Alzheimer’s disease (AD) is the most common cause of dementia in the elderly, wherein, the accumulation of amyloid β (Aβ) peptide as cytotoxic oligomers leads to neuropathologic changes. Transgenic mice with brain Aβ plaques immunized with aggregated Aβ have reduced amyloid burden and improved cognitive functions. However, such active immunization in humans led to a small but significant occurrence of meningoencephalitis in 6% AD volunteers due to Aβ induced toxicity. In an attempt to develop safer alternative vaccines, the design of a highly soluble peptide homologous to Aβ (Aβ-EK), that has a reduced amyloidogenic potential while maintaining the major immunogenic epitopes of Aβ is reported. More importantly, this homologue has been shown to be non-toxic, as this peptide failed to exert any observable effect on erythrocytes. The results of the present study suggests that immunization with non-toxic Aβ derivative may offer a safer therapeutic approach to AD, instead of using toxic Aβ fibrils.

Keywords: Amyloid β, Aggregation, Alzheimer’s disease, ELISA, Peptide homologue, Toxicity, Vaccine

Although these results provide promise for using immunomodulation as a general approach to treat AD, immunization with fibrillar Aβ may not be advisable for humans because of its potential toxicity. It is possible that it can co-deposit on existing amyloid plaques leading to increased toxicity, and may actually promote plaque formation. Secondly, cell-mediated autoimmunity could have resulted in the inflammatory response leading to the symptoms of meningoencephalitis. In view of these possibilities, the peptides that are not fibrillogenic/toxic and not identical to endogenous Aβ should reduce the likelihood of these serious side effects. A soluble peptide homologous to Aβ that has a reduced ability to adopt to a β-sheet conformation, while maintaining the major immunogenic epitopes of Aβ peptide has been designed. Accordingly, the peptide contained I→E and V→K substitutions at positions 32 and 36 respectively. Bulk amounts of this peptide homologue (Aβ-EK) were obtained by using bacterial expression system. The recombinant Aβ-EK was evaluated for its fibrillogenic potential and toxicity.
Materials and Methods

Materials—Oligonucleotides were custom synthesized by Chromous Biotech Pvt Ltd, Bangalore. Deep vent DNA polymerase, plasmid pMAL-c4X, BamH1 and EcoR1 restriction enzymes, T4 DNA ligase, amylose resin and factor Xa were purchased from New England Biolabs, UK. Isopropyl thiogalactoside (IPTG), goat anti-rabbit IgG conjugated to horse radish peroxidase, 3,3',5,5'-tetramethylbenzidine, bovine serum albumin (fraction V) and thioflavin T were from Sigma Aldrich, Bangalore. High-binding microtitre plates were purchased from Greiner Labortechnik Ltd, Germany. The native Aβ used in the present study was recombinantly expressed and purified as described earlier. Rabbit anti-Aβ antibodies available in the laboratory were raised and characterized previously. All other reagents used were of analytical grade and locally obtained.

Synthesis, cloning and expression of Aβ-EK—The approach adopted in the synthesis of Aβ-EK gene was similar as described earlier. Briefly, the nucleotide sequence was divided into four oligonucleotides of 45 bases length. Two end primers of 25-nucleotide length were employed to introduce the recognition sites for EcoR1 and BamH1 restriction enzymes at 5' and 3' ends, respectively. Overlapping PCR were performed for 20 cycles. The final PCR product was purified through Genei Quick PCR purification columns as per manufacturer’s instructions (Bangalore Genei, Bangalore). Bacterial cultures, plasmid purification, and transformations were performed using standard protocols. An aliquot of the purified DNA was subcloned into pMAL-c4X vector. The recombinant plasmid with correctly incorporated Aβ-EK sequence was used for transformation of competent E. coli K12 cells (New England BioLabs, UK). Inductions were achieved with 1 mM IPTG. The recombinant protein was purified to homogeneity by a single step amylose affinity chromatography. Vector encoded fusion tag of the recombinant protein was cleaved by factor Xa digestion. Homogeneity of the purified peptide was ascertained by 4-20 % gradient SDS-PAGE analysis.

Enzyme linked immunosorbent assay—The retention of Aβ specific B-cell epitopes in the peptide homologue Aβ-EK was assessed by direct ELISA. Briefly, 2 µg of either Aβ or Aβ-EK was coated in a high binding microtitre plate. After blocking the unoccupied sites in the antigen layered wells with 0.3 % (w/v) BSA, they were incubated with serially diluted rabbit polyclonal anti-native Aβ antiserum. The antigen-antibody complexes were visualized by adding horse radish peroxidase conjugated goat anti-rabbit IgG followed by 3,3',5,5'-tetramethylbenzidine as the chromogen. The colour developed was measured at 450 nm in an automated ELISA reader (Tecan).

Preparation of amyloid fibrils—Recombinantly expressed Aβ or Aβ-EK were incubated as 1 mg/ml solutions in 50 mM HEPES buffer, pH 7.4 with 50 mM NaCl for 15 days at 37°C. At regular intervals, 0.5 ml of the aliquot was added to 2.5 ml of the reaction buffer containing 30 µM thioflavin T, mixed and the fluorescence was monitored in a Shimadzu (RF 5301PC) spectrofluorimeter at an excitation wavelength of 440 nm and an emission wavelength of 492 nm.

Toxicity studies—The toxic nature of these peptides was demonstrated on human erythrocytes as described elsewhere. Towards this, normal human blood was collected into EDTA-coated vacutainers. The cells were separated by centrifugation at 8000 rpm for 20 min at room temperature. They were washed thrice by centrifugation with freshly prepared saline and counted. Erythrocyte suspension (20 µl; 5000 cells/ml) was spotted on a clean glass slide. To this, 100 µg of pre-aggregated form of either Aβ or Aβ-EK were added and the resultant morphological changes were monitored over a period of 30 min. They were viewed under phase contrast inverted microscope (Olympus).

Results and Discussion

Design of Aβ homologue Aβ-EK—Clinical trials using preaggregated Aβ (AN-1792) in conjunction with the Th1 adjuvant QS-21 in AD volunteers highlighted the importance of a directed immune response. Many factors, such as antigen, adjuvant, and delivery systems can be modified to elicit specific cellular and humoral responses. Given the distinct location of B and T cell epitopes within Aβ, new immunogens can be designed with substitutions in the irrelevant C-terminus without any alterations in those residues (4-10) required for binding to Aβ. Native wild type Aβ is a 42 amino acid long hydrophobic peptide with an inherent capacity to form aggregated fibrils. Recently published 3D structure of Aβ revealed that residues 1-17 which harbour the neutralizing B-cell epitope (4-10 AA) are disordered.
that contains two intermolecular, parallel, in-register β-sheets that are formed by residues 18-26 (β1) and 31-42 (β2). At least two molecules of Aβ are required to achieve the repeating structure of a protofilament. Intermolecular side chain contacts are formed between the odd numbered residues of strand β1 (L17, F19 and A21) of the n<sup>th</sup> molecule and the even numbered residues of strand β2 (I32, L34 and V36) of the (n-1)<sup>th</sup> molecule. The loop region of residues 27-30 is connected to sheet β1 by means of the intermolecular salt bridge D23-K28.

In the present study, an attempt has been made to disrupt these intermolecular interactions by introducing substitutions of I→E at position 32 and V→K of residue 36 in the β2 strand. It is anticipated that these substitutions introduced at i→i+4 locations would raise an additional possibility of salt bridge interactions with the side chains. These alterations may introduce helical conformation and increase the hydrophilicity, rendering the homologue less toxic with decreased amyloidogenic potential.

The sequence of this engineered peptide Aβ-EK (Fig. 1) has been subjected to PHD algorithm<sup>18</sup> using NPSA web server (Table 1) for secondary structure predictions. Peptides with high β-sheet content are more toxic and more likely to form fibrils than the peptides with low β-sheet content. Bioinformatic analysis has indicated that Aβ-EK peptide has much less β-sheet content than the native Aβ (Table 1).

Assessment of fibril formation by thioflavin T assay—Fibril formation of Aβ and Aβ-EK was measured after 3, 6, 9, 12 and 15 days of incubation at 37°C. Aβ formed fibrils within 3 days of incubation itself whereas Aβ-EK gradually formed aggregates after prolonged incubation, albeit to a much lesser extent (Fig. 3).

Influence of Aβ-EK on human erythrocytes—It has recently been shown that intact erythrocytes, upon treatment with aggregated Aβ can undergo transient shape changes which are detectable in the light microscope<sup>16</sup>. These changes include a “cup” shaped morphology as early as 2 min after Aβ treatment. By

| Table 1—The predicted percentages of α-helix, β-sheet and random coil as calculated by the PHD algorithm |
|-------------------|-----------------|----------------|
|                   | α-Helix | β-Sheet | Random coil |
| Aβ                | 0       | 57.14   | 42.86       |
| Aβ-EK             | 28.57   | 35.71   | 35.71       |

Fig. 1—Sequence of the peptides used in the present study. The substituted amino acids are underlined in the modified peptidyl sequence. Positions of β-strands are indicated.
10-12 min, cell lysis begins and by 25 min, cells lyse, form pores in the cell membrane, release the hemoglobin and leaving behind the membrane “ghost”. The effect of Aβ-EK on human erythrocytes was examined and compared with the morphological changes produced by native Aβ. As shown in Fig. 4, 100 µg of Aβ-EK in aqueous solution produced no significant changes in erythrocyte shape at 30 min compared with the untreated control cells, by which time the Aβ treated erythrocytes were already undergoing lysis and forming membrane pores. It is known that pore formation in neuronal membranes by Aβ amyloid is a critical step in Aβ-induced neurotoxicity. Lack of any observable effects on erythrocytes by Aβ-EK strongly suggests the non-toxic nature of this homologous peptide.

In summary, a homologue of Aβ with reduced amyloidogenic potential and much lower risk of
toxicity in humans has been generated. The above results demonstrated that it is possible to design a peptide antigen using rational design principles such that modifications introduced do not disturb its inherent immunological potential. Efforts are currently underway to extend these studies to rodent models to explore the applicability of the engineered peptide as a safer alternative vaccine than native Aβ, while maintaining the therapeutic potential of immunization for AD.

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References