyloid burden (diffuse and cored plaques), as well as potentially toxic forms of amyloid, soluble A β ₄₂ and A β ₄₀ peptides in the brains of immunized 3xTg-AD mice. Importantly the reduction of amyloid plaques in the brains of immune 3xTg-AD mice led to a reduction in astrogliosis and microglial activation, and did not increase the incidence of cerebral microhemorrhages. In concordance with our previous data [86, 114] no T cells (CD3⁺, CD4⁺, or CD8⁺ positive) were detected in the brains of immunized or control 3xTg-AD mice [115].

In summary, we developed an efficient and simple DNA-based AD vaccine strategy that uses the self-B cell epitope from A β , a non-self promiscuous T cell epitope (PADRE), and a strong molecular adjuvant, MDC. We propose that this DNA epitope vaccine has the potential to be safe and effective in humans because: (i) it will induce strong antibody responses to A β without generation of autoreactive Th cells; (ii) it uses PADRE, a promiscuous synthetic Th epitope that is known to be very effective in the general human population; (iii) it uses human MDC that will activate anti-inflammatory Th2-type cells specific to the foreign antigen that is not expressed in human brain. Safety and immunology studies in large animals with the goal toward achieving effective humoral immunity and the lowest rate of adverse events should help to translate our DNA epitope vaccine to human clinical trials.

We have also tried other approaches in the DNA epitope vaccine design to enhance anti-A β antibody production. For example, we have engineered a DNA epitope vaccine that expresses 3A β 1-11, PADRE, and 3 copies of C3d (3C3d), a component of complement as a molecular adjuvant, designed to significantly enhance the uptake of the immunogen by APCs. The following section provides the rationale for selecting C3d as a molecular adjuvant for an additional DNA epitope vaccine candidate. A major function of C' is the opsonization of antigens/immune complexes. This is mediated by the covalent attachment of activated complement C3 fragments (C3d and C3dg) to the antigen, which links the innate and the adaptive immune responses by targeting antigen to specific C' receptors type 1 (CD35) and type 2 (CD 21) [116, 117]. The C3d and C3dg fragments of activated C3 become covalently attached to targets and bind to CD21 receptor on APCs [118, 119]. This CD21 molecule is co-expressed on APC as a non-covalent complex with CD19. CD19 functions as a specialized membrane adaptor protein for antigen-specific B-cell receptor (BCR) [120, 121]. The activation of CD19 induces the phosphorylation of this molecule, which results in the activation of lipid and protein kinases and subsequent increases in Ca²⁺ influx [122, 123]. It has been demonstrated that following antigen binding, the BCR moves into cholesterol/sphingolipid-rich membrane microdomains, so-called "lipid rafts" [124, 125]. More recently, translocation of both CD19/CD21 complex and BCR into lipid rafts were shown to occur after binding of cells to an antigen tagged with C3d. Importantly, CD19/CD21 complex significantly prolongs BCR residency in lipid rafts and also signaling through this antigenic receptor [126]. Little is known about the mechanism of BCR translocation into lipid rafts; however, it is clear that oligomerization of BCR is crucial for both signal transduction and trafficking [125]. Immunization of mice with 3A β 1-11-PADRE epitope vaccine alone generated only moderate levels of anti-A β antibodies and a pro-inflammatory T helper (Th1 phenotype) cellular immune response. However, the addition of 3C3d to the vaccine construct significantly augmented the anti-A β humoral immune response and, importantly, shifted the cellular immune response towards the potentially safer anti-inflammatory Th2 phenotype [115].

Not all approaches utilizing DNA immunization have been successful. McLaurin and colleagues [127] tried to use apoptosis to stimulate Th2-biased cellular immune responses to A β immunization. Thus, they sought to investigate whether immunization using a DNA vaccine encoding A β in conjunction with an attenuated caspase could generate therapeutically effective levels of anti-A β antibodies. However, they found that plasmids encoding A β and an attenuated caspase were less effective at reducing amyloid pathology than a plasmid encoding A β alone. Moreover, use of A β with an Arctic mutation (E22G) as an immunogen was also less effective than wild-type A β . While only low levels of IgG and IgM were generated in response to immunization with a plasmid encoding wild-type A β , these antibody titers were sufficient to reduce plaque load and insoluble A β 42 levels. Clearance of A β was most effective when antibodies were directed against N-terminal epitopes of A β . Moreover, immunization also reduced cerebral amyloid angiopathy in TgCRND8 mice. Finally, high-molecular-weight oligomers and A β trimers were significantly reduced with immunization. Thus, immunization with a plasmid encoding A β alone drives an attenuated immune response that is sufficient to clear amyloid pathology in a mouse model of AD [127].

Recently Rosenberg and colleagues [74] described a very novel DNA A β 42 trimer immunization protocol, which was designed to produce specific Th2-type antibody response. Their goal was to compare the immune response in wild-type mice after immunization with either DNA A β 42 trimer or A β 42 peptide. Wild-type mice received either DNA A β 42 trimer immunization administered with gene gun or intraperitoneal injection of human A β 42 peptide with Quil A as the adjuvant. DNA A β 42 trimer immunization resulted in antibody titers with an average titer of 15 ug per milliliter. DNA A β 42 trimer induced

mostly an IgG1 antibody response indicative of a Th2-type immune response. The peptide-immunized mice showed a mixed Th1/Th2 immune response. In this preliminary study in a wild-type mouse model, DNA A β ₄₂ trimer immunization protocol produced a Th2 immune response and appeared to have low potential to cause an inflammatory T-cell response [74].

In order to overcome the limitations of A β as an immunogen and to improve the safety (removing the A β self T cell epitope) of the vaccine design we have incorporated technology developed to improve the immune response to bacterial capsular polysaccharides. Previously, rationally designed strings of promiscuous CD4(+) T cell epitopes (polyepitope) were shown to reverse age-related defects in immune response to vaccines and to enhance the humoral response to T cell epitope-deficient capsular polysaccharides, which had previously limited their use as vaccines, especially in children under 2 years of age [128]. Initial attempts to overcome the poor immunogenicity of capsular polysaccharides utilized immuno-conjugates, such as diphtheria toxoid or tetanus toxin and the diphtheria mutant (CRM197), similar to the vaccine construct being used by Elan and Johnson & Johnson in the ongoing active immunization clinical trial (ACC-001), which consists of a novel A β immuno-conjugate that draws on Wyeth conjugate technology that was previously described above. However, Grandi, Del Giudice and colleagues designed, constructed and tested multiple polyepitope vaccines, which eliminate the carrier protein B cell epitopes, which may be recognized by the immune system as the predominate B cell epitopes in the immuno-conjugate, possibly diminishing the antibody response to the capsular polysaccharides in their case, and to the A β B cell epitope in A β immuno-conjugates. For example, they constructed three recombinant carrier proteins constituted by strings of 6, 10 or 19 human CD4(+) T cell epitopes (N6, N10, N19) from various pathogen-derived antigens, including TT and proteins from *Plasmodium falciparum*, influenza virus, and HBV. Importantly the polyepitope constructs were deficient in B cell epitopes. Each of these T cell epitopes utilized in the vaccine design was defined as universal in that it binds to many human MHC class II molecules. The data indicate that rationally designed recombinant polyepitope proteins represent excellent candidates for the development and clinical testing of new conjugate vaccines [128]. Subsequently, they showed that the combined conjugate vaccines enhanced immunogenicity with the N19 polyepitope as a carrier protein. The N19 polyepitope, consisting of a sequential string of universal human CD4(+)-T-cell epitopes, was tested as a carrier protein in a formulation of combined glycoconjugate vaccines containing the capsular polysaccharides of multiple *Neisseria meningitidis* serogroups. Good antibody responses to all four polysaccharides were induced by a single immunization of mice with N19-based conjugates. After two immunizations the N19 conjugates elicited antibody titers comparable to those induced after three doses of glycoconjugates containing CRM197 as the carrier protein. Compared to cross-reacting material based constructs, lower amounts of N19-MenACWY conjugates still induced high bactericidal titers to all four polysaccharides. Moreover, N19-multiple serogroups-conjugated constructs induced faster and higher antibody avidity maturation against meningococcal C PS than cross-reacting material based conjugates. Particular relevant for targeting the humoral response to the actual therapeutic target, N19-specific antibodies did not cross-react with the pathogen protein from which N19 epitopes were derived. Thus the N19 polyepitope strategy not only represents a strong and valid option for the generation of improved or new combined glycoconjugate vaccines [129], but quite possibly for designing an effective anti-A β vaccine. We are currently testing both the polyepitope (T helper epitopes) carrier protein design, as well as investigating whether T memory cells specific for pathogens that were formed in young animals can be used to provide Th support in elderly animals to amplify the anti-A β antibody responses to immunization with a DNA epitope vaccine containing the dominate A β B cell epitope and a string of promiscuous Th epitopes from childhood vaccines and commonly encountered pathogens.

THE POTENTIAL OF PRIME BOOST TO FURTHER ENHANCE DNA VACCINES FOR ANTI-A β IMMUNOTHERAPY

Although DNA vaccines have many advantages over peptide-protein vaccines, they typically induce somewhat weaker immune responses in large animals and humans than in mice. Utilizing a DNA to prime the immune response followed by a more conventional peptide/protein boost can dramatically enhance the immune response to a DNA vaccine.

Fukuchi and colleagues [71] first demonstrated that an adenovirus vector, AdPEDI-(A β ₁₋₆)11, which encodes 11 tandem repeats of A β ₁₋₆ can induce anti-inflammatory Th2 immune responses in mice. They then investigated whether a DNA prime-adenovirus boost regimen could elicit a more robust Th2 response using AdPEDI-(A β ₁₋₆)11 and a DNA plasmid encoding the same antigen. All mice administered the DNA prime-adenovirus boost regimen were positive for anti-A β antibody, but only 4 out of 7 mice immunized with only AdPEDI-(A β ₁₋₆)11 developed anti-A β antibody. The mean anti-A β titer induced by the DNA prime-adenovirus boost regimen was approximately 7-fold greater than that by AdPEDI-(A β ₁₋₆)11 alone. Furthermore, anti-A β antibodies induced by the DNA prime-adenovirus boost regimen were predominantly of the IgG1 isotype. These results indicate that the DNA prime-adenovirus boost regimen can enhance Th2-biased responses with AdPEDI-(A β ₁₋₆)11 in mice and suggest that heterologous prime-boost strategies may make AD immunotherapy more effective in reducing accumulated A β [71].

The focus of our recent study in gene therapy was to further enhance anti-A β antibody responses by developing an improved DNA vaccination protocol utilizing a prime-boost regimen, in which we primed the mice using our DNA vaccine, followed by a booster injection with recombinant protein antigen. We used our DNA epitope vaccine followed by boosting with recombinant protein and showed that priming with DNA followed by boosting with a homologous recombinant protein vaccine significantly increases the anti-A β antibody responses without changing the IgG1 profile of the humoral immune response. Furthermore, the antibody responses generated by this prime-boost regimen were long lasting and the antibodies possessed a higher avidity for binding to the A β ₄₂ peptide than with DNA immunization alone. Thus, we showed that a heterologous prime-boost regimen could be an effective protocol for developing a potent anti-A β immunotherapy response with possible translational potential [130].

DNA VACCINE DELIVERY TECHNOLOGY

The nature of immune responses generated following vaccination with DNA depends on a number of key factors, such as the dose, route, delivery method, and vaccination schedule employed. Direct delivery of naked DNA vaccine *via* a standard needle injection does not result in very efficient uptake of the DNA by the cells surrounding the site of injection. However, various technologies have been developed to overcome this limitation associated with DNA immunization. Immunization through the skin *via* a gene gun is a reliable and reproducible method of DNA vaccine delivery and has been shown to be capable of inducing protective immunity in laboratory animal models. The Helios gene gun system delivers DNA to the epidermis using helium-driven bombardment of DNA-coated gold microparticles. Recent clinical trial results with gene gun delivery have provided encouragement that delivery technologies can enhance immune responses to DNA vaccines [131]. However, gene gun delivery is limited primarily to the skin, which may not be optimal for some antigens, and the DNA dose range that can be administered is limited when compared to the doses that can be delivered into muscle.

Electroporation (EP) represents a promising approach to facilitate advancing DNA vaccination from laboratory studies in small rodents to translation to large animals and human clinical trials [132]. EP can robustly increase delivery of DNA into cells, thereby amplifying the amount of the vaccine immunogen that is expressed in the tissue. In addition, EP has an adjuvant-like effect in tissues that enhances the potency of DNA vaccines. With recent developments in EP systems for muscle or skin DNA delivery, the safety, tolerability, reproducibility and clinically acceptable administration of DNA vaccines has advanced significantly. EP-mediated delivery of DNA vaccines is now being tested for safety and immunogenicity in several Phase I clinical trials (www.clinicaltrials.gov).

SUMMARY

The focus of this review is on the advantages of DNA vaccination for anti-A β immunotherapy, and the major hurdles, such as immunosenescence, selection of appropriate molecular adjuvants, universal T cell epitopes and the possibly of using a polyepitope design based on existing memory T cells in the general population that were generated in response to childhood or seasonal vaccines, as well as various infections. Ultimately, we believe that the further refinement of our AD DNA epitope vaccines, possibly combined with a prime boost regime will facilitate translation to human clinical trials in either very early AD, or preferably in preclinical stage individuals identified by validated AD biomarkers.

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ABBREVIATIONS

Aβ	Amyloid- β
AD	Alzheimer's disease Amyloid- β
APC	Antigen presenting cell
APP	Amyloid precursor protein
BCR	B-cell receptor
CNS	Central nervous system
EP	Electroporation
HBV	Hepatitis B virus
IL4	Interleukin-4
MAP	Multiple antigen peptide
MDC	Macrophage derived chemokine
PRCA	Pure red cell aplasia
Tg	Transgenic
TT	tetanus toxin

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