DIAGNOSTIC ANTIBODY ASSAY

The present invention pertains to novel diagnostic assays for the diagnosis of amyloidosis, a group of disorders and abnormalities associated with amyloid protein such as Alzheimer's disease and related aspects. In particular, an antibody assay is provided.

Amyloidosis is not a single disease entity but rather a diverse group of progressive disease processes characterized by extracellular tissue deposits of a waxy, starch-like protein called amyloid, which accumulates in one or more organs or body systems. As the amyloid deposits accumulate, they begin to interfere with the normal function of the organ or body system. There are at least 15 different types of amyloidosis. The major forms are primary amyloidosis without known antecedent, secondary amyloidosis following some other condition, and hereditary amyloidosis.

Secondary amyloidosis occurs during chronic infection or inflammatory disease, such as tuberculosis, a bacterial infection called familial Mediterranean fever, bone infections (osteomyelitis), rheumatoid arthritis, inflammation of the small intestine (granulomatous ileitis), Hodgkin's disease and leprosy.

Amyloid deposits include amyloid P (pentagonal) component (AP), a glycoprotein related to normal serum amyloid P (SAP), and sulphated glycosaminoglycans (GAG), complex carbohydrates of connective tissue. Amyloid protein fibrils, which account for about 90% of the amyloid material, comprise one of several different types of proteins. These proteins are capable of folding into so-called "beta-pleated" sheet fibrils, a unique protein configuration which exhibits binding sites for Congo red resulting in the unique staining properties of the amyloid protein.

Many diseases of aging are based on or associated with amyloid-like proteins and are characterized, in part, by the buildup of extracellular deposits of amyloid or amyloid-like material that contribute to the pathogenesis, as well as the progression of the disease. These diseases include, but are not limited to, neurological disorders such as mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer's dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome, , Lewy body dementia, , hereditary cerebral hemorrhage with amyloidosis (Dutch type); the Guam Parkinson-Dementia complex. Other diseases which are based on or associated with amyloid-like proteins are progressive supranuclear palsy, multiple sclerosis; Creutzfeld Jacob disease, Parkinson's disease, HIV-related dementia, ALS (amyotropic lateral sclerosis), Adult Onset Diabetes; senile cardiac amyloidosis; endocrine tumors, and others, including macular degeneration.

Although pathogenesis of these diseases may be diverse, their characteristic deposits often contain many shared molecular constituents. To a significant degree, this may be attributable to the local activation of pro-inflammatory pathways thereby leading to the concurrent deposition of activated complement components, acute phase reactants, immune modulators, and other inflammatory mediators (McGeer et al., Tohoku J Exp Med. 174(3): 269-277 (1994)).

Recently, accumulating evidence demonstrates involvement of N-terminal modified Aβ peptide variants in Alzheimer's disease. Aiming biopsies display a presence of Aβ 1-40 and Aβ 1-42 not only in the brain of Alzheimer's patients but also in senile plaques of unaffected individuals. However, N-terminal truncated and pyroGlu modified Aβ N3pE-40/Aβ N3pE-42 is almost exclusively engrained within plaques of Alzheimer's disease patients, making this Aβ variant an eligible diagnostic marker and a potential target for drug development.

At present, several commercial manufacturers offer ELISA kits which allow a detection of Aβ 1-40 / 1-42 and Aβ N3pE-40/Aβ N3pE-42 in the low picogramm (pg) range.

The brains of Alzheimer's disease (AD) patients are morphologically characterized by the presence of neurofibrillary tangles and by deposits of Aβ peptides in neocortical brain structures (Selkoe, D.J. & Schenk, D. Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. Annu. Rev. Pharmacol. Toxicol. 43, 545-584 (2003)). Aβ peptides are liberated from the amyloid precursor protein (APP) after sequential cleavage by β- and γ-secretase. The γ-secretase cleavage results in the generation of Aβ 1-40 and Aβ 1-42 peptides, which differ in their C-termini and exhibit different potencies of aggregation, fibril formation and neurotoxicity (Shin, R.W. et al. Amyloid beta-protein (Abeta) 1-40 but not Abeta 1-42 contributes to the experimental formation of Alzheimer disease amyloid fibrils in rat brain. J. Neurosci. 17, 8187-8193 (1997); Iwatsubo, T. et al. Visualization of Abeta 42(43) and Abeta 40 in senile plaques with end-specific Abeta monoclonals: evidence that an initially deposited species is Abeta 42(43). Neuron 13, 45-53 (1994); Iwatsubo, T., Mann, D.M., Odaka, A., Suzuki, N. & Ihara, Y. Amyloid beta protein (Abeta) deposition: Abeta 42(43) precedes Abeta 40 in Down syndrome. Ann. Neurol. 37, 294-299 (1995); Hardy, J.A. & Higgins, G.A. Alzheimer's disease: the amyloid cascade hypothesis. Science 256, 184-185 (1992); Roßner, S., Ueberham, U., Schliebs, R., Perez-Polo, J.R. & Bigl, V. The regulation of amyloid precursor protein metabolism by cholinergic mechanisms and neurotrophin receptor signaling. Prog. Neurobiol. 56, 541-569 (1998)). In addition to C-terminal variability, N-terminally modified Aβ peptides are abundant (Saido, T.C. et al. Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques. Neuron 14, 457-466 (1995) ; Russo, C. et al. Presenilin-1 mutations in Alzheimer's disease. Nature 405, 531-532 (2000); Saido, T.C., Yamao, H., Iwatsubo, T. & Kawashima, S. Amino- and carboxyl-terminal

heterogeneity of beta-amyloid peptides deposited in human brain. Neurosci. Lett. 215, 173-176 (1996)). It appears that a major proportion of the Aβ peptides undergoes N-terminal truncation by two amino acids, exposing a glutamate residue, which is subsequently cyclized into pyroglutamate (pE), resulting in Aβ3(pE)-42 peptides (Saido, T.C. et al. Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques. Neuron 14, 457-466 (1995) ; Saido, T.C., Yamao, H., Iwatsubo, T. & Kawashima, S. Amino- and carboxyl-terminal heterogeneity of beta-amyloid peptides deposited in human brain. Neurosci. Lett. 215, 173-176 (1996)). Alternatively, pE may be formed following β'-cleavage by BACE1, resulting in Aβ N11(pE)-42 (Naslund, J. et al. Relative abundance of Alzheimer A beta amyloid peptide variants in Alzheimer disease and normal aging. Proc. Natl. Acad. Sci. U. S. A. 91, 8378-8382 (1994); Liu, K. et al. Characterization of Abeta11-40/42 peptide deposition in Alzheimer's disease and young Down's syndrome brains: implication of N-terminally truncated Abeta species in the pathogenesis of Alzheimer's disease. Acta Neuropathol. 112, 163-174 (2006)). In particular Aβ N3(pE)-42 has been shown to be a major constituent of Aβ deposits in sporadic and familial Alzheimer's disease (FAD) (Saido, T.C. et al. Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques. Neuron 14, 457-466 (1995) ; Miravalle, L. et al. Amino-terminally truncated Abeta peptide species are the main component of cotton wool plaques. Biochemistry 44, 10810-10821 (2005)).

The Aβ N3pE-42 peptides coexist with Aβ 1-40/1-42 peptides (Saido, T.C. *et al.* Dominant and differential deposition of distinct beta-amyloid peptide species, Abeta N3pE, in senile plaques. *Neuron* 14, 457-466 (1995) ; Saido, T.C., Yamao, H., Iwatsubo, T. & Kawashima, S. Amino- and carboxyl-terminal heterogeneity of beta-amyloid peptides deposited in human brain. *Neurosci. Lett.* 215, 173-176 (1996)), and, based on a number of observations, could play a prominent role in the pathogenesis of AD. For example, a particular neurotoxicity of Aβ N3pE-42 peptides has been outlined (Russo, C. *et al.* Pyroglutamate-

modified amyloid beta-peptides--AbetaN3(pE)--strongly affect cultured neuron and astrocyte survival. *J. Neurochem.* 82, 1480-1489 (2002) and the pE-modification of N-truncated Aβ peptides confers resistance to degradation by most aminopeptidases as well as Aβ-degrading endopeptidases (Russo, C. *et al.* Pyroglutamate-modified amyloid beta-peptides--AbetaN3(pE)--strongly affect cultured neuron and astrocyte survival. *J. Neurochem.* 82, 1480-1489 (2002); Saido, T.C. Alzheimer's disease as proteolytic disorders: anabolism and catabolism of beta-amyloid. *Neurobiol. Aging* 19, S69-S75 (1998)). The cyclization of glutamic acid into pE leads to a loss of N-terminal charge resulting in accelerated aggregation of Aβ N3pE compared to the unmodified Aβ peptides (He, W. & Barrow, C.J. The Abeta 3-pyroglutamyl and 11-pyroglutamyl peptides found in senile plaque have greater beta-sheet forming and aggregation propensities in vitro than full-length A beta. *Biochemistry* 38, 10871-10877 (1999); Schilling, S. *et al.* On the seeding and oligomerization of pGlu-amyloid peptides (in vitro). *Biochemistry* 45, 12393-12399 (2006)). Thus, reduction of Aβ N3pE-42 formation should destabilize the peptides by making them more accessible to degradation and would, in turn, prevent the formation of higher molecular weight Aβ aggregates and enhance neuronal survival.

However, for a long time it was not known how the pE-modification of Aβ peptides occurs. Recently, it was discovered that glutaminyl cyclase (QC) is capable to catalyze Aβ N3pE-42 formation under mildly acidic conditions and that specific QC inhibitors prevent Aβ N3pE-42 generation *in vitro* (Schilling, S., Hoffmann, T., Manhart, S., Hoffmann, M. & Demuth, H.-U. Glutaminyl cyclases unfold glutamyl cyclase activity under mild acid conditions. *FEBS Lett.* 563, 191-196 (2004) ; Cynis, H. *et al.* Inhibition of glutaminyl cyclase alters pyroglutamate formation in mammalian cells. *Biochim. Biophys. Acta* 1764, 1618-1625 (2006)).

Lewy body dementia (LBD) is a neurodegenerative disorder that can occur in persons older than 65 years of age, and typically causes symptoms of cognitive (thinking) impairment and abnormal

behavioral changes. Symptoms can include cognitive impairment, neurological signs, sleep disorder, and autonomic failure. Cognitive impairment is the presenting feature of LBD in most cases. Patients have recurrent episodes of confusion that progressively worsen. The fluctuation in cognitive ability is often associated with shifting degrees of attention and alertness. Cognitive impairment and fluctuations of thinking may vary over minutes, hours, or days. Lewy bodies are formed from phosphorylated and nonphosphorylated neurofilament proteins; they contain the synaptic protein alpha-synuclein as well as ubiquitin, which is involved in the elimination of damaged or abnormal proteins. In addition to Lewy Bodies, Lewy neurites, which are inclusion bodies in the cell processes of the nerve cells, may also be present. Amyloid plaques may form in the brains of patients afflicted with DLB, however they tend to be fewer in number than seen in patients with Alzheimer's disease. Neurofibrillary tangles, the other micropathological hallmark of AD, are not a main characteristic of LBD but are frequently present in addition to amyloid plaques.

Amyotrophic lateral sclerosis (ALS) is characterized by degeneration of upper and lower motor neurons. In some ALS patients, dementia or aphasia may be present (ALS-D). The dementia is most commonly a frontotemporal dementia (FTD), and many of these cases have ubiquitin- positive, tau-negative inclusions in neurons of the dentate gyrus and superficial layers of the frontal and temporal lobes.

Inclusion-body myositis (IBM) is a crippling disease usually found in people over age 50, in which muscle fibers develop inflammation and begin to atrophy — but in which the brain is spared and patients retain their full intellect. Two enzymes involved in the production of amyloid-β protein were found to be increased inside the muscle cells of patients with this most common, progressive muscle disease of older people, in which amyloid-β is also increased.

Another disease that is based on or associated with the accumulation and deposit of amyloid-like protein is macular degeneration. Macular degeneration is a common eye disease that

causes deterioration of the macula, which is the central area of the retina (the paper-thin tissue at the back of the eye where light-sensitive cells send visual signals to the brain). Sharp, clear, "straight ahead" vision is processed by the macula. Damage to the macula results in the development of blind spots and blurred or distorted vision. Age-related macular degeneration (AMD) is a major cause of visual impairment in the United States and for people over age 65 it is the leading cause of legal blindness among Caucasians. Approximately 1.8 million Americans of age 40 and older have advanced AMD, and another 7.3 million people with intermediate AMD are at substantial risk for vision loss. The government estimates that by 2020 there will be 2.9 million people with advanced AMD. Victims of AMD are often surprised and frustrated to find out how little is known about the causes and treatment of this blinding condition.
There are two forms of macular degeneration: dry macular degeneration and wet macular degeneration. The dry form, in which the cells of the macula slowly begin to break down, is diagnosed in 85 percent of macular degeneration cases. Both eyes are usually affected by dry AMD, although one eye can lose vision while the other eye remains unaffected. Drusen, which are yellow deposits under the retina, are common early signs of dry AMD. The risk of developing advanced dry AMD or wet AMD increases as the number or size of the drusen increases. It is possible for dry AMD to advance and cause loss of vision without turning into the wet form of the disease; however, it is also possible for early-stage dry AMD to suddenly change into the wet form.

The wet form, although it only accounts for 15 percent of the cases, results in 90 percent of the blindness, and is considered advanced AMD (there is no early or intermediate stage of wet AMD). Wet AMD is always preceded by the dry form of the disease. As the dry form worsens, some people begin to have abnormal blood vessels growing behind the macula. These vessels are very fragile and will leak fluid and blood (hence 'wet' macular degeneration), causing rapid damage to the macula.

The dry form of AMD will initially often cause slightly blurred vision. The center of vision in particular may then become blurred and this region grows larger as the disease progresses. No symptoms may be noticed if only one eye is affected. In wet AMD, straight lines may appear wavy and central vision loss can occur rapidly.

Diagnosis of macular degeneration typically involves a dilated eye exam, visual acuity test, and a viewing of the back of the eye using a procedure called fundoscopy to help diagnose AMD, and – if wet AMD is suspected – fluorescein angiography may also be performed. If dry AMD reaches the advanced stages, there is no current treatment to prevent vision loss. However, a specific high dose formula of antioxidants and zinc may delay or prevent intermediate AMD from progressing to the advanced stage. Macugen® (pegaptanib sodium injection), laser photocoagulation and photodynamic therapy can control the abnormal blood vessel growth and bleeding in the macula, which is helpful for some people who have wet AMD; however, vision that is already lost will not be restored by these techniques. If vision is already lost, low vision aids exist that can help improve the quality of life.

One of the earliest signs of age-related macular degeneration (AMD) is the accumulation of extracellular deposits known as drusen between the basal lamina of the retinal pigmented epithelium (RPE) and Bruch's membrane (BM). Recent studies conducted by Anderson et al. have confirmed that drusen contain amyloid beta. (Experimental Eye Research 78 (2004) 243 - 256).

The aim of the present invention is to establish a highly sensitive and concomitantly robust detection technique that allows quantitative determination of Aβ variants, in particular pGlu-Aβ peptides, in biological samples, e.g. liquor or serum samples, preferably serum samples. This is a tremendous challenge, taking the low abundance of Aβ peptides in blood into account. Having such a detection technique available is,

however, a prerequisite for studying efficacy of small molecule inhibitors in drug screening programs.

The present invention provides novel methods and compositions comprising highly specific and highly effective antibodies, including chimeric antibodies and fragments thereof, including partially or fully humanized antibodies and fragments thereof, having the ability to specifically recognize and bind to specific epitopes from a range of β-amyloid antigens, in particular pGlu-Aβ peptides, which may be presented to the antibody in a monomeric, dimeric, trimeric, etc, or a polymeric form, in form of an aggregate, fibers, filaments or in the condensed form of a plaque. The antibodies enabled by the teaching of the present invention are particularly useful for diagnosis of amyloidosis, a group of diseases and disorders associated with amyloid plaque formation including secondary amyloidosis and age-related amyloidosis including, but not limited to, neurological disorders such as Alzheimer's Disease (AD), Lewy body dementia, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type); the Guam Parkinson-Dementia complex; as well as other diseases which are based on or associated with amyloid-like proteins such as progressive supranuclear palsy, multiple sclerosis; Creutzfeld Jacob disease, hereditary cerebral hemorrhage with amyloidosis Dutch type, Parkinson's disease, HIV-related dementia, ALS (amyotropic lateral sclerosis), Adult Onset Diabetes; senile cardiac amyloidosis; endocrine tumors, and others, including macular degeneration, to name just a few.

To meet all demands mentioned above, an ELISA based technique would be especially preferable. The task was started with Aβ N3pE ELISA, because for this Aβ variant an ELISA system is already commercially available (Human Amyloid β (N3pE) Assay Kit -IBL, Code No. 27716), which is to be used as reference and internal quality control. Capturing of the Aβ N3pE-40 peptide was done with the hAβ (x-40) ELISA (HS) from TGC (The Genetics Company, Inc.,Wagistrasse 23, 8952 Schlieren, Zurich area

Switzerland), which should facilitate and expedite the process of development.

Summary of the invention

The present invention pertains in particular to antibodies or variants thereof, which are characterized in that they bind to Aβ-peptide with a high affinity. Said high affinity means in the context of the present invention an affinity of a K_D value of 10^{-7} M or better, preferably a K_D value of 10^{-8} M or better, and even more preferably a K_D value of 10^{-9} M - 10^{-12} M.

In particular the antibody is preferably a monoclonal antibody and is selected from the following group

Aβ 5-5-6
Aβ 6-1-6
Aβ 17-4-3
Aβ 24-2-3

The antibody according to the present invention is especially useful in a diagnostic method to detect amyloidosis, in particular Alzheimer's disease.

Description of the Figures

Figure 1

A) Detection of 10 ng/ml amyloid β N3pE-40 by increasing concentrations of pGlu-6166 antibody (clone 12-1).

B) Determination of the highest concentration of pGlu-6166 antibody (clone 12-1) required for detection of 10 ng/ml amyloid β N3pE-40.

Figure 2

Dot Blot analysis of hybridoma cell culture supernatants of individual IgG producing clones.

Figure 3
PepSpot Analysis of pGlu-6166 Hybridoma Cell Clones and IBL-Aβ N3pE antibody.

Figure 4
12% SDS-PAGE of 20µg pGlu-6166 antibody and hybridoma cell culture supernatants.

Figure 5
Biacore analysis of hybridoma cell culture supernatants. An overlay of monitored binding courses is illustrated graphically.

Figure 6
Sensograms of interaction of anti-AβN3pE antibody clone 6-1-6 with AβpE3-40.

Figure 7
Sensograms of interaction of anti-AβN3pE antibody clone 24-2-3 with AβpE3-40.

Figure 8
N3pE-ELISA for clone 6-1-6, standard curve of AβpE3-40.

Figure 9:
Sensograms of N3pE antibody clone 6-1-6.

Figure 10
Quantification of AβpE3-42 using the method of neutralization by 1:20 dilution in EIA buffer, pH titration with 860 µl 3.5 M Tris.

Figure 11
Stained brain sections form Alzheimer's disease (AD) patients (A) Brain of a sporadic AD (SAD) patient stained with anti-Aβ antibody 6E10, recognizing total Aβ,

(B) Brain of a sporadic AD (SAD) patient stained with N3pE antibody clone 24-2-3, recognizing AβpE3-x,
(C) Brain of a familial AD (FAD) patient stained with N3pE antibody clone 24-2-3, recognizing AβpE3-x.

Detailed description of the Invention

Definitions

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity. The antibody may be an IgM, IgG (e.g. IgG_1, IgG2, IgG3 or IgG4), IgD, IgA or IgE, for example. Preferably however, the antibody is not an IgM antibody.

"Antibody fragments" comprise a portion of an intact antibody, generally the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments: diabodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e. the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to "polyclonal antibody" preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies can frequently be advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The "monoclonal" indicates the character

of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Köhler et al., Nature, 256:495 (1975), or may be made by generally well known recombinant DNA methods. The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature, 352:624-628 (1991) and Marks et al., J. Mol. Biol., 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include chimeric antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity.

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')2 or other antigen-binding subsequences of antibodies) which contain a minimal sequence derived from a non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences.

These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature, 321:522-525 (1986), Reichmann et al, Nature. 332:323-329 (1988): and Presta, Curr. Op. Struct. Biel., 2:593-596 (1992). The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_D) in the same polypeptide chain (V_H - V_D). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in Hollinger et al., Proc. Natl. Acad. Sol. USA, 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural

environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the expressions "cell", "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and culture derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, this will be clear from the context.

The terms "polypeptide", "peptide", and "protein", as used herein, are interchangeable and are defined to mean a biomolecule composed of amino acids linked by a peptide bond.

The terms "a", "an" and "the" as used herein are defined to mean "one or more" and include the plural unless the context is inappropriate.

The language "diseases and disorders which are caused by or associated with amyloid or amyloid-like proteins" includes, but is not limited to, diseases and disorders caused by the presence or activity of amyloid-like proteins in monomeric, fibril, or polymeric state, or any combination of the three. Such diseases and disorders include, but are not limited to, amyloidosis, endocrine tumors, and macular degeneration.

The term "amyloidosis" refers to a group of diseases and disorders associated with amyloid plaque formation including, but not limited to, secondary amyloidosis and age-related amyloidosis such as diseases including, but not limited to, neurological disorders such as Alzheimer's Disease (AD), including diseases or conditions characterized by a loss of cognitive memory capacity such as, for example, mild cognitive impairment (MCI), sporadic Alzheimer’s disease, Lewy body dementia, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type); the Guam Parkinson-Dementia complex, familial forms of Alzheimer’s disease like Familial British Dementia (FBD) and Familial Danish Dementia (FDD); as well as other diseases which are based on or associated with amyloid-like proteins such as progressive supranuclear palsy, multiple sclerosis; Creutzfeld Jacob disease, Parkinson's disease, HIV-related dementia, ALS (amyotropic lateral sclerosis), inclusion-body myositis (IBM), Adult Onset Diabetes, and senile cardiac amyloidosis; and various eye diseases including macular degeneration, drusen-related optic neuropathy, and cataract due to beta-amyloid deposition.

"Amyloid β, Aβ or /β-amyloid" is an art recognized term and refers to amyloid β proteins and peptides, amyloid β precursor protein (APP), as well as modifications, fragments and any functional equivalents thereof. In particular, by amyloid β as used herein is meant any fragment produced by proteolytic cleavage of APP but especially those fragments which are involved in or associated with the amyloid pathologies including, but not limited to, $A\beta_{1-38}$, $A\beta_{1-40}$, $A\beta_{1-42}$. The amino acid sequences of these Aβ peptides are as follows:

Aβ 1-42 (SEQ ID NO. 1):

Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala

Aβ 1-40 (SEQ ID NO. 2):

Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val

Aβ 1-38 (SEQ ID NO. 3):

Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly

"pGlu-Aβ" or "Aβ N3pE" refers to N-terminally truncated forms of Aβ, that start at the glutamic acid residue at position 3 in the amino acid sequence of Aβ, and wherein said glutamic acid residue is cyclized to form a pyroglutamic acid residue. In particular, by pGlu-Aβ as used herein are meant those fragments which are involved in or associated with the amyloid pathologies including, but not limited to, pGlu-$A\beta_{3-38}$, pGlu-$A\beta_{3-40}$, p-Glu-$A\beta_{3-42}$.

The sequences of the N-terminally truncated forms of Aβ, $A\beta_{3-38}$, $A\beta_{3-40}$, $A\beta_{3-42}$ are as follows:

Aβ 3-42 (SEQ ID NO. 4):

Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala

Aβ 3-40 (SEQ ID NO. 5):

Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val

Aβ 3-38 (SEQ ID NO. 6):
Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly

In particular the present invention pertains to the following items:

1. Antibody, characterised in that it binds to Aβ peptides or variants thereof, preferably with high affinity.

2. Antibody according to item 1, wherein said high affinity means a dissociation constant (K_D) value of 10^{-7} M, or better.

3. Antibody according to item 1 or 2, wherein said antibody is a monoclonal antibody.

4. Antibody according to any of the preceding items, wherein the variable part of the light chain of said antibody has a nucleotide sequence selected from SEQ ID NOs: 49, 53, 57 and 61, or an amino acid sequence selected from SEQ ID NOs: 50, 54, 58, and 62.

5. Antibody according to any of the preceding items, wherein the variable part of the heavy chain of said antibody has a nucleotide sequence selected from SEQ ID NOs: 51, 55, 59 and 63, or an amino acid sequence selected from SEQ ID NOs: 52, 56, 60 and 64.

6. Antibody according to any of the preceding items, wherein the variable part of the light chain of said antibody has the nucleotide sequence of SEQ ID NO: 49 or the amino acid sequence of SEQ ID NO: 50, and wherein the variable part of the heavy chain of said antibody has the nucleotide sequence of SEQ ID NO: 51, or the amino acid sequence of SEQ ID NO: 52.

7. Antibody according to any of the preceding items, wherein the variable part of the light chain of said antibody has the nucleotide sequence of SEQ ID NO: 53 or the amino acid sequence of SEQ ID NO: 54, and wherein the variable part of the heavy chain of said antibody has the nucleotide sequence of SEQ ID NO: 55, or the amino acid sequence of SEQ ID NO: 56.

8. Antibody according to any of the preceding items, wherein the variable part of the light chain of said antibody has the nucleotide sequence of SEQ ID NO: 57 or the amino acid sequence of SEQ ID NO: 58, and wherein the variable part of the heavy chain of said antibody has the nucleotide sequence of SEQ ID NO: 59, or the amino acid sequence of SEQ ID NO: 60.

9. Antibody according to any of the preceding items, wherein the variable part of the light chain of said antibody has the nucleotide sequence of SEQ ID NO: 61 or the amino acid sequence of SEQ ID NO: 62, and wherein the variable part of the heavy chain of said antibody has the nucleotide sequence of SEQ ID NO: 63, or the amino acid sequence of SEQ ID NO: 64.

10. Antibody according to any of the preceding items, wherein said antibody is selected from the following group:

Aβ 5-5-6	(Deposit No. DSM ACC 2923)
Aβ 6-1-6	(Deposit No. DSM ACC 2924)
Aβ 17-4-3	(Deposit No. DSM ACC 2925)
Aβ 24-2-3	(Deposit No. DSM ACC 2926)

or functional variants thereof.

11. Antibody according to any of the preceding items, wherein said antibody is Aβ 6-1-6 (Deposit No. DSM ACC 2924).

12. Antibody according to any of the preceding items, wherein said antibody is Aβ 24-2-3 (Deposit No. DSM ACC 2926).

13. Antibody according to any of the preceding items, wherein said antibody is a humanized or chimeric antibody, or an antibody fragment which retains the high affinity.

14. Antibody according to any of the preceding items for use in the detection of Aβ peptide or variants thereof.

15. Antibody according to item 14, wherein said variants are selected from the following group:

pGlu-$A\beta_{3-38}$
pGlu-$A\beta_{3-40}$
pGlu-$A\beta_{3-42}$, and
pGlu-$A\beta_{3-x}$ variants,

wherein x is an integer between 10 and 42; preferably 18 and 42, more preferably 30 and 42.

16. Antibody according to any of the preceding items, which is human.

17. Antibody according to any of the preceding items, which is a diabody or a single chain antibody which retains the high affinity.

18. Antibody according to any of the preceding items, which binds to the epitope bound by the antibodies defined in item 15.

19. Antibody according to any of the preceding items, which has the complementarity determining regions of the antibodies as defined in item 15.

20. Antibody according to any of the preceding items, which is labeled.

21. Antibody according to any of the preceding items, which is immobilised on a solid phase.

22. Antibody obtainable from any one of hybridoma cell lines DSM ACC 2923, DSM ACC 2924, DSM ACC 2925, DSM ACC 2926.

23. Composition comprising the antibody as defined in any of the preceding items.

24. Composition according to item 23 for the treatment, prevention or delay of amyloidosis.

25. Composition according to item 23 or 24, wherein said amyloidosis is a neurodegenerative disease selected from the group consisting of mild cognitive impairment, Alzheimer's disease and neurodegeneration in Down Syndrome.

26. Composition according to item 23 or 24, wherein said amyloidosis is sporadic Alzheimer's disease or a Familial Alzheimer’s dementia.

27. Composition according to item 26, wherein said Familial Alzheimer’s dementia is Familial British Dementia or Familial Danish Dementia.

28. Hybridoma cell line DSM ACC 2923.

29. Hybridoma cell line DSM ACC 2924

30. Hybridoma cell line DSM ACC 2925.

31. Hybridoma cell line DSM ACC 2926.

32. Use of the antibody as defined in any one of items 1 to 22 or the composition as defined in any one of items 23 to 27in a diagnostic or therapeutic method.

33. The use according to item 32 for the diagnosis of an amyloid-associated disease or condition.

34. The use according to item 33, wherein said amyloidosis is a neurodegenerative disease selected from the group consisting of mild cognitive impairment, Alzheimer's disease and neurodegeneration in Down Syndrome.

35. The use according to item 33, wherein said amyloidosis is sporadic Alzheimer's disease or a Familial Alzheimer's dementia.

36. The use according to item 35, wherein said Familial Alzheimer's dementia is Familial British Dementia or Familial Danish Dementia.

37. *In vitro* diagnostic method for the diagnosis of an amyloid-associated disease or condition, in particular Alzheimer's disease, comprising the following steps:

contacting an antibody according to any one of items 1 to 22 with a sample from a subject suspected to be afflicted with said disease or condition, and

detecting binding of the antibody to a pGlu-amyloid protein, preferably pGlu-Aβ peptide from the sample.

38. Diagnostic kit, comprising the antibody as defined in any one of items 1 to 22, and instructions for use, and - optionally - (a) further biologically active substance(s).

39. The diagnostic kit of item 32, wherein said further biological substance is an inhibitor of glutaminy cyclase.

40. An oligonucleotide selected from the group consisting of SEQ ID Nos: 23 to 48.

The antibodies of the invention may be useful for the diagnosis of amyloidosis.

The antibodies of the invention may be used as affinity purification agents. In this process, the antibodies are immobilised on a solid phase such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody is contacted with a sample containing the Aβ -peptide (or fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Aβ-peptide, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0 that will release the Aβ-peptide from the antibody.

Anti-Aβ-peptide antibodies may also be useful in diagnostic assays for Aβ-peptide, e.g. detecting its occurrence in specific cells, tissues, or serum. Thus, the antibodies may be used in the diagnosis of amyloidosis, in particular a neurodegenerative disease selected from the group consisting of mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer's dementias (FAD) such as Familial British Dementia (FBD) and Familial Danish Dementia (FDD) and neurodegeneration in Down Syndrome; preferably Alzheimer's disease.

For diagnostic applications, the antibody typically will be labelled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S, ^{14}C, ^{125}I, ^{3}H, and ^{131}I. The antibody can be labeled with the radioisotope using the techniques described in *Current Protocols in Immunology*, Volumes 1 and 2, Gütigen et al., Ed., Wiley-Interscience. New York, New York. Pubs., (1991) for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are

available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in *Current Protocols in Immunology*, *supra* for example. Fluorescence can be quantified using a fluorimeter.

(c) Various enzyme-substrate labels are available. The enzyme generally catalyses a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (e.g, firefly luciferase and bacterial luciferase; U.S. Patent No, 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase. 0-galactosidase, glucoamylase, lysozyme, saccharide oxidases (e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan et al., Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in Methods in Enzym (ed Langone & H. Van Vunakis), Academic Press, New York, 73: 147-166 (1981).

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (e.g. orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));
(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and

(iii) β -D-galactosidase (β -D-Gal) with a chromogenic substrate (e.g. p-nitrophenyl-β -D-galactosidase) or the fluorogenic substrate 4-methylumbellifery1-β -D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (e.g. digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (e.g. anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

The Aβ-antibody needs not be labeled, and the presence thereof can be detected using a labeled antibody, which binds to the Aβ-antibody.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies A Manual of Techniques*, pp.147-158 (CRC Press. Inc., 1987)

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of Aβ peptide in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the

competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one preferable type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tissue sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

Diagnostic Kits

As a matter of convenience, the antibody of the present invention can be provided in a kit, i.e., a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labelled with an enzyme, the kit will include substrates and cofactors required by the enzyme (e.g. a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (e.g. a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution

will provide a reagent solution having the appropriate concentration.

The diagnostic kit according to the invention may contain a further biologically active substance as described below. Especially preferred for the use in the diagnostic kit are inhibtors of glutaminyl cyclase.

The diagnostic kit of the invention is especially useful for the detection and diagnosis of amyloid- associated diseases and conditions, in particular neurodegenerative diseases selected from the group consisting of mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer's dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome; preferably Alzheimer's disease.

The present invention pertains in particular to antibodies which are characterized in that they bind to Aβ-peptide with a high affinity. The present invention also pertains to antibodies which are characterised in that they bind to Aβ-peptides or variants thereof with a high affinity. Said high affinity means in the context of the present invention an affinity of a K_D value of 10^{-7} M or better, preferably a K_D value of 10^{-8} M or better, and even more preferably a K_D value of 10^{-9} M - 10^{-12} M. Thereby, the inventive antibodies bind to Aβ-peptide with a higher affinity than previously known antibodies.

In particular the antibody is preferably a monoclonal antibody and is selected from the following group

Aβ 5-5-6	(DSM ACC 2923)
Aβ 6-1-6	(DSM ACC 2924)
Aβ 17-4-3	(DSM ACC 2925)
Aβ 24-2-3	(DSM ACC 2926)

The antibody according to the present invention is especially useful in a diagnostic method to detect amyloidosis, in particular a neurodegenerative disease selected from the group consisting of mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer's dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome; preferably Alzheimer's disease.

According to a preferred embodiment, the antibody can be humanised or is a chimeric antibody or is a human antibody.

Further, the antibody as selected from the above-mentioned group can also be a functional variant of said group.

In the context of the present invention, a variant of an p-Glu-Aβ peptide is in particular

pGlu-Aβ_{3-38},
pGlu-Aβ_{3-40},
pGlu-Aβ_{3-42}

Further variants of Aβ peptides are all pGlu-Aβ_{3-x} variants, which have been shown to accumulate in the brain as a consequence of Alzheimer's disease or preceding Alzheimer's disease. X is defined as an integer between 10 and 42, e.g. in the above pGlu-Aβ_{3-42}, "42" would be the integer for "x".

In the context of the present invention a "functional variant" of the inventive antibody is an antibody which retains the binding capacities, in particular binding capacities with high affinity to a pGlu-Aβ_{3-x} peptide or functional variant thereof. The provision of such functional variants is known in the art and encompasses the above-mentioned possibilities, which were indicated under the definition of antibodies and fragments thereof.

In a preferred embodiment, the antibody is an antibody fragment, as defined above.

In a further preferred embodiment, the inventive antibody is an antibody which binds to the epitope which is bound by the antibodies as defined above, in particular antibody 5-5-6, antibody 6-1-6, antibody 17-4-3 and antibody 24-2-3.

In a further preferred embodiment, the antibody of the invention is an antibody which has the complementarity-determining regions (CDRs) of the above-defined antibodies. Preferably, the antibody can be labeled; possible labels are those as mentioned above and all those known to a person skilled in the art of diagnostic uses of antibodies in particular.

Preferably, the antibody is immobilized on a solid phase.

The present invention also concerns an antibody which is obtainable from hybridoma cell line 6-1-6 (DSM ACC 2924).

The present invention also relates to a composition which comprises the antibody as defined above. In particular, said composition is a composition for a diagnostic use, especially for the diagnosis of a neurodegenerative disease selected from the group consisting of mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer’s dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome; preferably Alzheimer’s disease; in particular by detection of Aβ peptide or variants thereof in a biological sample.

In another embodiment, the antibody according to the invention and as described herein before or a fragment thereof, exhibits an binding affinity to an Aβ oligomer, fiber, fibril or filament which is at least 2 times, particularly at least 4 times, particularly at least 10 times, particularly at least 15 times,

more particularly at least 20 times, but especially at least 25 times higher than the binding affinity to an Aβ monomer.

In still another embodiment, an antibody or a fragment thereof or a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof is provided as described herein before, which antibody substantially binds to aggregated Aβ, including Aβ plaques, in the mammalian, particularly the human brain but, preferably, does not show any significant cross-reactivity with amyloid precursor protein (APP).

In another aspect of the invention, the antibody or a fragment thereof or the chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof is provided as described herein before, which antibody substantially binds to soluble polymeric amyloid, particularly amyloid β (Aβ), including Aβ monomers, in the mammalian, particularly the human brain but, preferably, does not show any significant cross-reactivity with amyloid precursor protein (APP).

The present invention relates also to humanized forms of the antibodies as defined above, compositions comprising said humanized antibodies and the use of said compositions for the treatment of amyloidosis, especially for the treatment of neurodegenerative disease in a mammal, in particular in a human. Said neurodegenerative disease is in particular selected from the group consisting of mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer's dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome. Preferably, said neurodegenerative disease is Alzheimer's disease.

The present invention is also directed to the following hybridoma cell lines 5-5-6, 6-1-6, 17-4-3 and 24-2-3.

The present invention also pertains to the use of the antibody or the composition comprising the antibody, both as defined

above, for use in an *in vitro* diagnostic method. In particular, this diagnostic method is directed to diagnosis of a neurodegenerative disease selected from the group consisting of mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer's dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome; preferably Alzheimer's disease; especially by detecting an Aβ peptide or variants thereof in a biological sample.

Preferably, said sample is a serum sample.

According to another preferred embodiment, said sample is a liquor or cerebrospinal fluid (CSF) sample.

In a particularly preferred embodiment, the present invention pertains to the following method:

In vitro or *in situ* diagnostic method for the diagnosis of an amyloid-associated disease or condition, preferably Alzheimer's disease, comprising the following steps:
contacting an antibody according to the invention with a sample, preferably selected from a serum, liquor or CSF sample, most preferably a serum sample; or a specific body part or body area of a subject suspected to be afflicted with said condition or disease, and
detecting binding of the antibody to a pGlu-amyloid protein, preferably pGlu-Aβ peptide, from the sample.

More particularly, the invention relates to a method of diagnosis of an amyloid-associated disease or condition, preferably Alzheimer's disease, comprising detecting the immunospecific binding of an antibody or an active fragment thereof to a pGlu-amyloid protein, preferably pGlu-Aβ peptide, in a sample or in situ which includes the steps of
(a) bringing the sample or a specific body part or body area suspected to contain the amyloid protein into contact with an

antibody, particularly a monoclonal antibody according to the invention, or a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to the invention and as described herein before, and/or a functional part thereof, which antibody binds a pGlu-Aβ peptide;
(b) allowing the antibody and/or a functional part thereof, to bind to the pGlu-Aβ peptide to form an immunological complex;
(c) detecting the formation of the immunological complex; and
(d) correlating the presence or absence of the immunological complex with the presence or absence of pGlu-Aβ peptide in the sample or specific body part or area.

Also comprised is a method of determining the extent of amyloidogenic plaque burden in a tissue and/or body fluids comprising
(a) obtaining a sample representative of the tissue and/or body fluids under investigation;
(b) testing said sample for the presence of amyloid protein with an antibody, particularly a monoclonal antibody according to the invention, or a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to the invention and as described herein before, and/or a functional part thereof;
(c) determining the amount of antibody bound to the protein; and
(d) calculating the plaque burden in the tissue and/or body fluids.

In particular, the invention relates to a method of determining the extent of amyloidogenic plaque burden in a tissue and/or body fluids, wherein the formation of the immunological complex in step c) is determined such that presence or absence of the immunological complex correlates with presence or absence of amyloid protein, in particular pGlu-Aβ peptides.

In still another embodiment, the invention relates to a composition comprising the antibody according to the invention, or a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to the invention and as

described herein before including any functionally equivalent antibody or any derivative or functional parts thereof, in a therapeutically effective amount, in particular a composition which is a pharmaceutical composition optionally further comprising a pharmaceutically acceptable carrier.

In another embodiment of the invention, said composition comprises the antibody in a therapeutically effective amount. Further comprised by the invention is a mixture comprising an antibody, particularly a monoclonal antibody according to the invention, or a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to the invention and as described herein before including any functionally equivalent antibody or any derivative or functional parts thereof, in a therapeutically effective amount and, optionally, a further biologically active substance and/or a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

In particular, the invention relates to a mixture, wherein the further biologically active substance is a compound used in the medication of amyloidosis, a group of diseases and disorders associated with amyloid or amyloid-like protein such as the Aβ protein involved in neurodegenerative diseases selected from the group consisting of mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer’s dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome; preferably Alzheimer’s disease.
In another embodiment of the invention, the other biologically active substance or compound may also be a therapeutic agent that may be used in the treatment of amyloidosis caused by amyloid β or may be used in the medication of other neurological disorders.

The other biologically active substance or compound may exert its biological effect by the same or a similar mechanism as the

antibody according to the invention or by an unrelated mechanism of action or by a multiplicity of related and/or unrelated mechanisms of action.

Generally, the other biologically active compound may include neutron-transmission enhancers, psychotherapeutic drugs, acetylcholine esterase inhibitors, calcium-channel blockers, biogenic amines, benzodiazepine tranquillizers, acetylcholine synthesis, storage or release enhancers, acetylcholine postsynaptic receptor agonists, monoamine oxidase-A or -B inhibitors, N-methyl- D-aspartate glutamate receptor antagonists, non-steroidal anti-inflammatory drugs, antioxidants, and serotonergic receptor antagonists.
More particularly, the invention relates to a mixture comprising at least one compound selected from the group consisting of compounds effective against oxidative stress, anti-apoptotic compounds, metal chelators, inhibitors of DNA repair such as pirenzepin and metabolites, 3- amino-1-propanesulfonic acid (3 APS), 1,3-propanedisulfonate (1,3PDS), α-secretase activators, β- and γ -secretase inhibitors, tau proteins, neurotransmitter, ß-sheet breakers, attractants for amyloid beta clearing / depleting cellular components, inhibitors of N-terminal truncated amyloid beta including pyroglutamated amyloid beta 3-42, such as inhibitors of glutaminyl cyclase, anti-inflammatory molecules, or cholinesterase inhibitors (ChEIs) such as tacrine, rivastigmine, donepezil, and/or galantamine, Ml agonists and other drugs including any amyloid or tau modifying drug and nutritive supplements, and nutritive supplements, together with an antibody according to the present invention and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.
The invention further relates to a mixture, wherein the compound is a cholinesterase inhibitor (ChEIs), particularly a mixture, wherein the compound is one selected from the group consisting of tacrine, rivastigmine, donepezil, galantamine, niacin and memantine.

In a further embodiment, the mixtures according to the invention may comprise niacin or memantine together with an antibody according to the present invention and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

In a further embodiment, the mixtures according to the invention may comprise a glutaminyl cyclase inhibitor together with an antibody according to the present invention and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

Preferred inhibitors of glutaminyl cyclase are described in WO 2005/075436, in particular examples 1-141 as shown on pp. 31-40. The synthesis of examples 1-141 is shown on pp. 40-48 of WO 2005/075436. The disclosure of WO 2005/075436 regarding examples 1-141, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/055945, in particular examples 1-473 as shown on pp. 46-155. The synthesis of examples 1-473 is shown on pp. 156-192 of WO 2008/055945. The disclosure of WO 2008/055945 regarding examples 1-473, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/055947, in particular examples 1-345 as shown on pp. 53-118. The synthesis of examples 1-345 is shown on pp. 119-133 of WO 2008/055947. The disclosure of WO 2008/055947 regarding examples 1-345, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/055950, in particular examples 1-212 as shown on pp. 57-120. The synthesis of examples 1-212 is shown on pp. 121-128 of WO 2008/055950. The disclosure of WO 2008/055950 regarding

examples 1-212, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO2008/065141, in particular examples 1-25 as shown on pp. 56-59. The synthesis of examples 1-25 is shown on pp. 60-67 of WO2008/065141. The disclosure of WO2008/065141 regarding examples 1-25, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/110523, in particular examples 1-27 as shown on pp. 55-59. The synthesis of examples 1-27 is shown on pp. 59-71 of WO 2008/110523. The disclosure of WO 2008/110523 regarding examples 1-27, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/128981, in particular examples 1-18 as shown on pp. 62-65. The synthesis of examples 1-18 is shown on pp. 65-74 of WO 2008/128981. The disclosure of WO 2008/128981 regarding examples 1-18, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/128982, in particular examples 1-44 as shown on pp. 61-67. The synthesis of examples 1-44 is shown on pp. 68-83 of WO 2008/128982. The disclosure of WO 2008/128982 regarding examples 1-44, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/128983, in particular examples 1-30 as shown on pp. 64-68. The synthesis of examples 1-30 is shown on pp. 68-80 of WO 2008/128983. The disclosure of WO 2008/128983 regarding examples 1-30, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/128984, in particular examples 1-36 as shown on pp. 63-69. The synthesis of examples 1-36 is shown on pp. 69-81 of WO 2008/128984. The disclosure of WO 2008/128984 regarding examples 1-36, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/128985, in particular examples 1-71 as shown on pp. 66-76. The synthesis of examples 1-71 is shown on pp. 76-98 of WO 2008/128985. The disclosure of WO 2008/128985 regarding examples 1-71, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

Further preferred inhibitors of glutaminyl cyclase are described in WO 2008/128986, in particular examples 1-7 as shown on pp. 65-66. The synthesis of examples 1-7 is shown on pp. 66-73 of WO 2008/128986. The disclosure of WO 2008/128986 regarding examples 1-7, their synthesis and their use as glutaminyl cyclase inhibitors is incorporated herein by reference.

In still another embodiment of the invention mixtures are provided that comprise "atypical antipsychotics" such as, for example clozapine, ziprasidone, risperidone, aripiprazole or olanzapine for the treatment of positive and negative psychotic symptoms including hallucinations, delusions, thought disorders (manifested by marked incoherence, derailment, tangentiality), and bizarre or disorganized behavior, as well as anhedonia, flattened affect, apathy, and social withdrawal, together with an antibody, particularly a monoclonal antibody according to the invention, but particularly a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to the invention and as described herein and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

In a specific embodiment of the invention, the compositions and mixtures according to the invention and as described herein

before comprise the antibody and the biologically active substance, respectively, in a therapeutically effective amount.

Other compounds that can be suitably used in mixtures in combination with the antibody according to the present invention are described in WO2008/065141 (see especially pages 37/38), including PEP-inhibitors (pp. 43/44), LiCl, inhibitors of dipeptidyl aminopeptidases, preferably inhibitors of DP IV or DP IV-like enzymes (see pp. 48/49); acetylcholinesterase (ACE) inhibitors (see p. 47), PIMT enhancers, inhibitors of beta secretases (see p. 41), inhibitors of gamma secretases (see pp. 41/42), inhibitors of neutral endopeptidase, inhibitors of phosphodiesterase-4 (PDE-4) (see pp. 42/43), TNFalpha inhibitors, muscarinic M1 receptor antagonists (see p. 46), NMDA receptor antagonists (see pp. 47/48), sigma-1 receptor inhibitors, histamine H3 antagonists (se p. 43), immunomodulatory agents, immunosuppressive agents or an agent selected from the group consisting of antegren (natalizumab), Neurelan (fampridine-SR), campath (alemtuzumab), IR 208, NBI 5788/MSP 771 (tiplimotide), paclitaxel, Anergix.MS (AG 284), SH636, Differin (CD 271, adapalene), BAY 361677 (interleukin-4), matrix-metalloproteinase-inhibitors (e.g. BB 76163), interferon-tau (trophoblastin) and SAIK-MS; beta-amyloid antibodies (see p.44), cysteine protease inhibitors (see p. 44); MCP-1 antagonists (see pp. 44/45), amyloid protein deposition inhibitors (see 42) and beta amyloid synthesis inhibitors (see p. 42), which document is incorporated herein by reference.

In another embodiment, the invention relates to a mixture comprising the antibody, particularly a monoclonal antibody according to the invention, or a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to the invention and as described herein before and/or the biologically active substance in a therapeutically effective amount.

The invention further relates to the use of an antibody, particularly a monoclonal antibody according to the invention,

but particularly a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to the invention and as described herein before and/or a functional part thereof and/or a pharmaceutical composition, or a mixture comprising said antibody, for the preparation of a medicament for treating or alleviating the effects of amyloidosis, a group of diseases and disorders associated with amyloid plaque formation including secondary amyloidosis and age-related amyloidosis such as diseases including, but not limited to, neurological disorders such as Alzheimer's Disease (AD), Lewy body dementia, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type); the Guam Parkinson-Dementia complex; as well as other diseases which are based on or associated with amyloid-like proteins such as progressive supranuclear palsy, multiple sclerosis; Creutzfeld Jacob disease, Parkinson's disease, HIV-related dementia, ALS (amyotropic lateral sclerosis), Adult Onset Diabetes; senile cardiac amyloidosis; endocrine tumors, and others, including macular degeneration.

Also comprised by the present invention is a method for the preparation of an antibody, particularly a monoclonal antibody according to the invention, but particularly a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to the invention and as described herein before and/or a functional part thereof and/or a pharmaceutical composition, or a mixture comprising said antibody and/or a functional part thereof, particularly in a therapeutically effective amount, for use in a method of preventing, treating or alleviating the effects of amyloidosis, a group of diseases and disorders associated with amyloid plaque formation including secondary amyloidosis and age-related amyloidosis such as diseases including, but not limited toneurodegenerative diseases such as mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer’s dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome; Lewy body dementia,

hereditary cerebral hemorrhage with amyloidosis (Dutch type); the Guam Parkinson-Dementia complex; as well as other diseases which are based on or associated with amyloid-like proteins such as progressive supranuclear palsy, multiple sclerosis; Creutzfeld Jacob disease, Parkinson's disease, HIV-related dementia, ALS (amyotropic lateral sclerosis), Adult Onset Diabetes; senile cardiac amyloidosis; endocrine tumors, and others, including macular degeneration comprising formulating an antibody, particularly a monoclonal antibody according to the invention, but particularly a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to the invention in a pharmaceutically acceptable form.

Further comprised by the present invention is a method for preventing, treating or alleviating the effects of amyloidosis, a group of diseases and disorders associated with amyloid plaque formation including secondary amyloidosis and age-related amyloidosis such as diseases including, but not limited to, neurological disorders such as mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer's dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome; Lewy body dementia, hereditary cerebral hemorrhage with amyloidosis (Dutch type); the Guam Parkinson-Dementia complex; as well as other diseases which are based on or associated with amyloid-like proteins such as progressive supranuclear palsy, multiple sclerosis; Creutzfeld Jacob disease, Parkinson's disease, HIV-related dementia, ALS (amyotropic lateral sclerosis), Adult Onset Diabetes; senile cardiac amyloidosis; endocrine tumors, and others, including macular degeneration by administering an antibody and/or a functional part thereof, but particularly a humanized antibody and/or a functional part thereof, or a composition or mixture comprising such an antibody and/or a functional part thereof, to an animal or a human affected by such a disorder comprising administering the antibody in a therapeutically effective amount.

It is also an object of the invention to provide a method for the treatment of amyloidosis, a group of diseases and disorders associated with amyloid plaque formation including secondary amyloidosis and age-related amyloidosis including, but not limited to, neurodegenerative diseases such as mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer's dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome; particularly a disease or condition characterized by a loss of cognitive memory capacity by administering to an animal, particularly a mammal or a human, an antibody, particularly a pharmaceutical composition according to the invention and as described herein.

In a specific embodiment the invention provides a method for retaining or increasing cognitive memory capacity but, particularly, for restoring the cognitive memory capacity of an animal, particularly a mammal or a human, suffering from memory impairment by administering to an animal, particularly a mammal or a human, an antibody, particularly a pharmaceutical composition according to the invention and as described herein before.

It is a further object of the invention to provide a therapeutic composition and a method of producing such a composition as well as a method for the treatment of amyloidosis, a group of diseases and disorders associated with amyloid plaque formation including secondary amyloidosis and age-related amyloidosis including, but not limited to, neurodegenerative diseases such as mild cognitive impairment (MCI), Alzheimer's disease (AD), like for instance sporadic Alzheimer's disease (SAD) or Familial Alzheimer's dementias (FAD) like Familial British Dementia (FBD) and Familial Danish Dementia (FDD), neurodegeneration in Down Syndrome; particularly a disease or condition characterized by a loss of cognitive memory capacity, using an antibody according to the invention and as described herein before.

In particular, the invention relates to the treatment of an animal, particularly a mammal or a human, suffering from an amyloid-associated condition characterized by a loss of cognitive memory capacity that leads to the retention of cognitive memory capacity.

EXAMPLES

1. Material and Methods

1.1 Production of antibodies

Mice

For the production of hybridomas, female BALB/C mice (Charles River) of 8 weeks age were used.

Myeloma cell line

For the generation of the hybridomas, the myeloma cell line SP2/0-Ag14 from Deutsche Stammsammlung von Mikoorganismen und Zellkulturen was used.

Antigen

The peptide pGlu-6166 (sequence pGlu-FRHDSGC, SEQ ID NO: 65) was used.

Strategy

As an immunogen, the peptide was coupled to bovine thyroglubulin (BTG, SIGMA) via maleimid groups from three different linkers. The three linkers of different length were used from N-[e-maleimidocaproyloxy] succinimide ester (EMCS), succiminidyl-4-(N-maleimidomethyl)-cyclohexan-1-carboxy-(6-amidocaproate) (LCSMCC) and N-[b-maleimidopropyloxy] succinimide ester (BMPS).

For the detection of the generated antibodies, the same peptide was conjugated to bovine serum albumine (BSA, SIGMA) via maleimid groups from succinimidyl-6-[(b-maleimido-propionamido)hexanoate] (SMPH).

Methods

Conjugation of the peptide for immunization

Conjugation was performed in two steps via SH-groups from the cystein residue of the peptide.

1. Maleoylation of the carrier protein

2 to 5 mg of the respective linker (50 mg/ml in N-methylpyrrolidone, NMP) was added to 2 ml of the carrier protein solution (10 mg/ml in 0.1 mM $NaHCO_3$, pH 8.0). The reaction mixture was incubated for 1 h at room temperature (RT). The reaction mixture was thereafter desalted using a Sephadex G-50 column (1.5 x 14 cm), which was equilibrated with 50 mM sodium phosphate, 250 mM NaCl, pH 6.8.

2. Coupling of the maleoylated BTG with the peptide

250 µl of the peptide solution (10 mg/ml in Aqua bidest) were mixed with 2 ml of a solution containing the maleoylated carrier proteins (2.5 mg/ml) in 50 mM sodium phosphate, 250 ml NaCl, pH 6.8 and incubated for 2 h at 4 °C and further 4 h at RT. Unreacted maleimid groups were blocked by addition of 2-mercaptoethanole up to a concentration of 10 mM and over night incubation at 4 °C. The resulting conjugate was dialysed against 10 mM sodium phosphate, 150 mM NaCl, pH 7.5 at 4 °C (3 times buffer exchange, MW cut-off 10.000).

Conjugation of the Peptide for ELISA

Conjugation was performed in two steps via SH-groups from the cystein residue of the peptide.

1. Maleoylation of the carrier protein

2 mg SMPH (50 mg/ml in N-methylpyrrolidone, NMP) was added to 2 ml of the carrier protein solution (BSA, 10 mg/ml in 0.1 mM $NaHCO_3$, pH 8.0). The reaction mixture was incubated for 1 h at room temperature (RT). The reaction mixture was thereafter desalted using a Sephadex G-50 column (1.5 x 14 cm), which was equilibrated with 50 mM sodium phosphate, 250 mM NaCl, pH 6.8.

2. Coupling of the maleoylated BTG with the peptide

100 µl of the peptide solution (10 mg/ml in 50 mM sodium phosphate, 250 mM NaCl, pH 6.8) were mixed with 1 ml of a solution containing the maleoylated carrier proteins (2.5 mg/ml) in 50 mM sodium phosphate, 250 ml NaCl, pH 6.8 and incubated for 2 h at 4 °C and further 4 h at RT. Unreacted maleimid groups were blocked by addition of 2-mercaptoethanole up to a concentration of 10 mM and over night incubation at 4 °C. The resulting conjugate was dialysed against 10 mM sodium phosphate, 150 mM NaCl, pH 7.5 at 4 °C (3 times buffer exchange, MW cut-off 10.000).

Immunization

Five mice were immunized intraperitoneally for 39 days. For immunization, a water-in-oil emulsion consisting of equal parts of the antigen solution (consisting of equal parts of the three different peptide-BTG-conjugates) and complete or incomplete Freundt's adjuvants was used.

Fusion

Three of the immunized mice were sacrificed by CO2 incubation. Spleens were taken and homogenized under sterile conditions. Spleen cells and myeloma SP2/0 cells were washed several times in Dulbecco's Modified Eagle Medium (DMEM, SIGMA) and fused in the ratio of 2,3 spleen cells : 1 SP2/0 cell using polyethylenglycole 3350 (1 ml 50 % (w/v)). Further handling of the fused hybridomas was performed according to standard methodologies.

ELISA

An IgG directed ELISA was used to screen the cell culture supernatant. The test was performed in 96-well polystyrol microtiter plates (Greiner, Cat. No. 655061). The plates were coated with the BSA-pGlu-6166 peptide. 100 µl undiluted cell culture supernatant was added to each well and incubated for 1 h at RT. Supernatant from SP2/0 cells was used as negative

control. Supernatant from the spleen cells was used as positive control.
Positive wells were detected using goat-anti-mouse IgG, which was conjugated with alkaline phosphatase. The optical density (OD) was measured in a Dynex Opsys MR Microplate Reader at 405 nm.

Selection of stable antibody producing hybridoma cells
Cells from positive wells were transferred to 24-well plates and cultivated for several days. Cells were again transferred and tested for BSA-pGlu—6199 binding and cross-reactivity in the ELISA. Positive wells were used for cryo-conservation of the hybridoma cell lines.

Cloning via limited dilution
Two consecutive cloning steps were performed in order to separate antibody producing cells from non-producing cells and to assure that the selected cells are monoclonal. Both cloning steps were performed according to the method of limited dilution.

Cryoconservation
Selected hybridomas were cryo-conserved using DMSO and standard methods.

1.2. ELISA Assays

Capturing of Aβ N3pE-40 was performed using hAβ (x-40) ELISA (HS) from TGC (The GENETICS Company; Switzerland), basically according to the manufacturer's instructions.

Biotinylated detection antibodies for Aβ N3pE (pGlu-6166) were generated. Where indicated, IBL HRP-conjugated Aβ N3pE antibody was used as positive control (available only in combination with the IBL ELISA Human Amyloid β (N3pE) Assay Kit). Corresponding Aβ N3pE-40 peptides (50 µg aliquots in Hexafluoroisopropanol(HFIP) stored at -80°C) were synthesized. Shortly before use, HFIP was evaporated and the peptide was diluted with 100 mM Tris/HCl pH 10,4 to 1 µg/µl. This stock

solution was diluted further with TGC antibody diluent. Subsequent capturing and detection was carried out according to manufacturer's instructions.

1.3. PepSpot™ Analysis

Specificity and biological integrity of Aβ N3pE antibodies and cell culture supernatants was determined by using the PepSpot™ technology of JPT Peptide Technologies GmbH, Volmerstrasse 5 (UTZ), 12489 Berlin, Germany.

Corresponding PepSpot™ membranes were prepared at JPT. The principle of this method was introduced and described before by Kramer et al. 1997 Cell 91, 799-809.

For analysis, membranes were blocked for two hours with TBST-M (10mM Tris-Hcl, pH 7.5, 150 mM NaCl, 0.005% Tween20 + 5% skimmed milk) at room temperature with gentle shaking. Membranes were incubated over night at 4°C on a rocking platform with the individual cell culture supernatants diluted in equal volumes of TBST-M. Secondary anti-mouse antibody conjugated with alkaline phosphatase was used for signal detection, following standard procedures.

1.4. DotBlot Analysis

A simple DotBlot protocol was accomplished to obtain information about the sensitivity of Aβ N3pE antibody and cell culture supernatants towards the respective native peptide. To that end, Aβ N3pE-40 peptide in descending concentrations (1000 ng - 8 ng) was spotted onto small pieces of nitrocellulose membrane, and subsequent experimental procedure was performed as for the PepSpot™ membranes.

1.5. SDS PAGE

12% SDS Polyacrylamide gels were cast following standard protocols. 15µl of cell culture supernatants and 10 µg of biotinylated antibody were separated on a 12% SDS polyacrylamide gel. Electrophoresis was carried out for 2 hours at 100 V constant.

1.6. BIACORE Analysis

Aβ N3pE-40 peptide (positive control) and Aβ N3E-40 peptide (negative control) were coupled on a Biacore CM5 Chip. Unmodified chips are used to determine blank values. Association and dissociation of biotinylated antibody diluted from 20 µg/ml to 1 µg/ml in TGC diluent was monitored to allow for subsequent determination of the respective KD value. In this way also binding characteristics of the individual cell culture supernatants were determined.

1.6.1 Affinity of AβN3pE specific antibody clone 6-1-6 and 24-2-3

The purified antibody clone 6-1-6 was diluted in HBS-EP buffer (Biacore) down to 100, 50, 30, 20, 15, 10, 7, 4, 2, 1 nM. The affinity was determined using a Biacore 3000 with CM5-Chip, on which AβpE3-40 was immobilized. The system was run with 30 µl/min. Measured bulk effects and unspecific binding to the chip surface were corrected by subtraction of the signal of flow cell 4, at which Aβ pE3-40 was immobilized, and the empty flow cell 3. The association (10 min) was obtained by injection of 300 µl of each concentration. The dissociation was observed over 10 min. Remaining antibody molecules were removed by injection of 5 µl 0.1 M HCL. For every antibody concentration the association and dissociation was recorded. The determination of the association and dissociation rate and the dissociation constant was performed by a global simultaneously fit of association and dissociation phase for all recorded antibody concentrations using the "Bivalent analyte" model.

1.7. Sequencing antibody variable regions

Cultivation of Hybridoma cells:

Hybridoma cells were grown in D-MEM (+ L-Glutamin, + Na-Pyruvat, 4,5g/l Glucose, Gibco) with the addition of 15% FBS, 1% MEM-NEA (non essential amino acids, Gibco), 50µg/ml Gentamycin (Gibco)

and 50µM β-mercaptoethanol at 37°C and 5% CO_2. Subcultivation occurred after 3-4 days depending on cell density. Cells were seeded in a concentration of 0.5 x 10^6 cells/ml, splitting occurred at a cell density of 2-5 x 10^6 cells/ml.

cDNA Synthesis and Reverse Transcription:
Total RNA was isolated from 2 x 10^6 cells according to the manual of the NucleospinRNA Isolation Kit (Macherey-Nagel). 100 ng RNA were applied for cDNA synthesis by using Oligo $(dT)_{15}$ primer (Promega) and SuperScript III Reverse Transcriptase (Invitrogen).

PCR-Amplification of Heavy and Light Chain Variable Regions:
Heavy chain variable regions were amplified from the template cDNA by using Phusion™ High-Fidelity DNA Polymerase (NEW ENGLAND BioLabs) with the primer MHCG1 (in case of clone 5-5-6 and 6-1-6) and MHCG2b (clone 17-4-3 and 24-2-3) in combination with primers MHV1-12. For amplification of light chain variable regions the primer MKC in combination with the primers MKV1-MKV11 were used. Primer sequences are shown in table 1.

Cloning of PCR products in pJET1.2:
Heavy and light chain variable regions, amplified by PCR, were cloned into pJET1.2/blunt vector according to the protocol of CloneJET™ PCR Cloning Kit (Fermentas). Sequencing occurred with pJET1.2 sequencing primers.

Table 1: Primer sequences for PCR-amplification of heavy and light chain variable regions

Name	**Sequence**	**SEQ ID NO.**
MKV1	ATGAAGTTGCCTGTTAGGCTGTTGGTGCTG	23
MKV2	ATGGAGWCAGACACACTCCTGYTATGGGTG	24
MKV3	ATGAGTGTGCTCACTCAGGTCCTGGSGTTG	25
MKV4	ATGAGGRCCCCTGCTCAGWTTYTTGGMWTCTTG	26
MKV5	ATGGATTTWCAGGTGCAGATTWTCAGCTTC	27
MKV6	ATGAGGTKCYYTGYTSAGYTYCTGRGG	28

MKV7	ATGGGCWTCAAGATGGAGTCACAKWYYCWGG	29
MKV8	ATGTGGGGAYCTKTTTYCMMTTTTTCAATTG	30
MKV9	ATGGTRTCCWCASCTCAGTTCCTTG	31
MKV10	ATGTATATATGTTTGTTGTCTATTTCT	32
MKV11	ATGGAAGCCCCAGCTCAGCTTCTCTTCC	33
MKC	ACTGGATGGTGGGAAGATGG	34
MHV1	ATGAAATGCAGCTGGGGCATSTTCTTC	35
MHV2	ATGGGATGGAGCTRTATCATSYTCTT	36
MHV3	ATGAAGWTGTGGTTAAACTGGGTTTTT	37
MHV4	ATGRACTTTGGGYTCAGCTTGRTTT	38
MHV5	ATGGACTCCAGGCTCAATTTAGTTTTCCTT	39
MHV6	ATGGCTTGTCYTRGSGCTRCTCTTCTGC	40
MHV7	ATGGRATGGAGCKGGRTCTTTMTCTT	41
MHV8	ATGAGAGTGCTGATTCTTTTGTG	42
MHV9	ATGGMTTGGGTGTGGAMCTTGCTATTCCTG	43
MHV10	ATGGGCAGACTTACATTCTCATTCCTG	44
MHV11	ATGGATTTTGGGCTGATTTTTTTTATTG	45
MHV12	ATGATGGTGTTAAGTCTTCTGTACCTG	46
MHCG1	CAGTGGATAGACAGATGGGGG	47
MHCG2b	CAGTGGATAGACTGATGGGGG	48

1.8 Application of antibody clone 6-1-6 for N3pE ELISA

A 96-well maxisorb plate (Nunc) was coated with capture antibody by incubation of 100 µl per well of 2 µg/ml anti-Aβ antibody 4G8, diluted in D-PBS, overnight at 4°C. The plated was sealed. The coating solution was removed and the surface of the plate was blocked with 200 µl per well PIERCE Protein-free ELISA-Blocker (without Tween-20) for 2 hours at room temperature. Afterwards the plate was washed with 6-times with TBS+0.05 % (v/v) Tween-20. Remaining washing solution was removed by tapping of the plate. The AβpE3-40 standard peptide was diluted in PIERCE Protein-free ELISA-Blocker (with Tween-20) down to 200, 100, 50, 25, 12.5, 6.25, 3.125 pg/ml. 100 µl of every concentration and 100 µl of dilution buffer (Blank) were pipetted on the plate.

The plate was sealed and incubated at 4°C for 2 hours. Afterwards the plate was washed 6-times with TBS + 0.05 % (v/v) Tween-20. Remaining washing solution was removed by tapping of the plate. 100 µl of the detection antibody-enzyme conjugate solution, which contains 1 µg/ml AβN3pE specific antibody clone 6-1-6 and 2 µg/ml Streptavidin-HRP conjugate (Sigma) solved in PIERCE Protein-free ELISA-Blocker (with Tween-20), was pipetted in each well. The plate was sealed and incubated at 4°C for 1 hour. Afterwards the plate was washed 6-times with TBS + 0.05 % (v/v) Tween-20. Remaining washing solution is removed by tapping of the plate. In every well 100 µl SureBlue substrate solution (KPL) are pipetted and the plate was incubated in the dark at room temperature for 30 min. The reaction was stopped by addition of 100 µl per well of 1 M H_2SO_4. The absorbance was measured with TECAN Sunrise at 450 nm corrected by the absorbance at 540 nm.

1.9 Investigation of cross reactivity, analyzed via ELISA and Surface-Plasmon-Resonance (SPR)

ELISA:

A 96-well maxisorb plate (Nunc) was coated with capture antibody by incubation of 100 µl per well of 2 µg/ml anti-Aβ antibody 4G8, diluted in D-PBS, overnight at 4°C. The plated was sealed. The coating solution was removed and the surface of the plate was blocked with 200 µl per well PIERCE Protein-free ELISA-Blocker (without Tween-20) for 2 hours at room temperature. Afterwards the plate was washed with 6-times with TBS+0.05 % (v/v) Tween-20. Remaining washing solution was removed by tapping of the plate. The AβpE3-40 standard peptide and other Aβ-Peptides (2-40, 3-40, 4-40, 1-42, 3-42 and pE11-40) were diluted in PIERCE Protein-free ELISA-Blocker (with Tween-20) down to 800, 400, 200, 100, 50, 25, 12.5. 100 µl of every concentration and 100 µl of dilution buffer (Blank) were pipetted on the plate. The plate

was sealed and incubated at 4°C for 2 hours. Afterwards the plate was washed 6-times with TBS + 0.05 % (v/v) Tween-20. Remaining washing solution was removed by tapping of the plate. 100 µl of the detection antibody-enzyme conjugate solution, which contains 1 µg/ml AβN3pE specific antibody clone 6-1-6 and 2 µg/ml Streptavidin-HRP conjugate (Sigma) diluted in PIERCE Protein-free ELISA-Blocker (with Tween-20), was pipetted in each well. The plate was sealed and incubated at 4°C for 1 hour. Afterwards the plate was washed 6-times with TBS + 0.05 % (v/v) Tween-20. Remaining washing solution was removed by tapping of the plate. In every well 100 µl SureBlue substrate solution (KPL) were pipetted and the plate was incubated in the dark at room temperature for 30 min. The reaction was stopped by addition of 100 µl per well of 1 M H_2SO_4. The absorbance was measured with TECAN Sunrise at 450 nm corrected by the absorbance at 540 nm.

SPR:

Beside different Aβ species also the cross reactivity to other pGlu-Peptide, which occur in the human body, was determined. This was made by surface plasmon resonance. Following peptides or there N-terminal region of them were immobilized on the surface of CM5-Chips:
MCP1, MCP2, big gastrin, gonadoliberin, neurotensin, orexin A, fibronectin, collagen 1 and TRH. As positive control also the binding to AβpE3-40 was analyzed. The N3pE antibody clones 6-1-6 and 24-2-3 were diluted in HBS-EP (Biacore) down to 25 µg/ml. The binding was observed using a Biacore 3000 with several CM5-Chips, on which the respective peptides (on flow cell 2, 3 and 4 was immobilized. The system was run with 20 µl/min. Measured bulk effects and unspecific binding to the chip surface were corrected by subtraction of the signal of flow cell 2, 3 and 4, at which the tested peptides was immobilized, and the empty flow

cell 1. The association (9 min) was obtained by injection of 180 µl of antibody clones 6-1-6 and 24-2-3, respectively. The dissociation was observed over 9 min. Remaining antibody molecules were removed by injection of 5 µl 0.1 M HCL. For every interaction of the antibody with the different peptides the association and dissociation was recorded. The cross reactivity was determined by evaluation of the association phase concerning rate and signal at the end. The values for all pGlu-Peptides compared with the signal for AβpE3-40.

1.10 Optimization and validation of N3pE ELISA for brain analysis

Our developed N3pE ELISA should be used for analysis of AβpE3-42 concentration in brain of transgenic mice. Generally, hemisphere and brainstem were separately analyzed concerning AβpE3-42 content. Mouse brain was homogenized in 500 µl 2 %SDS solution with protease inhibitor in Precelly (Peqlab) homogenizer using ceramic beads. The suspension was pipetted off from the beads and transferred into centrifuge tube. Beads were washed again with 250 µl 2 %SDS solution with protease inhibitor and solution transferred into the centrifuge tube. The 750 µl SDS brain suspension was sonificated on crashed ice for 20 sec. The sample was centrifuged for 1 hour at 4°C with 75000xg. Afterwards the supernatant was removed, aliquoted and stored until ELISA analysis at -80°C. The remaining SDS insoluble pellet was mixed with 150 µl 70 % formic acid and sonificated on crashed ice for 20 sec. Immediately after sonification the solution was neutralized with 2850 µl 1 M Tris, which was the old method, or 2850 µl EIA buffer (PBS+10 mg/ml BSA + 0.05 % Tween-20) + 860 µl 3.5 M Tris for neutralization, representing the new method. The formic acid fraction samples were stored until ELISA at -80°C. The N3pE ELISA was performed by following protocol:

A 96-well maxisorb plate (Nunc) was coated with capture antibody by incubation of 100 µl per well of 2 µg/ml anti-Aβ antibody 4G8, diluted in D-PBS, overnight at 4°C. The plated was sealed. The coating solution was removed and the surface of the plate was blocked with 200 µl per well PIERCE Protein-free ELISA-Blocker (without Tween-20) for 2 hours at room temperature. Afterwards the plate was washed with 6-times with TBS+0.05 % (v/v) Tween-20. Remaining washing solution was removed by tapping of the plate. The AβpE3-42 standard peptide was diluted in PIERCE Protein-free ELISA-Blocker (with Tween-20) (old method) or EIA buffer (new method) down to 1029.2, 514.6, 257.3, 128.65, 64.32, 31.16, 16.08 pg/ml. 100 µl of every concentration and 100 µl of dilution buffer (Blank) were pipetted on the plate. The SDS samples were thawed, diluted 1:25 and 1:100, respectively, in PIERCE Protein-free ELISA-Blocker (with Tween-20) (old method) or EIA buffer (new method) and pipetted on ELISA plate. The formic acid samples (old method: formic acid / Tris; new method: formic acid / EIA buffer / Tris) were thawed and undiluted pipetted on ELISA plate. The plate was sealed and incubated at 4°C for 2 hours. Afterwards the plate was washed 6-times with TBS + 0.05 % (v/v) Tween-20. Remaining washing solution was removed by tapping of the plate. 100 µl of the detection antibody-enzyme conjugate solution, which contains 1 µg/ml AβN3pE specific antibody clone 6-1-6 and 2 µg/ml Streptavidin-HRP conjugate (Sigma) solved in PIERCE Protein-free ELISA-Blocker (with Tween-20), was pipetted in each well. The plate was sealed and incubated at 4°C for 1 hour. Afterwards the plate was washed 6-times with TBS + 0.05 % (v/v) Tween-20. Remaining washing solution was removed by tapping of the plate. In every well 100 µl SureBlue substrate solution (KPL) are pipetted and the plate was incubated in the dark at room temperature for 30 min. The reaction was stopped by addition of 100 µl per well of 1 M H_2SO_4.

The absorbance was measured with TECAN Sunrise at 450 nm corrected by the absorbance at 540 nm.

1.11 Application of N3pE antibody clones for immunohistochemistry

Formalin-fixed and paraffin-embedded sections from human brain (cortex) were treated as follows:

1. Deparaffinizing and rehydrating sections (immobilized on slides):
    a. Incubation of slides in Histoclear or xylene for 3 minutes
    b. Remove cleaning solution
    c. Incubation of slides again in Histoclear or xylene for 3 minutes
    d. Incubation of slides in Histoclear or xylene 1:1 with 100 % ethanol for 3 minutes
    e. Incubation of slides in 100 % ethanol for 3 minutes, remove solution
    f. Incubation of slides again in 100 % ethanol for 3 min
    g. Incubation of slides in 95 % ethanol for 3 minutes
    h. Incubation of slides in 70 % ethanol for 3 minutes
    i. Incubation of slides in 50 % ethanol for 3 minutes
    j. Incubation of slides in destilled water for 3 minutes

2. Quenching endogenous peroxidase activity:
Incubation of slides with 99 ml methanol + 1 ml 30 % hydrogen peroxide for 10 minutes at room temperature.

3. Washing the slides with water: 2x 5 minutes

4. Removing water in individual slides and place slides on slide rack in a humidity chamber to prevent sections from drying.

Cover section with 88 % formic acid at room temperature for 10 minutes under fume hood. Rinse in water several times and allow to shake in a water-filled staining dish for 10 minutes.

5. Blocking in 10% horse serum for 20 minutes at room temperature.

6. Shaking off (or aspirate) blocking solution and apply primary antibody (N3pE antibody clone 6 or 24) for overnight at 4°C.

7. Washing slides separately with TBS for 10 minutes to avoid dragging from one slide to another.

8. Addition of biotinylated secondary antibody (goat anti-mouse from Vector Laboratories): 9 ml TBS, 1 ml goat serum, 45 µl 2nd antibody). Incubate 30 minutes at room temperature.

9. Washing slides separately with TBS for 10 minutes to avoid dragging from one slide to another.

10. Addition of ABC-solution (10 ml TBS, 100 µl horse serum, 90 µl component A, 90 µl component B). Incubate 30 minutes at room temperature.

11. Washing slides with 50 mM Tris: 2x 10 minutes

12. Color reaction: Incubation of sections with DAB solution (20 mg DAB from Sigma in 100 ml 50 mM Tris, filtered, and add 33 µl 33 % hydrogen peroxide). Using microscope to observe color reaction. The reaction product is brown colored. Stop the reaction by putting slides into staining dishes containing water.

13. Washing slides with water for 10 minutes

14. Counterstaining with hematoxylin, washing with water.

15. Dehydrating and clearing: Follow step 1 in reverse order (e.g. water, ethanols to 100 % histoclean)

16. Coverslip with permount (Fisher Scientific). Drying slides on air. Clean slides with razor blade and ethanol.

2. Results

2.1 Production of antibodies

Six clones were isolated that stably produce antibodies against the pGlu-6166-BSA peptide: clones 1-8-12, 5-5-6, 6-1-6, 12-1-8, 17-4-3 and 24-2-3. These clones were subject to further characterization.

2.2. Determination of Required Antibody Concentration:

The intensity of signals in the ELISA assays correlates not only with the concentration of analyte/Aβ variant but is also strongly dependent on the concentration of deployed antibody. Since Aβ variants are only present in low concentration in serum samples it is necessary to determine antibody concentrations that are able to detect low concentrations of the corresponding Aβ variants. Commercially available Aβ ELISA kits have a specified detection limit towards Aβ in the low pg range. In standard curves the highest concentration is usually 500 pg/ml. General information about deployed antibody concentration is typically lacking in data sheets / instruction manuals, however, due to further information as derivable from the general literature 1 µg / ml of antibody is used as default.

In a first series of experiments it was not possible to detect 500 pg/ml Aβ N3pE-40 with the corresponding pGlu-6166 12-1

biotin-conjugated antibody. In fact, relatively high Aβ N3pE concentrations (10 ng/ml) were required to obtain signals with 1 µg/ml antibody (see figure 1A: middle bar). The intensity of this signal was tremendously enhanced by increasing the antibody concentration to 10 µg/ml (see Figure 1A: left-hand bar). Until 20 µg / ml antibody the intensity of signal can be further elevated (see Figure 1B). Beyond this concentration no further increase in signal intensity can be achieved. Therefore, 20 µg/ml antibody was used to determine the detection limit for the pGlu-6166 antibody.

2.3. DotBlot Analysis

The Aβ N3pE-x antibody pGlu-6166 was chosen in the screening process because the original cell clone (designated 12-1-8) exhibited strong binding towards the peptide taken for immunization and very low cross reactivity (see table 2).

Table 2: Screening results demonstrating signals in ELISA assays obtained with several hybridoma cell clone supernatants.

Clone	N3pE-BSA	isoDAE-BSA	N3E-BSA	N11E-BSA	N11pE-BSA
1-8-12	1.787	0.012	0.142	0.011	0.005
5-5-6	1.649	0.015	0.126	0.004	0.006
6-1-6	1.377	0.013	0.125	0.007	0.014
12-1-8	2.123	0.005	0.009	0.001	0.005
17-4-3	1.915	0.007	0.320	0.003	0.004
24-2-3	1.768	0.014	0.218	0.003	0.002
positive control	1.824	1.227	1.596	1.243	1.346
negative control	0.045	0.005	0.008	0.001	0.003

No screening step, dealing with the full length, native Aβ N3pE-40 peptide had been included so far. Therefore the pool of

available pGlu6166 hybridoma cell clones was screened for clones expressing antibodies which might exhibit a higher affinity for the native Aβ N3pE-40 peptide.

As seen in figure 2, cell clones could be identified which indeed exhibit higher sensitivity towards the native full length Aβ N3pE-40 peptide. Whereas the pGlu-6166 antibody clone 12-1 could only detect 1 µg peptide, clones 6-1-6 and 24-2-3 also gave signals with as little as 8 ng peptide. Hence, clones 6-1-6 and 24-2-3 are 125 times more sensitive. With these clones, a detection limit of 8 pg/ml Aβ N3pE-40 peptide in a corresponding ELISA could be attainable.

2.4. PepSpot Analysis

Specificity was checked next by PepSpot analysis to compare biotinylated Aβ N3pE-x antibody pGlu-6166 with hybridoma cell clones. In table 3, all peptides are listed which correspond to spots on the PepSpot membrane. As seen in figure 3 pGlu-6166 clones 6-1-6 and 24-2-3 do not produce more cross signals than pGlu-6166 antibody clone 12-1. All clones investigated recognized primarily spot number 6 – the specific Aβ N3pE-x spot (pEFRHD…, i.e. SEQ ID No: 12), followed by spots number 5 (EFRHD…SEQ ID No: 11) and 7 (FRHD… SEQ ID No: 13). A faint signal was also attained with spot number 4 (AEFRHD… SEQ ID No: 10).

Table 3: Sequences of Aβ peptides spotted onto PepSpot™ Membranes (JPT Peptide Technologies GmbH) and detection by pGlu-6166 hybridoma cell clones

No.	**Peptide Sequence Aβ 1-40 (SEQ ID No:2)** DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	**IBL-AK**	**5-5-6**	**6-1-6**	**17-4-3**	**24-2-3**	**12-4**	**SEQ ID No.**
1	KMDAEFRHDSGYE	-	-	-	-	+/-	-	7
2	DAEFRHDSGYEVH	-	-	-	-	+/-	-	8

3	iDAEFRHDSGYEVH	-	-	-	-	+/-	-	9
4	AEFRHDSGYEVHH	-	+/-	+/-	-	+/-	+/-	10
5	EFRHDSGYEVHHQ	-	+	+	+	+	+	11
6	**pEFRHDSGYEVHHQ**	-	+++	+++	+++	+++	+++	12
7	FRHDSGYEVHHQK	+	+	+	+	+	+	13
8	GYEVHHQKLVFFA	-	-	-	-	-	-	14
9	EVHHQKLVFFAED	-	-	-	-	-	-	15
10	pEVHHQKLVFFAED	-	-	-	-	-	-	16
11	VHHQKLVFFAEDV	-	-	-	-	-	-	17
12	DAEFRHiDSGYEVH	-	-	-	-	-	-	18
13	iDSGYEVHHQKLVF	-	-	-	-	-	-	19
14	LVFFAEDVGSNKG	-	-	-	-	-	-	20
15	GSNKGAIIGLMVG	-	-	-	-	-	-	21
16	AIIGLMVGGVV	-	-	-	-	-	-	22

pE in table 3 means pGlu, pyroglutamate.
iD in table 3 means isoAsp, isoaspartate.

2.5. SDS-PAGE Analysis

Biological integrity of the Aβ-N3pE antibody and hybridoma cell culture supernatants was determined roughly by SDS-PAGE analysis (for details see Material & Methods *supra*).

As seen in Figure 4, all samples loaded onto the gel revealed sharp bands without smears, indicating the integrity of the pGlu-6166 12-1 antibody and the hybridoma cell clone supernatants.

2.6. BIACORE Analysis

With DotBlot analysis significant differences in sensitivity toward the Aβ N3pE-40 peptide of hybridoma cell clone supernatants compared to biotinylated pGlu-6166 antibody were diagnosed. However, with this method only an end point result is monitored. Biacore analysis on the other hand allows timewise resolution of the binding course of a given antibody.

In order to check whether the poor binding of the pGlu-6166 12-1 antibody was a result of a low association to the Aβ N3pE-40 peptide, a Biacore analysis was performed as described in Materials and Methods, *supra*.

Monitoring binding courses of increasing concentrations of pGlu-6166 antibody allowed for calculation of a KD value of 30nM. A comparison of the hybridoma cell clone supernatant 12-1 with cell clone supernatant 6-1-6 revealed striking differences in binding characteristics. The association of clone 6-1-6 was approximately 5 times higher than that observed with clone 12-1. Most markedly, however, is the difference in dissociation behavior. Whereas clone 6-1-6 hardly dissociates from the Aβ N3pE-40 peptide, 12-1 is readily washed off within a few minutes. Hence, the poor binding of clone 12-1 is very likely to be the consequence of the observed "off-rate". This assumption is further supported by the finding that clone 24-3-2, which gives particularly advantageous results in the DotBlot analysis, exhibits a very slow association to the Aβ N3pE-40 peptide but – in contrast to clone 12-1 – has no observable "off-rate" (see also Figure 5).

2.6.1 Affinity of AβN3pE specific antibody clone 6-1-6 and 24-2-3

For N3pE antibody clone 6-1-6 the association rate, dissociation rate and dissociation constant was calculated by a global fit of all sensograms shown in figure 6.

The association rate was calculated with 1.67e5 $M^{-1}s^{-1}$, the dissociation rate with 2.63e-4 s^{-1} and the dissociation constant with 1.57 nM.

For N3pE antibody clone 24-2-3 the association rate, dissociation rate and dissociation constant was calculated by a global fit of all sensograms shown in figure 7.

The association rate was calculated with 3.25e3 $M^{-1}s^{-1}$, the dissociation rate with 3.29e-4 s^{-1} and the dissociation constant with 101 nM.

2.7 Sequencing antibody variable regions

The following sequences were identified:

2.7.1 Clone 5-5-6

Variable part light chain, nucleotide sequence (SEQ ID NO: 49)

ATGGTGTCCTCAGCTCAGTTCCTGTTTCTGTTAGTGCTCTGGATTCAGGAAACCAACGGT
GATGTTGTGATGACCCAGACTCCACTCACTTTGTCGGTTACCATTGGACAACCAGCCTCT
ATCTCTTGCAAGTCAAGTCAGAGCCTCTTATATAGTGATGGAAAAACCTATTTGAATTGG
TTATTACAGAGGCCAGGCCAGTCTCCAATGCGCCTAATCTATCTGGTGTCTAAACTGGAC
TCTGGAGTCCCTGACAGGTTCACTGGCAGTGGATCAGGAACAGATTTTACACTGAAAATC
AGCAGAGTGGAGGCTGAGGATTTGGGAGTTTATTACTGCGTGCAAGGTACACATTTTCCA
TTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAAACGGGCTGATGCTGCACCAACTGTA
TCCATCTTCCCACCAT

Variable part light chain, protein sequence (SEQ ID NO: 50)

MVSSAQFLFLLVLWIQETNGDVVMTQTPLTLSVTIGQPASISCKSSQSLLYSDGKTYLNW
LLQRPGQSPMRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCVQGTHFP
FTFGSGTKLEIKRADAAPTVSIFPP

Variable part heavy chain, nucleotide sequence (SEQ ID NO. 51)

ATGGGATGGAGCGGGGTCTTTCTCTTCCTCCTGTCAGGAACTGCAGGTGTCCACTCTGAG
GTCCAGCTGCAACAGTCTGGACCTGAGCTGGTGAAGCCTGGAGCTTCAATGAAGATATCC
TGCAAGGCTTCTGGTTACTCATTCACTGGCTATACCATGAACTGGGTGAAGCAGAGCCAT
GGAAAGAACCTTGAGTGGATTGGACTTATTAATCCTTACAGTGGTGTTACTAGGTACAAC
CAGAAATTCAAGGGCAAGGCCACATTAATTGTAGACAAGTCATCCAGCACAGCCTACATG
GAGCTCCTCAGTCTGACATCTGAGGACTCTGCAGTCTATTATTGTACAAGAGAGGCTAAA
CGGGAGTGGGACGAGACTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCCAAA
ACGACACCCCCATCTGTCTA

Variable part heavy chain, protein sequence (SEQ ID NO: 52)

MGWSGVFLFLLSGTAGVHSEVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQSH
GKNLEWIGLINPYSGVTRYNQKFKGKATLIVDKSSSTAYMELLSLTSEDSAVYYCTREAK
REWDETYWGQGTLVTVSAAKTTPPSV

2.7.2 Clone 6-1-6

Variable part light chain nucleotide sequence (SEQ ID NO: 53)

ATGGTGTCCACAGCTCAGTTCCTGTTTCTGTTAGTGCTCTGGATTCAGGAAACCAACGGT
GATGTTGTGATGACCCAGACTCCACTCACTTTGTCGGTTACCATTGGACAACCAGCCTCT
ATCTCTTGCAAGTCAAGTCAGAGCCTCTTATATAGTGACGGAAAAACCTATTTGAATTGG
TTATTACAGAGGCCAGGCCAGTCTCCAATGCGCCTAATCTATCTGGTGTCTAAACTGGAC
TCTGGAGTCCCTGACAGGTTCACTGGCAGTGGATCAGGAACAGATTTTACACTGAAAATC
AGCAGAGTGGAGGCTGAGGATTTGGGAGTTTATTACTGCGTGCAAGGTACACATTTTCCA
TTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAAACGGGCTGATGCTGCACCAACTGTA
TCCATCTTCCCACCATCCAG

Variable part light chain, protein sequence (SEQ ID NO: 54)

MVSTAQFLFLLVLWIQETNGDVVMTQTPLTLSVTIGQPASISCKSSQSLLYSDGKTYLNW
LLQRPGQSPMRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCVQGTHFP
FTFGSGTKLEIKRADAAPTVSIFPPS

Variable part heavy chain, nucleotide sequence (SEQ ID NO: 55)

ATGGGATGGAGCGGGGTCTTTATCTTCCTCCTGTCAGGAACTGCAGGTGTCCACTCTGAGGTCC
AGCTGCAACAGTCTGGACCTGAGCTGGTGAAGCCTGGAGCTTCAATGAAGATATCCTGCAAGGC
TTCTGGTTACTCATTCACTGGCTACACCATGAACTGGGTGAAGCAGAGCCATGGAAAGAACCTT
GAGTGGATTGGACTTATTAATCCTTACAATGGTGTTACTAGGTACAACCAGAAGTTCAAGGGCA
AGGCCACATTAATTGTAGACAAGTCATCCAGCACAGCCTACATGGAGCTCCTCAGTCTGACATC
TGAGGACTCTGCAGTCTATTACTGTACAAGAGAGGCTAAACGGGAGTGGGACGAGACTTACTGG
GGCCAAGGGACTCTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCATCTGTCTATCCACTG

Variable part heavy chain protein sequence (SEQ ID NO: 56)

MGWSGVFIFLLSGTAGVHSEVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQSH
GKNLEWIGLINPYNGVTRYNQKFKGKATLIVDKSSSTAYMELLSLTSEDSAVYYCTREAK
REWDETYWGQGTLVTVSAAKTTPPSVYPL

2.7.3 Clone 17-4-3

Variable part light chain, nucleotide sequence (SEQ ID NO: 57)

ATGAAGTTGCCTGTTAGGCTGTTGGTGCTGGTGTTCTGGATTCCTGTTTCCAGCAGTGATGTTG
TGATGACCCAGACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGCAG
ATCTAGTCAGAGCCTTGTACACAGTGATGGAAACACCTATTTACATTGGTACCTGCAGAAGCCA
GGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGT
TCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCT
GGGAGTTTATTTCTGCTCTCAAAGTACACATGTTCCTCCGACGTTCGGTGGAGGCACCAAGCTG
GAAATCAAACGGGCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGT

Variable part light chain protein sequence (SEQ ID NO: 58)

MKLPVRLLVLVFWIPVSSSDVVMTQTPLSLPVSLGDQASISCRSSQSLVHSDGNTYLHWY
LQKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPP
TFGGGTKLEIKRADAAPTVSIFPPSS

Variable part heavy chain nucleotide sequence (SEQ ID NO: 59)

ATGGACTTTGGGCTCAGCTTACTTATTTTTGTCCTTATTTTAAAAGGTGTCCAGTGTGAG
GTGAAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGGGTCCCGGAAACTCTCC
TGTGCAGCCTCTGGATTCACTTTCAGTGACTACGGAATGGCGTGGGTTCGACAGGCTCCA
GGGAAGGGGCCTGAGTGGGTAGCATTCATTAGTAATTTGGCATATAGTATCTACTATGCA
GACACTGTGACGGGCCGATTCACCATCTCTAGAGAGAATGCCAAGAACACCCTGTACCTG
GAAATGAGCAGTCTGAGGTCTGAGGACACAGCCATGTACTACTGTGCAAGGTATGACTAC
GATAATATCTTGGACTATGTTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCC
TCAGCCAAAACAACACCCCCATCAGTCTATCCACTG

Variable part heavy chain protein sequence (SEQ ID NO: 60)

MDFGLSLLIFVLILKGVQCEVKLVESGGGLVQPGGSRKLSCAASGFTFSDYGMAWVRQAP
GKGPEWVAFISNLAYSIYYADTVTGRFTISRENAKNTLYLEMSSLRSEDTAMYYCARYDY
DNILDYVMDYWGQGTSVTVSSAKTTPPSVYPL

2.7.4 Clone 24-2-3

Variable part light chain nucleotide sequence (SEQ ID NO: 61)

ATGAAGTTGCCTGTTAGGCTGTTGGTGCTCTGGATTCAGGAAACCAAGGGTGATGTTGTGCTGA
CCCAGACTCCACTCACTTTGTCGGTTACCATTGGACAACCAGCCTCTATCTCTTGCAAGTCAAG
TCAGAGCCTCTTATATAGTAATGGAAAAACCTATTTGAATTGGTTATTACAGAGGCCAGGCCAG
TCTCCAAAGCGCCTAATCTATGTGGTGTCTAAACTGGACTCTGGAGTCCCTGACAGGTTCACTG

GCAGTGGATCAGGAACAGATTTTACACTGAAAATCAGCAGAGTGGAGGCTGAGGATTTGGGAGT
TTATTATTGCGTGCAAGGTACACATTTTCCATTCACGTTCGGCTCGGGGACAAAGTTGGAAATA
AAACGGGCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGT

Variable part light chain protein sequence (SEQ ID NO : 62)
MKLPVRLLVLWIQETKGDVVLTQTPLTLSVTIGQPASISCKSSQSLLYSNGKTYLNWLLQ
RPGQSPKRLIYVVSKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCVQGTHFPFTF
GSGTKLEIKRADAAPTVSIFPPSS

Variable part heavy chain nucleotide sequence (SEQ ID NO: 63)
ATGGGATGGAGCGGGGTCTTTCTCTTCCTCCTGTCAGTAACTGAAGGTGTCCACTCCCAGGTTC
AGCTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCTGGGTCCTCAGTGAAGATTTCCTGCAAGGC
TTCTGGCTATATATTCAATAACTACTGGATAAACTGGGTGAAGCAGAGGCCTGGTCAGGGTCTT
GAGTGGATTGGACAGATTTATCCTGGAGATGGTGATACTAACTACAATGGGAAGTTCAAGGGTA
AAGCCACACTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCAGCTCAGCAGCCTAACATC
TGAGGACTCTGCGGTCTATTTCTGTGCAAGAGAGGGATATATTGTTTATTGGGGCCAAGGGACT
CTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCATCTGTCTATCCACTG

Variable part heavy chain protein sequence (SEQ ID NO : 64)
MGWSGVFLFLLSVTEGVHSQVQLQQSGAELVRPGSSVKISCKASGYIFNNYWINWVKQRP
GQGLEWIGQIYPGDGDTNYNGKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYFCAREGY
IVYWGQGTLVTVSAAKTTPPSVYPL

2.8 Application of antibody clone 6-1-6 for N3pE ELISA

The final N3pE ELISA protocol was tested concerning limit of quantification (LOQ) and signal-to-noise ratio (S/N). The standard curve of the ELISA is shown in figure 8.
The shape of the standard curve looks very good especially for the low concentration range, which shows a nearly linear dependency of the absorbance. Based on this standard curve the LOQ is determined with 3.125 pg/ml with a S/N = 1.3.

2.9 Investigation of cross reactivity, analyzed via ELISA and SPR

ELISA:

The cross reactivity to other Aβ variants was determined using our N3pE-ELISA. The raw date are shown in table 4.

Table 4: Raw date of N3pE-ELISA with clone 6-1-6: Test of cross reactivity

Concentration (pg/ml)	**pE3-40 (28.04.)**	pE3-40(21.04.)	2-40	3-40	4-40	1-42	3-42	pE11-40
800	**1.8280**	1.806	0.048	0.090	0.055	0.053	0.052	0.047
400	**0.8750**	0.912	0.045	0.065	0.044	0.048	0.052	0.044
200	**0.4350**	0.484	0.044	0.057	0.046	0.048	0.049	0.048
100	**0.2290**	0.248	0.045	0.052	0.045	0.047	0.050	0.048
50	**0.1330**	0.143	0.044	0.048	0.044	0.050	0.045	0.045
25	**0.0820**	0.086	0.044	0.046	0.044	0.048	0.047	0.048
12.5	**0.0570**	0.063	0.039	0.042	0.040	0.043	0.042	0.046
0	**0.0410**	0.040	0.038	0.065	0.061	0.059	0.063	0.066

Only for AβpE3-40 a dependency of the absorbance from the concentration was observed. All tested Aβ variants have shown cross reactivity below 1 %, except of Aβ3-40. The signal (corrected by the blank) for 800 pg/ml was about 2.7 % of the signal for AβpE3-40. This is a very good value, considering that the N-terminus of both peptides have the same amino acids, except the first one, this is cyclized in the case of AβpE3-40. Overall, the Aβ N3pE antibody clone 6, which is generally used for ELISA, is very high specific for the N-terminus of Aβ-peptides starting with pGlu at position 3.

SPR:

The cross reactivity of clones 6-1-6 and 24-2-3 to other non-Aβ pGlu peptides was analyzed by surface plasmon resonance. Instead of AβpE3-40, which shows a typical binding sensogram, all other tested pGlu peptides have shown nearly no interaction with clones 6-1-6 and 24-2-3, respectively, see also figure 9 (data only shown for clone 6-1-6). The sensograms of the pGlu peptides were compared with the sensogram for AβpE3-40. The estimated

cross reactivities were all below 1 %. A summary of all analyzed peptides are shown in table 5.

Table5: Estimated Cross reactivity of clones 6-1-6 and 24-2-3 to other pGlu-peptides

pGlu Peptides	% cross reactivity
MCP-1	<1
MCP-2	<1
Big Gastrin	<1
Gonadoliberin	<1
Neurotensin	<1
Orexin A	<1
Fibronectin	<1
Collagen 1	<1
TRH	<1

All experiments have confirmed the fact that N3pE antibody clone 6-1-6 and 24-2-3 are specific for the N-terminal epitope of AβpE3-x. Neither other pGlu N-termini were recognized nor other Aβ peptide variants, which do not bear an N-terminal pE residue.

2.10 Optimization and validation of N3pE ELISA for brain analysis

AβpE3-42 concentration in mouse brainstem was analyzed dependent on the used method. The samples were derived from from transgenic mice (tg) overexpressing human AβQ3-42 in the brain, which is cyclized by QC to AβpE3-42. Compared were samples from heterozygous transgenic mice (tg het) and homozygous transgenic mice (tg hom) and from wildtype, non-transgenic mice (wt). The mice used for sample generation were produced as described in WO2009034158.

For all further experiments samples and standards were diluted in the EIA buffer. In a next step, the neutralization method for

analyzing formic acid fraction samples was optimized, i.e. the neutralization was N3pE ELISA. The resulting and herein developed N3pE ELISA works well, it detected significant levels of human AβpE3-42 in brains of the tg hom mice, significantly lower levels of human AβpE3-42 in brains of the tg het mice and no human AβpE3-42 in brains of the wt mice (see figure 10). The ELISA according to the present invention delivers high signals and consequently a very acceptable LOQ and is thus suitable for analysis of formic acid samples, in particular of formic acid brain samples.

2.11 Application of N3pE antibody clones for immunohistochemistry

With the N3pE antibodies of the present invention, AβpE3-x was stained in brain sections of patients in the late stage of sporadic Alzheimer's disease (SAD) and familial forms of Alzheimer's disease (FAD), i.e. patients which bear a mutation in the presenilin 1 (PS1) gene. The stained brain sections are shown in figue 11. Figure 11 shows that the N3pE antibodies of the present invention are suitable for immunohistochemistry. The antibodies specifically detect pGlu-Aβ in brain of SAD and FAD patients. The N3pE antibodies show no background signals on the images, which proves the specific binding shown by ELISA and SPR analysis.

3. Deposits

Monoclonal antibodies specifically recognizing Aβ N3pE-x, were generated. Currently all corresponding monoclonal antibodies expressing hybridoma cell lines 5-5-6, 6-1-6, 17-4-3, and 24-2-3 have been deposited in accordance with the Budapest Treaty and are available at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig, DE, with a deposit date of June 17, 2008, and with the respective deposit numbers

(clone 5-5-6) : DSM ACC2923
(clone 6-1-6) : DSM ACC2924
(clone 17-4-3) : DSM ACC2925
(clone 24-2-3) : DSM ACC2926.

Specificity of those antibodies for their respective target sequences could be confirmed. For Aβ N3pE-x, high affinity antibody clones could be identified that should give strong signals in an ELISA set up with an expected detection limit in the low pg range.

4. Summary

The main aim of the present invention was the establishment of a highly sensitive and robust detection technique that allows quantitative determination of Aβ variants in biological samples.

Preferably, an ELISA based technique should be pursued. The task was started with Aβ N3pE ELISA, because for this Aβ variant an appropriate ELISA system was already commercially available (IBL). This system was used as reference and internal quality control.

Applicability of the pGlu-6166 antibody in the chosen ELISA assay set up was investigated. To obtain clearly measurable signals, high antibody concentrations needed to be deployed (20 µg/ml). High affinity Aβ N3pE-x antibody clones could be identified. A detection limit in the low pg range (3-8 pg/ml) can be achieved with these clones.

PCT

Print Out (Original in Electronic Form)
(This sheet is not part of and does not count as a sheet of the international application)

0-1	**Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)**	
0-1-1	Prepared Using	PCT Online Filing Version 3.5.000.204 MT/FOP 20020701/0.20.5.9
0-2	**International Application No.**	
0-3	**Applicant's or agent's file reference**	PBD 00070/WO

1	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
1-1	**page**	68
1-2	**line**	5
1-3	**Identification of deposit**	
1-3-1	Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Inhoffenstr. 7B, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	17 June 2008 (17.06.2008)
1-3-4	Accession Number	DSMZ DSM ACC2926
1-5	**Designated States for Which Indications are Made**	all designations
2	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
2-1	**page**	68
2-2	**line**	4
2-3	**Identification of deposit**	
2-3-1	Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
2-3-2	Address of depositary institution	Inhoffenstr. 7B, D-38124 Braunschweig, Germany
2-3-3	Date of deposit	17 June 2008 (17.06.2008)
2-3-4	Accession Number	DSMZ DSM ACC2925
2-5	**Designated States for Which Indications are Made**	all designations

PCT

Print Out (Original in Electronic Form)
(This sheet is not part of and does not count as a sheet of the international application)

3	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
3-1	**page**	68
3-2	**line**	3
3-3	**Identification of deposit**	
3-3-1	Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
3-3-2	Address of depositary institution	Inhoffenstr. 7B, D-38124 Braunschweig, Germany
3-3-3	Date of deposit	17 June 2008 (17.06.2008)
3-3-4	Accession Number	DSMZ DSM ACC2924
3-5	**Designated States for Which Indications are Made**	all designations
4	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
4-1	**page**	68
4-2	**line**	2
4-3	**Identification of deposit**	
4-3-1	Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
4-3-2	Address of depositary institution	Inhoffenstr. 7B, D-38124 Braunschweig, Germany
4-3-3	Date of deposit	17 June 2008 (17.06.2008)
4-3-4	Accession Number	DSMZ DSM ACC2923
4-5	**Designated States for Which Indications are Made**	all designations

FOR RECEIVING OFFICE USE ONLY

0-4	**This form was received with the international application:** (yes or no)	yes
0-4-1	Authorized officer	Claudepierre, Mireille

FOR INTERNATIONAL BUREAU USE ONLY

0-5	**This form was received by the international Bureau on:**	
0-5-1	Authorized officer	

Claims

1. Antibody, characterised in that it binds to A peptides or variants thereof, preferably with high affinity.

2. Antibody according to claim 1, wherein said high affinity means a dissociation constant (K_D) value of 10^{-7} M, or better.

3. Antibody according to claim 1 or 2, wherein said antibody is a monoclonal antibody.

4. Antibody according to any of the preceding claims, wherein the variable part of the light chain of said antibody has a nucleotide sequence selected from SEQ ID NOs: 49, 53, 57 and 61, or an amino acid sequence selected from SEQ ID NOs: 50, 54, 58, and 62.

5. Antibody according to any of the preceding claims, wherein the variable part of the heavy chain of said antibody has a nucleotide sequence selected from SEQ ID NOs: 51, 55, 59 and 63, or an amino acid sequence selected from SEQ ID NOs: 52, 56, 60 and 64.

6. Antibody according to any of the preceding claims, wherein the variable part of the light chain of said antibody has the nucleotide sequence of SEQ ID NO: 49 or the amino acid sequence of SEQ ID NO: 50, and wherein the variable part of the heavy chain of said antibody has the nucleotide sequence of SEQ ID NO: 51, or the amino acid sequence of SEQ ID NO: 52.

7. Antibody according to any of the preceding claims, wherein the variable part of the light chain of said antibody has the nucleotide sequence of SEQ ID NO: 53 or the amino acid sequence of SEQ ID NO: 54, and wherein the variable part of the heavy chain of said antibody has the nucleotide sequence

of SEQ ID NO: 55, or the amino acid sequence of SEQ ID NO: 56.

8. Antibody according to any of the preceding claims, wherein the variable part of the light chain of said antibody has the nucleotide sequence of SEQ ID NO: 57 or the amino acid sequence of SEQ ID NO: 58, and wherein the variable part of the heavy chain of said antibody has the nucleotide sequence of SEQ ID NO: 59, or the amino acid sequence of SEQ ID NO: 60.

9. Antibody according to any of the preceding claims, wherein the variable part of the light chain of said antibody has the nucleotide sequence of SEQ ID NO: 61 or the amino acid sequence of SEQ ID NO: 62, and wherein the variable part of the heavy chain of said antibody has the nucleotide sequence of SEQ ID NO: 63, or the amino acid sequence of SEQ ID NO: 64.

10. Antibody according to any of the preceding claims, wherein said antibody is selected from the following group:

Aβ 5-5-6 (Deposit No. DSM ACC 2923)
Aβ 6-1-6 (Deposit No. DSM ACC 2924)
Aβ 17-4-3 (Deposit No. DSM ACC 2925)
Aβ 24-2-3 (Deposit No. DSM ACC 2926)

or functional variants thereof.

11. Antibody according to any of the preceding claims, wherein said antibody is Aβ 6-1-6 (Deposit No. DSM ACC 2924).

12. Antibody according to any of the preceding claims, wherein said antibody is Aβ 24-2-3 (Deposit No. DSM ACC 2926).

13. Antibody according to any of the preceding claims, wherein said antibody is a humanized or chimeric antibody, or an antibody fragment which retains the high affinity.

14. Antibody according to any of the preceding claims for use in the detection of Aβ peptide or variants thereof.

15. Antibody according to claim 14, wherein said variants are selected from the following group:

pGlu-$A\beta_{3-38}$
pGlu-$A\beta_{3-40}$
pGlu-$A\beta_{3-42}$, and
pGlu-$A\beta_{3-x}$ variants,

wherein x is an integer between 10 and 42; preferably 18 and 42, more preferably 30 and 42.

16. Antibody according to any of the preceding claims, which is human.

17. Antibody according to any of the preceding claims, which is a diabody or a single chain antibody which retains the high affinity.

18. Antibody according to any of the preceding claims, which binds to the epitope bound by the antibodies defined in claim 15.

19. Antibody according to any of the preceding claims, which has the complementarity determining regions of the antibodies as defined in claim 15.

20. Antibody according to any of the preceding claims, which is labeled.

21. Antibody according to any of the preceding claims, which is immobilised on a solid phase.

22. Antibody obtainable from any one of hybridoma cell lines DSM ACC 2923, DSM ACC 2924, DSM ACC 2925, DSM ACC 2926.

23. Composition comprising the antibody as defined in any of the preceding claims.

24. Composition according to item 23 for the treatment, prevention or delay of amyloidosis.

25. Composition according to item 23 or 24, wherein said amyloidosis is a neurodegenerative disease selected from the group consisting of mild cognitive impairment, Alzheimer's disease and neurodegeneration in Down Syndrome.

26. Composition according to item 23 or 24, wherein said amyloidosis is sporadic Alzheimer's disease or a Familial Alzheimer’s dementia.

27. Composition according to item 26, wherein said Familial Alzheimer’s dementia is Familial British Dementia or Familial Danish Dementia.

28. Hybridoma cell line DSM ACC 2923.

29. Hybridoma cell line DSM ACC 2924

30. Hybridoma cell line DSM ACC 2925.

31. Hybridoma cell line DSM ACC 2926.

32. Use of the antibody as defined in any one of items 1 to 22 or the composition as defined in any one of items 23 to 27in a diagnostic or therapeutic method.

33. The use according to item 32 for the diagnosis of an amyloid-associated disease or condition.

34. The use according to item 33, wherein said amyloidosis is a neurodegenerative disease selected from the group consisting of mild cognitive impairment, Alzheimer's disease and neurodegeneration in Down Syndrome.

35. The use according to item 33, wherein said amyloidosis is sporadic Alzheimer's disease or a Familial Alzheimer's dementia.

36. The use according to item 35, wherein said Familial Alzheimer's dementia is Familial British Dementia or Familial Danish Dementia.

37. *In vitro* diagnostic method for the diagnosis of an amyloid-associated disease or condition, in particular Alzheimer's disease, comprising the following steps:
contacting an antibody according to any one of items 1 to 22 with a sample from a subject suspected to be afflicted with said disease or condition, and
detecting binding of the antibody to a pGlu-amyloid protein, preferably pGlu-Aβ peptide from the sample.

38. Diagnostic kit, comprising the antibody as defined in any one of items 1 to 22, and instructions for use, and - optionally - (a) further biologically active substance(s).

39. The diagnostic kit of item 32, wherein said further biological substance is an inhibitor of glutaminy cyclase.

40. An oligonucleotide selected from the group consisting of SEQ ID Nos: 23 to 48.