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Single-domain antibodies recognize selectively small oligomeric forms of amyloid β, prevent Aβ-induced neurotoxicity and inhibit fibril formation

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ABSTRACT

Neurotoxic oligomers of amyloid β (Aβ) peptide have been incriminated in the pathogenesis of Alzheimer's disease. Further exploration of this issue has been hampered to this date by the fact that all previously described anti-Aβ antibodies are unable to discriminate between the different conformations of the peptide (oligomers, protofibrils and fibrils). Here, we describe the generation of novel camelid single-chain binding domains (VHHs) that recognizes specifically low molecular-weight (MW) oligomers. Three VHH specific for Aβ were obtained from an immunized alpaca phage display library. Two were able to recognize selectively intraneuronal Aβ oligomers; furthermore, one of them, V31-1, prevented Aβ-induced neurotoxicity and inhibited fibril formation. This study confirms that VHHs may recognize non-conventional epitopes and illustrates their potential for the immunodiagnostic of diseases due to protein accumulation.

1. Introduction

Alzheimer's disease (AD) is a progressive, irreversible brain disorder characterized neuropathologically by the extracellular accumulation of amyloid β (Aβ) peptide and the intracellular accumulation of tau protein. The Aβ peptide made of 39–42 amino acids forms insoluble aggregates, which constitute the core of the senile plaques. However, these fibrillar aggregates may not play the main role in pathogenesis. A soluble fraction of oligomeric Aβ has indeed been isolated in the human brain (Kuo et al., 1996). These soluble oligomers are toxic to neurons in culture. Oligomeric Aβ, also called ADDLs (Aβ-derived diffusible ligands), inhibit hippocampal long-term potentiation before killing neurons at nanomolar concentrations. Depending on conditions, ADDL preparations can contain predominantly trimers–hexamers, with larger structures of up to 24 mers. ADDLs show important regionally selective neurotoxicity, sparing cerebellar neurons while selectively killing hippocampal neurons and pyramidal neurons of the entorhinal cortex (Haass and Selkoe, 2007). In APP transgenic mice, low amounts of soluble oligomeric forms of Aβ are able to disrupt, transiently and potently, learned behavior without inducing permanent neurological deficits; trimers and, to a lesser extent, dimers and tetramers have been reported as being particularly active (Cleary et al., 2005; Townsend et al., 2006).

Accurate markers of AD are increasingly needed as therapeutics become available. A reliable assay for Aβ oligomers would be particularly useful. However, conventional antibodies raised against oligomers recognize both soluble forms and fibrils (Lacor et al., 2004; Lambert et al., 2007; Lee et al., 2006). Alternative "binders" which could recognize non-conventional epitopes could help discriminate between the different conformations of Aβ.

A significant proportion of camelid antibodies are homodimeric IgGs, which interact with the antigen via a single heavy-chain binding domain devoid of light chain, referred to as VHH (Hamers-Casterman et al., 1993). The recombinant VHH is a minimal-sized, intact antigen-binding domain. Because of the absence of VL, the VHHs attain a higher structural flexibility than VH domains associated with VLs. Furthermore, the complementarity determining regions (CDRs) of VHHs, and especially CDR3, are statistically longer than those of conventional VH-VL antibodies (Muyldermans et al., 2001). Small size and increased plasticity appear to endow VHHs with unique potentialities: for instance, several VHHs are capable of inhibiting enzymatic activity by interacting with the active site cavity of enzymes such as α-amylase, carbonic anhydrase and hen

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egg lysozyme (De Genst et al., 2006; Desmyter et al., 2002, 1996). These features may allow camelid VHHs to recognize unique epitopes that are poorly immunogenic for conventional antibodies. In the present study, we analysed whether VHHs were capable of recognizing non-conventional epitopes of the Aβ amyloid peptide.

2. Materials and methods

2.1. Materials

Aβ 1–42 and the different Aβ peptides used were purchased from Bachem. The anti-Aβ 8–17 monoclonal antibody 6F/3D (Dako) recognizes synthetic amyloid peptides in dot and western blots. It also specifically stains all type of amyloid deposits in AD brains. The anti-Aβ 22–35 rabbit polyclonal antibodies ref ab62658 were from Abcam.

2.2. Subjects

Human cortical brain tissue was obtained from the GIE Neuro-CEB of Hôpital de la Pitié-Salpétrière, Paris, France. Postmortem brain tissue was examined from representative neurologically AD patients (*Braak stage VI*) (Braak and Braak, 1991).

2.3. Antigen preparation and induction of a humoral immune response in alpaca

Aβ42 (1 mg) was dissolved in 450 μl H_2O and vortexed. 50 μl PBS 10× was added and the mixture was incubated overnight at 37 °C. 150 μl of the mixture was mixed with 150 μl of Freund complete adjuvant for the first immunization, and with 150 μl of Freund incomplete adjuvant for the following immunizations.

One young adult male alpaca (*Lama pacos*) was immunized at days 0, 21 and 35 with 200 μg of the immunogen. The alpaca was bled then immunized twice at 15 days interval. The immune response was monitored by titration of serum samples by ELISA on coated Aβ42. The bound alpaca antibodies were detected with polyclonal rabbit anti-alpaca IgGs (obtained by immunizing rabbits with alpaca IgGs isolated on protein A and protein G columns (Muyldermans et al., 1994)).

2.4. Library construction and expression of VHH

The blood of the immunized animal was collected and the peripheral blood lymphocytes were isolated by centrifugation on a Ficoll (Pharmacia) discontinuous gradient and stored at −80 °C until further use. Total RNA and cDNA was obtained as previously described (Lafaye et al., 1995). DNA fragments encoding VHH domains were amplified by PCR using CH2FORTA4 and VHBACKA6 primers, which respectively anneal to the 3′ and 5′ flanking region of VH genes (Arbabi Ghahroudi et al., 1997). The amplified product of approximately 600 bp was subjected to a second round of PCR using either the primers VHBACKA4 and VHFOR36 or the primers VHBACKA4 and LH (5′GGACTAGTT*GCGGCCGC*TGGTTGTGGTTTTGGTGTCTTGGG-3′) specific of the long hinge homodimeric antibody. The primers were complementary to the 5′ and 3′ ends of the amplified product and incorporated SfiI and NotI restriction sites at the ends of the VHH genes. The PCR products were digested and ligated into phage vector pHEN 1. The resulting library was composed of two sublibraries, one derived from VHH DNA-encoding genes with no hinge and the other from long hinge antibody genes. Phages were produced and isolated using both sublibraries, and subsequently pooled.

The library was panned against Aβ as previously described (Cardoso et al., 2000). Nunc Immunotubes (Maxisorp) tubes were coated overnight at 4 °C with the antigen (5 μg/ml) in PBS. Phages (10^{12} transducing units) were panned by incubation with the coated tubes for 2 h at 37 °C with gentle agitation. The blocking agent was changed at every round: 2% skimmed milk, 3% BSA and 0.5% gelatin were respectively used. Following panning, phage clones were screened by standard ELISA procedures using a HRP/anti-M13 monoclonal antibody conjugate (GE Healthcare) for detection. After the third round of panning, a further round was performed but HB2151 *E. coli* cells were infected instead of TG1.

The coding sequence of the selected VHHs in vector pHEN1 was subcloned in vector pET22 using the NcoI and NotI restriction sites. Transformed *E. coli* BL21(DE3) cells expressed VHHs in the periplasm after induction by IPTG 1 mM for 3 h at 20 °C. Periplasmic extracts were obtained according to the manufacturer's instructions (Novagen, Darmstadt, Germany). Purified VHHs were obtained by IMAC using a chelating agarose column charged with Ni^{2+} (Superflow Ni-NTA, Qiagen Ltd, UK) according to the manufacturer's instructions. The purity of the final preparation was evaluated by SDS-PAGE with Coomassie staining and by Western blot.

The DNA sequences of the VHH genes are available from EMBL/Genbank/DDBJ databases under the following accession numbers: V31-1: AM922479; 61-3: AM922480; L35: AM922481; L1-3: AM922482.

2.5. Obtention of VHH-CH2 protein

The alpaca Immunoglobulin CH2 domain was amplified by RT-PCR using primer CH2-Fwd-Not (5′-TGTGTGGCGGCCGCAGCC-CCTGAGCTCC-3′) and CH2-Rev-Xho (5′-ACGCTACTCGAGTTTG-GCCTTGGAGAT-3′). These primers contain respectively a NotI and a XhoI site allowing the cloning of CH2 domain in vector pET22 in frame with the VHH gene. The expression and the purification of the recombinant protein VHH-CH2 is described in the previous paragraph.

2.6. Preparation of Aβ monomers and protofibrils

One milligram samples of Aβ42 powder were dissolved in 500 μl of hexafluoroisopropanol (Sigma), gently stirred at 4 °C for 7 days, sonicated for 10 min using a Branson ultrasonic bath sonicator and then centrifuged for 10 min at 16,000 × *g*. Fifty microgram aliquots of peptides were lyophilised and stored at −20 °C. Aliquots were dissolved in double distilled water or in PBS pH 7.4 and incubated at 37 °C until use. To an aliquot of Aβ peptides, an equal volume of LDS sample buffer (NuPage, Invitrogen) was added and then incubated at 100 °C for 10 min before electrophoresis.

2.7. Amyloid extraction from brain tissue

Amyloid extraction was performed as previously described (Delacourte et al., 2002). Briefly, a total of 50 mg of brain tissue was homogenized in 500 μl of pure formic acid. One hundred microliters were evaporated under nitrogen, solubilized in 100 μl of the LDS sample buffer (NuPage, Invitrogen) supplemented with 2% β-mercaptoethanol and boiled 10 min before electrophoresis.

2.8. Immunoblots

Following separation by polyacrylamide gel electrophoresis (PAGE) using NuPAGE Novex 4–12% Bis–tris gel (Invitrogen), semi-dry transfer onto Hybond-C (Amersham) and western blotting were carried out using the Xcell II blot module (Invitrogen). Prior to the immunochemical reaction, membranes were blocked in a