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Site-specific UBITh® amyloid-β vaccine for immunotherapy of Alzheimer's disease

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Abstract

The UBITh® AD immunotherapeutic vaccine for Alzheimer's disease uses an amyloid-β (Aβ) immunogen having two designer peptides that have been engineered to elicit anti-N terminal $A\beta_{1-14}$ antibodies while minimizing potential for the generation of adverse anti-Aβ immune responses. The vaccine has been further designed for minimization of inflammatory reactivities through the use of a proprietary vaccine delivery system that biases Th2 type regulatory T cell responses in preference to Th1 pro-inflammatory T cell responses. In vitro studies and in vivo studies in small animals, baboons and macaques show that anti-Aβ antibodies are generated with the expected N-terminus site-specificity, and that these antibodies have functional immunogenicities to neutralize the toxic activity of Aβ and promote clearance of plaque deposition. The antibodies appear to draw Aβ from the CNS into peripheral circulation. Results indicate that the UBITh® AD vaccine did not evoke anti-Aβ cellular responses in a transgenic mouse model for AD. The vaccine was safe and well tolerated in adult Cynomolgus macaques during a repeat dose acute and chronic toxicity study.

Keywords: Alzheimer disease immunotherapy; Aβ vaccine; APP transgenic mice; Non-human primate toxicology

1. Introduction

United Biomedical Inc. (UBI) is developing an immunotherapeutic vaccine for Alzheimer's disease (AD) by targeting a specific peptide domain of amyloid-β (Aβ). Aβ was selected as the target antigen for our vaccine based on accumulating evidence in support of the Amyloid Cascade Hypothesis that places the accumulation of Aβ at the initiating step for AD [1].

Immunizations with Aβ immunogens [2–4] or passive administration of anti-Aβ antibodies [5–7], dramatically attenuated Aβ plaques and behavior deficits in transgenic mouse models for AD. Increased titers of mouse anti-Aβ antibodies were necessary for the observed reductions in plaque burdens and AD-like signs [8].

Further support for the Amyloid Hypothesis and the consequent efficacy of anti-Aβ antibody responses comes from clinical studies with an aggregated $A\beta_{1-42}$ (AN-1792) vaccine (Elan Pharmaceuticals) [4]. The Phase IIa clinical study was not powered for efficacy but some observations favor the view that the AN-1792 immunotherapeutic vaccine provided for the removal of Aβ deposits from the human brain through an antibody action mode, with at least a partial modification of the neuropathology of AD and slowed cognitive decline [8–10]. Unfortunately, 18 patients out of 298 given the AN-1792 vaccine in the Phase II clinical trial developed treatment-related meningoencephalitis and the manufacturer suspended the trial [11,12]. However, antibodies did not seem to be implicated in the inflammation as there was no correlation of adverse events with the generation of anti-Aβ antibodies. Immunohistochemistry studies associated the meningoencephalitis with extensive T lymphocyte infiltration, particularly with meningeal blood vessels affected by

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cerebral amyloid angiopathy [13,14]. The Aβ immunogen of AN-1792 is a complex aggregation of full-length synthetic $A\beta_{1-42}$. As such, both B and T cell epitopes are found on aggregated $A\beta_{1-42}$. Among these are B cell-activating epitopes on the N terminal segment of $A\beta_{1-42}$ that elicit antibody responses in humans and mice; and, several T cell-activating epitopes have been mapped on the amino acids of $A\beta_{1-42}$ beyond residue 16 [15,16]. These T cell sites may be responsible for adverse autoimmune inflammatory responses [11–13]. Moreover, the effects of T cell autoimmunity may have been exacerbated by the selection of a Th1-biased adjuvant composition for the AN-1792 vaccine that included QS-21 and polysorbate-80 [9].

The immunogenicity of the proposed UBITh® AD immunotherapeutic vaccine has been optimized by replacing the intrinsic self Th epitopes of the AN-1792 antigen with foreign UBITh® epitopes, while further minimizing the potential problem of undesirable inflammatory T cell reactivities by use of: (1) an N-terminus Aβ immunogen designed to have B cell-specific epitopic characteristics only and (2) a proprietary vaccine delivery vehicle based on a stabilized immunostimulatory complex admixed with an adjuvanting mineral salt suspension, designed to bias a preference for regulatory Th2 responses rather than Th1 pro-inflammatory T cell responses.

In the present study, functional immunogenicity and specificity analyses in normal guinea pigs, two species of non-human primates and a hAPP transgenic mouse model of the anti-Aβ antibody response to the UBITh® AD immunotherapeutic vaccine are described. This novel, proprietary vaccine formulation has improved safety features by design, as supported by a repeat dose toxicity study in macaques.

2. Materials and methods

2.1. Peptide synthesis

Peptide immunogens for vaccines and peptide antigens for ELISA were synthesized using automated solid-phase synthesis with F-moc chemistry using terminus and side chain-protected amino acids, cleaved from the resin and deblocked the functional groups on the amino acid side chains with TFA. Peptides were purified by preparative HPLC and characterized by MALDI-ToF mass spectrometry, amino acid analysis and reverse-phase HPLC.

2.2. Formulation of UBITh® AD immunotherapeutic vaccine

The $A\beta_{1-14}$ peptide immunogens, p3102 and p3075, are cationic at physiological pH's. The addition of polyanionic CpG oligonucleotide (ODN) results in charge neutralization and the immediate "self-assembly" of immunostimulatory complexes (ISC) in solution. The stoichiometry of the molar charge ratios of cationic peptide:anionic CpG determines the degree of association. The UBITh® AD vaccine was prepared in stages: The ISC was prepared in water-for-injection with an equimolar mixture of the two UBITh® Aβ peptides with a molar charge ratio to CpG ODN of 1.5:1. To the preformed ISC was sequentially added the aluminum mineral salt, a saline solution for tonicity and a preservative.

2.3. Animals

Protocols involving Duncan-Hartley guinea pigs (8–12 weeks of age; Covance Research Laboratories, Denver, PA, USA), adult male baboons (*Papio anubus*, 8–10 years of age; University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA), adult male and female Cynomolgus macaques (∼4 years of age; Beijing Jo-Inn New Drug Research Center, Beijing, China) and hAPP751 transgenic mice and their littermates (14 ± 2 weeks of age, JSW-Research GmbH, Graz, Austria) were performed under approved IACUC applications at the contracted animal facility as well as at UBI, as sponsor.

2.4. hAPP transgenic mouse model

The hAPP751 transgenic (tg+) mice constitutively over-express human amyloid precursor protein (hAPP) containing the London (V717I) and Swedish (K670M/N671L) double mutations, under the regulatory control of the murine Thy-1 promoter [17,18]. The $A\beta_{1-42}$ deposition occurs as early as 3–4 months of age with the appearance of mature plaques in the frontal cortex and at 5–7 months of age, plaque formation extends to the hippocampus, thalamus and olfactory region in the hAPP751 tg+ mice. The effects of intramuscular vaccinations over a 16 week period were observed for antibody response by ELISA assay of serum, and for brain amyloid deposition and brain plaque load, as well as for evidence of increased levels of cellular reactivity (e.g., T cell infiltration, microglial cell activation) in the brain by immunostaining and by biochemical extractions.

2.5. Serological assays

2.5.1. Solid-phase enzyme-linked immunoassay (ELISA) for detection of antibodies to synthetic peptides

Purified Aβ peptide domains, UBITh® peptides or carrier protein KLH were individually coated on 96-well plates at 5 μg/mL and dried overnight. Serum samples were serially diluted 10-fold with a starting dilution of 1:100. Briefly, 100 μL samples of diluted animal sera were incubated in the wells for 60–90 min at 37 °C, washed with PBS and incubated for 60 min at 37 °C with horseradish peroxidase-conjugated recombinant protein A/G. The plates were washed again with PBS and incubated with chromagen (3,3′,5,5′-tetramethylbenzidine) plus hydrogen peroxide as substrate for 15 min at 37 °C and then washed again; the reactions were stopped with H_2SO_4. The antibody ELISA titers, expressed in $\log_{10}$, were determined using an automated plate reader at absorbance, $A_{450\,nm}$. The UBI Aβ_{1-28} antibody ELISA test has been validated for specificity, reproducibility and robustness.

Specificity analyses of anti-Aβ antibody were determined by hAPP 10-mer epitope mapping. Briefly, ELISA plates (96-well) were coated with individual hAPP 10-mer peptides (0.5 μg per well) and then 100 μL serum samples (1:100 dilution in PBS) were incubated in 10-mer plate wells in duplicate following the steps of the antibody ELISA method described above. Specificity analyses of baboon anti-Aβ antibody were also pre-absorbed with Aβ_{1-10} peptide (DAEFRHDSGY), Aβ-modified synthetic peptides with substitutions at the N-terminus, or in addition, with non-relevant control peptide and then tested by anti-Aβ_{1-28} ELISA.

2.5.2. Solid-phase enzyme-linked immunoassay for detection of β-amyloid antigens

A high sensitivity Aβ_{1-40} immunoassay (Invitrogen^{TM}—BioSource^{TM} Cytokines & Signaling, Camarillo, CA, USA) was used to determine the concentration of Aβ in serum, plasma and CSF in Cynomolgus macaques following kit instructions. The Aβ_{1-42} levels in plasma, CSF and chemical extractions of brain tissue from hAPP751 transgenic mice were determined following immunoassay kit instructions (The Genetics Company Inc., Zurich-Schlieren, Switzerland).

2.5.3. In vitro neurotoxicity assay for inhibition of fibrillogenesis and protection from Aβ$_{1-40}$-mediated toxicity by anti-Aβ antibody

The neurotoxicity assays employed rat pheochromocytoma cell line, PC-12, and aged solutions of the Aβ_{1-40} peptide, as previously described by Solomon et al. [19]. The peptide solution was characterized for fibrillar formation by Congo Red binding. On days 6 and 9 the solution bound equivalent amounts of the dye as shown by absorbance, $A_{540\,nm}$. This observation provided evidence for formation of toxic Aβ_{1-40} aggregates; the day 9 preparation was tested for toxicity to PC-12 cells.

PC-12 cells were grown in tissue culture and suspended into assay medium and placed into the wells of a 96-well round bottom tissue culture plates, 5×10^3 cells/well in 100 μL. The toxicity of the 37 °C-incubated peptide (i.e., aggregated Aβ_{1-40}) and a freshly prepared peptide (i.e., non-aggregated) was tested at 25 and 6.5 μM in duplicates. Controls were PC-12 cells with assay medium only. The plates were incubated for 48 h at 37 °C in a CO_2 incubator. Toxicity to the cells was determined by the Promega CytoTox 96® Cytotoxicity Assay. Lysis was determined by absorbance, $A_{492\,nm}$ and results were presented as the percentage of cytotoxicity compared to 100% lysis.

2.6. Immunohistochemical analysis

Normal adult human tissues (PhenoPath Laboratories Inc., Seattle, WA, USA) and brain specimens from cases with Alzheimer's disease (Dr. Felicia Gaskin, University of Virginia, Charlottesville, VA, USA) were obtained from post-mortum and/or surgical pathology specimens. Cynomolgus macaque tissue specimens and hAPP transgenic mouse brain specimens (JSW-Research) were obtained at necropsy. Tissues were either snap-frozen in liquid nitrogen, submerged in cold OCT embedding compound and cryo-sectioned or they were formalin-fixed, paraffin-embedded and sections prepared by standard procedures.

Indirect immunofluorescence analysis of cryopreserved tissue sections were performed with preimmune and hyperimmune serum from guinea pigs, hAPP transgenic mice, baboons and macaques or with commercially available murine monoclonal antibodies and fluorochrome-conjugated secondary antibodies. Indirect immunoperoxidase staining using an avidin-biotin enhanced commercially available kit was performed on cryopreserved tissue sections of normal adult tissues using purified guinea pig anti-Aβ IgG, or on brain sections from control and UBITh® AD vaccine-treated macaques using commercially available monoclonal antibodies detecting CD3, CD11b, GFAP and specific Aβ epitopes. The immunohistochemical analyses were conducted according to standard pathology laboratory procedures.

2.7. Lymphocyte proliferation analysis and cytokine analysis

Peripheral blood mononuclear cells (PBMC) from baboons and from Cynomolgus macaques were isolated by Ficoll-hypaque gradient centrifugation. For peptide-induced proliferation and cytokine production, cells (2×10^5 per well) were cultured alone or with individual peptide domains added (including, Aβ_{1-14}, Aβ_{1-42}, UBITh®, non-relevant peptide). Mitogens (PHA, PWM, Con A) were used as positive controls. On day 6, 1 μCi of ^{3}H-thymidine (^{3}H-TdR) was added to each of three replicate culture wells. After 18 h of incubation, cells were harvested and ^{3}H-TdR incorporation was determined. The stimulation index (S.I.) represents the cpm in the presence of antigen divided by the cpm in the absence of antigen; a S.I. > 3.0 was considered significant.

Cytokine analyses (IL2, IL6, IL10, IL13, TNFα, IFNγ) from the Cynomolgus macaque PMBC cultures were performed on aliquots of culture medium alone or in the presence of peptide domains or mitogens. Monkey-specific cytokine sandwich ELISA kits (U-CyTech Biosciences, Utrecht, The Netherlands) were used to determine the concentration of individual cytokines following kit instructions.

3. Results and discussion

3.1. Description of UBITh® AD immunotherapeutic vaccine product

The Aβ_{1-14}–UBITh® peptide immunogens (p3102, p3075) are comprised of two well-defined, site-specific Aβ synthetic peptides. Each peptide consists of a highly active UBITh® helper T cell epitope [20–25] covalently linked through a spacer to the first 14 amino acids of the N-terminus of Aβ, as the target B cell epitope.

The UBITh®1 and UBITh®2 epitopes are idealized T helper (Th) cell designs based on and modified from Th sites on measles virus F protein and hepatitis B surface antigen, respectively [21]. Previously, the UBITh® peptide domains have been effective when synthetically linked to peptide domains for the HIV receptor on T cells, high affinity binding site on IgE and foot-and-mouth disease virus capsid [22–25]. These designed UBITh® epitopes are promiscuous and highly potent Th epitopes derived from viruses. They are expected to provide broader and stronger T cell help than the incidental intrinsic T helper epitopes of aggregated $A\beta_{1-42}$, which may improve immunogenicity in an elderly population. Moreover, as foreign T helper cell sites they further optimize the peptide antigen response and are unlikely to have cross-reactivities to human Aβ peptides or to hAPP thereby reducing the danger of T cell-mediated autoimmune reactions. The N-terminal Aβ site of the UBITh® immunogens is an immunodominant target for effective anti-Aβ aggregate antibodies [5,19], and is not known to contain intrinsic Aβ T cell epitopes [15,16]. Unlike the $A\beta_{1-42}$ fibril immunogen of the AN-1792 vaccine, the N-terminal $A\beta_{1-14}$ peptide cannot itself act to seed fibrillogenesis [26], for additional vaccine safety considerations. Another additional safety feature of the UBITh® vaccine technology is that the responses to chimeric UBITh® anti-self immunogens are reversible and must be maintained by repeated immunizations [22–25]. The $A\beta_{1-14}$–UBITh® immunogens are well-defined chemical entities manufactured from amino acids by automated peptide synthesis, enabling reproducible characterization and manufacture.

For the UBITh® AD vaccine formulation process, the two $A\beta_{1-14}$–UBITh® peptide immunogens, prepared in equimolar ratio, are mixed with a proprietary CpG ODN which results in the spontaneous formation of an immunostimulatory complex in solution. This novel particulate system comprising CpG and immunogen was designed to take advantage of the generalized B cell mitogenicity associated with CpG ODN use, yet promote balanced Th1/Th2 type responses [27,28].

The CpG ODN in our vaccine formulation are 100% bound to immunogen in a process mediated by electrostatic neutralization of opposing charge, resulting in the formation of micron-sized particulates. The particulate form allows for a significantly reduced dosage of CpG from the conventional use of CpG adjuvants, less potential for adverse innate immune responses, and facilitates alternative immunogen processing pathways including professional antigen presenting cells (APC). Consequently, the UBITh® AD vaccine formulations are novel conceptually and offer potential advantages by promoting the stimulation of immune responses by alternative mechanisms [28].

3.2. *Preliminary immunogenicity and specificity analyses of UBITh® Aβ peptide immunogens in guinea pigs*

During the discovery phase of this project [20], five groups of guinea pigs were immunized by intramuscular route at weeks 0, 2, 4 with either $A\beta_{1-28}$ synthetic peptide alone, $A\beta_{1-14}$ synthetic peptide alone or $A\beta_{1-14}$ linked with a UBITh® epitope or conjugated to a KLH carrier protein, at 100 μg per 0.5 mL dose. Montanide ISA 51 (Seppic Inc., Fairfield, NJ, USA) was used as the adjuvant for a water-in-oil emulsion type of vaccine formulation. Serum samples were collected at weeks 0, 4, 6, 8 and tested by ELISA against $A\beta_{1-42}$, UBITh® peptide or KLH carrier protein.

ELISA results from week 4 sera (Table 1) showed the immunogenicity and the specificity of $A\beta_{1-14}$ immunogens for Aβ and the requirement of the $A\beta_{1-14}$ site for extrinsic T cell help, provided by either a UBITh® epitope or the less effective KLH carrier protein. The $A\beta_{1-14}$ peptide alone did not generate anti-Aβ titers above background level by ELISA test; in contrast, the $A\beta_{1-28}$ peptide immunogen had intrinsic Th epitopes, to provide T cell help sufficient for the generation of an anti-Aβ antibody response. Table 1 also summarizes tissue immunostaining of the anti-Aβ anti-

Table 1
Immunogenicity of Aβ derived peptides in guinea pigs[a]

Immunogen	Adjuvant	ELISA titer ($\log_{10}$)[b]		Immunohistology of AD brain[c]	
		$A\beta_{1-42}$	UBITh®1 peptide or KLH	Plaque[d]	TSBV[e]
$A\beta_{1-28}$	ISA 51	3.40 ± 0.29	NA[f]	+3	+5
$A\beta_{1-14}$	ISA 51	0.97 ± 0.25	NA	Neg	Neg
$A\beta_{1-14}$–UBITh®1	ISA 51	4.09 ± 0.29	0.04 ± 0.03	+4	+6
$A\beta_{1-14}$–KLH	ISA 51	3.34 ± 0.16	4.90 ± 0.23	+2	+4
Preimmune sera	None	< 0.50	NA	Neg	Neg

[a] Sera from week 4 were used for testing.
[b] $\log_{10}$ values > 2.00 are scored as positive anti-Aβ titers.
[c] Immunostaining intensity of AD brain cryosections scored by Dr. Felicia Gaskin, University of Virginia, Charlottesville, VA, USA.
[d] Aβ plaques and cores.
[e] TSBV, thioflavin-S-positive blood vessels.
[f] NA, not applicable.

body generated from each vaccine formulation at week 4. The antibodies with N-terminal specificity bound to the amyloid plaques in tissue sections of human brain cortex from a case diagnosed with Alzheimer's disease. Note that the immune response to the prototype $A\beta_{1-14}$–UBITh®1 peptide immunogen had greater sera titers for Aβ peptide and greater recognition for the amyloid deposits in AD human brain sections than did antibody responses to $A\beta_{1-28}$ and KLH-linked $A\beta_{1-14}$ immunogens. Antibody titers to the UBITh® peptide alone were not detected ($\log_{10} < 0.5$) whereas antibody titers to the KLH carrier protein alone were strong ($\log_{10} \sim 5.0$), showing that the carrier protein directs much of the antibody response to itself.

3.3. Immunogenicity studies of prototype UBITh® AD vaccine formulations in baboons

In Part A of the protocol, four adult male baboons were immunized at 0, 3 and 6 weeks with $A\beta_{1-14}$–UBITh® immunogens (300 μg total peptide dose) complexed into proprietary ISC and formulated with aluminum mineral salt adjuvants. The ISC/mineral salt formulations resulted in strong anti-Aβ antibody responses in all animals (Fig. 1A). No adverse injection site reactions were noted.

The aims for Part B of the protocol were: (1) to monitor safety and injection site reactogenicity of repeated exposure at the target clinical dose and four-fold higher dose, (2) to monitor immunogenicity in a dose escalation study and (3) to evaluate the kinetics of the recall antibody response. These

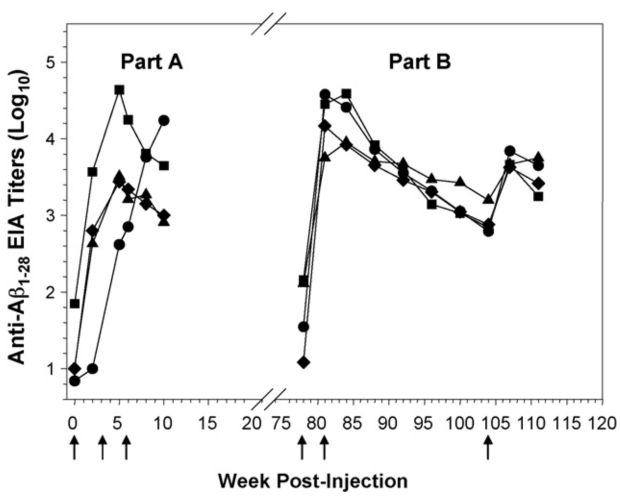

Fig. 1. Immunogenicity study in adult baboons, *P. anubus*. (A) Individual baboons immunized at 0, 3, 6 weeks (arrows) with 300 μg per dose of the UBITh® AD vaccine formulated in mineral salts (♦, ▲, ●, ■) and assayed for anti-Aβ antibody titers by ELISA. Note that three of four baboons generated anti-Aβ antibody titers after the first immunization. (B) Individual baboons immunized after a 72 week rest period, at 78, 81 and 104 weeks (arrows) with 300 μg (low dose, ●, ■) or 1200 μg (high dose, ♦, ▲) of the UBITh® AD vaccine formulated in mineral salts and assayed for anti-Aβ antibody titers. Note that all four baboons developed strong anti-Aβ antibody responses after a single vaccine boost. At the end of the 2-year study period, all four baboons remained healthy and active.

animals had been rested for 72 weeks. In the interim, serum levels of anti-Aβ antibodies had diminished by 10–100-fold (Fig. 1B). At 78 and 81 weeks post-initial injection, four animals were administered vaccines in either 300 μg peptide doses to animal nos. 564 and 565 or 1200 μg doses to animal nos. 556 and 561. The recall responses rapidly restored peak antibody titers in all four baboons. By week 104, antibody titers had begun to decline and the animals were again restored to peak titers by booster doses at week 104. The kinetics of the serum anti-Aβ responses were determined at weeks 0, 2, 5, 6, 8, 10, 78, 81, 84, 88, 92, 96, 100, 104 and 107 by anti-$A\beta_{1-28}$ peptide ELISA. No injection site reactions were noted in animals receiving the 300 μg dose. However, some redness and inflammation were noted at the sites of injection for the baboons receiving the high dose (1200 μg) at week 78 only; this transient reaction was fully resolved within one week. No other adverse events or safety concerns were reported throughout the 2 years that the baboons were evaluated.

3.4. In vitro evaluation of UBITh® AD vaccine for functional immunogenicity

The neurotoxicity assay using rat pheochromocytoma cell line, PC-12, and aged solutions of the $A\beta_{1-40}$ peptide characterized to be toxic were used to evaluate the functional efficacy of the antibody response to the UBITh® AD vaccine.

Aged $A\beta_{1-40}$ peptide solution was tested for toxicity on PC-12 cells following a one-hour pre-incubation in the presence of guinea pig or baboon anti-Aβ sera from the animal immunization protocols. The anti-Aβ sera were tested at 1:30 and 1:90 dilutions. Final results were presented as percentage inhibition of $A\beta_{1-40}$ fibril aggregation and percentage protection of PC-12 cells from $A\beta_{1-40}$ fibril-mediated cytotoxicity (Fig. 2A and B). The preimmune sera from week 0 of both immunization experiments were included as controls. The immune guinea pig sera and baboon sera from weeks 5 and 8, at both the 1:30 and 1:90 dilutions, provided significant inhibition of fibrillogenesis and protection of PC-12 cells from the $A\beta_{1-40}$-mediated toxicity, in comparison to the preimmune sera. These results establish functional neutralizing activity against toxic $A\beta_{1-40}$ peptide for the antibodies evoked by immunization with UBITh® amyloid-β peptide immunogens.

3.5. In vivo evaluation of UBITh® AD vaccine for functional immunogenicity in hAPP transgenic mouse model

The effects of the UBITh® AD vaccine on brain morphology were evaluated in a small pilot study of young transgenic mice over-expressing hAPP751 with the Swedish and the London mutations. $A\beta_{1-42}$ deposition occurs as early as 3–4 months of age with the appearance of mature plaques in the frontal cortex and at 5–7 months of age, plaque formation extends to the hippocampus, thalamus and olfactory region

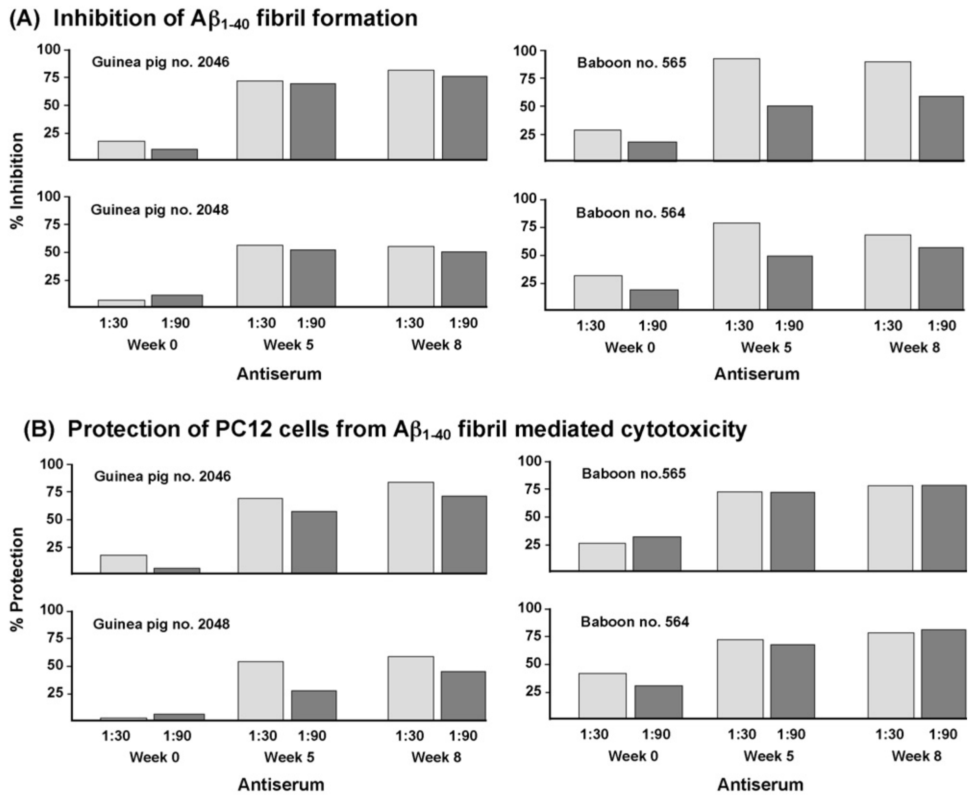

Fig. 2. Effect of guinea pig (nos. 2046 and 2048) and baboon (nos. 565 and 564) sera (collected at weeks 0, 5, 8) in inhibiting $A\beta_{1-40}$ fibril formation (A) and in protection of PC-12 cells from $A\beta_{1-40}$ mediated cytotoxicity (B). Refer to Sections 2.5.3 and 3.4 for experimental details.

in the hAPP751 tg+ mice. Three doses of the UBITh® AD vaccine or placebo vaccine (aluminum mineral salt) were administered at 0, 3 and 12 weeks. Cryocut tissue sections from the right hemisphere of the transgenic mice having high anti-Aβ antibody titers were evaluated using monoclonal antibody 4G8 (anti-$A\beta_{18-22}$) to determine Aβ deposition and plaque load in the cortex and hippocampus and compared with untreated control tg+ mice (Fig. 3). Clearance of the plaques, especially of the less intensely staining diffuse plaques, is striking. The immunostained brain sections also showed significantly reduced neuritic pathology in the immunized mice. Cryocut tissue sections also were evaluated for percent relative microglial cell activation using anti-CD11b antibody and for T cell infiltration using anti-CD3 antibody. No evidence for increased immune cell activation in the brains of the AD vaccine-treated tg+ animals when compared with the untreated control tg+ animals was revealed. The left brain hemisphere (including the bulbus olfactorius) of each animal was chemically extracted with Tris-buffered saline, Triton X-100 detergent, SDS detergent and formic acid and assayed to account for both fibril and soluble Aβ oligomers. Quantitative $A\beta_{1-42}$ ELISA of each extraction confirmed the decreased levels of Aβ deposition in tg+ responder animals of the experimental group receiving the UBITh® AD vaccine when compared to the untreated tg+ control group. The reduction in plaques and Aβ deposition and the lack of immunological activation in the brain

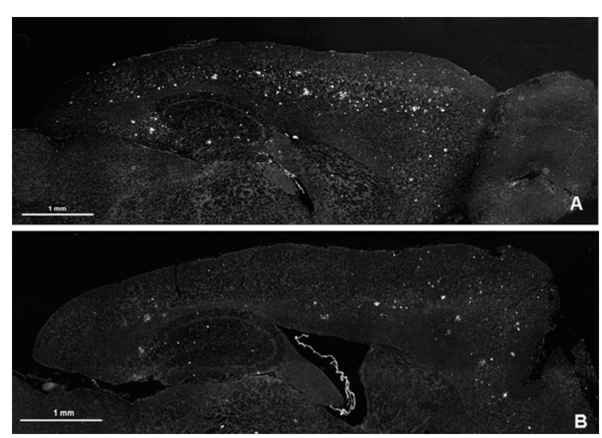

Fig. 3. Indirect immunofluorescence staining of $A\beta_{1-42}$ plaque deposition in the cortex and hippocampus from the right brain hemispheres, visualized with monoclonal antibody 4G8 (Signet®). This image comparison between an untreated hAPP751 transgenic control mouse (A, upper panel) and UBITh® $A\beta_{1-14}$ vaccine-treated transgenic mouse (B, lower panel) from the same layer shows significant decreased Aβ immunostaining in the vaccine-treated "anti-Aβ antibody responder" animal after three immunizations. Comparison of biochemically extracted fractions from the left brain hemispheres of the same untreated versus vaccine-treated mice also showed decreased $A\beta_{1-42}$ levels in animals responding after immunization.

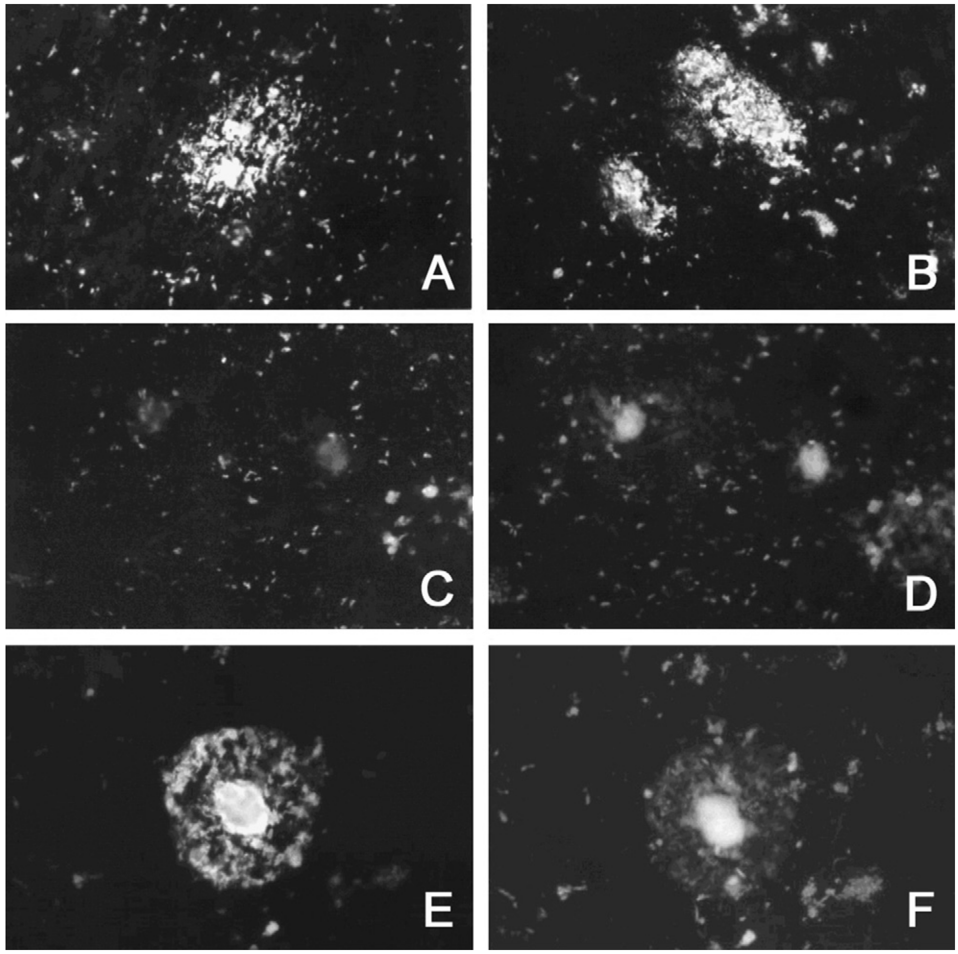

Fig. 4. Indirect immunofluorescence analyses of Aβ plaques or cores in frozen sections of human cerebrum, from a case diagnosed with Alzheimer's disease. Hyperimmune guinea pig anti-Aβ_{1-14} serum (no. 2300) immunostains plaques (A and B); preimmune baboon serum (no. 565) is negative (C); hyperimmune baboon anti-Aβ_{1-14} serum (no. 565) immunostains plaques and cores (E). Serial brain sections stained with thioflavin-S-positive stain for amyloid (D and F).

compartment seen in this pilot study are indications for the efficacy and safety of the UBITh® AD immunotherapeutic vaccine.

3.6. Safety evaluation of antibody response to UBITh® AD vaccine by immunohistochemistry

An immunohistopathology study using preimmune and hyperimmune guinea pig IgG was performed on cryostat sections of adult normal human tissues in order to monitor for specificity and undesirable antibody autoreactivities. The panel of human tissues was screened for immunoreactivity with purified anti-Aβ_{1-14} IgG from guinea pigs immunized with the UBITh® AD immunotherapeutic vaccine and compared to preimmune purified IgG from the same animals. The immunostaining patterns observed on adult normal tissue sections, were reviewed by certified clinical pathologists at PhenoPath Laboratories. Except for weak positive immunoreactivity of some muscle tissues (e.g., endometrium), all adult human tissues tested were negative other than strong positive reactivity on senile plaques in one of three adult cerebrum specimens and positive immunostaining of cerebral fluid within spinal cord samples.

The anti-Aβ antibodies generated from guinea pigs and baboons immunized with the UBITh® AD vaccine bound to deposited Aβ plaques and plaque cores of human cerebrum from a case with Alzheimer's disease by immunofluorescence (Fig. 4). It was observed that immunostaining with the guinea pig antibodies also recognized Aβ deposits in blood vessels. Preadsorption of the hyperimmune guinea pig IgG with the Aβ_{1-14} peptide or a non-related peptide, followed by immunostaining on cryosections of AD brain, confirmed the Aβ specificity of the antibody.

3.7. Epitope mapping of antibody response to UBITh® AD vaccine for safety

ELISA tests using plates coated with Aβ_{1-14}, Aβ_{1-28}, Aβ_{10-28}, Aβ_{24-43}, UBITh®1 and UBITh®2 peptides as the solid-phase antigens were evaluated for the specificity of the antibody response to the UBITh® AD immunotherapeutic vaccine in the sera from the immunized guinea pigs

Table 2
Specificity analyses of hyperimmune anti-Aβ sera from guinea pigs and baboons

Serological reagent	Antibody reactivity[a]						
	$A\beta_{1-14}$	C–$A\beta_{1-14}$	$A\beta_{1-28}$	$A\beta_{10-28}$	$A\beta_{24-43}$	UBITh®1	UBITh®2
mAb 6E10 (aa 3–8)	±	++	++	−	−	−	−
mAb 4G8 (aa 18–22)	−	−	+++	+++	−	−	−
Guinea pig anti-Aβ	+++	+++	++++	−	−	−	−
Baboon anti-Aβ (Part A)[b]	++	+++	++++	−	−	−	−
Baboon anti-Aβ (Part B)[b]	+++	++++	++++	−	−	−	−

[a] Antibody reactivity: ++++ for ELISA titer ≥4.0 $\log_{10}$; +++ for ≥3.0; ++ or + for <3.0; negative (−) for <2.0.
[b] Baboon serum samples from week 8 (Part A) and week 84 (Part B) were used for antibody ELISA tests.

and baboons. High titer anti-Aβ antibodies evoked by the vaccine were detected with the $A\beta_{1-14}$ and $A\beta_{1-28}$ antigens (Table 2); however, there was concern that the $A\beta_{1-28}$ peptide was also detecting additional antibodies due to "B cell epitope spreading" beyond amino acid 14, a source of potentially adverse cross-reactivities. To address this concern, hyperimmune guinea pig antisera and hyperimmune baboon antisera were also tested with $A\beta_{10-28}$ and $A\beta_{24-43}$ peptides by ELISA.

Briefly, mAb 6E10 (binds $A\beta_{3-8}$) and mAb 4G8 (binds $A\beta_{18-22}$) were included as positive and negative control reagents for comparison. The ELISA titers indicate that epitope spreading was not detected in the hyperimmune samples tested. The hyperimmune sera showed enhanced binding to the $A\beta_{1-28}$ peptide but did not react with $A\beta_{10-28}$ and $A\beta_{24-43}$. The hyperimmune sera did not react with UBITh® peptide domains.

In a fine epitope mapping method to localize the predominant antibody binding site(s) to specific residues within the target region, 24 overlapping 10-mer peptides were synthesized around the N-terminal aspartic acid residue "D" of the $A\beta_{1-14}$ peptide sequence and the adjacent region of the human amyloid-β peptide precursor protein (hAPP), to cover the entire length of $A\beta_{1-14}$ plus adjoining hAPP positions (Table 3). These nested peptides were used individually to coat microtiter wells as solid-phase immunoadsorbents for ELISA tests. The positive control ELISA plate was coated with $A\beta_{1-28}$. They were tested for antibody binding with the sera from the four immunized baboons, from weeks 0, 10, 84 and 111. Baboon sera were serially diluted and assayed on plates coated with a 10-mer peptide at 5 μg/mL. As expected, peptide p3411 (DAEFRHDSGY) representing the N-terminus 10-mer of $A\beta_{1-14}$, reacted strongly with immune sera from all four baboons. The "D" in position 1 of $A\beta_{1-14}$ was key to antibody specificity. Deletion of "D" or modification of position 1 to "E" (glutamic acid) resulted in severely reduced binding to the 10-mer peptides, indicating the high specificity of the baboon antibodies for $A\beta_{1-10}$ and the low likelihood for the occurrence of antibody recognition sites cross-reactive to the UBITh® Aβ immunogens elsewhere on $A\beta_{1-42}$ peptide or its precursor. In sum, these antibody epitope findings demonstrated that the antibodies induced by the UBITh® AD immunotherapeutic vaccine candidate were directed specifically to the N-terminal domain of Aβ, not to sites beyond residue 14 and not to the UBITh® domains.

3.8. Repeat dose acute and chronic toxicity of UBITh® AD vaccine in macaques

Normal adult Cynomolgus macaques were chosen to evaluate safety, toxicity and immunogenicity of the UBITh® AD vaccine. Eighteen macaques, half male and half female, were randomly divided into six animals per group. Group 1 (placebo) was injected with the aluminum mineral salt adjuvant only, Group 2 (150 μg/dose) and Group 3 (750 μg/dose) were injected with the UBITh® AD vaccine at low and high dose levels of the $A\beta_{1-14}$–UBITh® peptide immunogens. A total of six doses were administered 3 weeks apart at 0, 3, 6, 9, 12, 15 weeks post-initial injection (wpi) by intramuscular route (deltoid muscle). At week 15 + 1 day, one half of the animals in each group were sacrificed for the acute-phase study; after an additional 12 weeks (27 wpi) of continuous observation after the last immunization, the remaining nine animals were sacrificed for the recovery phase evaluation.

3.8.1. Physical parameters

All 18 vaccinated macaques appeared active, with smooth fur and displayed normal behavior. No allergic skin symptoms were observed. Body weight, body temperature, electrocardiogram, hematology, clinical chemistry and urine analysis were not affected significantly after the placebo and experimental animals were vaccinated.

3.8.2. Pathology

Observations of the normal adult macaques that received either the placebo or experimental UBITh® AD vaccine showed no administration-related pathological changes in the organs and tissues of the vaccinated animals. The exam included systemic necropsy, organ weights, deviations of viscera and histological examination (H&E staining) of 30+ paraffin-embedded organs and tissues including: brain (cerebrum, cerebellum, brain stem), meninges, spinal cord (cervical, thoracic, lumbar), eyeball, pituitary, thymus, thyroid, salivary gland, esophagus, stomach, duodenum, jejunum, ileum, rectum, liver, bile duct, kidney, bladder, adrenal gland, spleen, pancreas, trachea, lung, heart (aorta),

Table 3
Epitope mapping by ELISA using 10-mer peptides between residues 662 and 693 of human amyloid precursor protein (hAPP), including $A\beta_{1-23}$ residues

Peptide Code	Peptide Sequence	ELISA Titer Results[a] Weeks post immunization 0	10	84	111
p3402	TEEISEVKMD	0.23	0.26	0.16	0.15
p3403	EEISEVKMDA	0.24	0.30	0.21	0.22
p3404	EISEVKMDAE	0.23	0.25	0.16	0.14
p3405	ISEVKMDAEF	0.20	0.20	0.15	0.13
p3406	SEVKMDAEFR	0.21	0.24	0.16	0.13
p3407	EVKMDAEFRH	0.19	0.23	0.16	0.14
p3408	VKMDAEFRHD	0.27	0.46	0.48	0.55
p3409	KMDAEFRHDS	0.26	0.36	0.41	0.51
p3410	MDAEFRHDSG	0.26	0.32	0.16	0.17
p3411	**DAEFRHDSGY**	0.26	**3.26**	**3.65**	**3.21**
p3412	AEFRHDSGYE	0.25	0.35	0.25	0.19
p3413	EFRHDSGYEV	0.24	0.30	0.18	0.18
p3414	FRHDSGYEVH	0.20	0.40	0.22	0.30
p3415	RHDSGYEVHH	0.23	0.34	0.27	0.41
p3416	HDSGYEVHHQ	0.21	0.40	0.18	**1.14**
p3417	DSGYEVHHQK	0.32	0.78	0.64	0.76
p3430	SGYEVHHQKL	0.13	0.49	0.51	0.78
p3431	GYEVHHQKLV	0.13	0.27	0.19	0.12
p3432	YEVHHQKLVF	0.16	0.43	0.15	0.22
p3433	EVHHQKLVFF	0.17	0.26	0.13	0.13
p3434	VHHQKLVFFA	0.16	0.23	0.12	0.11
p3435	HHQKLVFFAE	0.19	0.22	0.13	0.12
p3436	HQKLVFFAED	0.18	0.21	0.13	0.13
p2085a	$A\beta_{1-28}$ **DAEFRHDSGYEVHH**QKLVFFAEDVGSNK	0.12	**3.26**	**3.57**	**3.32**

[a]Briefly, ELISA plates coated with hAPP 10-mer peptides (0.5 μg peptide per well) and hyperimmune baboon sera collected from weeks 0, 10, 84 and 111 were tested by individual 10-mer peptide coated wells. ELISA titers ($\log_{10}$) were calculated for each of the assays.

mammary gland, testicle (with epididymis), prostate, ovary (with Fallopian tube), uterus, sterum (bone and marrow), nerves (optic, sciatic, brachial), lymph nodes (mesenteric, iliac, tonsil), skeletal and smooth muscle and injection sites (with local blood vessels and subcutaneous tissue).

Immunohistochemical analysis of snap-frozen brain sections (frontal lobe, temporal lobe, hippocampus) from these normal macaques also noted that CD3+, CD4+, CD8+ T-lymphocytes and activated microglia (CD11b+ cells) or neurogliocyte hyperplasia were not detected in the brain tissue. Brain sections were histochemically stained for nerve fibers and immunostained for glial fibrillary acidic protein (GFAP); no obvious differences were noted between the experimental groups and placebo group. Immunostaining for Aβ antigen (mAb 6E10, Signet®) in brain cortex and hippocampus showed positive immunostaining of Aβ plaques in the frontal lobe of the cortex and in the neural plasma of the hippocampus in two macaques [placebo animal (no. 65 male) and high dose vaccine animal (no. 75 female)]; the Aβ deposition was considered an idiopathic change and not related to the UBITh® AD vaccine treatment.

The injection sites of the macaques in the placebo and experimental groups did not present with any reactions by visual examination during the 0 wpi (weeks post-initial immunization) to 12 wpi period of the study. No behavioral changes or signs of muscular weakness were reported in any of the vaccinated macaques. Microscopic examination of injection site reactogenicity in the

Table 4
Levels of $A\beta_{1-40}$ peptide detected in serum and cerebral spinal fluid (CSF) of Cynomolgus macaques

Animal group	$A\beta_{1-40}$ levels (pg/mL)[a] (mean ± S.D.)					
	Serum				CSF	
	0 wpi ($n=6$)	15 wpi ($n=6$)	21 wpi ($n=3$)	25.5 wpi ($n=3$)	15 wpi ($n=3$)	27 wpi ($n=3$)
Placebo control	61.7 ± 12.7	63.0 ± 16.8	63.7 ± 12.2	52.7 ± 2.6	56.4 ± 6.0	59.7 ± 5.9
Low dose vaccine	53.9 ± 6.3	127.4 ± 23.8	144.8 ± 17.4	158.0 ± 38.1	63.9 ± 6.5	67.6 ± 5.4
High dose vaccine	56.8 ± 7.7	138.2 ± 18.9	144.5 ± 22.5	118.0 ± 20.9	57.5 ± 4.9	54.3 ± 2.9

[a] $A\beta_{1-40}$ levels determined by commercial immunoassay (Invitrogen™—BioSource™).

macaques was similar to other vaccines using mineral salt adjuvants [29].

3.8.3. Antibody response and $A\beta_{1-40}$ levels in serum and CSF

The kinetics of the vaccine response showed that four of six macaques in the low dose Group 2 (150 μg per 0.25 mL) and all six macaques in the high dose Group 3 (750 μg per 1.25 mL) generated antibody against the $A\beta_{1-14}$–UBITh® peptide immunogens after the first immunization. Both low dose and high dose animals sustained high titer antibody for the duration of the study (through week 27).

The fine specificity of the antibody response with sera from four macaques, immunized five times with the high dose AD vaccine indicated a strong specificity for N-terminal $A\beta_{1-10}$ peptide p3411 (DAEFRHDSGY), similar to that observed with immune sera from the earlier baboon study (Table 3). No additional reactivities to other 10-mer peptides are noted for any of the macaque samples tested.

The effects of UBITh® AD vaccine on Aβ levels in sera and CSF were determined using commercially available immunoassay kits (Table 4). The concentration of $A\beta_{1-40}$ after vaccination was determined in serum at 0, 15, 21 and 25.5 weeks and in CSF at the time of sacrifice (week 15 + 1 day or week 27). The $A\beta_{1-40}$ levels in serum were elevated in macaques receiving the UBITh® AD vaccine but normal levels were noted in animals receiving the placebo vaccine. In contrast, $A\beta_{1-40}$ levels maintained a steady state in the cerebral spinal fluid (CSF) of macaques receiving either the placebo or UBITh® AD vaccine. These results support the "Peripheral Sink Hypothesis" as the action mode for anti-Aβ antibodies whereby the antibodies promote the efflux of Aβ peptides from the brain to the peripheral circulatory system [7].

3.8.4. Cellular immune response

Peripheral blood mononuclear cell samples were isolated from whole blood collected at 15, 21 and 25.5 weeks and then cultured in the presence of various Aβ peptides. No proliferation responses by lymphocytes were observed when $A\beta_{1-14}$ peptide was added to culture medium. However, positive proliferation responses were noted when the $A\beta_{1-42}$ peptide was added to some PBMC cultures. The PBMC samples collected at 15, 21 and 25.5 weeks were also tested for cytokine secretion in the presence of Aβ peptides or PHA mitogen. As shown in Table 5, three cytokines (IL2, IL6, TNFα) showed detectable secretion in response to the full-length $A\beta_{1-42}$ peptide but not to the $A\beta_{1-14}$ peptide; up-regulation of cytokine secretion was not detected in the UBITh® AD vaccine-treated samples when compared to the placebo vaccine samples. Three other cytokines (IL10, IL13, IFNγ) tested in the presence of the Aβ peptides were below the assay detection limit in all PBMC cultures.

The macaques were immunized with the UBITh® AD vaccine having only the N-terminal $A\beta_{1-14}$ peptide immunogens with foreign T helper epitopes, without the $A\beta_{17-42}$ peptide domain, indicating that the positive proliferation results noted in the PBMC cultures in the presence of $A\beta_{1-42}$ peptide were not related to the UBITh® AD vaccine response, but rather were a background response to native Aβ. The presence of T cell epitopes on $A\beta_{17-42}$ was reported by Monsonego et al. [16]. These results support the safety of the UBITh® AD vaccine that has only $A\beta_{1-14}$ and foreign T helper epitopes, showing that it does not generate potentially inflammatory anti-self cell-mediated immune responses to Aβ peptides in the normal macaques. In contrast, the adverse events associated with encephalitis in the clinical trial studies of the AN-1792 vaccine were attributed in part, to the inclusion of T cell epitopes within the fibrillar/aggregated $A\beta_{1-42}$ immunogen of that vaccine [12].

Table 5
Cytokine concentration in macaque PBMC cultures after $A\beta_{1-14}$, $A\beta_{1-42}$ or PHA stimulation[a]

Cytokine	Vaccine dose	Cytokine concentration (pg/mL)		
		$A\beta_{1-14}$	$A\beta_{1-42}$	PHA
IL2	Placebo	BDL[b]	23.3 ± 13.1	90.6 ± 12.4
	150 μg	BDL	19.4 ± 9.7	96.1 ± 13.3
	750 μg	BDL	25.2 ± 11.8	97.5 ± 6.6
IL6	Placebo	BDL	23.1 ± 11.7	69.1 ± 12.0
	150 μg	BDL	15.0 ± 9.1	70.6 ± 15.7
	750 μg	BDL	23.4 ± 10.5	66.2 ± 7.3
TNFα	Placebo	BDL	9.2 ± 5.3	91.0 ± 29.1
	150 μg	BDL	7.9 ± 4.8	96.1 ± 22.2
	750 μg	BDL	7.8 ± 5.9	89.0 ± 13.7

[a] Peripheral blood mononuclear cells (PBMC) from six Cynomolgus macaques were cultured 24 h after the last immunization (15 wpi) in the absence or presence of Aβ peptides or PHA mitogen. Culture supernatants were tested for detectable concentrations of each cytokine (IL2, IL6 and TNFα) by commercial ELISA tests.

[b] BDL, below detection level.

4. Conclusions

The in vitro and in vivo experimental observations described in normal guinea pigs, hAPP transgenic mice and non-human primates and the repeat dose toxicity study in macaques support the potential safety and efficacy of the UBITh® AD vaccine as an immunotherapy for the prevention and stabilization of Alzheimer's disease.

The UBITh® AD vaccine enjoys several advantages by design for commercialization: (1) it is highly immunogenic, across diverse species including in two non-human primate species, and across genetic backgrounds, predictive of immunogenicity in human populations. (2) It elicits the type of anti-Aβ antibody response already proven by ourselves and others to be efficacious in hAPP transgenic mouse models and has the desired functional antigenicity. (3) It has N terminal-specificity to eliminate potential toxicity of an $A\beta_{1-42}$ fibril vaccine. The precise specificity of the anti-Aβ antibody recognition site for the N-terminus is predictive of low immunotoxicity. (4) It has minimal potential for undesirable autoimmune T cell cross-reactivities and T cell-mediated inflammation. There is little evidence for adverse cross-reactivities by the antibodies to other normal tissues and organs, as shown by immunohistopathology study. (5) It has two chemically defined synthetic UBITh® Aβ peptide immunogens that are reproducibly manufactured by a standardized method for solid-phase peptide synthesis. The immunogens are well-defined biochemical entities that are readily characterized by validated methods. (6) It is formulated with a non-reactogenic vaccine delivery vehicle that is safe and does not require harsh adjuvants. The particulate peptide:CpG immunostimulatory complex and aluminum mineral salt-based vaccine delivery vehicle biases a Th2 type (regulatory) T cell response in preference to a Th1 type (proinflammatory) T cell response. The results from the repeat dose toxicity study in macaques support its safety without evidence for immunotoxicity or overall toxicity. In addition (7) the UBITh® AD vaccine is readily scalable with a long-term stability profile.

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