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Influence of conformational antibodies on dissociation of fibrillar amyloid ß (Aß1-42) *in vitro*

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Many neurodegenerative diseases result due to the accumulation of misfolded proteins as amyloid fibrils. Although the protein components of these fibrils from different disease states differ considerably, they appear to share common structure. Among these conformational disorders, Alzheimer's disease (AD) and prion diseases exhibit significant overlap in their mechanism of pathogenesis. The present report demonstrates that antibodies directed against the prion protein repeat motif, Tyr-Tyr-Arg motif, recognize recombinantly expressed human amyloid ß (Aß) aggregates in enzyme linked immunosorbent assay. In addition, these antibodies dissociate the preformed aggregates of Aß *in vitro.* These findings illustrate an important property of conformation dependent antibodies viz., they specifically recognize the protein deposits associated with pathology and not the protein in normal tissue. These antibodies may benefit the development of approaches towards prevention and treatment of protein misfolding diseases.

Keywords: Aggregation, Amyloid ß, Antibodies, ELISA, Fluorescence, Thioflavin T

Numerous age-related neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), prion diseases such as Creutzfeldt-Jakob disease (CJD) belong to the family of 'conformational disorders' or 'protein misfolding disorders'[1]. They are characterized by accumulation of a host protein that undergoes a structural change increasing its ß-sheet content and rendering it toxic by deposition as amyloid fibrils. Although the individual protein components of these insoluble aggregates from various disease states differ considerably from one another in primary amino acid sequence, all amyloid fibrils share common features, including a high degree of ß-sheet organized in a polymeric arrangement known as 'cross ß-structure' which is capable of altering the spectroscopic properties of amyloidophilic fluorophores including Congo red and thioflavin S/T[2].

Among these conformational disorders, AD and CJD share several pathological, genetic and mechanistic similarities[3-5]. AD is characterized by the presence of extracellular senile plaques and intracellular neurofibrillary tangles within the afflicted brain. The major constituent of the plaques is the amyloid ß (Aß) peptide, derived from the sequential proteolytic processing of the amyloid precursor protein by ß- and γ-secretases[6]. Aß is a 40-43 amino acid peptide, which, in AD, self-assembles into toxic oligomers and fibrils that accumulate as plaques and deposits in the walls of meningocephalic vessels[7]. The same peptide can be detected in most physiological fluids, such as serum or cerebrospinal fluid.

The cellular prion protein (PrP^C) is a 209 amino acid, cell membrane anchored protein expressed at highest levels by neurons and follicular dendritic cells of the immune systems. In prion disease, this protein undergoes a transformation to toxic PrP^{Sc} [8]. Recently, Paramithiotis *et al*[9] generated antibodies in rabbits, goats and mice to a conserved Tyr-Tyr-Arg (YYR) motif, which is cryptic in PrP^C, becomes exposed upon conversion to PrP^{Sc}. These antibodies have been shown to selectively recognize the PrP^{Sc} isoform in a saturable and specific manner[9]. On similar lines, O'Nuallain and Wetzel[10] generated two conformation specific mAbs, WO1 and WO2, that bind to the amyloid fibril state of Aß but not to its soluble, monomeric state. In addition, these antibodies could recognize other disease-related amyloid fibrils and amyloid-like aggregates derived from other proteins of unrelated sequence, such as transthyretin, islet amyloid polypeptide, ß2-microglobulin, and polyglutamine but not to their native precursors[10].

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Based on these reports and considering the mechanistic similarities between AD and prion diseases, it was of interest to investigate the ability of conformation-specific PrP^{Sc} antibody on recognition, selectively of fibrillar Aß. Here, we demonstrate that polyclonal rabbit anti-YYR antibodies recognize the aggregated version of recombinantly expressed human Aß. Secondly, the Fab fragments of these antibodies dissociate the preformed fibrils of Aß *in vitro.*

Materials and Methods

Animals— Albino rabbits used for the production of polyclonal antibodies were from Institute's Central Animal Research Facility. They were fed pellet diet and water *ad libitum*. They were exposed to 12:12 h L:D schedule under controlled conditions of humidity and ambient temperature.

Materials-Restriction enzymes, T4 DNA ligase, 100 bp DNA ladder, plasmid pMAL-c4X, Deep vent DNA polymerase, amylose resin and Factor Xa were purchased from New England BioLabs (UK). Oligonucleotides were custom synthesized by Chromus Biotech Pvt Ltd (Bangalore). Aß synthetic peptide was custom synthesized by Xcyton Diagnostics Pvt Ltd (Bangalore) and its purity (>95%) was established by reverse phase HPLC. Agarose, ampicillin, phenylmethanesulfonyl fluoride (PMSF), isopropyl thiogalactoside (IPTG), urea, ß-mercaptoethanol, goat anti-rabbit IgG conjugated to horseradish peroxidase, 3,3',5,5'-tetramethyldiamino benzidine, bovine serum albumin (fraction V), Freund's complete and incomplete adjuvants (FCA/FIA), thioflavin T, and coomassie brilliant blue 'R'250 were from Sigma Aldrich (Bangalore). High-binding microtitre plates were purchased from Greiner Labortechnik Ltd. (Germany). By employing multiple antigen peptide (MAP) system, YYR tripeptide sequence was custom synthesized on a symmetric lysine core (Mimotopes, Australia). All other reagents used were of analytical grade and obtained locally.

Synthesis and cloning of Aß minigene— An *E. coli* optimized 135 bp nucleotide sequence encompassing the ORF of Aß was designed and synthesized as described earlier[11]. The final PCR product was analyzed on 2% agarose gel. The Aß minigene 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[12]. For directional cloning into the plasmid pMAL-c4X, the vector DNA and Aß minigene were separately double digested with *Bam*H1 and *Eco*R1 enzymes. The fragments were separated by 1% low melting agarose gel electrophoresis. The target fragments of DNA were excised from the gel and DNA extracted using Genei Spin extraction kit as per manufacturer's instructions. After extraction, the vector DNA and the Aß minigene insert were ligated and the ligation mixture was used to transform competent DH5α cells. The recombinant plasmid with correctly incorporated Aß sequence was used to transform competent *E. coli* K12 cells (New England BioLabs, UK). The transformants were selected by ampicillin resistance.

Expression and purification of recombinant Aß — One recombinant clone was inoculate into LB medium supplemented with 50 μg/ml ampicillin and grown at 37°C, till the OD_{600} reached 0.6. For induction of protein, IPTG was added to 1 m*M* final concentration and growth of the culture continued for an additional 2 h. Cells from 100 ml of induced culture were harvested by centrifugation for 10 min at 3500 rpm at 4°C, suspended in 5 ml of lysis buffer (50 m*M* Tris-Cl, *p*H 8.0 containing 300 m*M* NaCl, 5 m*M* β-mercaptoethanol, and 2 m*M* PMSF) on ice and disrupted by sonication (6 × 10 s cycles, on ice). The soluble fraction was separated from the insoluble fraction by centrifugation at 10000 rpm for 10 min at 4°C and the latter was dissolved in 15 ml lysis buffer containing 8*M* urea. After centrifugation at 10 000 rpm for 15 min at 20°C, the supernatant was dialyzed for 24 h against the dialysis buffer containing 20 m*M* Tris *p*H 7.4, 0.2 *M* NaCl and 1 m*M* EDTA. The dialysate was then loaded onto the amylose resin affinity matrix (1 × 5 cm) pre equilibrated with column buffer containing 20 m*M* Tris *p*H 7.4, 0.2 *M* NaCl and 1 m*M* EDTA at a flow rate of 10 ml/h. Bound proteins were eluted at a flow rate of 30 ml/h with column buffer containing 10 m*M* maltose. Fractions of 1 ml were collected and subjected to 10% SDS-PAGE analysis. For cleavage of the N-terminal fusion tag i.e. the maltose binding protein (MBP), the eluate was dialyzed against 20 m*M* Tris at *p*H 8 containing 2 m*M* $CaCl_2$ and 10 m*M* NaCl and subjected to digestion with Factor Xa enzyme (1 unit /50 μg of recombinant protein) for 6 h at 25°C. The digest was then loaded onto amylose

affinity matrix. The column unbound fraction containing the released Aβ was analyzed by 4-20% gradient SDS-PAGE.

Production of anti-YYR antibodies and their characterization—For primary injection, 1 mg MAP-YYR peptide was administered to rabbit in Freund's complete adjuvant. For subsequent boosters at 4-week intervals, half the dose of peptide emulsified in Freund's incomplete adjuvant was administered subcutaneously. After four boosters, the rabbit was bled, serum collected and stored at –20°C until use. This study was approved by Animal Ethics Committee of this Institute.

The immune recognition patterns of these sera towards MAP peptide was determined by direct ELISA. Peptide (2 µg) in phosphate buffered saline (PBS) was coated in a high binding ELISA plate. After blocking the unoccupied sites with 0.3 % (w/v) bovine serum albumin (BSA) in PBS, these wells were probed with serially diluted antisera. The antigen-antibody interaction was visualized by employing horse radish peroxidase conjugated goat anti-rabbit IgG or anti-mouse IgG and 3,3',5,5'-tetramethylbenzidine as the chromogen. The colour developed was monitored at 450 nm.

Preparation of Fab fragments of anti-YYR antibodies—Rabbit anti-YYR antiserum was dialyzed against 0.1 *M* phosphate, *p*H 8.0 and loaded onto protein A-Sepharose affinity column pre-equilibrated with the same buffer. The bound IgG was eluted with 0.1 *M* glycine, *p*H 3.0. Fractions (1 ml) were collected into tubes containing 20 µl of 2 *M* Tris (to immediately neutralize the *p*H). Fractions showing absorbance at 280 nm were pooled and dialyzed against PBS. Fab fragments of the affinity purified IgG were obtained by papain digestion[13]. The digest was passed through protein A-Sepharose column pre-equilibrated with 0.1 *M* phosphate, *p*H 8.0. The unadsorbed protein containing the Fab was collected. The homogeneity of the Fab was confirmed by 10% SDS-PAGE and immunoreactivity was ascertained by ELISA.

Preparation of Aß aggregates— Aβ aggregation[14] was performed at 25 µ*M* of Aβ in 50 m*M* phosphate buffer, *p*H 7.4 and 100 m*M* NaCl at 37°C for 5 days with agitation. After the incubation, 150 µl of Aβ aggregate mix was added to 2 ml of 50 m*M* glycine-NaOH buffer, *p*H 8.5 containing 5 µ*M* thioflavin-T. Fluorescence was measured in a Shimadzu (RF 5301PC) spectrofluorimeter (Ex: 446 nm; Em: 490 nm) immediately after making the mixture.

To evaluate the influence of anti-YYR antibody on dissociation of Aβ aggregates, the preformed Aβ aggregate (150 µl of the mix) was incubated with 1 µg of Fab fragment of IgG for 6 h at 37°C and this sample was taken for thioflavin-T binding studies.

Results and Discussion

Aß (1-42) peptide used in the present study was generated in bulk quantities by using bacterial expression system. In this, the minigene containing the ORF corresponding to Aß with *E. coli* preferred codons was synthesized using overlapping PCR technique[11]. The final PCR product was analyzed by 2% agarose gel electrophoresis. As anticipated, a band size of 155 nt was visualized upon ethidium bromide staining of the gel (Fig. 1a). After double digestion with *Bam*H1 and *Eco*R1 restriction enzymes, the minigene was ligated with pMAL-c4X expression vector pre-digested with the same enzymes. Incorporation of the correct sequence of the minigene into the vector was confirmed by DNA sequencing of the recombinant plasmid. This recombinant plasmid was used for transformation of competent EK12 cells. This plasmid allowed the expression of recombinant peptide with an N-terminal MBP tag and Factor Xa cleavage site. Expression of Aβ was achieved by transformation and induction of *E. coli* K12 cells with 1 m*M* IPTG for 2 h. SDS-PAGE analysis of the induced and uninduced cells extracted in electrophoresis loading buffer showed that a protein species with an anticipated molecular mass of approximately 46.5 kDa was produced in the induced

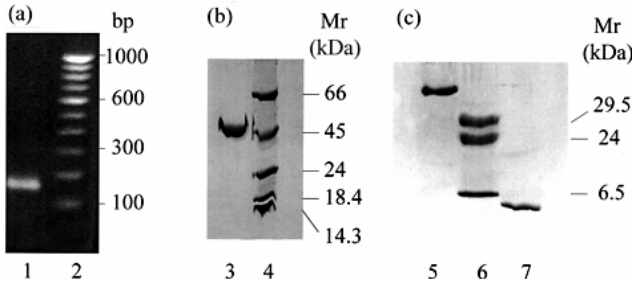

Fig. 1— Expression and purification of recombinant Aß. [(a) Shown are the final PCR product for the synthesis of Aß cDNA (lane 1) and the 100 bp DNA ladder as marker in lane 2. (b) 10% SDS-PAGE analysis of human Aß expressed in *E. coli*. Lane 3 contains 20 µg of the fusion protein and protein molecular weight markers loaded in lane 4. (c) 4-20% gradient SDS-PAGE analysis of Aß peptide after factor Xa cleavage. Shown are 20 µg of MBP-fusion protein in lane 5, low molecular weight markers in lane 6 and 20 µg of purified Aß in lane 7].

cells. Analysis of the pellet and soluble fractions of the induced cells revealed that the fusion protein is in the inclusion bodies (data not shown). Hence, 8 *M* urea was used to solubilize the insoluble fraction.

Using amylose resin it was possible to purify Aβ from 8 *M* urea soluble fraction to homogeneity (Fig. 1b) in a single step. Yields were about 10 mg of fusion protein/litre of bacterial culture. The N-terminal fusion tag of the recombinant protein was then subjected to Factor Xa digestion and the reaction mix was subjected to amylose resin chromatography. From the SDS-PAGE analysis of the column flow through (Fig. 1c), it is clear that the fusion protein underwent cleavage and the apparent molecular weight of processed Aβ was approximately 4 kDa (Fig. 1c). Final yields of recombinant Aβ were approximately 6 mg/L of bacterial culture.

Rabbit polyclonal anti-MAP-YYR antibodies used in the present study were generated against an exposed epitope in pathologically misfolded PrP^{Sc} protein which otherwise is cryptic in PrP^{c} protein[9]. On lines similar to the specificity exhibited by previously reported conformation sensitive antibodies (WO1 and WO2)[10], it was of interest to assess the ability of anti-YYR antibodies to recognize aggregated Aβ fractions by ELISA. In this assay, MAP peptide served as a positive control. It was interesting to note from the binding curves obtained (Fig. 2) that anti-YYR antibodies indeed recognize aggregated Aβ. However, these antibodies fail to show any significant binding to monomeric Aβ. These results demonstrate that anti-YYR antibodies are yet another conformation specific antibodies which can differentiate between native proteins and amyloid fibrils. These findings illustrate an important property of conformation dependent antibodies that can be used in their selection viz., they specifically recognize the protein deposits associated with pathology and not the protein in normal tissue. However further studies with other nongeneric amyloids are necessary to confirm these findings. Next, the effect of these antibodies was examined on destabilization of preformed Aβ fibrils *in vitro* by using fluorescence spectroscopy with thioflavin T. The data (Fig. 3) indicated that Fab fragments of anti-YYR antibodies significantly destabilized preformed fibrils of Aβ in thioflavin T fluorescence assay. The results show for the first time, that PrP^{Sc} specific antibody significantly destabilizes the preformed aggregation of Aβ *in vitro*.

Further studies include evaluation of the ability of these antibodies to bind to other non-generic amyloid fibrils and their influence on such fibrils will have to be investigated. However, the present data suggest possible application of anti-YYR antibodies in the detection of amyloid fibrils or in the modulation of their formation. There is now increasing evidence that amyloid fibrils and oligomeric intermediates have a common structure and pathway of aggregation[15]. This has been demonstrated from the discovery of antibodies that recognize generic epitopes on all types of amyloid fibrils[10] and soluble oligomers[16], independently of their amino acid sequences.

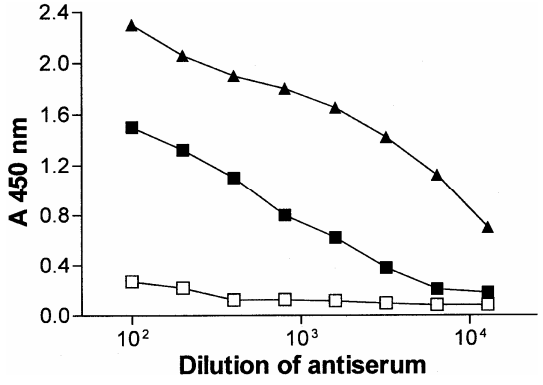

Fig. 2— Reactivity of rabbit polyclonal anti-YYR serum as determined by ELISA. [Antibody dilution curves obtained with 2 µg/ well of MAP-YYR peptide (▲), soluble Aß (■) and fibrillar Aß (□)].

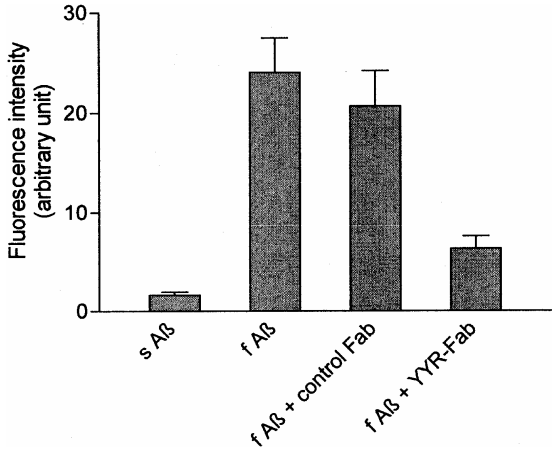

Fig. 3— Effect of Fab fragments of rabbit polyclonal anti-YYR antibodies on the dissociation of fibrillar Aß. [Fluorescence intensity values obtained with binding of thioflavin T to soluble Aß (sAß) or fibrillar Aß (fAß) in the presence of either Fab portion of anti-YYR IgG (YYR-Fab) or Fab fragments of pre-immune IgG (control Fab). Values as mean ± SD of duplicates from 3 different experiments].

Understanding the mechanism of action of these antibodies and their specificity is of high importance in the development of approaches towards prevention and treatment of conformational diseases.

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