J. Biochem. **143,** 475–486 (2008)
doi:10.1093/jb/mvm239

Immunoreactivity of Phage Library-derived Human Single-Chain Antibodies to Amyloid Beta Conformers *In Vitro*

Tomoki Yoshihara[1], Sho Takiguchi[1], Akifumi Kyuno[1], Koichi Tanaka[1], Sayaka Kuba[1], Shuhei Hashiguchi[1], Yuji Ito[1], Tadafumi Hashimoto[2], Takeshi Iwatsubo[2], Shinichiro Tsuyama[3], Toshihiro Nakashima[4] and Kazuhisa Sugimura[1,*]

[1]Department of Bioengineering, Faculty of Engineering, Kagoshima University, Korimoto 1-21-40, Kagoshima, Kagoshima 890-0065; [2]Department of Neuropathology and Neuroscinece, Tokyo University, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033; [3]The Second Department of Anatomy, Center of Chronic Viral Disease, Faculty of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima, Kagoshima 890-8544; and [4]Chemo-Sero-Therapeutic Research Institute, Kyokushi, Kikuchi, Kumamoto 869-1298, Japan

Received September 29, 2007; accepted December 11, 2007; published online January 2, 2008

The pathogenesis of Alzheimer's disease involves conformational changes of Aβ. A series of antibodies recognizing a distinct conformation of Aβ (snapshot antibody) is useful for both understanding the mechanism of molecular conversion and identifying diagnostic and therapeutic reagents. As Aβ with various conformations can be prepared *in vitro* under varying physicochemical conditions, snapshot antibodies can be isolated by directly binding to target molecules with antibody-displaying phages. We tested the feasibility of this idea. We show a feature of several Aβ-reactive antibodies isolated from our human single-chain Fv antibody-phage library and particularly report the characteristics of an scFv clone, B6, selected from the fibrillar $Aβ_{1–42}$-coated biopanning. B6 bound to fibrillar $Aβ_{1–42}$ as well as globulomer $Aβ_{1–42}$ but not to soluble $Aβ_{1–42}$ or $Aβ_{1–40}$. B6 inhibited $Aβ_{1–42}$ fibril formation with 600 nM IC_{50} in spite of being the monovalent scFv form. Epitope analysis suggested that the binding site might be located at the β2 sheet of the C-terminus of $Aβ_{1–42}$. Although it is believed that N-terminus-recognizing antibodies tend to show the capability to inhibit $Aβ_{1–42}$ fibrillation, B6 is the first human inhibitory antibody recognizing the C-terminus of $Aβ_{1–42}$.

Key words: amyloid beta 1–42, conformation, human antibody, single-chain variable fragment, scFv.

Abbreviations: $Aβ_{1–42}$, amyloid beta $_{1–42}$; AP, alkaline phosphatase; BN-PAGE, blue native PAGE; CBB, Coomassie brilliant blue; CDR, complementary determining region; DMSO, dimethyl sulphoxide; FR, framework; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HRP, horseradish peroxidase; IC_{50}, 50% inhibitory concentration; mAb, monoclonal antibody; scFv, single-chian variable fragment; ThT, Thioflavin T; Vh, variable domain of immunoglobulin heavy chain; Vl, variable domain of immunoglobulin light chain.

The accumulation of amyloid-beta (Aβ) peptide in the brain and its deposition as plaque plays a central role in the neuropathology of Alzheimer's disease (AD) (*1*). A primary component of amyloid plaque is Aβ, a short natively unfolded peptide with 40–42 amino acids that assembles into a number of different morphologies, including oligomers, protofibrils and insoluble fibrils. It has been demonstrated that non-fibrillar structures, including oligomers and amyloid-derived diffusible ligands (ADDLs), as well as protofibrils, are also neurotoxic *in vitro* (*2*, *3*). In accordance with these observations, *in vivo*, small, stable oligomers of $Aβ_{1–42}$ have been isolated from brain, plasma and cerebrospinal fluid and correlate with the severity of neurodegeneration on AD (*4*). Thus, recent studies have focused on whether soluble oligomers are active species that contribute to neurodegeneration and dementia. $Aβ_{1–40}$ or $Aβ_{1–42}$ was generated by splicing out from amyloid precursor protein (APP) by β-secretase followed by γ-secretase (*5–7*). An *in vitro* study demonstrated that $Aβ_{1–42}$ rapidly progressed to convert from the monomeric form to oligomers or fibril forms, whereas $Aβ_{1–40}$ generated the fibre form much more slowly (*8*, *9*). A pathohistological analysis employing $Aβ_{1–40}$ transgenic (Tg) and $Aβ_{1–42}$ Tg mice constructed from APP-knockout mice demonstrated that $Aβ_{1–42}$ Tg mice, but not $Aβ_{1–40}$ Tg mice, express high levels of the human $Aβ_{1–42}$ peptide, accumulate detergent-insoluble Aβ, and develop compact amyloid plaque, reactive gliosis, congophilic amyloid angiopathy and diffuse Aβ deposits in the brain (*10*). These results suggested that the oligomerization of $Aβ_{1–42}$ is critical for the AD pathology. However, as the oligomerization is a sequential consequence resulting from various conformers, the structure of the active species exerting neuronal toxicity has remained obscure. Although circular dichroism analyses detect the changing phenomenon and ThT detects the fibril forms but not ADDL or globulomers, these methodologies do not resolve the structure of the active species.

*To whom correspondence should be addressed. Tel: +81 99 285 8345, Fax: +81 99 258 4706, E-mail: kazu@be.kagoshima-u.ac.jp

Thus, a series of antibodies recognizing the distinct conformational change of Aβ would be useful for not only understanding the molecular assembly mechanism of fibrillation but also for implementing clinical applications.

Protein conformation can be controlled under various physicochemical conditions, such as pH, the ionic strength of the buffer solution and the presence of reducing agents and/or denaturing surfactant agents *in vitro*. Based on the direct confirmation of these molecular structures by electron microscopy or atomic force microscopy, the phage library can be directly reacted to the conformation-defined protein molecules in solution or immobilized at plastic plates. By capturing a specific phage clone, the isolation of a given conformation-specific antibody (snapshot antibody) clone may be attained.

In the present study, we evaluate whether the idea of a snapshot-antibody signature is feasible. We characterized the binding specificities of human scFvs derived from a phage library under a panning selection with various conformers of a soluble or fibrillar form of $A\beta_{1-42}$. In particular, we report on the characteristics of an scFv clone, B6, selected from the fibrillar $A\beta_{1-42}$-coated biopanning. B6 binds to fibrillar $A\beta_{1-42}$ as well as globulomer $A\beta_{1-42}$ in the absence of binding activity to soluble $A\beta_{1-42}$ or $A\beta_{1-40}$. B6 inhibited the $A\beta_{1-42}$ fibril formation with 600 nM IC_{50} in spite of being the monovalent scFv form. Epitope analysis suggested that the binding site might be located at the β2 sheet of the C-terminus of $A\beta_{1-42}$, although it is believed that N-terminus-recognizing antibodies tend to show the capability to inhibit $A\beta_{1-42}$ fibrillation. This human antibody is useful for both understanding the molecular basis of $A\beta_{1-42}$ fibril assembly and identifying diagnostic and therapeutic reagents of AD.

MATERIALS AND METHODS

Preparation of $A\beta_{1-42}$ Conformers—Synthetic $A\beta_{1-42}$ peptides or $A\beta_{1-40}$ peptides (Peptide Institute Inc., Osaka) were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Wako) at a concentration of 1 mg/ml and separated into aliquots in microcentrifuge tubes. HFIP was removed under vacuum in a Speed Vac, and the peptide film was stored at −80°C as described (*11*). Immediately prior to use, the HFIP-treated $A\beta_{1-42}$ was dissolved in dimethyl sulphoxide (DMSO) to 1 mg/ml and diluted with phosphate buffer saline (PBS) to the proper concentration. Fibrillar $A\beta_{1-42}$ and fibrillar $A\beta_{1-40}$ were prepared by incubating $A\beta_{1-42}$ or $A\beta_{1-40}$ at 37°C for 48 h for fibrillar $A\beta_{1-42}$ or 7 days for fibrillar $A\beta_{1-40}$. Globulomer $A\beta_{1-42}$ was prepared as described by Barghorn *et al*. (*12*). An HFIP-pre-treated $A\beta_{1-42}$-peptide film was re-suspended at a concentration of 5 mM in DMSO and sonicated for 20 s. It was diluted in PBS to 400 μM at a final concentration of 0.2% SDS by adding 1/10 vol 2% SDS. The globulomer $A\beta_{1-42}$ intermediate with 16/20-kDa resulted from incubation for 6 h at 37°C. The globulomer $A\beta_{1-42}$ with 38/48-kDa was generated by a further dilution with three volumes of H_2O and incubation for 18 h at 37°C.

Thioflavin T (ThT) Assay—Amyloid fibril formation was followed by a fluorometric ThT assay (*13*). Briefly, $A\beta_{1-42}$ was diluted to a final concentration of 2 μM in a 20-mM phosphate buffer, pH 7.2, containing 10 μM ThT. After a 2-min incubation at room temperature, the fluorescence of the ThT dye was measured at 482 nm using the excitation wavelength of 450 nm (Wallac 1420 ARVOsx: Wellesley, MA).

Electron Microscopy—A drop of the incubation mixture was placed on a single-hole copper grid and negatively stained with 2% aqueous uranyl acetate (Wako, Japan). Grid preparations were visualized by using an H-7000 transmission electron microscope operating at 75 kV (HITACHI, Tokyo).

Phage Library—Four kinds of a human scFv-displaying M13 phage library were constructed using the pCANTAB 5E phagemid vector as described previously (*14*). The *scFv* gene segments were constructed by ligating the *Vh* gene derived from the μ-chain cDNA with either Vκ-cDNA (μ/κ-library) or Vλ-cDNA (μ/λ-library) or the *Vh* gene derived from the γ-chain cDNA with either Vκ-cDNA (γ/κ-library) or Vλ-cDNA (γ/λ-library). The μ-library was composed of a μ/κ-library and a μ/λ-library at 5×10^{11} transforming units (TU) each, and the γ-library was a combination of the γ/κ-library and the γ/λ-library at 5×10^{11} TU each. The peptide-displaying phage libraries (Ph.D.-12) were purchased from New England Biolabs (Beverly, MA).

Biopanning—Biopanning was performed as described by Gejima *et al*. (*15*). Briefly, each well of a Cova-link plate (Nunc, Denmark) was activated by using dissuccinimidyl suberate [DSS with a spacer of $(CH_2)_6$] according to the manufacturer's instruction. After washing wells with distilled water, 100 μl of a 10 μg/ml $A\beta_{1-42}$ solution was added to the wells and incubated overnight at room temperature. The $A\beta_{1-42}$-coated plate was washed once with PBS containing 0.1% Tween-20 and blocked with either 0.5% gelatin for the first selection or 0.25% BSA for the second selection.

The phage library was incubated in an $A\beta_{1-42}$-coated plastic plate at room temperature for 1 h. After the plates were washed with PBS containing 0.1% Tween-20, the bound phages were eluted with 100 μl of 0.1 M glycin–HCl (pH 2.2) followed by immediate neutralization with 1 M Tris–HCl (pH 9.1). Phage amplification or preparation was performed as described (*14*).

In the case of the peptide-phage library, biopanning was performed as described (*16*, *17*). Briefly, each well of a Maxi-sorp plate (Nunc, Denmark) was coated with B6 scFv at 1,000 ng/100 μl in 0.1 M $NaHCO_3$ (pH 8.6). The isolated phage clones were amplified by infecting into *Escherichia coli* ER2738 cells.

Soluble scFv—The soluble scFv was prepared by infecting phage clones into *E. coli* HB2151 as described (*14*). The scFv fragments were purified with an RPAS purification module from the supernatant fraction according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

ELISA—ELISA was performed as described (*14*, *15*). Briefly, antigens (50 ng/40 μl/well) were adsorbed to a microtitre plate (Nunc, Denmark). Five micrograms per millilitre scFv clone or 0.5 μg/ml of BAM-10 (Sigma, Saint Louis, MO) was added to the wells followed by incubation with biotinylated anti-E tag mAb (Pharmacia, CA) at a dilution of 1:1,000 and detected by alkaline-phosphatase (AP)-conjugated strepavidin

(Vector Laboratories, Burlingame) or AP-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA) at a dilution of 1:1,000. Phage clones were detected by a biotinylated anti-M13 mAb (1:1,000, Pharmacia) in combination with AP-conjugated streptavidin. Absorbance was measured at 405 nm by the use of a microplate reader (NJ-2300; Nunc, Tokyo).

Electrophoresis—SDS–PAGE was carried out as described (*15*). Blue native PAGE (BN-PAGE) was performed as described (*18*). Globulomer $A\beta_{1-42}$ was subjected to BN-PAGE (4–16% gradient gel: Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The purified globulomer was obtained by slicing and crushing the gels containing globulomers in 1.7-ml tubes as described (*19*).

Immunoblotting Analysis—Non-denatured $A\beta_{1-42}$ conformers were subjected to SDS–PAGE (15%) and blotted to a PVDF membrane (Applied Biosystems) using a semi-dry electroblotter (Sartorius, Tokyo). After blocking with 5% skimmed milk, the conformers were detected using a combination of BAM-10 with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA) using ECL reagents (Pharmacia) on the image analyzer LAS-1000 (Fujifilm). The NEB pre-stained protein marker (NE Biolabs) was used as protein markers.

DNA Sequencing and Computer Analysis—The nucleotide sequence of the scFv genes was identified using the Dye Terminator Cycle Sequencing FS Ready Reaction kit (PE Applied Biosystems, Foster City, CA) with primers pCANTAB5-S1 (5′-CAACGTGAAAAAATTATTATTCGC-3′) and pCANTAB5-S8 (5′-GTAAATGAATTTTCTGTATGAGG-3′). Immunoglobulin V gene assignment was performed according to Kabat *et al*. (*20*, *21*). The germ-line V gene was assigned on the basis of the homology to a database (VBASE) of germ-line V genes compiled by Tomlinson *et al*. (*22*). The scFv nucleotide sequences were analysed by searching the IMGT/V-QUEST database using DNAPLOT software.

Competitive Absorption ELISA—This assay was performed according to a previously described method (*23*) with some modifications. Fibrillar $A\beta_{1-42}$ (100 ng/40 μl) was adsorbed to a microtitre plate, blocked with 0.5% gelatin/PBS for 8 h at 4°C, and washed with PBS containing 0.1% Tween-20 (PBST). The scFvs (200 ng/40 μl) was pre-incubated with 1,000 ng/10 μl of fibril or soluble $A\beta_{1-42}$ at 4°C for 8 h and then loaded onto fibrillar $A\beta_{1-42}$-coated microplate wells. After washing the plates with PBST, the binding scFv was detected with anti-E Tag mAb and AP-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA).

Fibril $A\beta_{1-42}$ Formation Inhibition Assay—An aliquot of the $A\beta_{1-42}$ stock solution was diluted to 20 μM in a 20-mM phosphate buffer, pH 7.2, in the absence or presence of varying concentrations of scFvs. The $A\beta_{1-42}$ fibrillation was monitored by periodically removing 10-μl aliquots from the incubation samples, followed by adding them to 90 μl of an 11-μM ThT solution (a 20-mM phosphate buffer, pH 7.2). The intensity of the fluorescence was monitored at an excitation wavelength of 450 nm and an emission wavelength of 482 nm by Wallac 1420 ARVOsx (Wellesley, MA).

Epitope Competition Assay—A microtitre plate was coated with B6 (100 ng/40 μl/well). Fibrillar $A\beta_{1-42}$ (100 ng/40 μl) was added to the well followed by incubation with 1 μg/ml BAM-10 (specific to the N-terminus of $A\beta_{1-42}$) or 8G7 (specific to the C-terminus of $A\beta_{1-42}$, CALBIOCHEM). The antibody was detected by AP-conjugated goat anti-mouse IgG.

RESULTS

Designation Profile of $A\beta_{1-42}$ Conformers—$A\beta_{1-42}$ peptide quickly converts the conformation leading to oligomers or fibril forms *in vitro* (Fig. 1Aa). This fibrillation was visualized by SDS–PAGE (Fig. 1Ab) and electron microscopy (Fig. 1Ac). SDS–PAGE showed that freshly prepared $A\beta_{1-42}$ contained small oligomers, such as dimers, trimers and tetramers, in addition to monomers. Fibrils were retained at the stacking gel phase (Fig. 1Ab and Ba, lane 3). Fibrillar $A\beta_{1-42}$ did not contain such oligomers at 48 h of incubation (Fig. 1Ac). The Aβ preparation at 0 h of this protocol is abbreviated as soluble $A\beta_{1-42}$, and the Aβ preparation after 48 h of incubation, as fibrillar $A\beta_{1-42}$. We generated the globulomer $A\beta_{1-42}$ as described [(*12*), Fig. 1B]. As this preparation contained various intermediates of oligomers (Fig. 1Ba), the distinctive globulomeric bands with nonamers to dodecamers (40–54 kDa) were purified under non-denaturing conditions using BN-PAGE (Fig. 1Bb). In our experiments, a rabbit anti-oligomer serum antibody, A11 (CHEMICON International, Inc., Temecula, CA), did not react to this preparation (data not shown). The reason for this lack of reaction is unknown. We used this preparation as globulomer $A\beta_{1-42}$ in this study.

scFvs Specific to Either Soluble or Fibrillar $A\beta_{1-42}$—By means of biopanning with the soluble $A\beta_{1-42}$ form, five scFv clones were established (Fig. 2A). These scFvs bound to both forms, soluble and fibrillar $A\beta_{1-42}$. They showed no binding activity to $A\beta_{1-40}$ irrespectively of whether it was the soluble or the fibril form. Furthermore, they did not show binding activity to the heat-denatured preparation in the immunoblotting analysis (Fig. 2D), indicating that they recognize the conformation of $A\beta_{1-42}$.

In cases of fibrillar $A\beta_{1-42}$-specific scFv selection, the fibrillar $A\beta_{1-42}$ was covalently conjugated to Cova-link plates with a chemical spacer moiety. This procedure might be useful to avoid a possible conformational change of fibrillar $A\beta_{1-42}$. Four fibrillar $A\beta_{1-42}$-specific scFv clones were established (Fig. 2B). Several distinct features were noted regarding their characterization. All four clones bound to fibrillar $A\beta_{1-42}$ with varying activities. When the colour-developing time for ELISA was fixed at 150 min, the reactivity of all these clones was found to be fibrillar $A\beta_{1-42}$-specific with no binding activity to soluble $A\beta_{1-42}$ or fibrillar $A\beta_{1-40}$. F10 scFv showed the highest binding activity to fibrillar $A\beta_{1-42}$; then B7, B6 and D1 scFvs did so in order. Among these clones, B6 scFv showed the most prominent specificity to fibrillar $A\beta_{1-42}$ in the absence of binding activity to a soluble form even at the longer time of colour developing in ELISA (Fig. 2C). These clones showed no significant

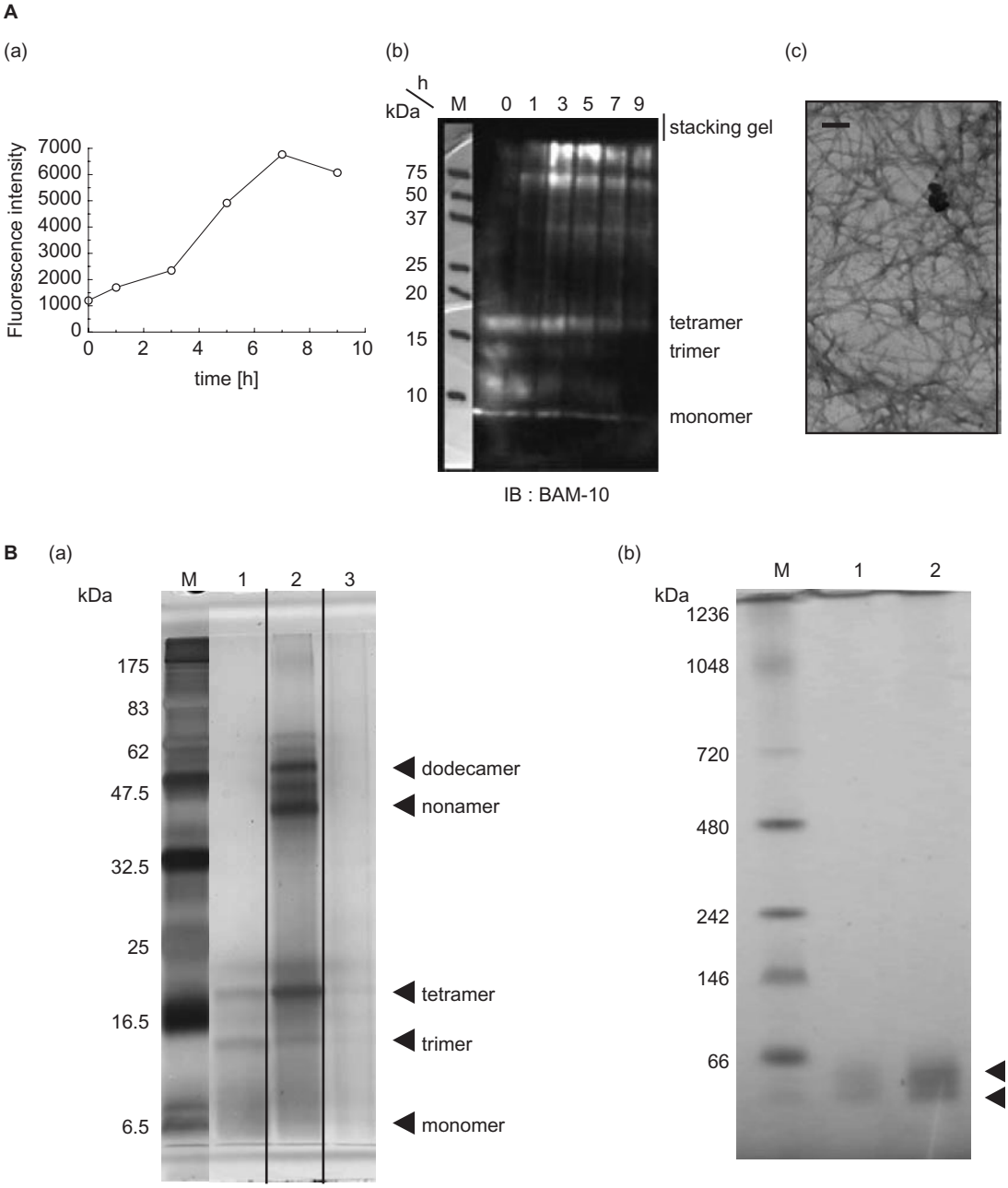


Fig. 1. **$A\beta_{1-42}$ conformers used in this study.** (A) $A\beta_{1-42}$ fibrillation was performed as described (*12*, *14*). This reaction was monitored either by (a) Thioflavin T assay, (b) western blotting (10% SDS–PAGE blotted with a murine anti-Aβ antibody, BAM-10) or (c) electron microscopy view of fibrillar $A\beta_{1-42}$ at 48 h. (B) Globulomer $A\beta_{1-42}$ (a) silver-stained 15% SDS–PAGE. Lane 1: soluble $A\beta_{1-42}$; lane 2: globulomer $A\beta_{1-42}$ (*13*); lane 3: fibrillar $A\beta_{1-42}$. (b) Blue native PAGE: the sample of Fig. 1B lane 2 was resolved. The arrows indicate the nonamer and dodecamer, lane 1: 2,250 ng, lane 2: 4,500 ng. Scale bar in picture: 100 nm.

binding activity to unrelated proteins. Immunoblotting analysis showed that these clones did not bind to heat-denatured fibrillar $A\beta_{1-42}$ forms (Fig. 2D), indicating that they are also conformation specific. On the contrary, a murine anti-Aβ mAb, BAM-10, bound equally to a soluble $A\beta_{1-42}$ and to globulomer and fibrillar $A\beta_{1-42}$ conformers and worked with western blot analysis (Figs 1B and 4C), suggesting that BAM-10 recognizes the primary amino acid sequence. A whole murine IgG, BAM-10, was used to estimate the amount of various $A\beta_{1-42}$ conformers coated on plastic plates.

DNA Sequencing of scFvs—The gene usage and amino acid sequences deduced from scFv DNA sequence data are presented in Fig. 3. All selected clones were unique in their variable heavy and light chains. It is of note that the *Vh* genes of B6 and B7 are very close to each other in the Vh3 family, and *Vl* genes are also close in B6, B7, D1 and F10 belonging to the Vl3 family.

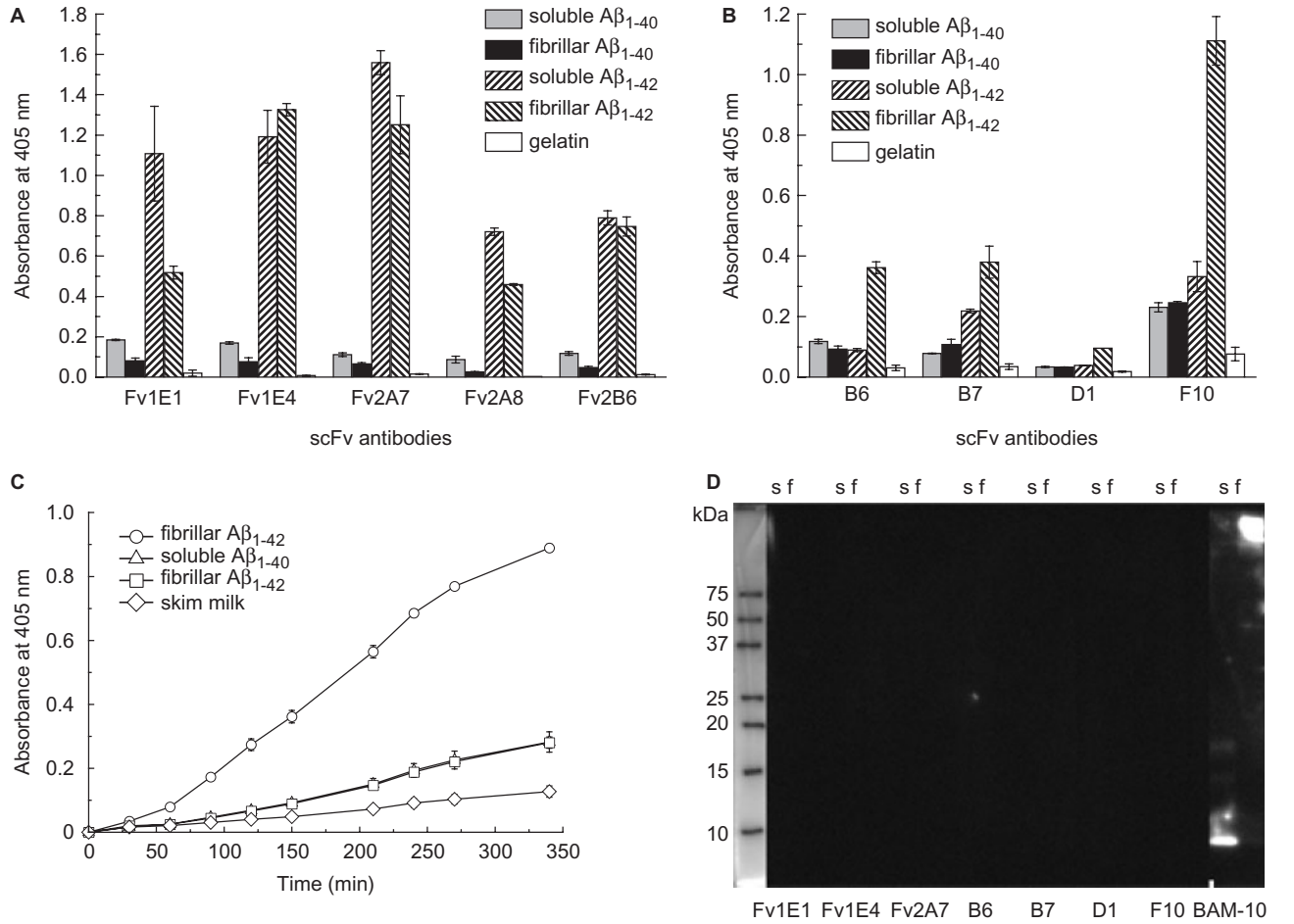


Fig. 2. **scFvs specific to either soluble or fiblillar Aβ$_{1–42}$.** The scFvs were purified by RPAS purification modules (Amersham Biosciences). (A) ELISA of scFv clones derived from the biopanning with soluble Aβ$_{1–42}$-coated plates: the reactivity of scFv (200 ng/40 μl/well) to fibrillar Aβ$_{1–42}$, soluble Aβ$_{1–42}$, fibrillar Aβ$_{1–40}$, soluble Aβ$_{1–40}$ or gelatin (100 ng/40 μl/well) was determined. (B) ELISA of scFv clones derived from the biopanning with fibrillar Aβ$_{1–42}$-coated plates, (C) time-course of ELISA of B6, (D) western blotting of heat-denatured soluble Aβ$_{1–42}$ (s) and fibril Aβ$_{1–42}$ (f). Ten percent SDS–PAGE was blotted with the selected scFvs or mouse BAM-10. The clone name is indicated below the panel of gel. All scFvs did not show significant binding activity to mouse IgG, gelatin or BSA in ELISA.

Fine Specificity of B6 scFv to Fibrillar Aβ$_{1–42}$ and Globulomer Aβ$_{1–42}$—Thus, we focused on the characterization of the binding specificity of B6 scFv. To confirm the binding specificity to fibrillar Aβ$_{1–42}$, we analysed the binding activity of B6 scFv to fibrillar Aβ$_{1–42}$ in solution in addition to its immobilized form on plastic plates. After pre-incubation of these B6 scFv with soluble or fibrillar Aβ$_{1–42}$ in solution, the binding activity of B6 was examined using fibrillar Aβ$_{1–42}$-coated plates. B6 scFv or BAM-10 lost the binding activity to fibrillar Aβ$_{1–42}$ when these scFvs were pre-incubated with fibrillar Aβ$_{1–42}$ (Fig. 4A). However, when B6 scFv was pre-incubated with soluble Aβ$_{1–42}$, B6 did not lose the binding activity to fibrillar Aβ$_{1–42}$ (Fig. 4B). In contrast, BAM-10 almost completely lost the binding activity to fibrillar Aβ$_{1–42}$. Thus, these results demonstrated the preference of the binding specificity of B6 to the fibrillar Aβ$_{1–42}$ form in the liquid phase.

As recent studies suggested that the Aβ$_{1–42}$ globulomer might be an active species in the neuropathology of AD, we tested the reactivity of B6 scFv using a globulomer preparation purified with BN-PAGE under a non-denatured condition. As shown in Fig. 4C, B6 scFv showed significant binding activity to the globulomer in addition to strong binding activity to the fibril. The amounts of coated Aβ$_{1–42}$ protein were confirmed by the binding of BAM-10.

Inhibitory Activity of B6 scFvs on Aβ$_{1–42}$ Fibrillation—Aβ$_{1–42}$ fibrillation was performed in the presence of various scFvs, particularly on B6 scFv at varying concentrations at the beginning of the Aβ$_{1–42}$ fibrillation reaction *in vitro* (Fig. 5). The scFv clones selected from soluble Aβ$_{1–42}$-coated plates hardly inhibited the Aβ$_{1–42}$ fibrillation except that Fv1E4 scFv showed a weak inhibitory activity (Fig. 5A and E). On the other hand, the scFv clones selected from fibril Aβ$_{1–42}$-coated plates markedly inhibited the Aβ$_{1–42}$ fibrillation (Fig. 5B). BAM-10 as an IgG form showed inhibition at 150 nM IC$_{50}$ (Fig. 5C and D). Compared with this result, B6 scFv exhibited 600 nM IC$_{50}$ in spite of its monovalent feature.

A Amino acid sequence of the Vh domains

clone		FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
Fv1E1	(IgG)	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	**S--YAIS**	WVRQAPGQGLEWMG	**RIIP--IFGIANYAQKFQG**	RVTITADKSTSTAYMELSSLRSEDTAVYYCAR	**GTTAS------PLDY**	WGQGTLVTVSS
Fv1E4	(IgG)	EVQLVESGTEVKKPGESLKISCKGSEYRFS	**S--YWIA**	WVRQMPGKGLDWMG	**LIYP--GDSDTRYSPSFQG**	QVTISADKSISTAYLQWSSLKASDTAMYYCAR	**HQLRGIAARSP-FDI**	WGQGTMVTVSS
Fv2A7	(IgM)	QVQLVQSGGGLVQPGRSLRLSCTASGFTFG	**D--YAMS**	WVRQAPGKGLEWVG	**FIRSKAYGGTTEYAASVKG**	RFTISRDDSKSIAYLQMNSLKTEDTAMYYCTR	**VRSGSY---YARPDY**	WGQGTLVTVSS
Fv2A8	(IgM)	QVQLQQSGAEVKKPGASVKVSCKASGYTFS	**S--YYMH**	WVRQAPGQGLEWMG	**IINP--SGGSTSYAQKFQG**	RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR	**PRYNWND--GDAFDI**	WGQGTMVTVSS
Fv2B6	(IgM)	EVQLVESGGGVVQPGRSLRLSCAASGFTFS	**D--YGMH**	WVRQAPGKGLEWVF	**LTSH--DGSNTKYADSVKG**	RFTISRDNSKNTVYLQMNSLRADDTAVYYCAN	**GFCGSNC---YYFHH**	WGRGTTVTVSS
B6	(IgM)	QVQLVQSGGGVVQPGGSLRLSCAASGFTFS	**R--YAMS**	WVRQAPGKGLEWVS	**AMSG--SGDTTYYADSVKG**	RFTISRDNSKKTVYLQMNRLRVEDTAIYYCAK	**DGRFRNRRS--DGFDT**	WGQGTLVTVSS
B7	(IgM)	QVQLVQSGGGLVQPGGSQRLSCAASGFGFS	**N--YAVS**	WVRQAPGTGLEWVA	**GVNG--GGQNTFYADSVKG**	RFTISRDNSKKTVYLQMNSLRVEDTAIYYCAK	**DGRFRNRRP--DGFDT**	WGQGTLVTVSS
D1	(IgM)	QVTLKESGGDLVKPGGSLRLSCAASGFTFR	**K--YYMA**	WIRQAPGKGPEWLS	**TISN--SGDIIDYADSVRG**	RFSISRDNAQKSLYLQMTSLRPDDSAIYYCAR	**EYFFS--------FDV**	WGRGTLVTVSS
F10	(IgM)	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	**S--YAIS**	WVRQAPGQGLEWMG	**RIIP--ILGIANYAQKFQG**	RVTITADKSTSTAYMELSSLRSEDTAVYYCAR	**AKRFAAARRGLDAFDI**	WGQGTMVTVSS

B Amino acid sequence of the Vl domains

clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
Fv1E1	LPVLTQPPSASGTPGQRVTISC	**SGSSSNIGTN-YVY**	WYQQLPGTAPKLLIY	**RN-----NQRPS**	GVPDRFSGSKSG--TSDSLAISGLRSEDEADYYC	**AVWD-ASLRGWL**	FGGGTQLTVL
Fv1E4	QSALTQPPSASGSPGQSVTISC	**TGTSGDVGRNNYVS**	WYQHHPGKAPKLMIY	**DV-----SKRPS**	GVSNRFSGSKSG--NTASLTISGLQAEDEADYYC	**SSYT-SSST-WV**	FGGGTKLTVL
Fv2A7	QAVLTQP---SGTPGQRVTLSC	**SGSSSNIGSN-TVY**	WYRQLPGTAPRLLIY	**SY-----NQRPS**	GVPDRFSGSKSG--TSASLAISGLRSEDEADYYC	**AAWD-DSLSGYV**	FGTGTKVTVL
Fv2A8	QTVVTQEPAVSVALGQTVTITC	**QG-DS-LRRY-YAS**	WYQQKPGQAPVLVVY	**GK-----NKRPS**	RIPARFSASSSR--NTASLTIHGARAEDEADYYC	**ESRDISGNHLYV**	FGTGTKVTVL
Fv2B6	QTVVTQEPSLTVSPGGTVTLTC	**GSSTGAVTSGHYPY**	WFQQKPGQAPRTLIY	**DT-----SNKHS**	WTPARFSGSLLG--GKAALTLSGAQPEDEAEYYC	**-LLSYSGVR--V**	FGGGTKLTVL
B6	SSELTQDPAVSVALGQTVRITC	**QG-DS-LRSY-YAS**	WYQQKPGQAPVLVIY	**GK-----DNRPS**	GIPDRFSGSSSG--NAASLTITGAQAEDEADYYC	**NSRDTSGNHL-V**	FGGGTKVTVL
B7	SSELTQDPNVSVALGQTVRITC	**QG-DT-LRNN-FPT**	WYQQKPGQAPVLVFY	**GK-----DNRPS**	GIPDRFSGSRSG--TTASLVITGAQAQDEADYYC	**NSRDSGGHHL-V**	FGGGTKLTVL
D1	SSELTQDPAVSVALGQTIRITC	**QG-DS-LRSY-YAS**	WYQQKPGQAPVLVIY	**GK-----NKRPS**	GIPDRISGSRSG--NTASLTITGAQAEDEADYYC	**NSRDSSGNHL-V**	FGGGTQLTVL
F10	SSELTQDPAVSVALGQTVRITC	**QG-DS-LRSY-YAS**	WYQQKPGQAPVLVIY	**GK-----NNRPS**	GIPDRFSGSSSG--NTASLTITGAQAEDEADYYC	**NSRDSSGNHL-V**	FGGGTKLTVL

C Variable gene usage

	Heavy chain (h)			Light chain (l)	
Clone#	V	D	J	V	J
Fv1E1	IGHV1-69*09	IGHD4-17*01	IGHJ4*02	IGLV1-47*01	IGLJ3*02
Fv1E4	IGHV5-51*01	IGHD6-6*01	IGHJ3*02	IGLV2-14*01	IGLJ3*02
Fv2A7	IGHV1-8*01	IGHD6-6*01	IGHJ4*02	IGKV3-20*01	IGKJ2*01
Fv2A8	IGHV3-49*03	IGHD1-26*01	IGHJ4*02	IGLV1-47*02	IGLJ1*01
Fv2B6	IGHV3-30*03	IGHD2-21*02	IGHJ2*01	IGLV7-46*01	IGLJ3*02
B6	IGHV3-23*04	IGHD6-6*01	IGHJ3*02	IGLV3-19*01	IGLJ3*02
B7	IGHV3-23*04	IGHD6-13*01	IGHJ3*02	IGLV3-19*01	IGLJ3*02
D1	IGHV3-11*01	IGHD2-21*01	IGHJ2*01	IGLV3-19*01	IGLJ3*02
F10	IGHV1-69*09	IGHD6-6*01	IGHJ3*02	IGLV3-19*01	IGLJ3*01

Fig. 3. **Amino acid sequence of the Vh and Vl domains of anti-Aβ_{1-42} scFvs.** The scFv nucleotide sequences were analysed by searching the IMGT/V-QUEST database using DNAPLOT software. The complementary determining regions (CDR1–CDR3) and the flanking regions (FR1–4) were deduced according to Kabat *et al*. Vh domains (A) and Vl domains (B). CDR regions are indicated in bold. (C) Variable gene usage was based on homology to a database (VBASE) of germ-line V-genes compiled by Tomlinson *et al*. (*23*).

IC_{50} values including other scFv clones are summarized in Fig. 5E.

To investigate the dynamics of Aβ_{1-42} conformation conversion, scFvs was added at various times after the onset of the Aβ_{1-42} fibrillation reaction and was monitored by a ThT reaction (Fig. 6A–D) and electron microscopic observation (Fig. 6E–J). Both assays showed consistent results, *i.e*. when B6 scFv was added to Aβ_{1-42} at 0 h, the Aβ_{1-42} fibril and aggregates were not at all observed after 30 h. When B6 scFv was added at 3 h, Aβ_{1-42} appeared to form a large sphere and an immature fibril. When B6 scFv was added to Aβ_{1-42} at 6 h, the pre-existing fibril did not decrease with further incubation, and a mature fibril was observed. In the same experiments, Fv2A7 (anti-Aβ_{1-42} scFv as a negative control, see Fig. 2A) or unrelated scFv (data not shown) showed no influence on Aβ_{1-42} fibrillation.

Epitope Analysis of B6 scFv—The murine antibody, BAM-10, recognizes the N-terminus, while 8G7 binds to the C-terminus of Aβ_{1-42} (*24*). In order to examine the binding site of B6 scFv on Aβ_{1-42}, we tested the competitive binding assay of B6 scFv to Aβ_{1-42} in sandwich ELISA. As shown in Fig. 8, B6 scFv did not compete with BAM-10, whereas it was competitive with 8G7 (Fig. 7).

The epitope of B6 scFv was also determined using Ph.D.-12 random peptide-displaying phage libraries. We isolated five B6-specific clones (Fig. 8A). The DNA sequences of four clones were identical. Therefore, two deduced amino acid sequences were elucidated (Fig. 8B). Homology search using CLUSTAL W ver. 3.1 suggested a weakly similar region at Aβ31-36. Thus, both epitope analyses suggested that B6 scFv might bind to the C-terminus of Aβ_{1-42}.

DISCUSSION

There are over a 100 anti-Aβ monoclonal antibodies commercially available. These antibodies are prepared using a completely animal-based, cell-hybridization approach; therefore, they are of murine-origin, and their specificity is directed mainly to the soluble form of Aβ. By employing an antibody-displaying phage library, we here demonstrated the feasibility of a snapshot antibody signature of the conformational conversion of protein molecule. We discuss two scFv clones; one is Fv1E4, which was selected with soluble Aβ_{1-42}-coated plates, and the other is B6, which was selected from fibrillar Aβ_{1-42}-coated plates.

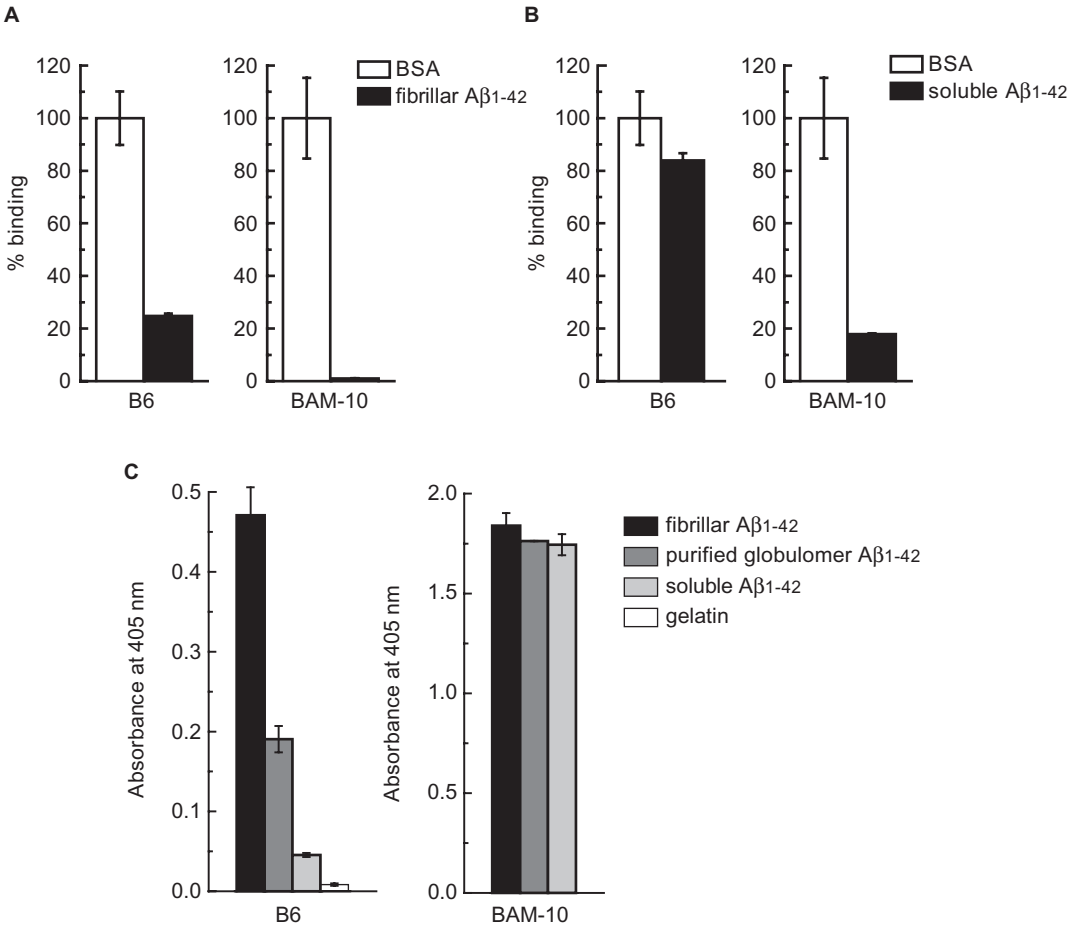
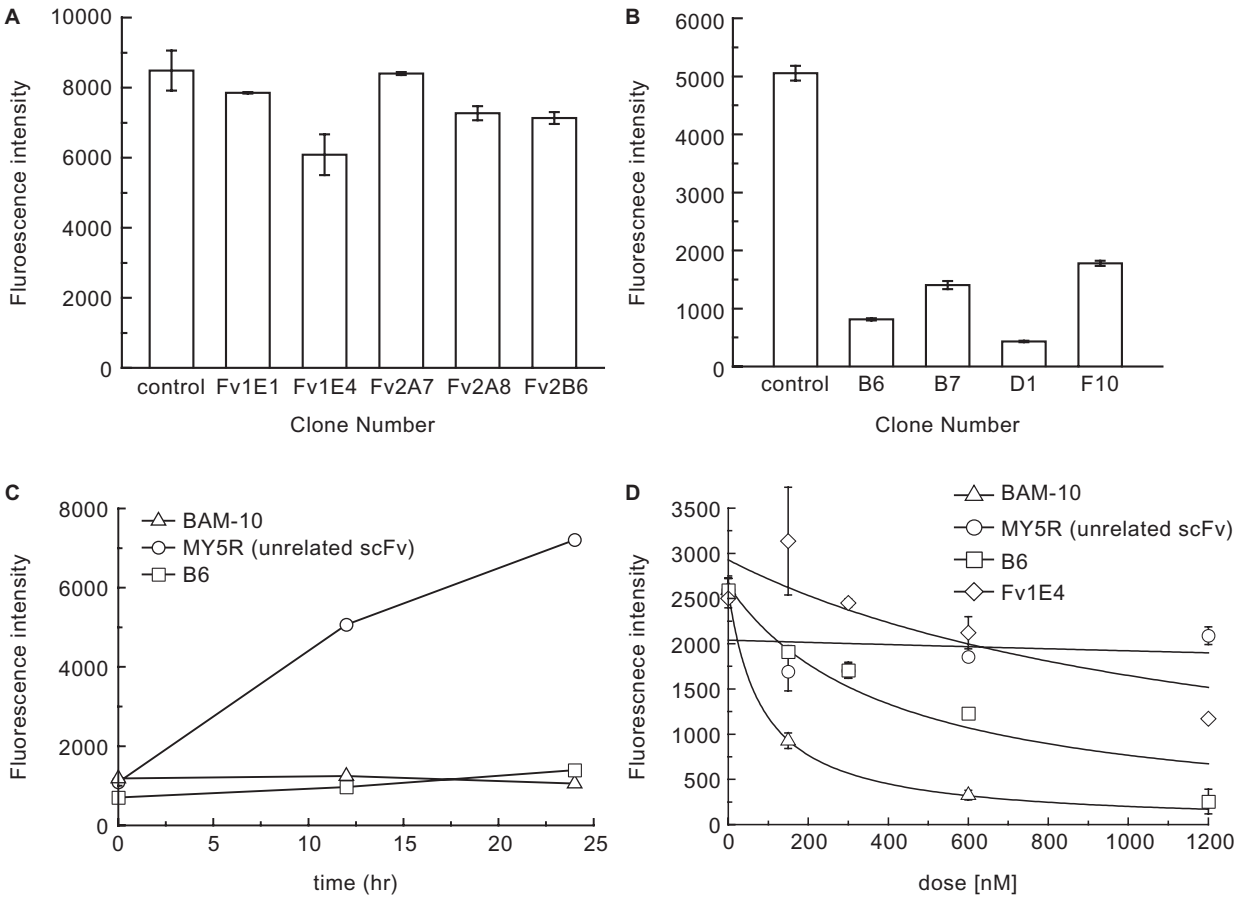


Fig. 4. **Binding specificity of scFv B6.** Competitive absorption ELISA was performed as described in MATERIALS AND METHODS section. Briefly, B6 scFv (200 ng/40 μl) pre-incubated with soluble or fibril $A\beta_{1-42}$ (1,000 ng) was added to a fibrillar $A\beta_{1-42}$-coated ELISA plate (100 ng/40 μl), and its binding activity was determined. Absorption of B6 scFv with fibrillar (A) or soluble $A\beta_{1-42}$ (B). B6 scFv was absorbed with fibrillar but not soluble $A\beta_{1-42}$, while BAM-10 was absorbed with both conformers. (C) Binding activity of B6 scFv to purified globulomer $A\beta_{1-42}$. ELISA was performed using the fibrillar, purified globulomer or soluble $A\beta_{1-42}$-coated plates as described. The amounts of $A\beta_{1-42}$ conformers coated on plastic plates were determined by BAM-10.

As noticed, a soluble Aβ preparation may contain marginal amounts of dimers, trimers, tetramers and low-molecular oligomers in addition to a monomer, as shown in SDS–PAGE (Fig. 1). Fv1E4 scFv binds to fibrillar $A\beta_{1-42}$ as well as to soluble forms, while B6 binds to fibrillar $A\beta_{1-42}$ but not to soluble forms. As shown in western blot analysis, Fv1E4 and B6 scFvs recognize the conformation but not the primary amino acid sequence of $A\beta_{1-42}$. Regarding the specificity, these scFvs failed to stain the formalin-fixed brain tissue of AD patients (data not shown). However, the *in vivo* immunoreactivity of these antibodies to the native $A\beta_{1-42}$ form still remains to be determined in an animal model.

In this study, we used two kinds of phage libraries, *i.e.* a μ-library or a γ-library, which were constructed from IgM mRNA or IgG mRNA, respectively. Six soluble $A\beta_{1-42}$-specific clones were selected from both μ- and γ-libraries, while fibrillar $A\beta_{1-42}$-specific clones were selected from the μ-library alone when 192 clones were examined. It is conceivable that the germ-line V gene may have affinity to the amyloid form, since V genes derived from the μ-chain cDNA are closer to the germ-line type relative to the γ-chain.

It is of note that soluble $A\beta_{1-42}$-selected scFvs, except Fv1E4, hardly inhibited the $A\beta_{1-42}$ fibrillation, whereas all fibrillar $A\beta_{1-42}$-selected scFvs, including B6, significantly showed an inhibitory effect against the fibrillation of $A\beta_{1-42}$ despite their monovalent nature (Fig. 5). This result suggests that the freshly prepared soluble $A\beta_{1-42}$ has not yet expressed the epitope involving the fibrillar assembly. When the inhibitory effect of each scFv clone was characterized by adding these scFvs on the course of the $A\beta_{1-42}$-fibrillation reaction at varying times after the onset of reaction, they showed a distinct influence of their own (Figs 5 and 6). The inhibitory effects (IE) of B6 or Fv1E4 scFv were detected at the early phase, fading away by around 3 h, although the IE of Fv1E4 were very weak ($IC_{50} > 1{,}400$ nM: Fig. 5E). The IE of BAM-10 was evident from 0 to 3 h but no longer significant at the point of 6 h. This result suggests the presence of the epitope-bearing conformers as fibrillar $A\beta_{1-42}$ intermediates at the scFv addition time. Active molecular species



E

clone	IC50
BAM-10	150 nM
B6	600 nM
B7	800 nM
D1	400 nM
F10	1000 nM
Fv1E4	1400 nM <

Fig. 5. **Inhibitory activity of B6 scFv on $A\beta_{1-42}$ fibrillation.** Fibrillation of $A\beta_{1-42}$ (20 μM) was monitored for 24 h by ThT fluorescence in the absence and presence of 1,200 nM antibodies selected either by soluble $A\beta_{1-42}$-coated plates (A) or fibril $A\beta_{1-42}$–coated plates (B). (C) Time course of fibrillation of $A\beta_{1-42}$ (20 μM) monitored for 24 h by ThT fluorescence in the absence and presence of 1,200 nM antibodies. BAM-10: murine whole IgG specific to Aβ, MY5R: scFv with unrelated specificity. (D) $A\beta_{1-42}$ fibrillation was determined by a ThT assay in the presence of varying concentrations of antibodies at 24 h. (E) The IC_{50} value of all antibodies tested on $A\beta_{1-42}$ fibrillation is summarized.

on neurodegenerative effects have been postulated as oligomers, such as ADDL or globulomers. In this regard, B6 scFv did not bind to ADDL [(*2*), data not shown], while it recognized globulomers (Fig. 4C). This result suggested that there were conformational differences between these oligomers. It is unknown whether B6 scFv could inhibit the assembly process towards globulomer formation *in vivo* because the binding activity of B6 scFv could not be examined in the SDS-containing refolding solution of globulomers *in vitro*. Thus, it is still unclear how different was the structure of intermediates of $A\beta_{1-42}$ assembling towards ADDL, globulomer or fibre. Furthermore, the cell cytotoxicity of ADDL, globulomers, or fibrils of $A\beta_{1-42}$ was also examined with an LDH assay (Promega, Madison, WI) or an MTT assay (Dojindo, Japan) using a human neuroblastoma cell line, SH-SY5Y, in the absence or presence of retinoic acid, according to the reports (*2*, *25*). As these *in vitro*

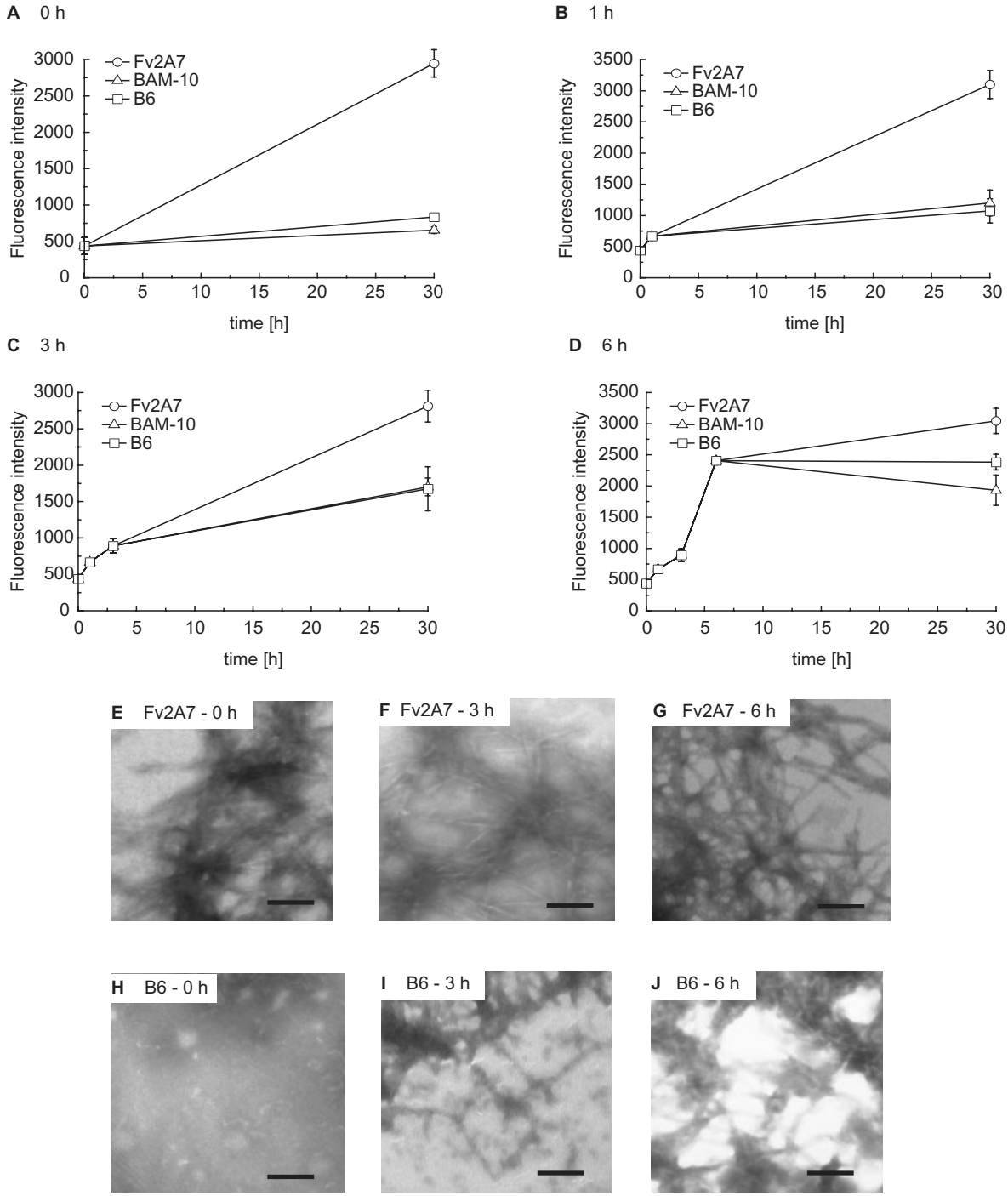


Fig. 6. **Time-dependent inhibitory activity of B6 scFv on $A\beta_{1-42}$ fibrillation.** The ThT assay was performed at 30 h after antibodies were added at 0, 1, 3 or 6 h after the onset of $A\beta_{1-42}$ fibrillation at a concentration of 1,200 nM (A–D). The same experiments were observed by EM (E–J) in the presence of Fv2A7 scFv (see Fig. 2A: E–G) or scFv B6 (H–J). Scale bar in picture: 100 nm.

refolding $A\beta_{1-42}$ conformers contained DMSO or SDS in their solution, we could not exclude the cytotoxicity due to the minute amount of possible contamination resulting from these denaturing chemical reagents. It is unknown why there has been inconsistency between our results and those from reported observations (*12*). We have not examined the influences of these preparations on long-term potentiation in *in vitro* or *in vivo* experiments.

Epitope analysis of B6 scFv suggested that B6 scFv might recognize the C-terminus of $A\beta_{1-42}$ (Fig. 7). In another experiment, 12E4 antibody which was established by immunizing last six amino acids of C-terminus

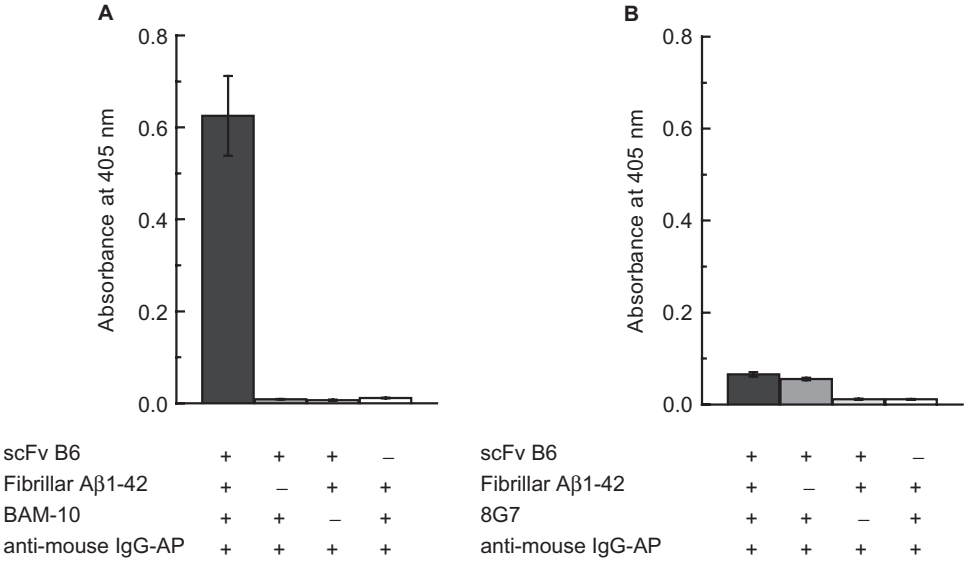


Fig. 7. **Epitope competition assay of B6 scFv.** An ELISA plate was coated with scFv B6 (100 ng/40 μl). Fibrillar $A\beta_{1–42}$ was added to the well followed by 1 μg/ml anti-$A\beta_{1–42}$ mAb, either BAM-10 (A) or 8G7 (B). The reactivity of B6, BAM-10 and 8G7 to fibrillar $A\beta_{1–42}$ was confirmed in the same experiments.

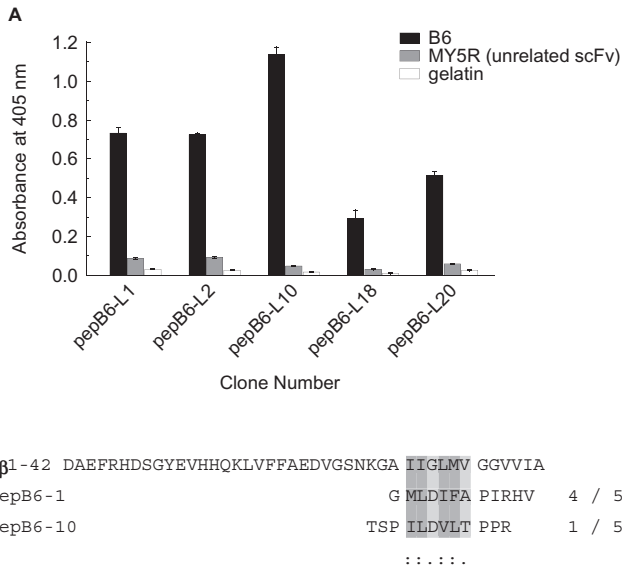


Fig. 8. **Epitope analysis of B6 scFv using a peptide-displaying phage library.** B6 scFv-specific peptide-displaying phage clones were isolated by biopanning as described (*17*, *18*). (A) B6 scFv-specific peptide-displaying phage clones. An ELISA plate was coated with B6 or MY5R scFv (unrelated clone: 50 ng/40 μl). The phage clones (4.0×10^{10} virions/40 μl) were added to the wells, and their binding was detected as described (*15*). (B) The amino acid sequence of B6-specific phage clones was determined by DNA sequencing. The homologous region between the $A\beta_{1–42}$ and scFv epitopes was searched using CLUSTAL W ver. 3.1. The mark indicated below the epitope sequence, ':' or '.' indicates the conserved or semi-conserved residues, respectively. Four of five clones (4/5) had identical peptide sequences (pepB6-1), and one (1/5: pepB6-10) was unique.

conjugated with KLH in mice competed with B6 scFv on the binding to $A\beta_{1–42}$ fibre-coated plastic plates whereas 6E10 antibody which was established by immunizing first 3–8 amino acids (EFRHDS) of N-terminus conjugated with carrier protein in mice did not (data not shown). Figure 8 suggested the involvement of Aβ31–36 at the β2 sheet of the C-terminus of $A\beta_{1–42}$. This structure is suggested to be involved in fibrillar elongation (*26*). These data indicated that B6 scFv inhibited fibril formation by means of capping the end point of the fibril. However, it is unlikely that this binding leads to the disaggregation of pre-formed fibrils and large aggregates, as no disaggregation activity of B6 scFv was observed when an $A\beta_{1–42}$ fibre prepared by 50 μM $A\beta_{1–42}$ peptide was incubated in the presence of a 6.4-μM concentration of B6 scFv (data not shown).

Regarding the therapeutic purpose, a promising strategy for reducing the level of Aβ in the brain is immunotherapy, where soluble Aβ-specific antibodies facilitate the clearance of Aβ although they bind to the normal Aβ peptide as well as the APP molecule (*27*). It was demonstrated that the active immunization of Aβ is effective in murine experimental models (*28*). However, in human cases, inflammatory side effects caused by primed T cells were observed (*29–31*).

An alternative potentially non-inflammatory approach to facilitate clearance is passive immunization, *i.e*. the administration of an antibody against the amyloid peptide (*32–34*). Thus, B6 scFv is ideal for both diagnostic and therapeutic purposes, as it is a complete human antibody specific to the active form of neurotoxic Aβ but not that of soluble Aβ detected in blood serum and normal APP in brain.

Snapshot antibodies including B6 and Fv1E4 should be examined regarding their influence on the pathogenesis of the mouse model of AD. If a distinctive Aβ intermediate molecule complexed with specific scFv is easily crystallized, X-ray crystallographic analysis

could be possible to define the transitional structure of Aβ. Based on the structural information of Aβ, we are able to pinpoint the neurotoxic structure and elucidate the flowchart of molecular conversion by testing the inhibiting or neutralizing neurodegenerative activity of these antibody clones.

REFERENCES

1. Katzman, R. and Saitoh, T. (1991) Advances in Alzheimer's disease. *FASEB J.* **5**, 278–286
2. Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., and Liosatos, M. (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc. Natl Acad. Sci. USA* **95**, 6448–6453
3. Roher, A.E., Chaney, M.P., Kuo, Y., Webster, S.D., Stine, W.B., and Havrkamp, L.J. (1996) Morphology and toxicity of Abeta-(1-42) dimer derived from neuritic and vascular amyloid deposits of Alzheimer's disease. *J. Biol. Chem.* **271**, 20631–20635
4. Sylvain, L., Ming, T.K., Linda, K., Rakez, K., Charles, G., Austin, Y., Michela, G., and Karen, H.A. (2006) A specific amyloid-β protein assembly in the brain impairs memory. *Nature* **440**, 352–357
5. Haass, C., Schlossmache, M., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, L.B., Liebergurg, I., Koo, H.E., Schenk, D., Teplow, B.D., and Selkoe, J.D. (1992) Amyloid β-peptide is produced by cultured cells during normal metabolism. *Nature* **359**, 322–325
6. Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., Mccormack, R., Wolfert, R., Selkoe, D., Lieberburg, I., and Schenk, D. (1992) Isolation and quantification of soluble Alzheimer's β-peptide from biological fluids. *Nature* **359**, 325–327
7. Shoji, M., Golde, T.E., Ghiso, J., Cheung, T.T., Estus, S., Shaffer, L.M., Cai, X.D., McKay, D.M., Tintner, R., and Frangione, B. (1992) Production of the Alzheimer amyloid beta protein by normal proteolytic processing. *Science* **258**, 126–129
8. Burdick, D., Soreghan, B., Kwon, M., Kosmoski, J., Knauer, M., Henschen, A., Yates, J., Cotman, C., and Glabe, C. (1992) Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. *J. Biol. Chem.* **267**, 546–554
9. Jarrett, J.T., Berger, E.P., and Lansbury P.T., Jr., (1993) The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* **32**, 4693–4697
10. McGowan, E., Pickford, F., Kim, J., Onstead, L., Eriksen, J., Yu, C., Skipper, L., Murphy, M. P., Beard, J., Das, P., Jansen, K., Delucia, M., Lin, W.L., Dolios, G., Wang, R., Eckman, C.B., Dickson, D.W., Hutton, M., Hardy, J., and Golde, T. (2005) Aβ42 is essential for parenchymal and vascular amyloid deposition in mice. *Neuron* **47**, 191–199
11. Stine, W.B. Jr, Dahlgren, K.N., Krafft, G.A., and LaDu, M.J. (2003) In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. *J. Biol. Chem.* **278**, 11612–11622
12. Barghorn, S., Nimmrich, V., Striebinger, A., Krantz, C., Keller, P., Janson, B., Bahr, M., Schmidt, M., Bitner, R.S., Harlan, J., Barlow, E., Ebert, U., and Hillen, H. (2005) Globular amyloid beta-peptide oligomer - a homogenous and stable neuropathological protein in Alzheimer's disease. *J. Neurochem.* **95**, 834–847
13. LeVine, H., III. (1993) Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. *Protein Sci.* **2**, 404–410
14. Hashiguchi, S., Nakashima, T., Nitani, A., Yoshihara, T., Yoshinaga, K., Ito, Y., Maeda, Y., and Sugimura, K. (2003) Human Fc epsilon RIalpha-specific human single-chain Fv (scFv) antibody with antagonistic activity toward IgE/Fc epsilon RIalpha-binding. *J. Biochem.* **133**, 43–49
15. Gejima, R., Tanaka, K., Nakashima, T., Hashiguchi, S., Ito, Y., Yoshizaki, K., and Sugimura, K. (2002) Human single-chain Fv (scFv) antibody specific to human IL-6 with the inhibitory activity on IL-6-signaling. *Hum. Antibodies* **11**, 121–129
16. Fukumoto, T., Torigoe, N., Kawabata, S., Murakami, M., Uede, T., Nishi, T., Ito, Y., and Sugimura, K. (1998) Peptide mimics of the CTLA-4-binding domain stimulate T-cell proliferation. *Nat. Biotechnol.* **16**, 267–270
17. Hamasaki, T., Hashiguchi, S., Ito, Y., Kato, Z., Nakanishi, K., Nakashima, T., and Sugimura, K. (2005) Human anti-human IL-18 antibody recognizing the IL-18-binding site 3 with IL-18 signaling blocking activity. *J. Biochem.* **138**, 433–442
18. Swamy, M., Siegers, G.M., Minguet, S., Wollschied, B., and Schamel, W.W.A. (2006) Blue native polyacrylamide gel electrophoresis (BN-PAGE) for the identification and analysis of multiprotein complexes. *Sci. STKE* **2006**, pl4
19. Hamasaki, T., Uchida, S., Yoshihara, T., Hashiguchi, S., Ito, Y., and Sugimura, K. (2007) Biopanning of antibody-phage clones using immunoplates coated with gel slices of electrophoresis: immunogel-biopaninng. *Biol. Pharm. Bull.* **30**, 1361–1364
20. Kabat, E.A. and Wu, T.T. (1991) Identical V region amino acid sequences and segments of sequences in antibodies of different specificities. Relative contributions of VH and VL genes, minigenes, and complementarity-determining regions to binding of antibody-combining sites. *J. Immunol.* **147**, 1709–1719
21. Johnson, G., Wu, T.T., and Kabat, E.A. (1995) SEQHUNT. A program to screen aligned nucleotide and amino acid sequences. *Methods Mol. Biol.* **51**, 1–15
22. Tomlinson, I.M., Williams, S.C., Ignatovich, O., Corbett, S.J., and Winter, G. (1996) *VBASE Sequencing Directory,* Medical Research Council Center for Protein Engineering, Cambridge
23. Hashimoto, T., Wakabayashi, T., Watanabe, A., Kowa, H., Hosoda, R., Nakamura, A., Kanazawa, I., Arai, T., Takio, K., Mann, D.M., and Iwatsubo, T. (2002) CLAC: a novel Alzheimer amyloid plaque component derived from a transmembrane precursor, CLAC-P/collagen type XXV. *EMBO J.* **21**, 1524–1534
24. Kotilinek, A.L., Bacskai, B., Westerman, M., Kawarabayashi, T., Younkin, L., Hyman, T.B., Younkin, S., and Ashe, H.K. (2002) Reversible memory loss in a mouse transgenic model of Alzheimer's disease. *J. Neurosci.* **22**, 6331–6335
25. Kayed, R., Head, E., Thompson, L.J., Mclntire, M.T., Milton, C.S., Cotman, W.C., and Glabe, G.C. (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* **300**, 486–489
26. Luhrs, T., Ritter, C., Adrian, M., Rieki-Loher, D., Bohrmann, B., Dobeli, H., Schubert, D., and Riek, R. (2005) 3D structure of Alzheimer's amyloid-beta (1-42) fibrils. *Proc. Natl Acad. Sci. USA* **102**, 17342–17347
27. Bard, F., Cannon, C., Barbour, R.L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Lieberburg, I., Motter, R., Nguyen, M., Soriano, F., Vasquez, N., Weiss, K., and Welch, B. (2000) Peripherally administered antibodies against amyloid β-peptide enter the central nervous

system and reduce pathology in a mouse model of Alzheimer disease. *Nat. Med.* **6**, 916–919
28. Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Liegerburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevert, C., Walker, S., Wogulis, M., Yednock, T., Games, D., and Seubert, P. (1999) Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* **400**, 173–177
29. Birmingham, K. and Frantz, S. (2002) Set back to Alzheimer vaccine studies. *Nat. Med.* **8**, 199–200
30. Bishop, G.M., Robinson, S.R., Smith, M.A., Perry, G., and Atwood, C.S. (2002) Call for Elan to publish Alzheimer's trial details. *Nature* **416**, 677
31. Check, E. (2002) Nerve inflammation halts trial for Alzheimer's drug. *Nature* **415**, 462
32. Mohajeri, M.H., Saini, K., Schultz, J.G., Wollmer, M.A., Hock, C., and Nitsch, R.M. (2002) Passive immunization against beta-amyloid peptide protects central nervous system (CNS) neurons from increased vulnerability associated with an Alzheimer's disease-causing mutation. *J. Biol. Chem.* **277**, 33012–33017
33. Wilcock, D.M., DiCarlo, G., Henderson, D., Jackson, J., Clarke, K., Ugen, K.E., Gordon, M.N., and Morgan, D. (2003) Intracranially administered anti-Abeta antibodies reduce beta-amyloid deposition by mechanisms both independent of and associated with microglial activation. *J. Neurosci.* **23**, 3745–3751
34. Bacskai, B.J., Kajdasz, S.T., McLellan, M.E., Games, D., Seubert, P., Schenk, D., and Hyman, B.T. (2002) Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy. *J. Neurosci.* **22**, 7873–7878