ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2011, *56*(4), 317-323

Dedicated to Professor Alexandru T. Balaban on the occasion of his 80th anniversary

CONFORMATION SPECIFIC THERAPEUTIC VACCINES AGAINST PROTEIN MISFOLDING DISEASES

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Received December 20, 2010

This is a brief review of our recent work concerning conformation-specific therapeutic vaccines, which have shown high efficacy against Alzheimer's Disease (AD), in animal models. The preparation of such vaccines, the determination of the antigen's conformation in the vaccine and the nature of the antibodies ellicited are discussed. The mechanism of the antibody action, in vitro and in vivo has been elucidated. The anti-amyloid vaccine is presently in a Phase I Clinical Trial on AD patients.

Liposomes have been shown in a large number of cases to act as powerful immune adjuvants, besides being also carriers for peptides.[1]

Studies in our laboratory had demonstrated that palmitoylated peptide sequences of the multi drug resistance (MDR1) protein, reconstituted in the bilayer of liposomes, when injected into mice elicited strong immune responses, breaking the immune tolerance to this self protein.[2-5] These results suggested that such an approach might work in the case of β-amyloid, yielding possibly a therapeutic vaccine against Alzheimer's disease.[6]

Alzheimer's disease (AD) is a progressive degenerative disorder of insidious onset characterized by memory loss, confusion, and a variety of cognitive disabilities. The major neuropathological change in the brains of AD patients is neuronal death, particularly in regions related to memory and cognition.[7] One of the major pathological features of AD is the abundant presence of amyloid plaques in the brain of affected individuals.[8] Intracellular bundles of paired helical filaments, composed largely of phosphorylated tau protein, accumulate in large amounts in dying neurons. On the neuron surfaces, insoluble aggregates of proteinaceous debris, termed amyloid, appear in the form of neuritic plaques and vascular amyloid deposits. The frequency and distribution of the neurofibrillar tangles and of the neuritic plaques appear to correlate well with the extent of cognitive impairment and other characteristic symptoms of AD.[9]

Amyloid plaques are formed by the β-amyloid peptide (Aβ), a 39- to 43-aa-long polypeptide that is mostly coiled and slightly α-helical in its benign soluble form and, on conformational transition into a mainly β-sheet secondary structure, spontaneously aggregates into insoluble deposits. Aβ is a physiological metabolite of the much larger amyloid precursor protein (APP), 695–770-aa-long, which undergoes sequential proteolysis.[10] The peptide may remain in solution as a random coil or an α-helix.[11] A mAb, raised against the sequence $A\beta_{1-16}$ of the amyloid protein, was shown *in vitro* to have a solubilizing effect on fibrils formed by the $A\beta_{1-42}$ amyloidogenic peptide.[11] The amyloid filaments obtained were similar to those found in amyloid plaques and cerebrovascular amyloid, assembled from chemically synthesized amyloid sequences under defined experimental conditions.[12]

The palmitoylated $A\beta_{1-16}$ sequence reconstituted in liposomes-lipid A, when injected i.p. into mice, including transgenic NORBA mice, which over-

express human APP resulting in amyloid plaque deposits on their pancreases, elicited significant titers of antiamyloid antibodies displaying therapeutic as well as prophylactic action in NORBA transgenic mice.

The palmitoylated $A\beta_{1–16}$ sequence reconstituted in liposomes containing lipid A and Alum injected i.p. to mice proved to be a strong antigen capable of eliciting a significant immune response. These results extend earlier observations made with the murine multidrug-resistance (MDR)1 sequences[2] by using also palmitoylated peptides reconstituted in liposomes–lipid A.

Antibody titers of 1:5,000–1:10,000 were detected about 12 weeks after the first inoculation and subsequent boostings at 2-week intervals. Schenk *et al.*[13] obtained similar titers of anti-$A\beta_{1–42}$ antibodies over an 11-month period, when the antigen used was the $A\beta_{1–42}$ sequence emulsified with the complete and subsequently incomplete Freund adjuvant. The titers of antiamyloid antibodies in mice inoculated with the palmitoylated $A\beta_{1-16}$ sequence reconstituted in liposomes/lipid A were measured by ELISA by using $A\beta_{1–42}$ as antigen. The antibodies proved effective in solubilizing *in vitro* Aβ fibers. Mouse sera containing irrelevant IgGs had no solubilizing effect on the Aβ fibers *in vitro*. This observation may be significant for the mechanism of action of these antibodies *in vivo*, because the amyloid fibers assembled in solution under the condition described are similar to those found in amyloid plaques.[12]

Deposition of amyloid plaques was observed in pancreas of transgenic NORBA mice.[14] Study of the Aβ production, accumulation, and recycling in the pancreas of transgenic NORBA mice indicated that Aβ deposits are formed in four types of pancreatic cells: acinar cells, macrophages infiltrating stroma, epithelial cells of pancreatic ducts, and blood monocytes/macrophages in the lumen of pancreatic cells. All these types of cells produce fibrillar amyloids. Amyloid production in acinar pancreatic cells starts in mice younger than 45 days, progresses in 2- to 7-month-old mice, and plateaus in the second year of life.[15]

Schenk *et al.*[13] reported that immunization with$A\beta_{1–42}$ with complete or/and incomplete Freund adjuvant reduced the development of AD-like pathology in PDAPP mice. In 18-month-old PDAPP mice, reduction of neuritic plaque burden was between 50 and 60% on vaccination,[13] although the burden was relatively low in absolute terms. The pancreas burden in the 16- and 17-month-old NORBA mice was very high, so that its reduction by 50% appears to be significant and correlates well with the *in vitro* solubilization of the preformed $A\beta_{1–42}$ fibers by the antisera.

Breaking the immune tolerance to Aβ by using palmitoylated $A\beta_{1–16}$ peptide reconstituted in liposomes appears to be quite efficient, as "therapeutic titers" are obtained rapidly, only 12 weeks after the first inoculation. Moreover, the amount of plaque removal/solubilization is high after immunization with this system: in the 9- and 15-month-old mice, the reduction of plaque burden is ≈50% compared with controls.

An extensive pathology study carried out with the vaccinated mice did not find any autoimmune lesions in lung, kidney, liver, adrenals, and pancreas of the NORBA mice 7 months after vaccination. The absence of the blood–brain barrier hurdle to be crossed by the antibodies to reach the pancreatic plaques in the NORBA mice may reduce the value of our animal model, though.

We further investigated the therapeutic effects of two different versions of $A\beta_{1–15}$ [17] liposome-based vaccines. Inoculation of APP-V717IxPS-1 (APPxPS-1) double-transgenic mice with tetrapalmitoylated amyloid 1–15 peptide (palm$A\beta_{1–15}$), or with amyloid 1–16 peptide (PEG-$A\beta_{1–16}$) linked to a polyethyleneglycol spacer at each end, and embedded within a liposome membrane, elicited fast immune responses with identical binding epitopes. Palm$A\beta_{1–15}$ liposomal vaccine elicited an immune response that restored the memory defect of the mice, whereas that of PEG-$A\beta_{1–16}$ had no such effect. Immunoglobulins that were generated were predominantly of the IgG class with palm$A\beta_{1–15}$, whereas those elicited by PEG-$A\beta_{1–16}$ were primarily of the IgM class. The IgG subclasses of the antibodies generated by both vaccines were mostly IgG2b indicating noninflammatory Th2 isotype. CD and NMR revealed predominantly β-sheet conformation of palm$A\beta_{1–15}$ and random coil of PEG-$A\beta_{1–16}$. We conclude that the association with liposomes induced a variation of the immunogenic structures and thereby different immunogenicities. This finding supports the hypothesis that Alzheimer's disease is a "conformational" disease, implying that antibodies against amyloid sequences in the β-sheet conformation are preferred as potential therapeutic agents.[16]

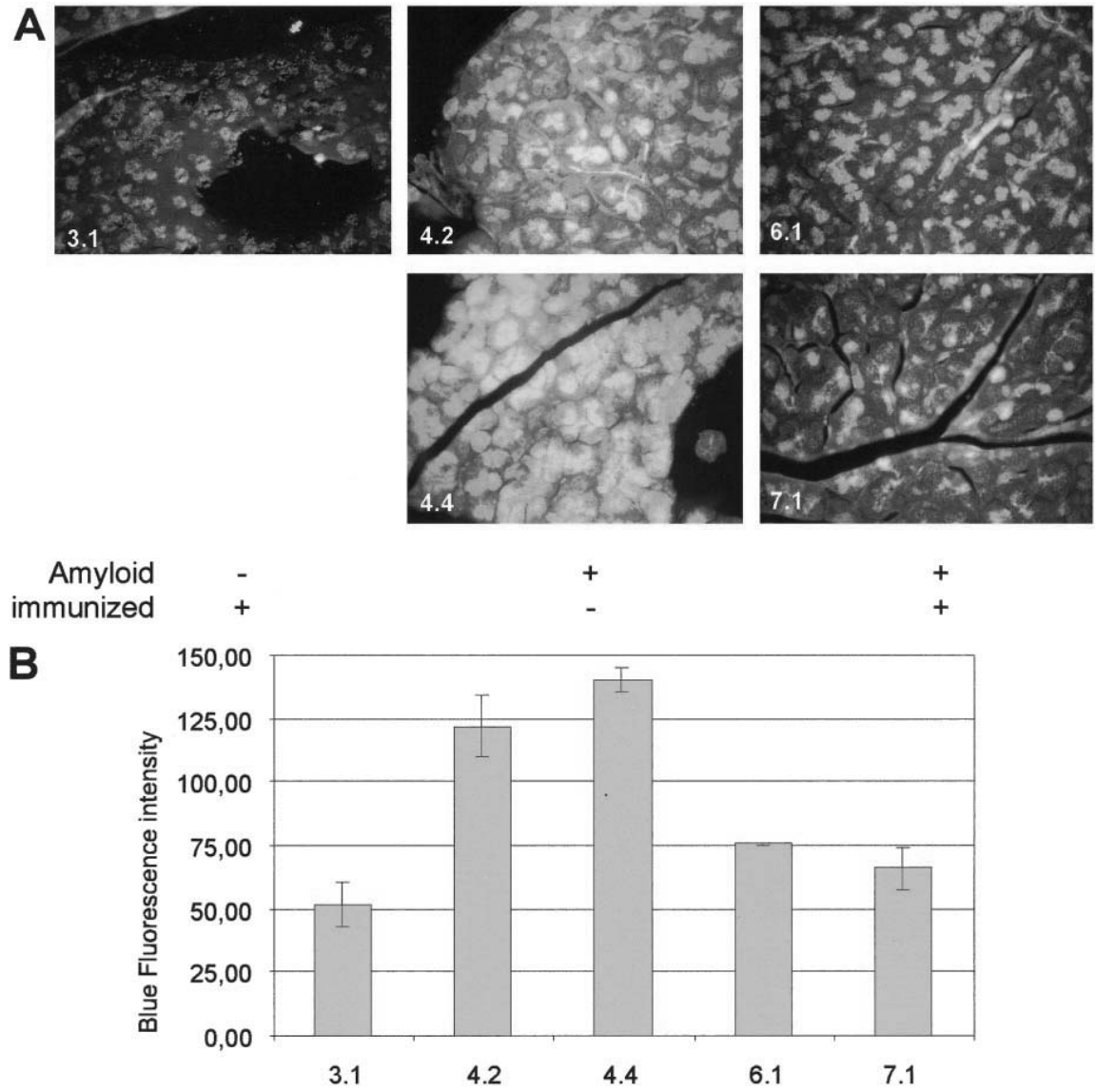

Fig. 1 – Histological study and quantitation of Aβ in thin sections of pancreases from vaccinated and unvaccinated NORBA transgenic mice, stained with ThT. (*A*) (3.1) 9-month-old mouse vaccinated 7 weeks after birth, without Aβ plaques; (4.4, 4.2) 14-month-old mice with fully developed Aβ plaques, unvaccinated; (9- and 15-month-old mice with fully developed Aβ plaques, vaccinated. (*B*) Quantitation of fluorescence in images in *A*. Three sections were used per treatment group, and treatments were unknown to the analyst. Washed sections were stained with a 1%ThT aqueous solution for 3 min. To remove excess fluorochrome from the background, sections were rinsed with water and incubated in 1% acetic acid for 20 min. Sections were washed with water extensively before analysis by fluorescence microscopy. Thin sections stained with ThT were analyzed with a Nikon Labophot Microscope by using a 100-watt mercury source and a Lucifer Yellow Filter Set (Chroma Technology). Images were captured with a Cool Snap Pro Digital Capture Kit (Media Cybernetics) by using a χ40 objective and an exposure time of 600 msec. Images were analyzed with IMAGE PRO PLUS v. 4.1 (Media Cybernetics). A uniform threshold setting of 567,000 pixels per image was maintained for all groups. Measurements included the total area of the fluorescent region and the mean intensity within the region. (with permission*, Proc. Natl. Acad. Sci. USA*, *Washington, DC).*

Design of Liposomal Vaccines and Analysis of the Conformation of the Reconstituted Antigens. To anchor the antigen $A\beta_{1–15}$ on the liposomal surface (ACI-24, Fig. 2A), we used a palmitoylated lysine tandem at each end of the peptide as described.[7] Sixteen-carbon palmitic acid has the appropriate length for stable insertion into the liposomal bilayer. In this construct, the peptide is closely apposed to the surface of the liposome. In an attempt to prolong the immune response, we synthesized the peptide $A\beta_{1-16}$ in which polyethylene glycol (PEG) spacers with 77 repetitive units were introduced between the peptide termini and liposomal anchors (ACI-01, Fig. 2A). It was envisaged that the PEG spacers might enhance liposome stability *in vivo.*[17]

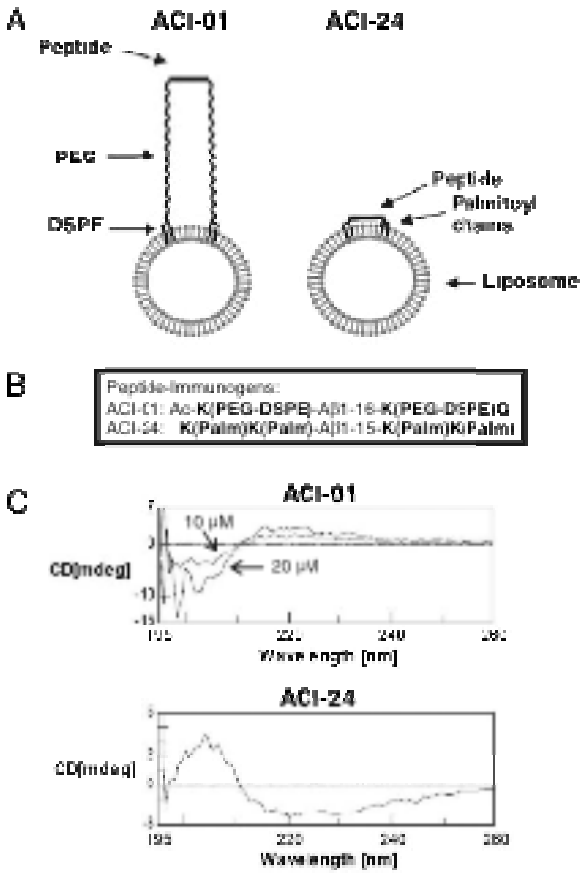

Fig. 2 – Design and biophysical characterization of the two liposomal vaccines containing peptide immunogens with the first 16 (ACI-01, $A\beta_{1–16}$) and 15 (ACI-24, $A\beta_{1–15}$) amino acids of the full length $A\beta_{1–42}$ peptide. (*A*) ACI-01 contains $A\beta_{1–16}$ flanked with one PEGylated lysine residue on each side that utilizes DSPE as liposomal anchor of the PEG chain. For ACI-24, two terminal palmitoylated lysine residues were covalently linked at each end of $A\beta_{1–15}$ to reconstitute and anchor the antigen into the liposome. (*B*) Sequence of the peptides integrated into the liposomal vaccines ACI-01 and ACI-24. (*C*) CD spectra of ACI-01 at 10 and 20 µM peptide concentration (*Upper*) and ACI-24 at 20 µM(lower) (with permission, *Proc. Natl. Acad. Sci.USA,Washington, DC*).[16]

Immunization with ACI-24 but Not ACI-01 Restores Cognitive Memory and Reduces Brain Amyloid Load. To analyze the effect of immunization on nonspatial, hippocampus-dependent cognitive memory in the APPxPS1 mouse model, a 3-month immunization was carried out consisting of six inoculations with ACI-01 or ACI-24, at 2-wk intervals. One group of mice received empty liposomes as control. The cognitive memory capacity of APPxPS-1 mice in the novel object recognition test was significantly increased by immunization with ACI-24 compared with APPxPS-1 mice immunized with ACI-24 (Fig. 3).

We investigated the effect of antigen conformation on the safety and efficacy of anti-β-amyloid liposome-based vaccines. The aim of the study was to enhance the effectiveness of vaccine therapy for AD. We developed a vaccine that preferentially generates antibodies against amyloid sequences in a β-sheet conformation. This construct (ACI-24) exhibited increased affinity for aggregated β-amyloid compared with ACI-01, an Ig isotype that enabled passage across the blood–brain barrier and a negligible inflammatory response.

These liposomal vaccines were highly immunogenic in APPxPS1 double-transgenic mice in terms of kinetics and antibody titers. After two intraperitoneal injections, and by 3 wk after the start of the immunization, significant levels of systemic anti-$A\beta_{1–42}$ antibodies were observed. The epitope of the resulting immunoglobulins was identical for ACI-01 and ACI-24, indicating its independence of the 16th amino acid used to create ACI-01. By way of comparison, a significant titer of Aβ-specific antibodies by intranasal administration of $A\beta_{1–15}$ tandempeptides with and without covalently linked T helper epitope was elicited only after 6 wk and six intranasal applications.[18] Peripheral administration of a $A\beta_{1–42}$ peptide immunogen likewise took 11 months to reach a "therapeutic titer",[18] comparable with that achieved by ACI-24 after only 3 months.

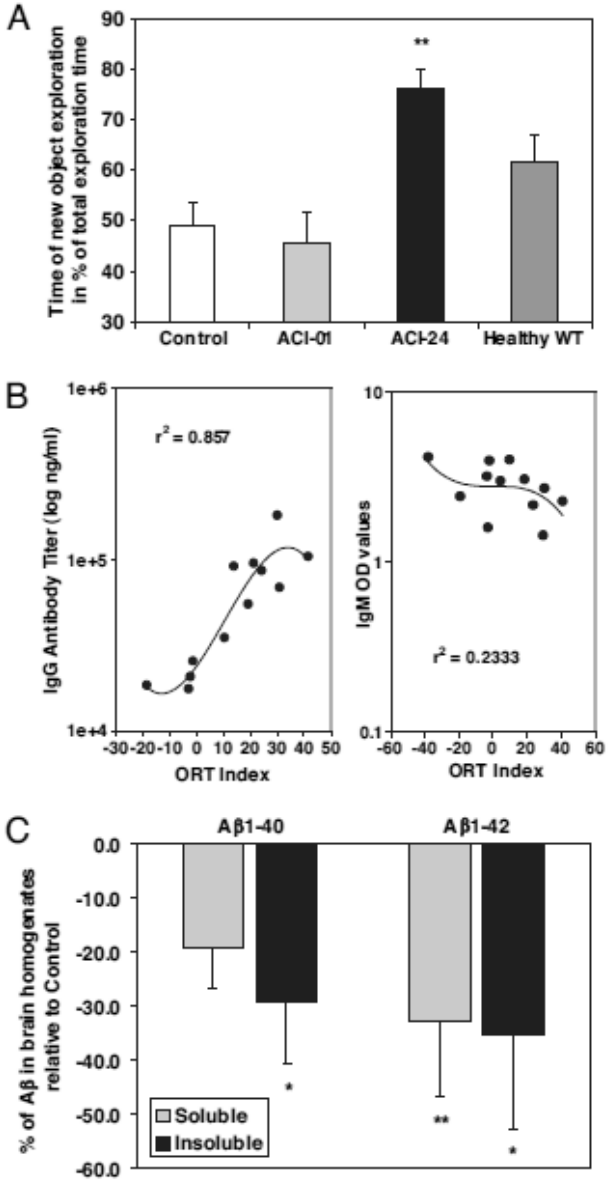

Fig. 3 – Effect of immunization of APPxPS-1 mice with ACI-01 and ACI-24 on memory capacity and brain amyloid load. (*A*) Analysis of cognition assessed by ORT of 6-month-old APPxPS-1 mice immunized with PEGylated (ACI-01) and palmitoylated (ACI-24) liposomal vaccines. Data are expressed in mean ±SEM in groups of 5–8 mice. (*B*) Analysis of individual correlation (nonlinear regression, order of three) of anti $A\beta_{1-42}$-specific IgG antibody titer (*Left*) and of anti $A\beta_{1-42}$-specific IgM antibody titer (*Right*) with ORT Index (ORT individual – ORT mean of control). (*C*) Analysis of soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ of brain homogenates of ACI-24 immunized APPxPS-1 mice compared with empty liposome-immunized control group by $A\beta_{1-40}$- and $A\beta_{1-42}$-specific ELISA. Data are percent means ±SD of the values of 7–8 mice. *, $P < 0.05$; **, $P < 0.01$ by ANOVA (Turkey–Kramer multiple-comparison test).[16] (with permission, *Proc. Natl. Acad. Sci USA, Washington, DC*).

Introduction of a spacer between the antigen peptide and the surface of the liposome appeared to have a major impact on the immune response. First, PEGylated ACI-01 elicited lower IgG antibody titers than palmitoylated ACI-24 and, second, the Aβ antigen is in a random coil conformation in ACI-01, whereas the antigen in ACI-24 is predominantly in β-sheet conformation. The resulting dominating IgG subclasses are the noninflammatory Th2 isotypes (IgG1 and IgG2b). This finding is in accordance with recently published results[19] indicating that vaccines that do not contain the strong T cell epitopes located in the C terminus[20,21] can induce predominantly Th2-associated antibodies (IgG1, Ig2b). Immunization of the APPxPS-1 double-transgenic mice with ACI-24 led to complete restoration of cognitive, nonspatial memory as measured by ORT. ACI-24-

immunized mice had a significantly improved memory over those vaccinated with ACI-01. Because the only significant structural difference between the two vaccines that could account for their different function *in vivo* is the linker chemistry, the resulting difference in conformation of the antigenic peptide seems most likely to be the key for the specificity and efficacy of the resulting antibodies. Both CD and MAS solid-state NMR spectroscopy indicated that the secondary structures of the ACI-01 and ACI-24 antigens exhibit significant differences when reconstituted into liposomes. Whereas, in aqueous solution, the $A\beta_{1-16}$ peptide, alone or in the context of the ACI-01 vaccine, exhibited predominantly random-coil conformation, a predominant β-sheet conformation was observed in the ACI-24 vaccine, probably because of closer proximity to the liposomal surface. At present, we can only speculate on the reason for the β-sheet-like structure of $A\beta_{1-15}$ on the liposome surface. Can electrostatic interactions or hydrogen bonding between the hydrophilic termini of the lipids and the peptide stabilize the structure? Alternatively, does the peptide form an intramolecular hairpin of two antiparallel β-strands with a β-turn stabilized at both ends by the fatty acid groups inserted into the membrane? An alternative model might be that several Aβ-peptides form a two-dimensional array of parallel or antiparallel β-strands that would be stabilized by hydrogen bonds between the strands, contact with the liposome surface, and fixation of the termini by fatty acids inserted into the liposome. The antibodies formed against such structures would be expected to more readily interact with the β-sheet structures of the amyloid oligomers or fibrils found in the brain than with nonaggregated $A\beta_{1-40/42}$.[22]

The memory restoration and higher specificity of antibodies for aggregated Aβ by ACI-24 points to an improved therapeutic effect of antibodies against amyloid sequences in β-sheet conformation that target Aβ in either oligomers or deposited plaques.[23] This assumption is supported by our findings that antibodies resulting from the ACI-24 vaccination were effective in decreasing insoluble amyloid deposits as well as soluble $A\beta_{1-42}$ in the brains of vaccinated mice.

An additional factor for the high biological activity of ACI-24 compared with ACI-01 appears to be related to the Ig classes of the anti-Aβ antibodies. Whereas ACI-24 elicited predominantly an IgG-based immune response, antibodies produced by immunization with ACI-01 were mainly of the IgM class. Although anti-Aβ IgM antibodies can contribute to the clearance of Aβ plaques in the brain, probably by the so-called "sink-effect",[24] we did not observe a significant improvement of memory impairment with the IgM anti-β-amyloid antibodies.

We did not detect any significant signs of inflammation, measured by the proinflammatory TNFα, IL-1β, IL-6, IFN-γ cytokines or by an increase of the MHCII marker for activated microglial cells or the GFAP marker for astrogliosis. The absence of induction of TNFα secretion in the brain of immunized mice is an additional important safety criterion. The lack of inflammation as indicated by the specific markers examined and specifically by the trend of a reduction in the number of activated microglial cells identifies the liposomal vaccine ACI-24 as a potential candidate for clinical investigations. Such investigation are currently underway.

The findings reported here demonstrate that the liposomal antigen vaccines examined in these investigations elicited therapeutic antibodies against β-amyloid only when the antigenic peptide was in a predominantly β-sheet conformation. This is substantiated by the restoration of the memory defect in the APPxPS1 mice to that of wild-type mice with this specific type of vaccination. The dependency of the therapeutic activity of the antibodies on antigen conformation provides support for the hypothesis that Alzheimer's disease is a "conformational" disease and that antibodies against amyloid sequences in β-sheet conformation will be preferred as therapeutic agents. The study also raises the question whether these observations apply specifically to the amyloid peptide vaccine or whether they can be applied to the development of vaccines against other proteins whose pathogenicity is linked to a particular conformation.[16]

List of Abbreviations

AD:	Alzheimer's Disease
APP:	amyloid precursor protein
Aβ:	β-amyloid
CD:	circular dichroism
GFAP:	glial fibrillary acidic protein
IL:	interleukin
IFN:	interferon
MAS NMR:	Magic Angle Spining Nuclear Magnetic Resonance
MHCII:	major histocompatibility class II protein
ORT:	Object Recognition Test
PEG:	polyethylene-glycol
TNF:	umor necrosis factor

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