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(54) Method of providing disease-specific binding molecules and targets

Verfahren zur Bereitstellung krankheitsspezifischer Bindungsmoleküle und -ziele

Procédé pour fournir des molécules de liaison et des cibles spécifiques à une maladie

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Description

Field of the Invention

[0001] The present invention relates to a novel method of providing specific binding molecules, particularly human antibodies as well as fragments, derivatives and variants thereof that recognize disease-associated epitopes, including neopeptides, of proteins which derive from native endogenous proteins, and which are prevalent in the body of a patient in a variant form and/or out of their normal physiological context.

BACKGROUND OF THE INVENTION

[0002] The success in generating monoclonal antibodies rests on the efficient and selective fusion of antigen-stimulated B cells with a murine myeloma cell line followed by selection of stable antibody producing hybrids as originally described by Köhler and Milstein, Nature 256 (1975), 495-497. However, the therapeutic utility of murine based antibodies in human is hampered by the human anti-mouse antibody (HAMA) response in view of their non-human origin. Approaches for making human or human-like monoclonal antibodies became available through genetic engineering. However, the methods hitherto available suffer from the drawback that they are not suitable to produce antibodies with the characteristics of those produced in the course of a physiological human immune response. Furthermore, such antibodies may not be specific enough because of cross-reactivity with other proteins and/or the target protein in context with normal physiological function. In case of Alzheimer's or Parkinson's disease, for example, antibodies that also cross-react with high affinity with physiological derivatives of amyloid precursor protein (APP) or alpha synuclein are considered to exhibit side effects related to the normal functions of the physiologic target structures. In this respect, an undesired autoimmune disease would downrightly be induced - a hardly calculable risk in the conceptual design of active immunization experiments employing protein structures that, in variant form, also occur physiologically. Side effects not related to the target structure are, for example, anaphylactic reactions, as are to be expected as undesired and dreaded side effects of the systemic administration of exogenous proteins. According to recent findings, this can also be the case in so-called humanized antibodies, which originally stem from non-human organisms, usually from mice. On the other hand, active immunization with pathological relevant antigens bears the considerable risk of patients developing antibodies and T cell responses which also recognize physiological variants of such proteins and in consequence lead to a dangerous and uncontrollable autoimmune response.

[0003] Thus, there is a need of providing agents which are specific for a target involved in a disorder and which are tolerated by the human body.

SUMMARY OF THE INVENTION

[0004] An object of the present invention is a method for identifying, validating and producing diagnostically and therapeutically useful binding molecules, in particular antibodies that are directed against pathologic variants of endogenous proteins, which method is characterized in the claims. More specifically, the present invention relates to a method of isolating a disease-associated protein-specific antibody comprising:

- (a) subjecting a sample obtained from a patient who is symptom-free, or who is clinically unusually stable, but who is affected with or at risk of developing a disorder to a specimen of pathologically altered cells or tissues with predetermined pathological characteristics; and
- (b) identifying and optionally isolating an antibody which binds to said specimen but not to corresponding cells or tissues without such pathological characteristics as it may be derived from a healthy subject.

[0005] Known is the fact that, in case of autoimmune diseases, antibodies are directed against autologous cells and proteins or other compounds such as glycolipids expressed by said cells while evading the known tolerance mechanisms. Also known is the fact that, in case of endogenous neoplastic developments, a cellular and humoral immunity to the neoplastic cells can develop and can thus effect an endogenous immunological protection mechanism against neoplastic tissue degeneration.

[0006] The present invention makes use of the surprising finding that antibodies can also be directed against pathophysiologically relevant variants of endogenous proteins, in particular against neopeptides, which are formed due to pathologically altered transcription, translation, or post-transcriptional or post-translational modification, or proteolytic processing, or aggregation. Such antibodies are directed against endogenous proteins which, owing to their new structure that deviates from the normal physiology, become pathophysiologically relevant by means of developing pathological effects. For reasons of immune tolerance, the antibodies connected with the corresponding immune response to neopeptides in such pathological variants do not normally exhibit any cross reactions against the physiologically functional

proteins, however, as opposed to the case of autoimmune diseases. This is because the formation of potentially cross-reactive antibodies is specifically suppressed by the known tolerance mechanisms, whereas the development of an immune response to pathological neoepitopes can escape tolerance.

[0007] Hence, the present invention relates to a novel approach of identifying diagnostically, therapeutically, and preventively active binding molecules, especially antibodies and antibody fragments from clinically preselected human subjects by means of interaction with identifiable pathological structures.

[0008] The present application thus discloses antibodies or antigen-binding fragments and similar antigen binding molecules which are capable of recognizing epitopes, including neoepitopes, of disease-associated proteins which derive from native endogenous proteins and are prevalent in the body of a patient in a variant form, e.g. as a pathological protein and/or out of their normal physiological context. Furthermore, the present application discloses compositions comprising said antibodies and to immunotherapeutic and immunodiagnostic methods using the same.

[0009] Furthermore, in antibody identification, the method according to the present invention can do without previous hypothesis on the identity of its molecular target structure, solely by means of its association with pathologically relevant structures. Besides the possibility of thus identifying molecular target structures hitherto unknown for specific diseases, a further advantage of antibodies that are exclusively directed against pathological structures is based on the fact that their pharmacodynamic availability is not negatively influenced by binding to non-diseased tissues in such a way that the antibody is buffered with respect to its concentration and sink effects thus hampering the determination of therapeutically effective concentrations. Furthermore, the antibody and binding molecules isolated in accordance with the method of the present invention are preferably characterized in that they react with the variant form of the disease-associated protein *in vivo* or with a cell or cell membrane, and on a section of the pathologically characterized diseased tissue, respectively, but not or to a significantly lesser extent with the physiological variant of the cognate protein; see also, e.g., Example 2.

[0010] The antigen-binding fragment of the antibody can be a single chain Fv fragment, an F(ab') fragment, an F(ab) fragment, and an F(ab')₂ fragment, or any other antigen-binding fragment. In a specific embodiment, *infra*, the antibody or fragment thereof is a human IgG isotype antibody.

[0011] Further embodiments of the present invention will be apparent from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012]

Fig. 1: Antibody against beta-amyloid. **A:** Human antibodies. **B:** Control staining with known antibody against human beta-amyloid. Clinically unusually stable patients with Alzheimer's disease contain antibodies to beta-amyloid plaques. Immunohistochemical staining with antibodies from clinically unusually stable patients on brain sections obtained from patients with pathologically confirmed Alzheimer's disease reveals antibodies that bind to beta-amyloid plaques confirmed by a known antibody against human beta-amyloid.

Fig. 2: Antibody against neurofibrillary tangles. **A:** Human antibodies. **B:** Control staining with known antibody against human tau. Healthy human subjects contain antibodies to neurofibrillary tangles. Immunohistochemical staining with antibodies from healthy subjects on brain sections obtained from patients with pathologically confirmed Alzheimer's disease reveals antibodies that bind to neurofibrillary tangles confirmed by a known antibody against human tau.

Fig. 3: Antibody against dystrophic neurites. **A:** Human antibodies. **B:** Control staining with known antibody against human tau. Healthy human subjects contain antibodies to dystrophic neurites. Immunohistochemical staining with antibodies from healthy subjects on brain sections obtained from patients with pathologically confirmed Alzheimer's disease reveals antibodies that bind to dystrophic neurites.

Fig. 4: Antibody against beta-amyloid. The figure shows specific binding of recombinant human NI-101.11 antibody that was isolated from a clinically unusually stable Alzheimer's disease patient to brain beta-amyloid plaques. Brain sections obtained from a patient with neuropathologically confirmed Alzheimer's disease were stained with recombinant human antibody at the indicated concentrations. Antibody binding to beta-amyloid plaques with concentrations of 50 pM suggest high affinity binding.

Fig. 5: Binding of recombinant human NI-101.11 antibody to beta-amyloid plaques is not competed by linear synthetic N-terminal Abeta polypeptides. Binding of the recombinant antibody against brain beta-amyloid (0.5 nM) cannot be competed by N-terminal Abeta-derived polypeptide representing positions 1 to 16 at concentrations up to 1 μM.

Fig. 6: Recombinant human NI-101.11 antibody recognizes a conformational Abeta epitope that is not present in monomeric Abeta. Binding of NI-101.11 to beta-amyloid plaques on brain sections can be competed by Abeta1-42 fibrils but not linear synthetic Abeta1-42 monomers.

Fig. 7: Recombinant human NI-101.11 antibody does not bind to linear, monomeric synthetic Abeta on Western blots. Preparations of monomeric Abeta were separated by non-denaturing PAGE. Blotted protein was probed with

human recombinant antibody against beta-amyloid and control antibodies against N-terminal linear Abeta sequences (6E10). No binding of NI-101.11 to monomeric Abeta was detected. This observation suggests that the antibody recognizes a conformational Abeta epitope.

Fig. 8: Human NI-101.11 antibody binds artificial amyloid fibrils prepared from synthetic Abeta1-42 peptides. Synthetic Abeta fibrils or monomeric synthetic Abeta coated onto ELISA plates at equal coating densities were incubated with recombinant human antibodies against brain beta-amyloid at the indicated concentrations. Binding activity of human antibody against brain beta-amyloid to artificial amyloid fibrils (open squares) is more than 100 times higher as compared to monomeric Abeta (filled squares). Control antibody 22C4 preferentially binds to monomeric Abeta (filled circles), and less well to fibrils (open circles). This suggests that NI-101.11 recognizes a conformational epitope which is also present on artificial amyloid fibrils prepared from synthetic Abeta peptides.

Fig. 9: Absent cross-reactivity of recombinant human NI-101.11 antibody to cellular full-length APP or with any of its physiological derivatives occurring in cultured cells. In contrast to the control antibody (6E10) that binds to cell-surface APP, binding of NI-101.11 to full-length APP present at cellular surfaces is absent. These data demonstrate absent cross-reactivity of NI-101.11 to physiological, cellular full-length APP.

Fig. 10A-C: Absence of binding of NI-101.11 to monomeric Abeta via size exclusion chromatography. Figures 10A and 10B show no binding of NI-101.11 or an unrelated control antibody to monomeric FITC-labeled Abeta 1-42 while Figure 10C shows prominent binding of antibody 22C4 that recognizes a linear epitope present in the C-terminus of Abeta.

Fig. 11: Competition ELISA showing that binding of antibody 6E10, an antibody directed against a linear epitope at the N-terminus of Abeta could be completely blocked upon pre-incubation with excess concentrations of monomeric Abeta peptides while pre-incubation with excess concentrations of these monomeric Abeta peptide preparations did not abolish NI-101.11 binding.

Fig. 12: Binding of NI-101.13A and NI-101.13B to brain sections obtained from Tg2676 transgenic mouse model of Alzheimer's disease.

Fig. 13: ELISA showing preferential binding of NI-101.13A and NI-101.13B to artificial amyloid fibrils as compared to monomeric Abeta.

Fig. 14A-B: Figure 14A shows the binding of recombinant NI-101.12 to synthetic Abeta1-42 peptide via ELISA. Figure 14B shows NI-101.12 binding was competed by excess Abeta1-42 peptide.

Fig. 15: Recombinant human NI-101.11 antibody against brain beta-amyloid crosses the blood brain barrier in a transgenic mouse model of Alzheimer's disease, and binds to brain beta-amyloid plaques *in vivo*.

Fig. 16A-B: Recombinant human NI-101.11 antibody improves abnormal cognitive behavior in a transgenic mouse model of Alzheimer's disease. 24 months old arcAbeta mice were treated weekly i.p. with 3 mg/kg antibody for 2 months. Y-maze behavioral testing was performed before and after completion of the treatment.

Fig. 17: Blood-brain barrier penetration and decoration of amyloid plaques by peripherally administered NI-101.11. NI-101.11 can cross the blood-brain barrier and bind to beta-amyloid deposits in NI-101.11 treated mice (left panel) whereas no such staining is visible in animals treated with the human control antibody (right panel). Recombinant human NI-101.11 antibody reduces brain beta-amyloid plaque load after systemic treatment for two months.

Fig. 18: Passive immunization with NI-101.11 reduces beta-amyloid load in arcAbeta mice. (A, B) Thioflavin S and Congo Red plaque load analyses reveal significant reductions of more than 50% compared to the control antibody treated animals (Mann-Whitney U; $p=0.02$ for cortex, $p=0.009$ for hippocampus for ThioS and $p=0.009$ for cortex and $p=0.04$ for hippocampus for Congo Red analysis). Scale bar: 200 μm . (C-E) Thioflavin S analysis reveals a significant reduction in beta-amyloid burden (C), number of beta-amyloid plaques (D) and average plaque size (E) in NI-101.11 treated arcAbeta mice compared to control treated animals. Mann-Whitney U statistics: $p=0.02$ for plaque area cortex; $p=0.009$ for plaque area hippocampus; $p=0.047$ for plaque number cortex; $p=0.047$ for plaque number hippocampus; $p=0.009$ for plaque size cortex; $p=0.009$ for plaque number hippocampus.

Fig. 19: Reduced beta-amyloid load is accompanied by decreased astrocytosis and microgliosis A) Quantification of anti-GFAP staining revealed a significant reduction in the number of reactive astrocytes in the cortex of NI-101.11 treated arcAbeta mice when compared to control treated transgenics. B) Quantification of Iba-1 staining showed a trend towards a reduced number of activated microglia in NI-101.11 treated mice in cortex and hippocampus. Scale bar: 200 μm .

Fig. 20: No increase of brain microhemorrhages after two months of treatment with recombinant human NI-101.11 antibody. 24 months old arcAbeta mice with proven massive congophilic amyloid angiopathy were treated weekly i.p. with 3 mg/kg antibody for 2 months. Representative picture of a brain microhemorrhage in arcAbeta mice revealed by Perl's Prussian blue staining (left). Quantitative analysis demonstrates a significantly elevated frequency of microhemorrhages in arcAbeta transgenic mice compared to their wildtype littermates. Chronic treatment with NI-101.11 did not result in increased frequency of microhemorrhages. Scale bar: 20 μm

Fig. 21: Recombinant human NI-101.11 antibody inhibits the formation of synthetic Abeta fibrils *in vitro*. The effect of recombinant human NI-101.11 antibody on the formation of Abeta fibrils was assayed by measuring Thioflavin

S bound to aggregated Abeta by fluorescence analysis.

Fig. 22: Antibody-mediated dose-dependent phagocytosis of FITC-Abeta1-42 fibrils by BV-2 microglial cells was measured upon inhibition of the scavenger receptor system. NI-101.11 triggers potent dose-dependent Fcgamma receptor-mediated phagocytosis of Abeta fibrils.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0013] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "an antibody," is understood to represent one or more antibodies. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0014] The terms "fragment," "variant," "derivative" and "analog" when referring to antibodies or antibody polypeptides include any polypeptides which retain at least some of the antigen-binding properties of the corresponding native binding molecule, antibody, or polypeptide. Fragments of polypeptides include proteolytic fragments, as well as deletion fragments, in addition to specific antibody fragments discussed elsewhere herein. Variants of antibodies and antibody polypeptides include fragments as described above, and also polypeptides with altered amino acid sequences due to amino acid substitutions, deletions, or insertions. Variants may occur naturally or be non-naturally occurring. Non-naturally occurring variants may be produced using art-known mutagenesis techniques. Variant polypeptides may comprise conservative or non-conservative amino acid substitutions, deletions or additions. Derivatives of neopeptide-specific binding molecules, e.g., antibodies and antibody polypeptides, are polypeptides which have been altered so as to exhibit additional features not found on the native polypeptide. Examples include fusion proteins. Variant polypeptides may also be referred to herein as "polypeptide analogs." As used herein a "derivative" of a binding molecule or fragment thereof, an antibody, or an antibody polypeptide refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Also included as "derivatives" are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For example, 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine.

[0015] Unless stated otherwise, the terms "disorder" and "disease" are used interchangeably herein. A "binding molecule" as used in the context of the present invention relates to antibodies, and fragments thereof.

[0016] The terms "antibody" and "immunoglobulin" are used interchangeably herein. An antibody or immunoglobulin is an antigen-binding molecule which comprises at least the variable domain of a heavy chain, and normally comprises at least the variable domains of a heavy chain and a light chain. Basic immunoglobulin structures in vertebrate systems are relatively well understood. See, e.g., Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988).

[0017] As will be discussed in more detail below, the term "immunoglobulin" comprises various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon (γ , μ , α , δ , ϵ) with some subclasses among them (e.g., $\gamma 1$ - $\gamma 4$). It is the nature of this chain that determines the "class" of the antibody as IgG, IgM, IgA, IgG, or IgE, respectively. The immunoglobulin subclasses (isotypes) e.g., IgG1, IgG2, IgG3, IgG4, IgA1, etc. are well characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of the instant invention. All immunoglobulin classes are clearly within the scope of the present invention, the following discussion will generally be directed to the IgG class of immunoglobulin molecules. With regard to IgG, a standard immunoglobulin molecule comprises two identical light chain polypeptides of molecular weight approximately 23,000 Daltons, and two identical heavy chain polypeptides of molecular weight 53,000-70,000. The four chains are typically joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the "Y" and continuing through the variable region.

[0018] Light chains are classified as either kappa or lambda (κ , λ). Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain.

[0019] Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used functionally. In this regard, it will be appreciated that the variable domains of both the light (VL) and heavy (VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CL) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention the numbering of the

constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the antibody. The N-terminal portion is a variable region and at the C-terminal portion is a constant region; the CH3 and CL domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

[0020] As indicated above, the variable region allows the antibody to selectively recognize and specifically bind epitopes on antigens. That is, the VL domain and VH domain, or subset of the complementarity determining regions (CDRs), of an antibody combine to form the variable region that defines a three dimensional antigen binding site. This quaternary antibody structure forms the antigen binding site present at the end of each arm of the Y. More specifically, the antigen binding site is defined by three CDRs on each of the VH and VL chains. Any antibody or immunoglobulin fragment which contains sufficient structure to specifically bind to an antigen is denoted herein interchangeably as an "antigen binding fragment" or an "immunospecific fragment."

[0021] In naturally occurring antibodies, the six "complementarity determining regions" or "CDRs" present in each antigen binding domain are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen binding domain as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the amino acids in the antigen binding domains, referred to as "framework" regions, show less inter-molecular variability. The framework regions largely adopt a β -sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β -sheet structure. Thus, framework regions act to form a scaffold that provides for positioning the CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to its cognate epitope. The amino acids comprising the CDRs and the framework regions, respectively, can be readily identified for any given heavy or light chain variable region by one of ordinary skill in the art, since they have been precisely defined (see, "Sequences of Proteins of Immunological Interest," Kabat, E., et al., U.S. Department of Health and Human Services, (1983); and Chothia and Lesk, J. Mol. Biol., 196:901-917 (1987)).

[0022] In the case where there are two or more definitions of a term which is used and/or accepted within the art, the definition of the term as used herein is intended to include all such meanings unless explicitly stated to the contrary. A specific example is the use of the term "complementarity determining region" ("CDR") to describe the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. This particular region has been described by Kabat et al., U.S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983) and by Chothia et al., J. Mol. Biol. 196:901-917 (1987), where the definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody or variants thereof is intended to be within the scope of the term as defined and used herein. The appropriate amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth below in Table I as a comparison. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

Table 1: CDR Definitions¹

	Kabat	Chothia
VH CDR1	31-35	26-32
VH CDR2	50-65	52-58
VH CDR3	95-102	95-102
VL CDR1	24-34	26-32
VL CDR2	50-56	50-52
VL CDR3	89-97	91-96
¹ Numbering of all CDR definitions in Table 1 is according to the numbering conventions set forth by Kabat <i>et al.</i> (see below).		

[0023] Kabat *et al.* also defined a numbering system for variable domain sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of "Kabat numbering" to any variable domain sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat et al., U.S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest" (1983). Unless otherwise specified, references to the numbering of specific amino acid residue positions in an antibody or antigen-binding fragment, variant, or derivative thereof are according to the Kabat numbering system.

[0024] Antibodies or antigen-binding fragments, immunospecific fragments, variants, or derivatives thereof include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized, primatized, or chimeric antibodies, single chain antibodies, epitope-binding fragments, e.g., Fab, Fab' and F(ab')₂, Fd, Fvs, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a VL or VH domain, fragments produced by a Fab expression library, and anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies disclosed herein). ScFv molecules are known in the art and are described, e.g., in US patent 5,892,019. Immunoglobulin or antibody molecules can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

[0025] In one embodiment of the method of the present invention, the antibody is not IgM or a derivative thereof with a pentavalent structure. Particular, in specific applications, especially therapeutic use, IgMs are less useful than IgG and other bivalent antibodies or corresponding binding molecules since IgMs due to their pentavalent structure and lack of affinity maturation often show unspecific cross-reactivities and very low affinity. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human patients. A human antibody is still "human" even if amino acid substitutions are made in the antibody, e.g., to improve binding characteristics.

[0026] The term "neoepitope" in accordance with the present invention denotes an epitope which is unique for a disease pattern and contained in or formed by a disorder-associated protein which is a pathological variant from an otherwise non-pathological protein and/or deviating from the physiology of the healthy state. Said pathophysiological variants can be formed by means of pathologically altered transcription, pathologically altered translation, post-translational modification, pathologically altered proteolytic processing, pathologically altered complex formation with physiological or pathophysiological interaction partners or cellular structures in the sense of an altered co-localization, or pathologically altered structural conformation - like for example aggregation, oligomerization or fibrillation - whose three- or four-dimensional structure differs from the structure of the physiologically active molecule. Moreover, a pathophysiological variant can also be characterized in that it is not located in its usual physiological environment or subcellular compartment. As an example, neoepitopes may be located in the pathologically conspicuous structures in the areas of brain tissues that obviously experience or have already experienced functional damage. Whether a given structure, for example cell or tissue, or protein displays a neoepitope can be verified by reversing the method described below for isolating and characterizing a disorder-associated protein specific binding molecule in that a binding molecule, for example antibody identified by said method is used to screen a sample for binding to the antibody, thereby determining the presence of a neoepitope.

[0027] The phrases "disease-associated protein specific" and "neoepitope specific" are used interchangeably herein with the term "specifically recognizing a neoepitope". As used herein terms such as "absence of cross-reactivity", "specific," "specifically recognizing," "specifically binding," "preferentially binding," and the like refer to the binding molecule's ability to discriminate between the neoepitope of a disorder-associated protein and the native protein in its wild type form and natural context. Thus, the binding molecule obtained in accordance with the method of the present invention has a preferential binding affinity to the neoepitope over the native protein antigen by a factor of at least two, preferably at least 5, usually more than by a factor of 10, particularly preferred by a factor of 50 and even more preferred higher than 100. Furthermore, the relative K_D of the binding molecule, e.g., antibody for the specific target epitope, e.g. neoepitope is preferably at least 10-fold less, more preferably at least 100-fold less or more than the K_D for binding that antibody to other ligands or to the native counterpart of the disease-associated protein.

[0028] By "specifically binds," or "specifically recognizes," used interchangeably herein, it is generally meant that a binding molecule, e.g., an antibody binds to an epitope via its antigen binding domain, and that the binding entails some complementarity between the antigen binding domain and the epitope. According to this definition, an antibody is said to "specifically bind" to an epitope when it binds to that epitope, via its antigen binding domain more readily than it would bind to a random, unrelated epitope. The term "specificity" is used herein to qualify the relative affinity by which a certain antibody binds to a certain epitope. For example, antibody "A" may be deemed to have a higher specificity for a given epitope than antibody "B," or antibody "A" may be said to bind to epitope "C" with a higher specificity than it has for related epitope "D."

[0029] By "preferentially binds," it is meant that the binding molecule, e.g., antibody specifically binds to an epitope more readily than it would bind to a related, similar, homologous, or analogous epitope. Thus, an antibody which "preferentially binds" to a given epitope would more likely bind to that epitope than to a related epitope, even though such an antibody may cross-react with the related epitope.

[0030] By way of non-limiting example, a binding molecule, e.g., an antibody may be considered to bind a first epitope preferentially if it binds said first epitope with a dissociation constant (K_D) that is less than the antibody's K_D for the second epitope. In another non-limiting example, an antibody may be considered to bind a first antigen preferentially if it binds the first epitope with an affinity that is at least one order of magnitude less than the antibody's K_D for the second epitope. In another non-limiting example, an antibody may be considered to bind a first epitope preferentially if it binds the first epitope with an affinity that is at least two orders of magnitude less than the antibody's K_D for the second epitope.

[0031] In another non-limiting example, a binding molecule, *e.g.*, an antibody may be considered to bind a first epitope preferentially if it binds the first epitope with an off rate ($k(\text{off})$) that is less than the antibody's $k(\text{off})$ for the second epitope. In another non-limiting example, an antibody may be considered to bind a first epitope preferentially if it binds the first epitope with an affinity that is at least one order of magnitude less than the antibody's $k(\text{off})$ for the second epitope. In another non-limiting example, an antibody may be considered to bind a first epitope preferentially if it binds the first epitope with an affinity that is at least two orders of magnitude less than the antibody's $k(\text{off})$ for the second epitope.

[0032] As used herein, the term "affinity" refers to a measure of the strength of the binding of an individual epitope with the CDR of a binding molecule, *e.g.*, an immunoglobulin molecule. See, *e.g.*, Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988) at pages 27-28. As used herein, the term "avidity" refers to the overall stability of the complex between a population of immunoglobulins and an antigen, that is, the functional combining strength of an immunoglobulin mixture with the antigen. See, *e.g.*, Harlow at pages 29-34. Avidity is related to both the affinity of individual immunoglobulin molecules in the population with specific epitopes, and also the valencies of the immunoglobulins and the antigen. For example, the interaction between a bivalent monoclonal antibody and an antigen with a highly repeating epitope structure, such as a polymer, would be one of high avidity.

[0033] By "subject" or "individual" or "animal" or "patient" or "mammal," is meant any subject, particularly a mammalian subject, *e.g.*, a human patient, for whom diagnosis, prognosis, prevention, or therapy is desired.

II. Methods to Identify Binding Molecules

[0034] The present invention generally relates to a method of isolating a monoclonal antibody specific for a disorder-associated pathological variant form of an endogenous protein as characterized in the claims including means and methods for discovering therapeutically efficient antibodies from clinically preselected human subjects. As demonstrated in the Examples, human antibodies against abnormal structures in human brain diseases can be isolated from phenotypically healthy, or clinically unusually stable patients and corresponding recombinant antibodies can be successfully used for the treatment, amelioration of pathology and prevention of impairment of brain function without substantial side effects. Clinical stability or non-progression of disease can be identified by way of example by measurement over time of clinical, *e.g.*, cognitive, status (by neuropsychological testing, for example); assessment of the global functional level; evaluation of the daily living capacities or behavioral deficits; volumetric analysis of brain structures; *in vivo* measurement of pathological deposits of abnormal proteins in brain (*e.g.* PET beta-amyloid imaging) or biochemical variables in body fluids (*e.g.* tau proteins, or Abeta peptides); and by comparison to the natural course/history of the disease. Thus, the present invention provides antibodies and binding molecules which are capable of recognizing neoepitopes of disease-associated proteins which derive from native endogenous proteins, and are prevalent in the body of a patient in a variant form, *e.g.* as a pathological protein and/or out of their normal physiological context. In particular, antibodies and antigen-binding fragments thereof are provided, which demonstrate the immunological binding characteristics and/or biological properties as outlined for the antibody illustrated in the Examples. Where present, the term "immunological binding characteristics," or other binding characteristics of an antibody with an antigen, in all of its grammatical forms, refers to the specificity, affinity, cross-reactivity, and other binding characteristics of an antibody. The present invention is based on the observation that in human subjects and patients preselected according to specific clinical criteria, who bear a risk of developing a neurological disorder like Alzheimer's disease due to their age, as related to the humoral defense, antibodies to endogenous pathophysiological variants like Abeta peptide aggregates, neurofibrillary tangles, dystrophic neurites and further cellular structures can also be found, which are neuropathologically characteristic for the disorder. These structures can be found alone or in combination with other pathological structures and can, as in case of the Abeta aggregates and the precursors of the neurofibrillary tangles, develop pathological effects. However, those antibodies specifically recognizing said structures exhibit no or significantly lower cross-reactivity to the normal physiologically functional forms of the proteins underlying the pathological structures, contrary to the known case of an autoimmune response.

[0035] Without intending to be bound by theory it is, based on the experiments performed in accordance with the present invention, believed that a disorder does not have to become manifest and phenotypically perceivable, as in case of an infection, in order to result in an experimentally measurable activity of the humoral immune system like the generation or activation of specific B cells or B memory cells. In case of tumor cells or differentiated cells, which have to be physiologically disposed of, mechanisms are already known, wherein partners like T4 helper cells and cytotoxic T cells and natural killer cells of the cellular immune system cooperate in the induction of apoptosis. It has to be assumed that, also in a healthy human, tumor cells or precursors thereof are formed on a daily basis by means of mutation. However, these are immediately driven to apoptosis by means of humoral and cellular mechanisms, so that a tumor is not detectable. In this sense, a "healthy, or clinically unusually stable, patient" is not understood to denote an individual in whom no disease events take place, but an individual in whom the diverse disease events are controlled by blocking or defense mechanisms before they phenotypically manifest themselves.

[0036] In view of the above, it was hypothesized in accordance with the present invention that it should be possible

to identify an antibody or an antibody-producing cell from specific patient collectives or healthy subjects, which have been preselected according to clinical criteria, without precognition of the molecular nature of a target structure epitope, e.g. neoepitope, wherein the antibody, in case of already prevailing tolerance in the donor organism - which is, for example, proved by the absence of autoimmunity - could successfully be employed against a disease in form of a recombinantly produced agent. Identifying such an antibody can thus be conducted without precognition of the molecular epitope of the target structure, but rather solely by means of binding to neoepitopes of pathologically conspicuous structures in clinico-pathologically well characterized tissue sections derived from human patients or from animal models of the respective disease.

[0037] Accordingly, in one embodiment the method of the present invention comprises:

(a) subjecting a sample obtained from a patient who is symptom-free, or who is clinically unusually stable, but who is affected with or at risk of developing a disorder or effectively suppressing the manifestation or outbreak of a disorder to a specimen of pathologically or physiologically altered cells or tissue of predetermined clinical characteristics; and

(b) identifying and optionally isolating a binding molecule which preferentially binds to said specimen but not or with significantly lower affinity to corresponding cells or tissues without such pathological characteristics as it may be derived from a healthy subject.

[0038] The method of the present invention can be performed as outlined in the Examples section with means well known to a person skilled in the art. For example, a liquid sample obtained from the patient can be passed through a first aperture of a duct which is in contact with the specimen target structure firmly held in an object holder, thereby allowing putative binding molecules present in the sample, either in a soluble form or expressed on the cell surface and membrane, respectively, to bind to said target structure. The liquid sample may contain for example lymphocytes and/or antibodies while the specimen may be a tissue section or a membrane coated with molecules or molecular combinations which are distinct for a pathological target structure.

[0039] Any non-binding matter can be removed via the second duct aperture. At the same time, the temperature of the object holder may be controlled by an object holder thermostat, for example at a temperature at which natural binding of the putative binding molecule to the neoepitope of the antigen specific for the specimen takes place in the human body. By way of the flowing motion, e.g. passing the liquid sample containing binding molecules, preferably at body temperature over the target structure natural systems of binding interactions can be simulated. However, other methods of incubating the sample with the specimen such as by means of a shaker or rotating table may be used as well. A particular advantage of the above-mentioned system is that it allows an interruption of metabolic processes at any time by decreasing the temperature of the object holder by means of the object holder thermostat. In doing so, the temperature of the object holder can be decreased to for example 2-104°C, in particular 4°C. A corresponding device that can be used in accordance with the method of the present invention is described in European patent application EP 1 069 431 A2. Hence, the method of the present invention will allow identification and characterization of the binding partners as well as at the same time to identify and characterize the molecular classes, molecular groups and/or molecular parts required for the binding process, *i.e.* the target structures of the specimen, which hitherto may be unknown. This will not only open up new possible ways of diagnosis, but will also provide a new test system for therapeutic approaches on a molecular level.

[0040] As a patient may qualify in accordance with the present invention a pool of healthy volunteers if specific surrogate markers predict a high probability of a status of a disease, which has surprisingly - and possibly due to a specific endogenous immune response - not become clinically manifest, however. In this sense, as related to diverse diseases, a healthy elderly human would be an individual, in which a neurodegenerative disease like Alzheimer's disease or Parkinson's disease is not yet clinically manifest, but in which the preclinical development of pathophysiological protein variants, *i.e.* disorder-associated proteins, and in the above-mentioned sense by means of early intervention of the humoral immune system with or without involvement of cellular components of the immune system, has been restricted or delayed to such an extent that, until the moment when the healthy, but not preclinical patient participated in the study, the clinical manifestation of the disease had not yet occurred. Preferably, inconspicuous volunteers in whom neither autoimmunological processes have been diagnosed nor other possible pathological conditions occur as side effects are recruited as donors for the sample in the first instance.

[0041] In principle samples from patients may be used, who have undergone an active immunization with variants of physiological proteins or peptides, wherein the antibody development has been boosted by the immunization. Antibodies, for example, can be identified and isolated from Alzheimer's disease patients that have been vaccinated with Abeta peptide. See for e.g., Hock et al., Nature Medicine 8:1280-1275 (2002), Hock et al., Neuron 38:547-554 (2003), and WO 2004/095031. However, it may be preferred to use samples from volunteers which have not received such immunization or corresponding medication concerning the disorder the variant pathological protein is associated with and/or causative of.

[0042] According to the present invention, samples of a patient, e.g. of individuals that have been clinically preselected are analyzed for the presence of binding molecules specifically recognizing specimen of pathologically conspicuous structures, for example in *ex vivo* tissue from clinico-pathologically characterized human patients or animal models like, for example, transgenic mice, or *in vitro* cell structures, or in pathological allogenic or xenogenic tissue. Preferably, said patient and/or as said subject providing the specimen are human, most preferably both.

[0043] The characteristic pathologically or physiologically altered sample, cell or tissue specimen is preferably displayed by optical detection after reaction with a binding molecule, e.g. antibody of the present invention. The specimen may be obtained as/from a cell sample, tissue section, cellular smear test, cell or tissue sample of an animal model of a human disease or *in vitro* cultured cell and tissue material. Preferably, at least one of said specimen is present in an object holder in form of a scan positions, wherein each scan position corresponds to a specific pathological structure and at least two of the scan positions are concomitantly exposed to detection of a reaction with the antibody. By way of example, antibodies to the neuropathological hallmarks of such neurodegenerative diseases as Alzheimer's disease, Parkinson's disease or tauopathies - including beta-amyloid plaques, Lewy bodies, neurofibrillary tangles and dystrophic neuritis can be detected on sections obtained from human post-mortem brain tissues with clinico-pathologically confirmed diagnoses of the respective disease entities. This is done by mounting small rods of paraffin embedded tissue on to glass slides which are then probed with samples of a patient or healthy donor. The detection of a reaction with the antibody is done following standard procedures of immunohistochemistry and microscopical scanning.

[0044] Multiple tissue microsections containing various human disease tissues can be assembled on the glass slide to form a multiple tissue microarray. Similarly, additional specimens from tissue samples of animal models of human disease, cellular smear or cells can be embedded in paraffin and mounted on glass slides alone or in combination with above described human post-mortem Alzheimer's disease brain tissue or tissue arrays. Thus, a single test position on the glass slide can comprise mixed arrays of tissue and other specimen displaying pathologically conspicuous structures.

[0045] In order to increase the throughput of the assay, more than one test position is mounted on a glass slide. Preferably, eight test positions are mounted onto glass slides in a two by four format fitting the 96 well or microtiter format. A more detailed description of this microtiter-compatible tissue microarray can be found *infra* in the supplementary methods section below.

[0046] As mentioned the sample to be analyzed may comprise a body fluid, a cell sample or the supernatant of a cell sample or a derivative thereof. Body fluids such as lumbar cerebrospinal fluid (CSF), plasma or urine can be collected following standard clinical procedures after informed consent of the patients. Most preferably, the sample comprises or is derived from B-cells or memory B-cells and/or comprises antibodies.

[0047] Preferably, said patient has been determined to be affected with a not yet manifested disorder or at risk to develop the disorder by the presence or absence of a surrogate marker, or by an unusually stable disease course. Clinical criteria are to be considered in connection with surrogate markers having either an increased probability of the occurrence or the manifestation of a disease according to the present invention in the sense of a preclinical condition, or, vice versa, proving the improbability of such a disease already having occurred as, for example, a genetic constellation promoting the disease is existent or an extreme exposition or way of living renders the phenotypical development of a disease probable. In the case of Alzheimer's disease this means that, according to the present invention, such human volunteers are searched for B cells or memory B cells against neuropathology-associated protein complexes like Abeta aggregates, oligomeric Abeta species and beta-amyloid fibrils in beta-amyloid plaques, for tau filaments in neurofibrillary tangles, for alpha synuclein in Lewy bodies or components hitherto not molecularly identified that, with respect to age, belong to a group of individuals in whom either the prevalence of Alzheimer's disease is particularly high or who originate genetically from a population bearing a high risk for Alzheimer's disease. These are, for example, persons older than 75 years having no or only marginal neuropsychologically measurable cognitive impairments, or in case of tumor indications, persons having highly indicative tumor markers, for example genetic tumor markers, but not suffering from the disease (Alloul et al., Arch. Gerontology Geriatrics 27 (1998), 189; Dunn et al., Immunity 21 (2004) 137-148). In case of mild cognitive impairment, these are patients that have remained clinically, neuropsychologically, or cognitively stable for years in spite of their annual statistical risk of more than 20% for developing a neurodegenerative disease that is clinically manifest and progressive in the further course. Thus, in one embodiment said surrogate marker is selected from the group consisting of old age, brain amyloid load, ApoE genotype, APP genotype, PS1 genotype, levels in body fluids of Abeta peptide, isoprostanes, Tau, and phospho-Tau.

[0048] A particular approach in employing the method according to the present invention is testing samples of B memory cells from clinically preselected volunteers against arrays of specimen of pathologically conspicuous tissues of entirely different or related primary diseases. In particular, such pathological tissues originate from human patients suffering from neurodegenerative diseases, protein-misfolding diseases, tissue amyloidoses and other diseases related to pathological deposits, autoimmune disorders, inflammatory diseases, hyperproliferative and neoplastic diseases, e.g., tumors, storage diseases and inclusion diseases as well as from animal models of human diseases, in particular from mice altered with pathologically relevant human genes.

[0049] In one particularly preferred embodiment, the present invention focuses on Alzheimer's disease. In this em-

bodiment, the sample is obtained from a subject preferably fulfilling the following criteria:

- a) being 65, preferably 70 and more preferably 75 years of age or older;
- b) having full cognitive capacity and good health; and
- c) having no clinical signs of dementia; or
- d) having unusually slow rates of progression of disease despite the presence of an established clinical diagnosis of probable Alzheimer's disease; or
- e) having unusually low conversion rates from Mild Cognitive Impairment (MCI) to full blown Alzheimer's disease.

[0050] The method of the present invention comprises the steps of:

- a) purifying B memory cells from a sample which has been identified to contain binding molecules, e.g. antibodies which preferentially bind to said specimen but not or with significantly lower affinity to corresponding cells or tissue of a healthy subject;
- b) obtaining the immunoglobulin gene repertoire encoding said antibodies from said B memory cells; and
- c) using said repertoire to express said antibodies, wherein step (b) comprises the steps of:
- d) obtaining mRNA from said memory B cells;
- e) obtaining cDNA from the mRNA of step (iv); and
- f) using a primer extension reaction to amplify from said cDNA the fragments corresponding to the heavy chains (HC) and the kappa/lambda light chains (LC) of said antibodies.

[0051] Methods of producing clones of an immortalized human B cell and B memory lymphocyte, comprising the step of transforming human B memory lymphocytes using Epstein Barr Virus (EBV) in the presence of a polyclonal B cell activator are summarized in international application WO2004/076677. This international application also describes methods for obtaining a nucleic acid sequence that encodes an antibody of interest, comprising the steps of preparing an immortalized B cell clone and obtaining/sequencing nucleic acid from the B cell clone that encodes the antibody of interest and further inserting the nucleic acid into or using the nucleic acid to prepare an expression host that can express the antibody of interest, culturing or sub-culturing the expression host under conditions where the antibody of interest is expressed and, optionally, purifying the antibody of interest. It goes without saying that the nucleic acid may be manipulated in between to introduce restriction sites, to change codon usage, and/or to add or optimize transcription and/or translation regulatory sequences. For example, nucleic acid sequences can be generated by back-translation of the polypeptide sequences using software such as vector NTI software to generate nucleic acid sequences that are codon-optimized and optimized for RNA stability. All this techniques are state of the art and can be performed by the person skilled in the art without undue burden. Additional methods of immortalizing human B cells are well known in the art, e.g., the construction of human hybridomas or human-murine chimeric hybridomas.

[0052] The present invention application also discloses a binding molecule which is capable of selectively recognizing an epitope of a disease-associated protein including a neoepitope of a disease-associated protein, which preferably can be obtained or validated by the method of the present invention described hereinbefore and illustrated in the Examples. Advantageously, the binding molecule does not substantially recognize said protein in its non-disorder-associated form; see also *supra*.

[0053] Means and methods for the recombinant production of binding molecules, in particular antibodies and mimics thereof as well as methods of screening for competing binding molecules, which may or may not be antibodies, are known in the art and are summarized, for example, in international application WO2006/103116 with respect to antibodies against beta-amyloid and the treatment/diagnosis of Alzheimer's disease.

[0054] However, as described herein, in particular with respect to therapeutic applications in human an antibody to be isolated in accordance with the method of the present invention is a human antibody. In this context, the variant pathological protein recognized by the antibody is preferably associated with a neurological disorder, preferably a disorder of the brain.

[0055] Moreover, as demonstrated in Examples 3 to 5 the binding molecule isolated in accordance with the method

of the present invention, in particular an antibody has several advantageous biological properties one or more of which have been accomplished by the present invention for the first time, e.g. it is capable of:

- (i) crossing the blood brain barrier, for example at the site of the pathological event;
- (ii) binding beta-amyloid plaques, cerebrovascular amyloid, diffuse Abeta deposits, neurofibrillary tangles, hyperphosphorylated tau, alpha-synuclein positive Lewy-bodies or protein aggregates associated with dystrophic neurites;
- (iii) removing beta-amyloid plaques in the brain and/or preventing the formation of amyloid plaques in the brain;
- (iv) substantially restoring normal behavior; and/or
- (v) causing no microhemorrhages.

[0056] The antibody or equivalent binding molecule isolated in accordance with the method of the present invention may be distinguished from other antibodies by one or more of the following properties, e.g. they are able to:

1. pass, at least in small amounts, the blood-brain barrier at the site of the pathological events;
2. bind to one or more pathophysiologically relevant extracellular or cellular structure;
3. lead to reduction of the pathophysiologically relevant structure *in vitro* or *in vivo*;
4. lead to reduction of the pathophysiologically relevant structure and to the reduction of a toxicity associated therewith;
5. lead to blocking or delaying a disease process;
6. lead to regeneration of cellular and organ-specific and organismic functions and possibly to a secondary prevention of the recurrence of the original pathophysiology after degradation of the toxicity connected with the pathophysiologically relevant structure; and/or
7. is not associated with increased microhemorrhages

[0057] Furthermore, the absence of cross-reactivity with physiological precursors or derivatives leads to the consequence that, firstly, the concentrations are predictable as *sink effects* in healthy tissue structures are avoided and, secondly, that autoimmune responses in the sense of undesired side effects are substantially missing. In addition, previous reports suggested an association of cerebral amyloid angiopathy (CAA) with compromised vascular reactivity in a transgenic mouse model with CAA (Mueggler et al., J Neurosci 22 (2002), 7218-24.). The severe CAA occurring in old arcA β mice (Knobloch et al., Neurobiol. Aging 28:1297-1306 (2007) epub July 31, 2006) might thus constrain the vasodilative flexibility of affected blood vessels. In accordance with the present invention it is prudent to expect that treatment with the antibodies obtained in accordance with the method of the present invention can improve vasoreactivity and cerebral blood flow in aged APP transgenic mice. This may be validated by using the arcA β mice model described in Knobloch et al. (2006), supra, and disclosed in US application US 2009/0117120 A1.

[0058] As an alternative to obtaining immunoglobulins directly from the culture of immortalized B memory cells, the immortalized cells can be used as a source of rearranged heavy chain and light chain loci for subsequent expression and/or genetic manipulation. Rearranged antibody genes can be reverse transcribed from appropriate mRNAs to produce cDNA. If desired, the heavy chain constant region can be exchanged for that of a different isotype or eliminated altogether. The variable regions can be linked to encode single chain Fv regions. Multiple Fv regions can be linked to confer binding ability to more than one target or chimeric heavy and light chain combinations can be employed. Once the genetic material is available, design of analogs as described above which retain both their ability to bind the de-sired target is straightforward. Methods for the cloning of antibody variable regions and generation of recombinant antibodies are known to the person skilled in the art and are described, for example, Gilliland et al., Tissue Antigens 47 (1996), 1-20; Doenecke et al., Leukemia 11 (1997), 1787-1792.

[0059] Once the appropriate genetic material is obtained and, if desired, modified to encode an analog, the coding sequences, including those that encode, at a minimum, the variable regions of the heavy and light chain, can be inserted into expression systems contained on vectors which can be transfected into standard recombinant host cells. A variety of such host cells may be used; for efficient processing, however, mammalian cells are preferred. Typical mammalian

cell lines useful for this purpose include, but are not limited to, CHO cells, HEK 293 cells, or NSO cells.

[0060] The production of the antibody or analog is then undertaken by culturing the modified recombinant host under culture conditions appropriate for the growth of the host cells and the expression of the coding sequences. The antibodies are then recovered by isolating them from the culture. The expression systems are preferably designed to include signal peptides so that the resulting antibodies are secreted into the medium; however, intracellular production is also possible.

[0061] Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')₂ fragments may be produced recombinantly or by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments). F(ab')₂ fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

[0062] DNA encoding desired monoclonal antibodies may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The isolated and subcloned hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into prokaryotic or eukaryotic host cells such as, but not limited to, *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells or myeloma cells that do not otherwise produce immunoglobulins. More particularly, the isolated DNA (which may be synthetic as described herein) may be used to clone constant and variable region sequences for the manufacture of antibodies as described in Newman et al., U.S. Pat. No. 5,658,570. Essentially, this entails extraction of RNA from the selected cells, conversion to cDNA, and amplification by PCR using Ig specific primers. Suitable primers for this purpose are also described in U.S. Pat. No. 5,658,570. As will be discussed in more detail below, transformed cells expressing the desired antibody may be grown up in relatively large quantities to provide clinical and commercial supplies of the immunoglobulin.

[0063] In another embodiment, lymphocytes can be selected by micromanipulation and the variable genes isolated. For example, peripheral blood mononuclear cells can be isolated from an immunized or naturally immune mammal, e.g., a human, and cultured for about 7 days *in vitro*. The cultures can be screened for specific IgGs that meet the screening criteria. Cells from positive wells can be isolated. Individual Ig-producing B cells can be isolated by FACS or by identifying them in a complement-mediated hemolytic plaque assay. Ig-producing B cells can be micromanipulated into a tube and the VH and VL genes can be amplified using, e.g., RT-PCR. The VH and VL genes can be cloned into an antibody expression vector and transfected into cells (e.g., eukaryotic or prokaryotic cells) for expression.

[0064] Alternatively, antibody-producing cell lines may be selected and cultured using techniques well known to the skilled artisan. Such techniques are described in a variety of laboratory manuals and primary publications. In this respect, techniques suitable for use in the invention as described below are described in Current Protocols in Immunology, Coligan et al., Eds., Green Publishing Associates and Wiley-Interscience, John Wiley and Sons, New York (1991). Once the nucleotide sequence and corresponding amino acid sequence of the antibody, or antigen-binding fragment, variant, or derivative thereof is determined, its nucleotide sequence may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, e.g., recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Sambrook et al., Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1990) and Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley & Sons, NY (1998), to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

V. Expression of Antibody Polypeptides

[0065] The present invention also involves a method for producing cells capable of expressing an antibody obtained in accordance with the method of the invention or its corresponding immunoglobulin chain(s) comprising genetically engineering cells with the polynucleotide or with the vector described above. The cells obtainable by the method of the invention can be used, for example, to test the interaction of the antibody with its antigen.

[0066] Following manipulation of the isolated genetic material to provide antibodies, or antigen-binding fragments, variants, or derivatives thereof in accordance with method of the invention, the polynucleotides encoding the antibodies are typically inserted in an expression vector for introduction into host cells that may be used to produce the desired quantity of antibody.

[0067] Recombinant expression of an antibody, or fragment, derivative or analog thereof, e.g., a heavy or light chain of an antibody which binds to a target molecule described herein. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or portion thereof (preferably containing the heavy or light chain variable domain), the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques,

and *in vivo* genetic recombination. In the method of the invention, thus, replicable vectors are used comprising a nucleotide sequence encoding an antibody molecule, or a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Pat. No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

[0068] In particularly preferred embodiments the cloned variable region genes are inserted into an expression vector along with the heavy and light chain constant region genes (preferably human) synthetic as discussed above. In one embodiment, this is effected using a proprietary expression vector of Biogen IDEC, Inc., referred to as NEOSPLA (disclosed in U.S. patent 6,159,730). This vector contains the cytomegalovirus promoter/enhancer, the mouse beta globin major promoter, the SV40 origin of replication, the bovine growth hormone polyadenylation sequence, neomycin phosphotransferase exon 1 and exon 2, the dihydrofolate reductase gene and leader sequence. This vector has been found to result in very high level expression of antibodies upon incorporation of variable and constant region genes, transfection in CHO cells, followed by selection in G418 containing medium and methotrexate amplification. Of course, any expression vector which is capable of eliciting expression in eukaryotic cells may be used in the present invention. Examples of suitable vectors include, but are not limited to plasmids pcDNA3, pHCMV/Zeo, pCR3.1, pEF1/His, pIND/GS, pRc/HCMV2, pSV40/Zeo2, pTRACER-HCMV, pUB6/V5-His, pVAX1, and pZeoSV2 (available from Invitrogen, San Diego, CA), and plasmid pCI (available from Promega, Madison, WI). In general, screening large numbers of transformed cells for those which express suitably high levels of immunoglobulin heavy and light chains is routine experimentation which can be carried out, for example, by robotic systems. Vector systems are also taught in U.S. Pat. Nos. 5,736,137 and 5,658,570. This system provides for high expression levels, e.g., > 30 pg/cell/day. Other exemplary vector systems are disclosed e.g., in U.S. Patent 6,413,777.

[0069] In other preferred embodiments the antibodies, or antigen-binding fragments, variants, or derivatives thereof may be expressed using polycistronic constructs such as those disclosed in United States Patent Application Publication No. 2003-0157641 A1. In these novel expression systems, multiple gene products of interest such as heavy and light chains of antibodies may be produced from a single polycistronic construct. These systems advantageously use an internal ribosome entry site (IRES) to provide relatively high levels of antibodies. Compatible IRES sequences are disclosed in U.S. Pat. No. 6,193,980. Those skilled in the art will appreciate that such expression systems may be used to effectively produce the full range of antibodies disclosed in the instant application.

[0070] More generally, once the vector or DNA sequence encoding a monomeric subunit of the antibody has been prepared, the expression vector may be introduced into an appropriate host cell. Introduction of the plasmid into the host cell can be accomplished by various techniques well known to those of skill in the art. These include, but are not limited to, transfection (including electrophoresis and electroporation), protoplast fusion, calcium phosphate precipitation, cell fusion with enveloped DNA, microinjection, and infection with intact virus. See, Ridgway, A. A. G. "Mammalian Expression Vectors" Vectors, Rodriguez and Denhardt, Eds., Butterworths, Boston, Mass., Chapter 24.2, pp. 470-472 (1988). Typically, plasmid introduction into the host is via electroporation. The host cells harboring the expression construct are grown under conditions appropriate to the production of the light chains and heavy chains, and assayed for heavy and/or light chain protein synthesis. Exemplary assay techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or fluorescence-activated cell sorter analysis (FACS), immunohistochemistry and the like.

[0071] The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody for use in the methods described herein. Thus, the invention includes host cells containing a polynucleotide encoding an antibody of the invention, or a heavy or light chain thereof, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below. Said host cell may be a prokaryotic or eukaryotic cell. The polynucleotide or vector which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained extrachromosomally. The host cell can be any prokaryotic or eukaryotic cell, such as a bacterial, insect, fungal, plant, animal or human cell. Preferred fungal cells are, for example, those of the genus *Saccharomyces*, in particular those of the species *S. cerevisiae*. The term "prokaryotic" is meant to include all bacteria which can be transformed or transfected with a DNA or RNA molecules for the expression of an antibody or the corresponding immunoglobulin chains. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, *E. coli*, *S. typhimurium*, *Serratia marcescens* and *Bacillus subtilis*. The term "eukaryotic" is meant to include yeast, higher plant, insect and preferably mammalian cells, most preferably HEK 293, NSO and CHO cells. Depending upon the host employed in a recombinant production procedure, the antibodies or immunoglobulin chains encoded by the polynucleotide may be glycosylated or may be non-glycosylated. Antibodies or the corresponding immunoglobulin chains may also include an initial methionine amino acid residue. A polynucleotide can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Furthermore, methods for preparing fused, operably linked genes and expressing them in, e.g., mammalian cells and bacteria are well-known in the art (Sambrook,

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression of the antibody or the corresponding immunoglobulin chains in eukaryotic or prokaryotic hosts. In general, expression vectors containing promoter sequences which facilitate the efficient transcription of the inserted polynucleotide are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of the transformed cells. Suitable source cells for the DNA sequences and host cells for immunoglobulin expression and secretion can be obtained from a number of sources, such as the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," Fifth edition (1985) Rockville, Maryland, U.S.A.). Furthermore, transgenic animals, preferably mammals, comprising the above described cells may be used for the large scale production of the antibody.

[0072] The transformed hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like; see, Scopes, "Protein Purification", Springer Verlag, N.Y. (1982). The antibody or its corresponding immunoglobulin chain(s) can then be isolated from the growth medium, cellular lysates, or cellular membrane fractions. The isolation and purification of the, e.g., recombinantly expressed antibodies or immunoglobulin chains may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibodies directed, e.g., against the constant region of the antibody. It will be apparent to those skilled in the art that the antibodies can be further coupled to other moieties for, e.g., drug targeting and imaging applications. Such coupling may be conducted chemically after expression of the antibody or antigen to site of attachment or the coupling product may be engineered into the antibody at the DNA level. The DNAs are then expressed in a suitable host system, and the expressed proteins are collected and renatured, if necessary.

[0073] Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the antibodies may then be used therapeutically (including extracorporally) or in developing and performing assay procedures.

[0074] The host cell may be co-transfected with two expression vectors, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain is advantageously placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, Nature 322:52 (1986); Kohler, Proc. Natl. Acad. Sci. USA 77:2197 (1980)). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

[0075] The above disclosure generally describes the present invention. Unless otherwise stated, a term as used herein is given the definition as provided in the Oxford Dictionary of Biochemistry and Molecular Biology, Oxford University Press, 1997, revised 2000 and reprinted 2003, ISBN 0 19 850673 2. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only.

EXAMPLES

[0076] The examples which follow further illustrate the invention. Detailed descriptions of conventional methods, such as those employed herein can be found in the cited literature; see also "The Merck Manual of Diagnosis and Therapy" Seventeenth Ed. ed by Beers and Berkow (Merck & Co., Inc. 2003).

[0077] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. For further elaboration of general techniques useful in the practice of this invention, the practitioner can refer to standard textbooks and reviews in cell biology and tissue culture; see also the references cited in the examples. General methods in molecular and cellular biochemistry can be found in such standard textbooks as Molecular Cloning: A Laboratory Manual, 3rd Ed. (Sambrook et al., Harbor Laboratory Press 2001); Short Protocols in Molecular Biology, 4th Ed. (Ausubel et al. eds., John Wiley & Sons 1999); DNA Cloning, Volumes I and II (Glover ed., 1985); Oligonucleotide Synthesis (Gait ed., 1984); Nucleic Acid Hybridization (Hames and Higgins eds. 1984); Transcription And Translation (Hames and Higgins eds. 1984); Culture Of Animal Cells (Freshney and Alan, Liss, Inc., 1987); Gene Transfer Vectors for Mammalian Cells (Miller and Calos, eds.); Current Protocols in Molecular Biology and Short Protocols in Molecular Biology, 3rd Edition (Ausubel et al., eds.); and Recombinant DNA Methodology (Wu, ed., Academic Press). Gene Transfer Vectors For Mammalian Cells (Miller and Calos, eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al., eds.); Immobilized Cells And Enzymes (IRL Press, 1986); Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Immu-

nochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (Weir and Blackwell, eds., 1986). Protein Methods (Bollag et al., John Wiley & Sons 1996); Non-viral Vectors for Gene Therapy (Wagner et al. eds., Academic Press 1999); Viral Vectors (Kaplitt & Loewy eds., Academic Press 1995); Immunology Methods Manual (Lefkovits ed., Academic Press 1997); and Cell and Tissue Culture: Laboratory Procedures in Biotechnology (Doyle & Griffiths, John Wiley & Sons 1998). Reagents, cloning vectors and kits for genetic manipulation referred to in this disclosure are available from commercial vendors such as BioRad, Stratagene, Invitrogen, Sigma-Aldrich, and ClonTech. General techniques in cell culture and media collection are outlined in Large Scale Mammalian Cell Culture (Hu et al., Curr. Opin. Biotechnol. 8 (1997), 148); Serum-free Media (Kitano, Biotechnology 17 (1991), 73); Large Scale Mammalian Cell Culture (Curr. Opin. Biotechnol. 2 (1991), 375); and Suspension Culture of Mammalian Cells (Birch et al., Bioprocess Technol. 19 (1990), 251); Extracting information from cDNA arrays, Herzel et al., CHAOS 11 (2001), 98-107.

[0078] The following experiments are illustrated and described with respect to antibody NI-101.11. However, the other antibodies of the NI 101 series, in particular NI 101.10 are structurally similar and thus may be expected to provide comparable results.

Supplementary Methods

Memory B cell display

[0079] Clinically carefully selected human subjects who are characterized by unusually positive clinical courses, e.g. absence of clinical signs of disease in the presence of risk factors, or stable courses of mild or prodromal signs with no disease development, or long-term non-progressors are recruited to provide peripheral blood lymphocytes as the starting material for the isolation of memory B cells. The strategy is based upon the concept established in infection immunology that a subject's memory B cell pool preserves the antibody specificities and possibly also antibody frequencies generated during previous antigen encounters (McHeyzer-Williams and Ahmed Curr. Opin. Immunol. 11 (1999), 172-179; Bernasconi et al., Science 298 (2002), 2199-202; Traggiai et al., Nat. Med. 10 (2004), 871-875). This concept was developed to explain adaptive immunity against infectious agents, as well as for the description of antibody-mediated immunity following a primary infection. According to this theory, the entire complement of antibodies against all antigens that had induced an antibody response in the subject's history, either naturally or upon vaccination, should be fully represented within the memory B cell pool. In accordance with the present invention this theory is applied to *endogenous* antigens generated as a result of *abnormal aggregation or conformation* of an otherwise physiological relevant protein, and that, as such, is not subject to the physiological immunologic tolerance, and, thus, can acquire antigenic properties and induce an immune response against the conformational *neo-epitopes (neoepitopes)*.

[0080] Memory B cells are isolated with surface markers including the pan B cell marker CD22, combined with negative selection of antigen-inexperienced B cells that expressed IgM, IgD, IgE, and IgA. With this technique, approximately 10.000 to 150.000 memory B cells can be obtained from 30ml of human blood. These are immortalized, for example with Epstein Barr Virus, and cultured oligo-clonally on irradiated human fibroblast feeder layers (Zubler et al., J Immunol. 134 (1985), 3662-3668; Traggiai et al., Nat. Med. 10 (2004), 871-875). To improve transformation and immortalization efficacy of antibody-secreting memory B cells, CpG 2006 which mimics the activities of bacterial un-methylated CpG-dinucleotides (Hartmann and Krieg J Immunol 164(2) (2000), 944-953) can be used.

Experimental protocol:

[0081] Selection of B cells from the bulk of PBL was performed using the MACS technology and CD22 microbeads (Miltenyi, Bergisch Gladbach, Germany). PBL were labeled with MACS anti human CD22, phycoerythrin-conjugated anti human IgD and APC-conjugated antibodies anti human IgM, IgA, CD3, CD8, CD56 (Becton Dickinson, Basel, Switzerland). CD22-positive cells were isolated using LS columns and the Midi MACS device (Miltenyi) followed by selection of phycoerythrin- and APC-negative cells using a MoFlo cell sorter (Dako, Fort Collins, USA). CD22-positive, IgM-, IgD-, IgA-, and IgE-negative B cells were then incubated with Epstein Barr Virus containing supernatant obtained from B95-8 cells and CpG 2006 (Sigma, Buchs, Switzerland) at a concentration of 2.5mg/l in B cell medium (RPMI 1640 supplemented with 10% fetal calf serum (Hyclone, Perbio, Lausanne, Switzerland). 5-50 cells were seeded per well in Costar round bottom 96 well plates (Corning, Vitaris, Baar, Switzerland) in B cell medium on 30.000 irradiated human PBL prepared from voluntary donors. Memory B cell cultures were maintained at 37°C and 5% CO₂ in a humidified cell culture incubator for 2-4 weeks after which time the conditioned medium of the cultures was assayed in ELISA and on tissue arrays.

Antibody screening

[0082] Antibodies in conditioned media are screened for binding to pathological epitopes including protein aggregates and abnormal, pathologically relevant structures on tissue sections obtained from human patients with pathologically confirmed diagnoses including, but not restricted to, Alzheimer's disease, or obtained from tissue sections of transgenic mouse models of human disease, or from tissue sections obtained from animal models of human disease including aged non-human primates, or by ELISA of aggregated synthetic peptide preparations. Abnormal, pathologic structures in the sense of this invention include, but are not restricted to, β -amyloid plaques, neurofibrillary tangles, alpha-synuclein aggregates in Lewy bodies, and protein aggregates deposited in dystrophic neurites. Human tissues are also used to exclude cross-reactivities of antibodies with normal cellular or supercellular tissue structures. Selected antibodies are further analyzed for class and light chain subclass determination. Selected pathology-relevant antibody messages from memory B cell cultures are transcribed by using RT-PCR, cloned and combined into expression vectors for recombinant production.

Experimental protocol:

[0083] Screening of B cell conditioned medium using microtiter-compatible tissue micro arrays.

Array production

[0084] Paraffin-embedded human post-mortem Alzheimer's disease brain tissues were cut into rods of 1-2 mm in diameter and 10mm in length. Four rods were embedded vertically in paraffin to form a square fitting the microtiter format of 9 by 9 mm. 5 μ m tissue slices were cut from this assembly with a microtome and two slices were mounted adjacent to each other onto glass slides resulting in an assembly of 2 by 4 rods that fit the 96-well microtiter format. Alternatively, tissues from APP transgenic mice were used to prepare the tissue arrays.

B cell screening

[0085] Conditioned medium from memory B cell cultures was transferred onto the tissue array slides using a multi-channel pipette and incubated for 2 h at room temperature. After a washing step the binding of human antibodies to tissue sections was analyzed using Cy 3-conjugated secondary antibodies to human IgG (Jackson ImmunoResearch Europe Ltd., Suffolk, UK). Analysis of fluorescence was performed on an inverted fluorescence microscope (Leica, Heerbrugg, Switzerland).

ELISA

[0086] 96 well half area Microplates (Corning) were coated with synthetic Abeta-peptide at a standard concentration of 1 μ g/ml in coating buffer (15mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.42) overnight at 4°C. Plates were washed and non-specific binding sites were blocked for 1h at RT with PBS containing 2% BSA (Sigma, Buchs, Switzerland). B cell conditioned medium was transferred from memory B cell culture plates to ELISA plates and was incubated for 2 h at room temperature. Binding of human antibodies was determined using horse radish peroxidase (HRP)-conjugated donkey anti-human IgG polyclonal antibodies (Jackson ImmunoResearch Europe Ltd., Cambridgeshire, UK) followed by measurement of HRP activity in a standard colorimetric assay.

Molecular cloning of antibodies displaying specificity of interest

[0087] Living B cells of selected memory B cell cultures are harvested using a cell sorter. mRNA is prepared and immunoglobulin heavy and light chain sequences are obtained using Ig-framework specific primers for all human variable heavy and light chain framework 1 (FR1) families as 5' primers in combination with primers specific for all human J-H segments as 3' primers (Marks et al., Mol. Biol. 222 (1991), 581-597). Alternatively, single-cell RT-PCR of single sorted cells from the memory B cell culture can be used as source of Ig heavy and light chain sequences (Babcook et al., Proc. Natl. Acad. Sci. USA 93 (1996), 7843-7848; Brezinschek et al., J. Immunol. 155 (1995), 190-202; Coronella et al., Nucleic Acids Research 28 (2000); Owens, et al., J. Immunol. 171 (2003), 2725-2733). Single cell sorting preserves the correct pairing of the immunoglobulin heavy and light chains of the antibody clones originally produced in the B cell culture.

[0088] Identification of the antibody clone with the desired specificity is performed by re-screening on microtiter compatible tissue microarray and ELISA upon recombinant expression of complete antibodies. Recombinant expression of complete IgG1 antibodies is achieved upon insertion of the variable heavy and light chain sequences "in the correct reading frame" into expression vectors that complement the variable region sequence with a sequence encoding a signal

peptide at the 5'-prime end and at the 3'-end with a sequence encoding the appropriate constant domain(s). To that end the primers contained restriction sites designed to facilitate cloning of the variable heavy and light chain sequences into antibody expression vectors. Heavy chain immunoglobulin are expressed by inserting the immunoglobulin heavy chain RT-PCR product in frame into a heavy chain expression vector bearing a signal peptide and the constant domains of human immunoglobulin. Kappa light chain immunoglobulin is expressed by inserting the kappa light chain RT-PCR-product in frame into a light chain expression vector providing a signal peptide and the constant domain 1 of human kappa light chain immunoglobulin. Alternatively, lambda light chain immunoglobulin is expressed by inserting the lambda light chain RT-PCR-product in frame into a lambda light chain expression vector providing a signal peptide and the constant domain 1 of human lambda light chain immunoglobulin.

[0089] Functional recombinant monoclonal antibodies are obtained upon co-transfection into HEK 293 cells (or any other appropriate recipient cell line) of a Ig-heavy-chain expression vector and a kappa or lambda Ig-light-chain expression vector. Recombinant human monoclonal antibody is subsequently purified from the conditioned medium using a standard Protein A column purification. Recombinant human monoclonal antibody can be produced in unlimited quantities using either transiently or stably transfected cells. Cell lines producing recombinant human monoclonal antibody can be established either by using the Ig-expression vectors directly or by re-cloning of Ig-variable regions into different expression vectors. Derivatives such as F(ab), F(ab)₂ and scFv can also be generated from these Ig-variable regions.

Experimental protocol:

RT-PCR of bulk B cells

[0090] Living cells as identified by their forward- and sideward light scattering properties of selected memory B cell cultures were sorted in aliquots of 100-2000 cells directly in 0.2 ml PCR tubes filled with 20 µl of RNeasy lysis buffer (Qiagen, Crawley, UK) using a MoFlo cell sorter. mRNA was prepared using the mRNA-Direct Micro Kit (Dyna, Invitrogen, Basel, Switzerland). cDNA was prepared using the "RT for PCR" Kit (Clontech Becton Dickinson, Basel, Switzerland) and PCR of immunoglobulin (Ig) heavy and light chain variable sequences was performed using the Advantage 2 PCR Kit (Clontech) using specific primers for all human variable heavy and light chain framework 1 (FR1) families as 5' primers in combination with primers specific for the constant domains of human Ig heavy or Ig kappa or Ig lambda light chains as 3' primers. Primers were purchased from Microsynth (Balgach, Switzerland).

[0091] A signal peptide that was used in all expression vectors was derived from the human immunoglobulin kappa light chain family 1 L5 sequence (MDMRVPAQLLGLLLWFGSRC; SEQ ID NO: 2) as described at V-Base and designed to provide the restriction site Xba 1 in order to facilitate the cloning of PCR amplified variable regions (ATGGACATGCGGGTGCCCGCCAGCTGCTGGGCTGCTGCTGCTGTGGTTCCCGGCTCTAGATGC; SEQ ID NO: 1). Xba1 was introduced by silent mutagenesis. As a 3' restriction site used for the cloning of variable heavy chain regions the restriction site Sal1 was introduced into C1 of IgG1 provided by the vector. Similarly, the restriction site BsiW1 was introduced into C1 of the kappa light chain and Xho1 was introduced into C1 of lambda light chain. Restriction digest of PCR products and ligation into recipient vectors was performed according to standard procedures. Plasmid DNA was prepared using standard kits (Qiagen, Hombrechtikon, Switzerland). Recipient vectors contained a CMV promoter for the expression of antibody genes in mammalian cells.

Single-cell RT-PCR

[0092] For single cell RT-PCR of Ig heavy and light chain variable regions from cultured B cells a modification of the method described by Owens *et al.* was used (Owens *et al.*, 2003). Single cells were deposited directly into each tube of an array of 0.2 ml PCR tubes using the MoFlo cell sorter. Each tube was prepared to contain 10 µl of RT buffer for Superscript II reverse transcriptase (Invitrogen). PCR tubes were shock frozen on dry ice and thawed immediately prior to RT-PCR. cDNA was prepared using random hexamers (Clontech) and Superscript II reverse transcriptase (Invitrogen). 1st round PCR of immunoglobulin heavy and light chain variable regions was performed using, as 5' primers, a set of primers that primed in all signal peptides conserved among Ig-variable region families. At the 3' position, single primers specific for the constant region of Ig C1 heavy chain or the Ig kappa- or Ig lambda light chain were used. 2nd round PCR was performed on PCR-products obtained during first round PCR using the primers as described for the bulk B cell RT-PCR. Cloning into recipient vectors was performed accordingly.

Alternative cellular cloning

[0093] Cloning was performed using the standard limited dilution method or by single cell deposition into 96 well culture plates using a cell sorter (MoFlo, Dako, Fort Collins, USA). For limiting dilution, cells of a memory B cell culture were harvested, passed through a 30 µm nylon mesh (Falcon, Becton Dickinson, Basel, Switzerland) resuspended in medium

and seeded onto 96 well plates or 384 well plates at a concentration of 0.3 cells per well.

[0094] For seeding with the cell sorter, the device was set to deposit one single cell (single 1 mode) per well directly in 96 well plates supplemented with B cell medium. The culture medium was supplemented with medium conditioned by activated T cells. Alternatively, 30.000 irradiated feeder cells were added to the medium.

Sequence analysis of immunoglobulin variable region sequences

[0095] Sequencing of cloned immunoglobulin variable region sequences was performed using primers specific for the CMV promoter present 5' of the inserted Ig variable region sequences in the expression vector. Alternatively, primers that primed in the constant domains of Ig heavy- and light chains were used. Sequences obtained were analyzed and aligned using Vector NTI software (Informax-Invitrogen). Plasmids containing sequences that encoded complete immunoglobulin variable regions in frame with the leader peptide and the constant domain were used for expression.

Expression of functional recombinant monoclonal antibodies

[0096] The antibodies can be produced in sufficient quantities by recombinant expression using technologies known in the art (Trill et al., Curr. Opin. Biotechnol. 6 (1995), 553-601). Recombinant human monoclonal antibody of up to 1 mg was produced upon transient transfection of 293 HEK cells. Recombinant human monoclonal antibody of up to 100 mg was produced upon stable transduction of 293 HEK cells or the murine NSO cells using recombinant lentivirus vectors.

Small scale production of human recombinant antibody by transient transfection

[0097] Ig-heavy chain vector and Ig-light chain vectors were co-transfected into HEK 293 cells using the standard calcium phosphate co-precipitation method. Recombinant antibodies were purified from the medium conditioned by transfected HEK 293 cells using protein A column purification (GE-Healthcare, Otelfingen, Switzerland).

Large scale production of human recombinant antibody by stable transduction

[0098] Here, a lentivirus-based transfection system was employed to generate stably transduced cell lines producing human recombinant antibody (Zufferey et al., J. Virol. 72 (1998), 9873-9880). HEK 293 cells were co-transduced with two distinct lentivectors one bearing an expression cassette for the Ig heavy chain, the other a cassette for the Ig light chain of a recombinant antibody. This method of transduction can be used in a broad range of mammalian cell lines such as CHO and NSO cells.

Validation in transgenic mouse models of human disease

[0099] Transgenic mice were generated as previously described (Knobloch et al., Neurobiol. Aging July 28 (2006)) on a hybrid background of C57B1/6 and DBA2. The test group was backcrossed once to C57B1/6. Mice were kept under standard housing conditions on a reversed 12h:12h light/dark cycle and had free access to food and water. The treatment groups were balanced for age (24 months at first testing, 26 months at 2nd testing) and gender. Mice are treated with antibodies (3mg/kg body weight) by once weekly intraperitoneal injections over a time period of 2 months resulting in 8 injections per animal.

Behavioral testing in the Y-maze

[0100] The spontaneous alternation rate is assessed using a Y-shaped plastic maze, with 40 x 20 x 10 cm arm sizes. During 5 min sessions, the sequences of arm entries are recorded; alternation was defined as successive entries into the three arms, in overlapping triplet sets. The percent alternation was calculated as the ratio of actual to possible alternations. After 2 months of antibody treatment, the mice are retested in the Y-maze. The experimenters are always kept blind for both treatments and genotypes during the whole experiment.

Blood-brain barrier penetration and binding to abnormal structures in the brain

[0101] To assess whether the selected antibodies or fragments thereof can penetrate the blood-brain barrier and bind to their abnormally aggregated or conformationally altered protein targets in the brain, an effective dose of the antibody is administered systemically, intraperitoneally, intravenously, intramuscularly, subcutaneously or intranasally to a transgenic animal that is characterized by unphysiological accumulation of the aggregated or conformationally altered protein target in the brain. Binding of the antibody to the pathology specific structures in the brain is then evaluated by immu-

nostaining with a labeled anti-human Ig secondary antibody followed by standard immunohistochemical detection.

Experimental protocol:

[0102] PS-1/APPswe transgenic model mice for Alzheimer's disease received two peripheral injections of 150 μ g NI-101.11 at day 1 and day 3. The mice were sacrificed 24 h after the second injection and perfused with PBS. Brains were frozen and tissue slices were prepared from frozen tissue using a cryotome. Presence of human antibody on cryostat sections was assayed by staining with Cy3-labeled anti human IgG antibody (Jackson ImmunoResearch Europe, Suffolk, UK). Localization of amyloid plaques was performed by co-staining the cryostat sections with the murine Abeta-specific control antibody 6E10 (available from Covance, Catalog Number SIG-39320) followed by FITC-labeled anti mouse IgG antibody. Alternatively, staining with Cy3-labeled anti human IgG antibody was used alone. Analysis of fluorescence was performed on an inverted fluorescence microscope (Leica).

Reduction of brain pathology

[0103] The effects of antibody treatment on the levels of aggregated or conformationally altered protein targets in the brain is assessed by systemic treatment or targeted brain delivery of the antibody (intracranial, intrathecal or intraventricular) and an unrelated antibody control to transgenic animals with characteristic unphysiological accumulation of the aggregated or conformationally altered protein target in the brain. The treatment effects is evaluated by immunostaining or histochemical staining of the altered or aggregated protein targets and measuring the area covered by such aggregates, aggregate size and aggregate number and biochemical quantification of the concentrations of the protein targets in different brain areas.

Absence of antibody-treatment related side effects

[0104] Potential target related adverse effects of the antibody-treatment will be assessed by systemic administration or targeted brain delivery of the antibody (intracranial, intrathecal or intraventricular) and an unrelated antibody control to transgenic animals with characteristic unphysiological accumulation of the aggregated or conformationally altered protein target in the brain. Potential side effects will be evaluated by immunostaining or histochemical staining (e.g. Prussian blue for microrhemorrhages, hematoxylin-eosin, activated white blood cells) and biochemical quantification (e.g. cytokine levels by ELISA).

Immunofluorescence staining of living cells

[0105] HEK 293 cells were transiently transfected with a vector that expresses human wild type APP fused at the intracellular C-terminus to the yellow fluorescent protein variant Citrine. 24 hours after transfection the cells were incubated with human recombinant antibodies or control antibodies at 4°C for 30 minutes. After a washing step the cell were fixed and surface-bound antibody was detected using Cy-3-labeled secondary antibodies to human or mouse IgG (Jackson ImmunoResearch). Analysis of fluorescence was performed on a confocal microscope (Leica).

Preparation of Abeta fibrils

[0106] Abeta peptide was purchased from Bachem (Bubendorf, Switzerland). Lyophilised peptide was reconstituted in TFA and resuspended in PBS immediately prior to its use as monomeric Abeta in the assays. Abeta fibrils were prepared by incubation of monomeric Abeta1-42 peptide at a concentration of 100 μ g/ml in PBS at 37°C for 24h. Monomeric Abeta peptide and fibril preparations were also used as substrate to coat ELISA plates.

Western blotting

[0107] Monomeric Abeta peptide was mixed with loading dye, heat denatured and 0.2 μ g was loaded per lane and separated on a gradient SDS-PAGE. Blots were incubated with primary antibody for 2h. Binding of primary human monoclonal antibody or mouse control antibody 6E10 was revealed using secondary anti human or anti mouse antibodies conjugated with horse radish peroxidase (HRP). Blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Fisher Scientific, Wohlen, Switzerland).

Competition of tissue amyloid plaque binding

[0108] Recombinant human NI-101.11 antibody was incubated for 2 h with Abeta peptide preparations. The anti-

body/Abeta preparations were then used for immunohistochemical staining of brain section obtained from a patient with neuropathologically confirmed Alzheimer's disease. 5 μ m cryo-sections were prepared, blocked with 4% BSA, 5% goat serum and 5% horse serum in PBS for 1 h at RT and stained with NI-101.11/Abeta preparations for 1 h at room temperature. After a washing step the binding of human antibodies to tissue sections was analyzed using Cy3-conjugated secondary antibodies to human IgG (Jackson ImmunoResearch Europe Ltd). Analysis of fluorescence was performed on an inverted fluorescence microscope (Leica, Heerbrugg, Switzerland).

Example 1

Detection of Human Antibodies against Abnormal Structures Prevalent in Human Brain Diseases

[0109] Antibodies from phenotypically healthy subjects, or clinically unusually stable patients with Alzheimer's disease were tested by immunohistochemistry on brain sections obtained from patients with pathologically confirmed Alzheimer's disease. Fig. 1A demonstrates the presence of antibodies in a clinically unusually stable patient that bind to beta-amyloid plaques as was confirmed by co-staining with a known antibody against human beta-amyloid (antibody 4G8; Fig. 1B). The presence in a healthy human subject of antibodies to neurofibrillary tangles in a tissue section obtained from a patient with Alzheimer's disease is shown in Figure 2A. This result was confirmed by co-staining with a known antibody against human tau (HT7). Figure 3A reveals the presence in a healthy human subject of antibodies against dystrophic neurites in a tissue section obtained from a patient with Alzheimer's disease. Control staining with known antibody against human tau (HT7) is depicted in Figure 3 B. These results demonstrate the presence in phenotypically healthy, or clinically unusually stable patients of antibodies against identifiable pathological structures in human tissue samples with histopathologically confirmed diagnoses.

Example 2

Recombinant Human Antibodies Maintain Specificity to Abnormal Structure *in vivo* and Recognize Conformational Epitope of Disease-Associated Beta-Amyloid Protein in Brain Amyloid Plaques but not the Physiological Precursor or Non-Pathogenic Derivative Thereof

[0110] Antibodies NI-101.11, NI-101.12, NI-101.13A and NI-101.13B were obtained from clinically unusually stable Alzheimer's disease patients with a significantly reduced rate of cognitive decline. Antibody isolation and recombinant production was performed as specified in supplementary methods.

NI-101.11

[0111] Recombinant NI-101.11 was tested for binding to brain beta-amyloid plaques (Fig. 4). Brain sections obtained from a patient with neuropathologically confirmed Alzheimer's disease were stained at the indicated concentrations. Antibody binding to beta-amyloid plaques with concentrations of 50 pM suggest high affinity binding. The binding of antibody NI-101.11 to beta-amyloid plaques at a concentration of 0.5 nM cannot be competed by addition of excess amounts of linear synthetic N-terminal Abeta-derived polypeptide representing positions 1 to 16 at concentrations of up to 1 μ M (Fig. 5). Furthermore, the binding of NI-101.11 to beta-amyloid plaques on brain sections at 8 nM concentration is competed by excess amounts Abeta1-42 fibrils (4 μ M) but not of linear synthetic Abeta1-42 monomers at 4 μ M concentration, suggesting that NI-101.11 recognizes a conformational epitope that is not present in monomeric Abeta (Fig. 6).

[0112] To further assess binding of human recombinant NI-101.11 antibody to linear, monomeric synthetic Abeta, preparations of monomeric Abeta were separated by non-denaturing PAGE. Blotted protein was probed with human recombinant NI-101.11 antibody and a control antibody against N-terminal linear Abeta sequences (6E10). While 6E10 produced prominent staining of monomeric Abeta peptide, no binding was detected for human NI-101.11 suggesting that NI-101.11 does not bind to the linear monomeric Abeta peptide but recognizes a conformational Abeta epitope. (Fig. 7)

[0113] The binding of recombinant NI-101.11 to artificial amyloid fibrils prepared from synthetic Abeta1-42 peptides and monomeric Abeta was determined by ELISA (Fig. 8). Synthetic Abeta fibrils or monomeric synthetic Abeta coated onto ELISA plates at equal coating densities were incubated with NI-101.11 at the indicated concentrations. Binding to artificial amyloid fibrils (open squares) is more than 100 times higher as compared to monomeric Abeta (filled squares). Control antibody 22C4 against the C-terminus of Abeta preferentially binds to monomeric Abeta (filled circles), and less well to fibrils (open circles). This suggests that NI-101.11 recognizes a conformational epitope which is also present on artificial amyloid fibrils prepared from synthetic Abeta peptides.

[0114] Cross-reactivity of recombinant human NI-101.11 antibody against cellular full-length APP or with any of its physiological derivatives was determined by cell binding assays (Fig. 9).

[0115] Live HEK 293 cells stably expressing human APP fused to Citrin as a marker were incubated for 30 min at 4°C, to prevent internalization, with the recombinant human NI-101.11 antibody or the control antibody 6E10 against N-terminal linear Abeta sequence. Citrin-positive signals indicate APP-expressing cells. In contrast to the control antibody (6E10) that binds to cell-surface APP in all cells expressing the fusion construct, no binding of recombinant human NI-101.11 antibody to full-length APP is detected. These data demonstrate absent cross-reactivity of NI-101.11 to physiological, cellular APP.

[0116] The lack of binding of NI-101.11 to monomeric Abeta was further demonstrated by size exclusion chromatography: No binding of NI-101.11 or an unrelated control antibody was observed to monomeric FITC-labeled Abeta1-42 (Fig. 10A, 10B). In contrast, antibody 22C4 directed against a linear epitope present in the C-terminus of Abeta co-eluted with FITC-Abeta1-42 monomers (Fig. 10 C).

[0117] In a competition ELISA, binding of 6E10, an antibody directed against a linear epitope at the N-terminus of Abeta, could be completely blocked upon pre-incubation with excess concentrations of monomeric Abeta1-16, Abeta1-28 and Abeta1-40 peptides. In contrast, pre-incubation with excess concentrations of linear Abeta peptides did not abolish NI-101.11 binding, suggesting that NI-101.11 requires a conformational epitope (Fig. 11).

NI-101.13A and 13B

[0118] Recombinant human antibodies NI-101.13A and 13B were tested for binding to brain sections obtained from an APP transgenic mouse model of Alzheimer's disease (Tg2576). NI-101.13A and NI-101.13B produced prominent staining of beta-amyloid plaques at 10 nM concentration (Fig. 12). The binding of recombinant NI-101.13A and NI-101.13B to artificial amyloid fibrils prepared from synthetic Abeta1-42 peptides and monomeric Abeta was determined by ELISA. Synthetic Abeta fibrils or monomeric synthetic Abeta coated onto ELISA plates at equal coating densities were incubated with NI-101.13A and NI-101.13B at the indicated concentrations. Preferential binding to artificial amyloid fibrils as compared to monomeric Abeta was observed for both antibodies tested. (Fig. 13)

NI-101.12

[0119] The binding of recombinant NI-101.12 to synthetic Abeta1-42 peptide was confirmed by ELISA (Fig. 14A). NI-101.12 binding at 133nM concentration was competed by excess of Abeta1-42 peptide (Fig 14 B).

Example 3

Recombinant Human Antibody against Brain Beta-Amyloid Crosses the Blood Brain Barrier in a Transgenic Mouse Model of Alzheimer's Disease, and Binds to Brain Beta-Amyloid Plaques *In vivo*

[0120] To determine whether recombinant human NI-101.11 antibody crosses the blood brain barrier and binds to brain beta-amyloid plaques *in vivo* transgenic PS-1/APP^{sw} Alzheimer's disease model mice received two peripheral injections of 150µg NI-101.11 at day 1 and day 3. Mice were sacrificed 24 h after the second injection and perfused with PBS. Brains were harvested and brain sections were stained with FITC-labeled antibodies against human IgG or with the mouse monoclonal Abeta antibody 6E10 followed by a FITC-labeled antibody against mouse IgG to confirm the presence of brain beta-amyloid plaques. Intense staining of amyloid plaques with anti-human IgG indicated that the recombinant human NI-101.11 antibody can cross the blood-brain barrier of transgenic mice and bind to brain beta-amyloid plaques in living animals (Fig. 15).

Example 4

Recombinant Human Antibody against Beta-Amyloid Improves Abnormal Cognitive Behavior and Confers Reduction of Beta-Amyloid Plaque Load, Astrogliosis and Microgliosis in a Transgenic Mouse Model of Alzheimer's Disease without Increasing the Frequency of Microhemorrhages

[0121] 24 months old arcAbeta mice and age-matched wild type littermates were treated weekly i.p. with 3 mg/kg recombinant human NI-101.11 antibody or an isotype-matched human control antibody for 2 months. To assess the treatment effect on abnormal behavior in the transgenic mice, Y-maze behavioral testing was performed before and after completion of the treatment. The spontaneous rate of alternation was assessed using a Y-shaped plastic maze, with 40 x 20 x 10 cm arm sizes. During 5 min sessions, the sequences of arm entries were recorded; alternation was defined as successive entries into the three arms, in overlapping triplet sets. The percent alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries - 2) multiplied by 100%. The Y-maze performance of untreated arcAbeta mice and wildtype littermate controls was compared using an unpaired t-test.

The nonparametric Kruskal-Wallis test was used to compare the improvement after treatment in all 4 groups. The nonparametric Mann-Whitney U test was chosen for pair-wise comparison of the different groups. Zero-performers (i.e. mice that did not leave the arm they were placed in) were excluded from the analysis.

[0122] As was observed in previous studies, untreated 24-months old arcAbeta mice were significantly impaired compared to their wildtype littermates (Fig. 16A, before treatment; unpaired t-test, $p=0.0007$).

[0123] NI-101.11 treated arcAbeta mice showed clearly enhanced alteration levels, comparable to NI-101.11 treated wildtype control mice after the 2 months treatment. Analysis of the improvement (i.e. performance after treatment minus performance before treatment) showed a significant difference between the four groups (Fig. 16 B, Kruskal-Wallis test; $p=0.03$). A pair-wise post-hoc analysis between all groups showed that NI-101.11 treated arcAbeta mice improved their cognitive performance significantly more than wildtype mice (Mann-Whitney U; $p=0.05$ NI-101.11 tg vs. NI-101.11 wt; $p=0.008$ NI-101.11 tg vs. control wt). This group of mice also showed a strong trend towards improved performance compared to the control antibody treated transgenic littermates (Mann-Whitney-U; $p=0.08$ NI-101.11 tg vs. control tg). All mice showed a ~10% improvement in performance in the re-testing, which was likely due to the familiar environment of the task.

[0124] The effects of chronic, 2 months NI-101.11 treatment on amyloid burden, astrogliosis and microgliosis were analyzed by quantitative histochemical and immunohistochemical analysis. To that end, mice were anesthetized after completion of the behavioral testing and perfused transcardially with PBS. One brain hemisphere was fixed in 4% paraformaldehyde and embedded in paraffin. 5 μ m sagittal sections were cut with a Leica RM 2135 microtome (Bannockburn, Illinois). Beta-amyloid plaque load in cortex and hippocampus was quantified on brain sections stained with Thioflavin S and Congo Red according to standard protocol. For immunohistochemistry, slices were dewaxed, blocked with 4% BSA, 5% goat serum and 5% horse serum in PBS for 1h at RT. Antibodies were incubated overnight at 4°C using the following dilutions: anti GFAP (Advanced Immunochemicals) 1: 500, anti IBA1 (WAKO) 1:500. 2nd fluorophore coupled antibodies were incubated at RT for 2h. 2-3 sections per mouse brain spaced 75 μ m apart were used for each staining. 2 images per section were taken at 10x magnification for cortex analysis (parietal and frontal region). The entire hippocampus area (5x magnification cropped to ROI) was taken for the hippocampus analysis. Automated image analysis was done with the software ImageJ.

[0125] Double staining of brain sections from immunized arcAbeta mice with 6E10 and anti-human IgG revealed binding of NI-101.11 to Abeta deposits (Fig. 17, left panel), indicating that NI-101.11 can cross the blood brain barrier and bind to brain beta-amyloid plaques. No such binding of human antibody to Abeta deposits was seen in control antibody treated arcAbeta mice (Fig. 17 right panel).

[0126] [0317] Chronic treatment with 3mg/kg of NI-101.11 resulted in a significant reduction of amyloid plaque load as was revealed by Thioflavin S and Congo Red staining. This reduction reached levels of greater than 50% in cortex and hippocampus compared to control antibody-treated arcAbeta mice (Fig. 18 A, B). In addition to the plaque area (Fig 18 C), significant reductions were also observed for the number of plaques (Fig. 18 D) and the average plaque size (Fig. 18 E).

[0127] To test whether chronic treatment with NI-101.11 affects the neuroinflammatory response in arcAbeta mice, reactive astrocytes and microglia were quantified after immunohistological staining. A reduction in the number of reactive astrocytes (anti GFAP-staining) was observed in the cortex of NI-101.11 treated arcAbeta mice compared to control antibody treated animals (Fig. 19A; Mann-Whitney-U; $p=0.047$). No change was detected in the hippocampus. Staining with an antibody against a marker of microglia and macrophages (anti-Iba1) also revealed a statistical trend towards reduced inflammation (Fig. 19 B; Mann-Whitney-U; $p=0.075$ for both cortex and hippocampus). The decrease in astrogliosis and microgliosis is in line with the reduced beta-amyloid load observed after NI-101.11 treatment.

[0128] Passive immunotherapy with certain monoclonal antibodies directed against Abeta can be associated with increased frequency of microhemorrhages in the brain (Pfeifer et al., Science 298 (2002), 1379; Wilcock et al., J Neuroinflammation 1 (2004), 24). To assess the effects of chronic therapy with NI-101.11, Perl's Prussian blue staining was performed on brain sections from arcAbeta and wild-type mice after chronic NI-101.11 treatment. This staining reveals the presence of hemosiderin, a breakdown product of hemoglobin, and marker of previous microhemorrhages (Fig. 20). In aged arcAbeta mice treated with a control antibody, the frequency of Prussian blue positive profiles was significantly elevated compared to wild-type littermates (Mann-Whitney-U; $p=0.001$). Treatment with the NI-101.11 did not lead to an increase the number of microhemorrhages when compared to control-antibody treated arcAbeta mice (Mann-Whitney-U; $p=0.347$) indicating that the beneficial therapeutic effects of NI-101.11 treatment occurred in the absence of this frequently observed side effect of passive Abeta immunotherapy.

Example 5

Recombinant Human Antibody against Brain Beta-Amyloid Inhibits the Formation of Synthetic Abeta Fibrils *In vitro*

[0129] The effect of recombinant human NI-101.11 antibody on the formation of Abeta-fibrils was assayed by measuring

Thioflavin S bound to aggregated Abeta by fluorescence analysis. Monomeric Abeta solutions were incubated at 37°C for 24 h in the presence of increasing concentration of NI-101.11. The formation of synthetic Abeta fibrils *in vitro* was inhibited by recombinant human NI-101.11 in a concentration dependent manner (Fig. 21).

Example 6

NI-101.11 Effects on Ex-vivo Phagocytosis of Abeta Fibrils by BV-2 Microglial-derived Cells

[0130] The effects of NI-101.11 on Fcgamma-receptor mediated phagocytosis of Abeta fibrils were studied in the BV-2 microglial-derived cell line. BV-2 cells were maintained in DMEM supplemented with 5% FBS, Pen/Step and glutamine. Cells were trypsinized and 120'000 BV-2 cells/well were seeded in flat bottom 24-well plates. After 12 h, the medium was replaced with 400 µl DMEM/F12/well supplemented with 20mM HEPES (pH 7.3), 1% BSA, 10 µg/ml Pen/Step. 100 µg/ml Fucoidan, an inhibitor of the scavenger receptor, was added 30 min prior to experiment. 50 µM FITC-labeled Abeta fibrils were pre-incubated with the indicated concentrations of antibodies for 30 min at 37°C, washed twice followed by centrifugation for 5 min at 14'000 x g. This suspension was added to the tissue culture plates. After 30 min BV-2 cells were washed twice with HBSS to remove unassociated fibrillar Abeta.

[0131] Cells were treated with 250 µg/ml trypsin/EDTA for 20 min at 4°C and washed twice by centrifugation at 500 x g for 5 min at 4°C. Cells were fixed for 20 min in FACS-Fix (PBS, 2%FA, 2% Glucose, 5 mM NaN) and washed twice with FACS wash (PBS, 5 µM EDTA, 0.2% BSA). Fluorescence (FL-1) of 10'000 cells was determined by FACS analysis (based on Webster SD et al, JI 2001).

[0132] Fcgamma receptor-dependent phagocytosis of FITC-labeled Abeta1-42 fibrils was measured upon inhibition of the scavenger receptor system. Comparative analysis of human NI-101.11 and a commercially available antibody directed to a linear epitope at the N-terminus of the Abeta peptide (6E10) demonstrated dose-dependent induction of phagocytosis of Abeta fibrils. The uptake of fibrils mediated by NI-101.11 is up to 3 fold higher than that observed for the 6E10 antibody (Fig. 22). These data indicate that NI-101.11 triggers potent dose-dependent Fcgamma receptor-mediated phagocytosis of Abeta fibrils by microglial cells.

Conclusion

[0133] As demonstrated in the above experiments performed in accordance with the present invention it was surprisingly possible to detect protective and therapeutically active antibodies and antibody producing B-cells in phenotypically healthy, asymptomatic human subjects, as well as in patients with unusually stable clinical disease courses despite a diagnosis of cognitive impairment or Alzheimer's disease. More specifically, a new class of human antibodies could be detected and isolated, which discriminate the physiologically functional form of an antigen, thereby minimizing the risk of autoimmunogenic side effects hitherto being a problem in immunotherapy. Thus, antibodies and equivalent binding molecules are provided that specifically recognize a variant of the antigen in a pathophysiologically relevant structure, which the antibody is supposed to bind in order to diminish its toxicity or to reduce its concentration or to promote its degradation, by means of, for example, making the pathogen for FcR-expressing macrophages or microglia cells visible and therefore to render it innocuous. As further demonstrated in the examples such antibodies are therapeutically effective and are capable of both suspending as well as preventing deleterious effects of abnormal pathological proteins and aggregates thereof without increasing the frequency of brain microhemorrhages.

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	Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly	
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25 Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg
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Claims

- 30 1. A method of isolating a monoclonal antibody specific for a disorder-associated pathological variant form of an endogenous protein comprising:
- 35 (a) subjecting a sample from a culture of memory B cells from a subject who is phenotypically healthy but affected with or at risk of developing a neurological disorder **characterized by** pathological protein structures of the pathological variant form of the endogenous protein, or a patient with a clinically unusually stable disease course of the disorder to a specimen displaying pathological structures of the pathological variant form of the endogenous protein;
- 40 (b) identifying in the sample the presence of an antibody which binds to said specimen but not to corresponding tissue of a healthy subject without such pathological structures;
- (c) obtaining the immunoglobulin gene repertoire for antibodies from said memory B cells of the sample which has been identified to contain antibodies which bind to said specimen but not to the corresponding tissue of a healthy subject without such pathological structures comprising the steps of:
- 45 (i) obtaining mRNA from said memory B cells;
- (ii) obtaining cDNA from the mRNA of step (i); and
- (iii) using primer extension reaction to amplify from said cDNA the fragments corresponding to the heavy chains (HC) and the kappa/lambda light chains (LC) of said antibodies;
- 50 (d) using the HC and LC sequences obtained in (c) to express said antibodies; and
- (e) isolating the antibody which specifically binds to pathologically altered tissue derived from a human patient or animal model of the disorder, wherein the tissue comprises pathological protein structures of the protein but not to tissue of a healthy subject without such pathological structures.
- 55 2. The method of claim 1, wherein the pathological tissue originates from human patients suffering from neurodegenerative diseases, protein-misfolding diseases, tissue amyloidosis or other diseases related to pathological deposits.
3. The method of claim 1 or 2, wherein said patient and subject, respectively, is a human.

4. The method of any one of claims 1 to 3, wherein said patient has been determined to be affected with a not yet manifested disorder or at risk to develop the disorder by the presence or absence of a surrogate marker, preferably wherein said surrogate marker is selected from the group consisting of old age, brain amyloid load, ApoE genotype, APP genotype, PS1 genotype, levels in body fluids of Abeta peptide, isoprostanes, Tau, and phospho-Tau.
5. The method of any one of claims 1 to 4, wherein said disorder is Alzheimer's disease.
6. The method of claim 5, wherein the sample is obtained from a patient fulfilling the following criteria:
 - (i) being 65 years of age or older;
 - (ii) having full cognitive capacity and good health; and
 - (iii) having no clinical signs of dementia; or
 - (iv) having unusually slow rates of progression of disease despite the presence of an established clinical diagnosis of probable Alzheimer's disease.

Patentansprüche

1. Verfahren zur Isolierung eines für eine krankheitsassoziierte pathologische Variante eines endogenen Proteins spezifischen monoklonalen Antikörpers umfassend:
 - (a) Aussetzen einer Probe aus einer Kultur von B-Gedächtniszellen aus einem Probanden der phänotypisch gesund ist, jedoch von einer neurologischen Erkrankung betroffen ist oder dem Risiko ausgesetzt ist eine neurologische Erkrankung zu entwickeln, die durch pathologische Proteinstrukturen der pathologischen Variante des endogenen Proteins gekennzeichnet ist, oder einem Patienten mit einem klinisch ungewöhnlich stabilen Krankheitsverlauf der Erkrankung, einem Präparat das pathologische Strukturen der pathologischen Variante des endogenen Proteins zeigt;
 - (b) Bestimmen in der Probe die Anwesenheit eines Antikörpers der an diese Probe bindet, aber nicht an das entsprechende Gewebe eines gesunden Probanden ohne solche pathologische Strukturen;
 - (c) Erlangen des Immunglobulin-Genrepertoires für Antikörper aus den B-Gedächtniszellen der Probe für die bestimmt wurde, dass sie Antikörper enthält die an diese Probe binden, jedoch nicht an das entsprechende Gewebe eines gesunden Probanden ohne solche pathologische Strukturen, umfassend die Schritte von:
 - (i) Erhalten von mRNA aus den B-Gedächtniszellen;
 - (ii) Erhalten von cDNA von der mRNA aus Schritt (i); und
 - (iii) Verwendung einer Primer-Verlängerungsreaktion, um von der cDNA die Fragmente, die den schweren Ketten (HC) und den Kappa/Lambda leichten Ketten (LC) der Antikörper entsprechen, zu amplifizieren;
 - (d) Verwendung der in (c) erhaltenen HC- und LC-Sequenzen, um die Antikörper zu exprimieren; und
 - (e) Isolierung des Antikörpers, der spezifisch an pathologisch verändertes Gewebe aus einem menschlichen Patienten oder Tiermodell für die Erkrankung bindet, wobei das Gewebe pathologische Proteinstrukturen des Proteins umfasst, jedoch nicht an Gewebe eines gesunden Probanden ohne solche pathologische Strukturen.
2. Verfahren nach Anspruch 1, wobei das pathologische Gewebe aus menschlichen Patienten stammt, die an neurodegenerativen Erkrankungen, Erkrankungen assoziiert mit Proteinfehlfaltung, Gewebe-Amyloidose oder anderen Erkrankungen die mit pathologischen Ablagerungen zusammenhängen leiden.
3. Verfahren nach Anspruch 1 oder 2, wobei der Patient beziehungsweise Proband ein Mensch ist.
4. Verfahren nach einem der Ansprüche 1 bis 3, wobei durch die Anwesenheit oder Abwesenheit eines Surrogatmarkers bestimmt wird, dass der Patient von einer noch nicht manifestierten Erkrankung betroffen ist oder dem Risiko ausgesetzt ist die Erkrankung zu entwickeln, vorzugsweise wobei der Surrogatmarker ausgewählt ist aus der Gruppe bestehend aus hohes Alter, Gehirn-Amyloid-Last, ApoE-Genotyp, APP-Genotyp, PS1-Genotyp, Level von Abeta-Peptid in Körperflüssigkeiten, Isoprostane, Tau und Phospho-Tau.
5. Verfahren nach einem der Ansprüche 1 bis 4, wobei die Erkrankung Alzheimer ist.
6. Verfahren nach Anspruch 5, wobei die Probe aus einem Patienten erhalten wurde der die folgenden Kriterien erfüllt:

- (i) dass er 65 Jahre oder älter ist;
- (ii) dass er volle kognitive Kapazität und einen guten Gesundheitszustand aufweist; und
- (iii) dass er keine klinischen Anzeichen von Demenz aufweist; oder
- (iv) dass er eine ungewöhnlich langsame Progressionsrate einer Erkrankung aufweist ungeachtet der Anwesenheit einer gestellten klinischen Diagnose von mutmaßlich Alzheimer.

Revendications

1. Procédé d'isolement d'un anticorps monoclonal spécifique d'une forme variante pathologique associée à un trouble d'une protéine endogène comprenant :

(a) la soumission d'un échantillon provenant d'une culture de lymphocytes B à mémoire provenant d'un sujet qui est en bonne santé d'un point de vue phénotypique mais affecté par ou à risque de développer un trouble neurologique **caractérisé par** des structures protéiques pathologiques de la forme variante pathologique de la protéine endogène, ou d'un patient présentant une évolution pathologique inhabituellement stable d'un point de vue clinique du trouble à un spécimen présentant des structures pathologiques de la forme variante pathologique de la protéine endogène ;

(b) l'identification dans l'échantillon de la présence d'un anticorps qui se lie audit spécimen mais pas au tissu correspondant d'un sujet en bonne santé sans de telles structures pathologiques ;

(c) l'obtention du répertoire des gènes des immunoglobulines pour les anticorps à partir desdits lymphocytes B à mémoire de l'échantillon qui a été identifié comme contenant des anticorps qui se lient audit spécimen mais pas au tissu correspondant d'un sujet en bonne santé sans de telles structures pathologiques comprenant les étapes suivantes :

- (i) l'obtention de l'ARNm desdits lymphocytes B à mémoire ;
- (ii) l'obtention de l'ADNc à partir de l'ARNm de l'étape (i) ; et
- (iii) l'utilisation de la réaction d'extension d'amorces pour amplifier à partir dudit ADNc les fragments correspondant aux chaînes lourdes (HC) et aux chaînes légères kappa/lambda (LC) desdits anticorps ;

(d) l'utilisation des séquences des HC et des LC obtenues en (c) pour exprimer lesdits anticorps ; et

(e) l'isolement de l'anticorps qui se lie spécifiquement au tissu pathologiquement modifié dérivé d'un patient humain ou d'un modèle animal du trouble, dans lequel le tissu comprend des structures protéiques pathologiques de la protéine mais pas au tissu d'un sujet en bonne santé sans de telles structures pathologiques.

2. Procédé selon la revendication 1, dans lequel le tissu pathologique provient de patients humains souffrant de maladies neurodégénératives, de maladies dues à un repliement incorrect des protéines, de l'amyloïdose tissulaire ou d'autres maladies associées à des dépôts pathologiques.

3. Procédé selon la revendication 1 ou 2, dans lequel ledit patient et sujet, respectivement, est un être humain.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel ledit patient a été déterminé comme étant affecté par un trouble qui ne s'est pas encore manifesté ou à risque de développer le trouble par la présence ou l'absence d'un marqueur de substitution, de préférence dans lequel ledit marqueur de substitution est choisi dans le groupe constitué d'un âge avancé, une charge d'amyloïde dans le cerveau, le génotype de l'ApoE, le génotype de l'APP, le génotype de la PSI, les taux dans les liquides corporels de peptide A-bêta, les isoprostanes, la Tau, et la phospho-Tau.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel ledit trouble est la maladie d'Alzheimer.

6. Procédé selon la revendication 5, dans lequel l'échantillon est obtenu à partir d'un patient répondant aux critères suivants :

- (i) ayant un âge de 65 ans ou supérieur ;
- (ii) présentant une capacité cognitive totale et une bonne santé ; et
- (iii) ne présentant aucun signe clinique de démence ; ou
- (iv) présentant des vitesses inhabituellement lentes de progression de la maladie en dépit de la présence d'un diagnostic clinique établi d'une maladie d'Alzheimer probable.

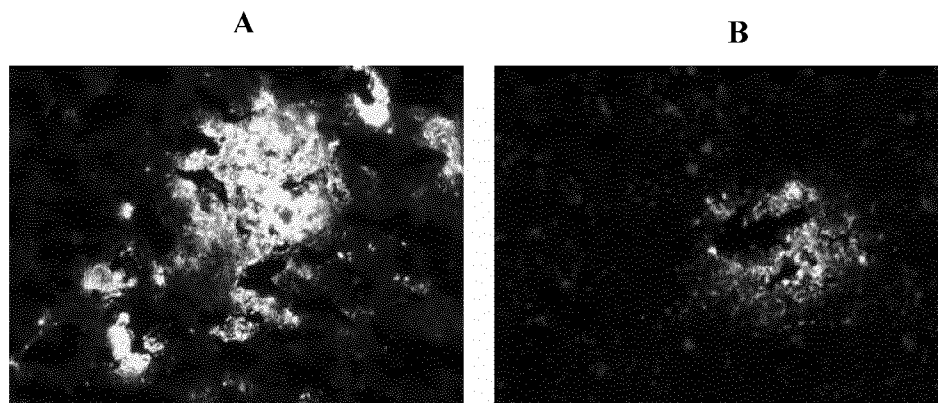


FIG. 1

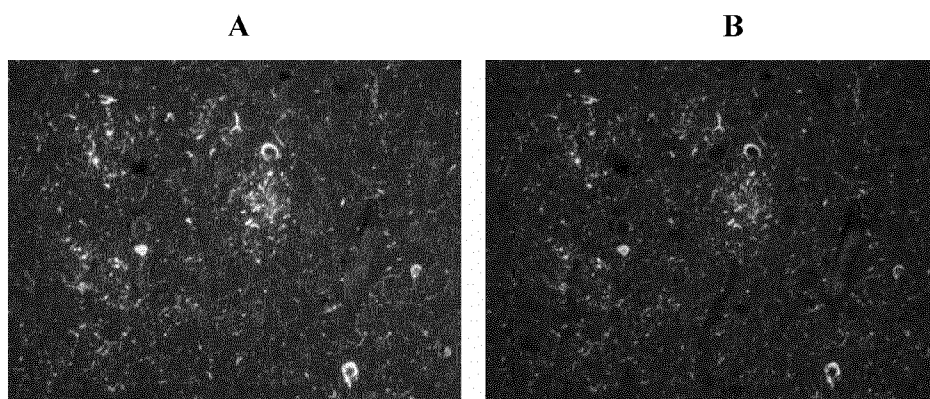


FIG. 2

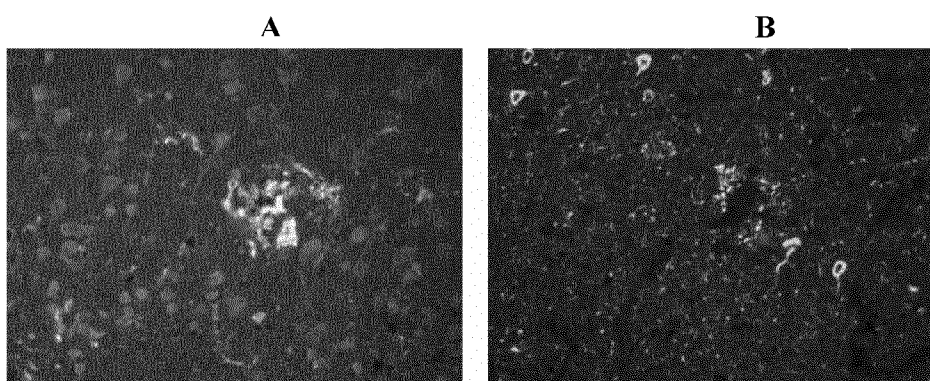


FIG. 3

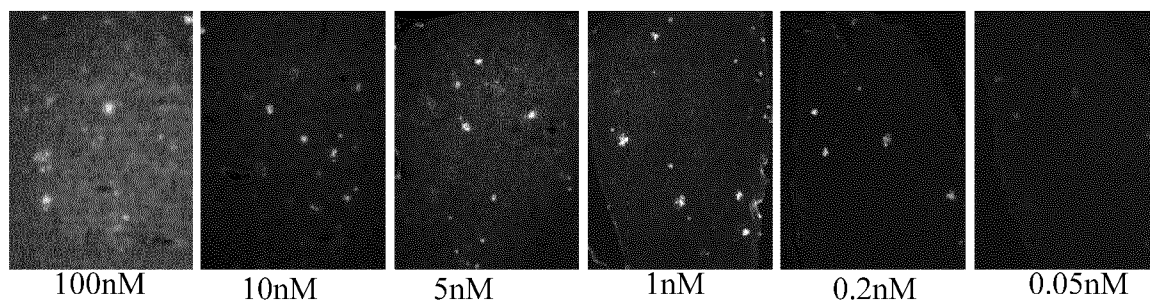


FIG. 4

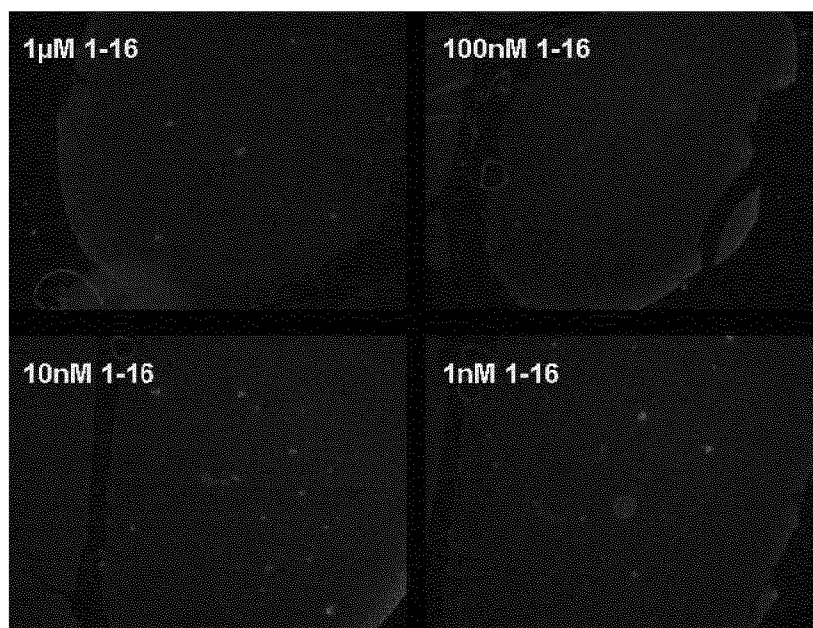


FIG. 5

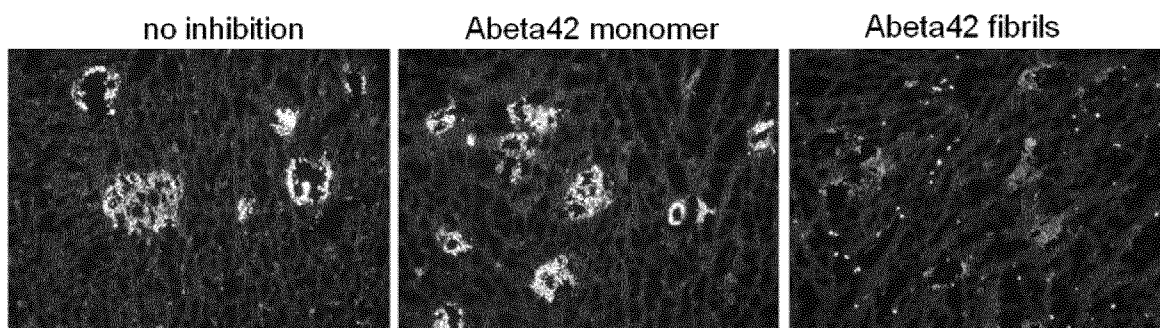


FIG. 6

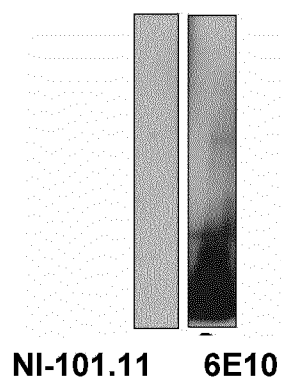


FIG. 7

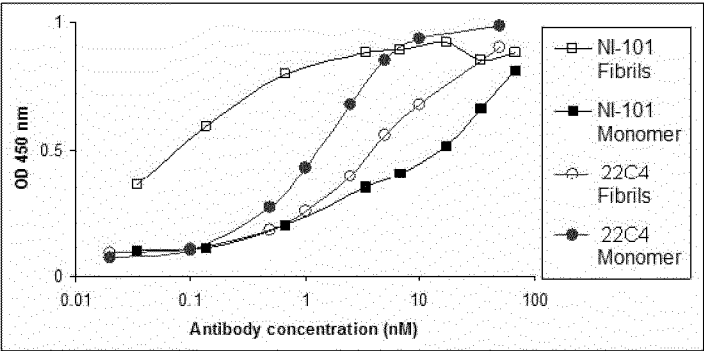


FIG. 8

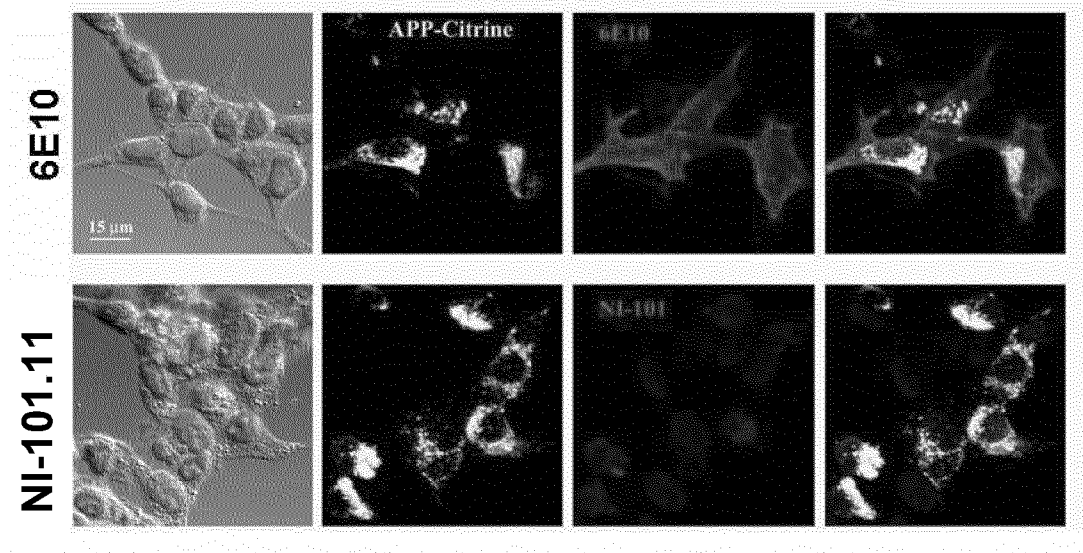
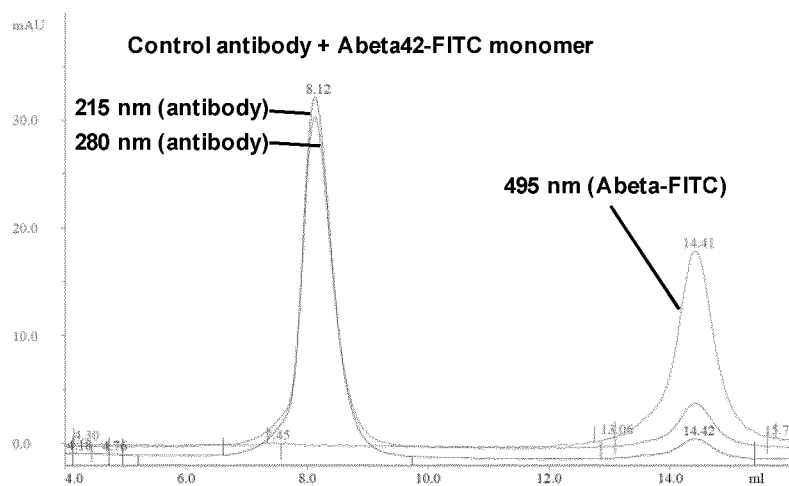
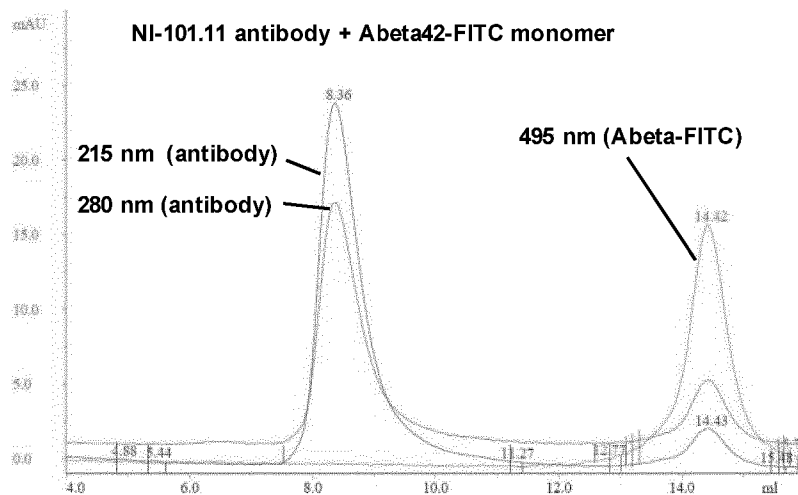


FIG. 9

A



B



C

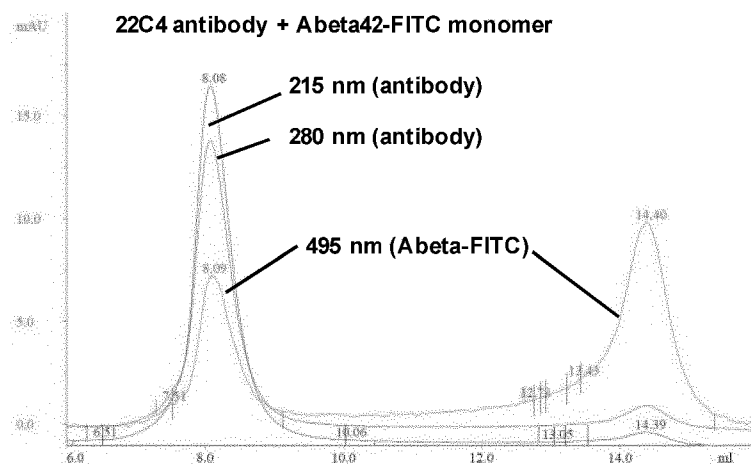


FIG. 10A-C

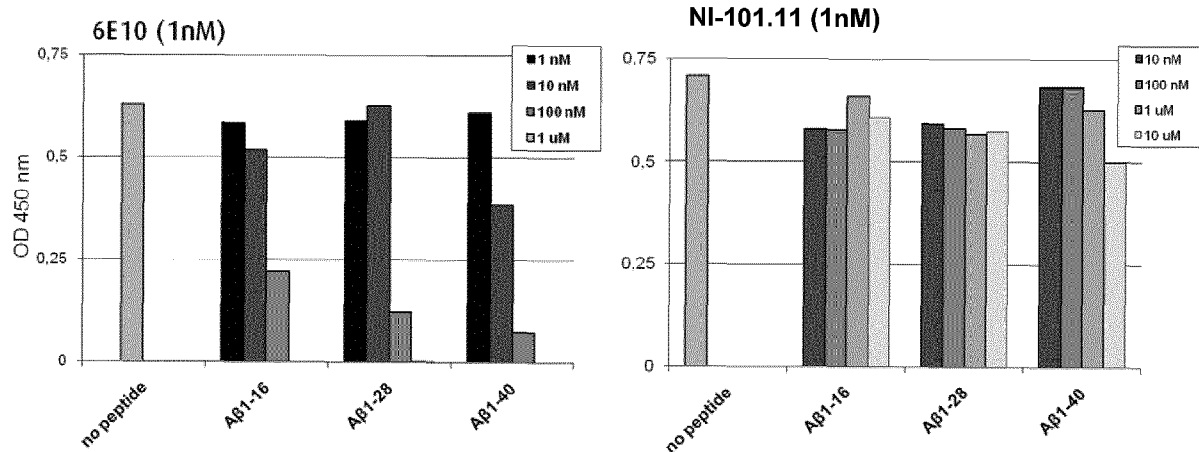


FIG. 11

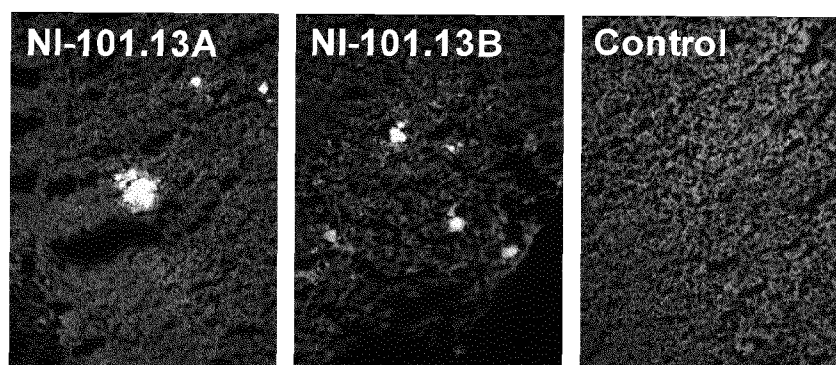


FIG. 12

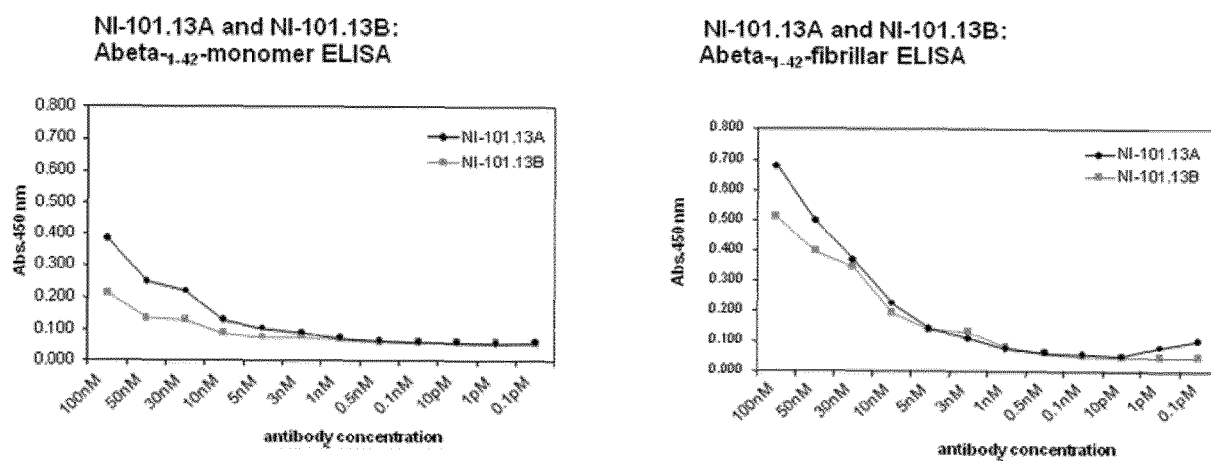


FIG. 13

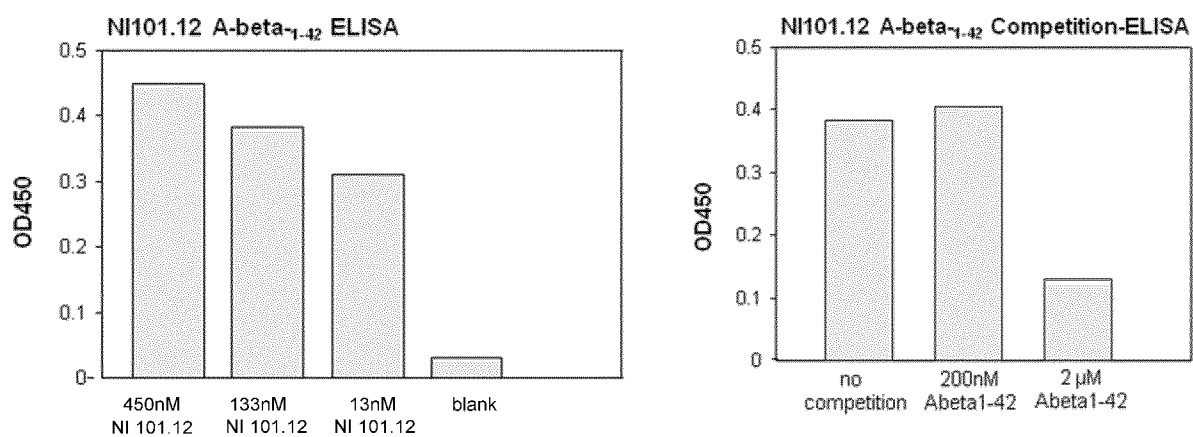


FIG. 14A-B

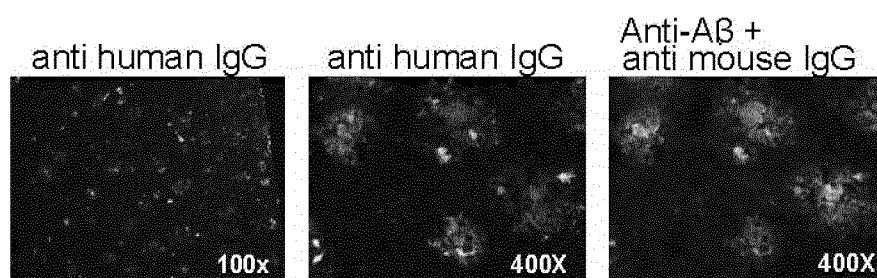


FIG. 15

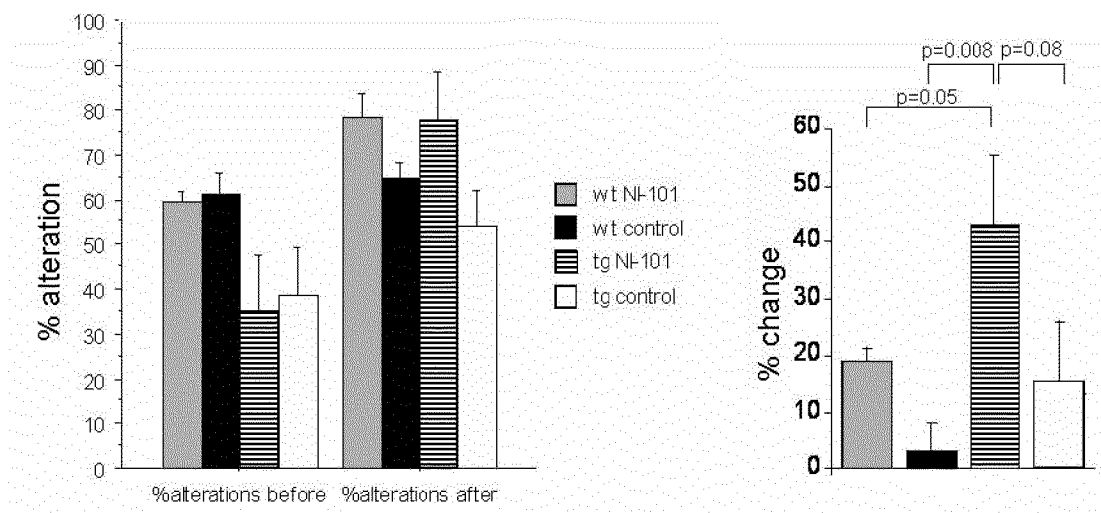


FIG. 16A-B

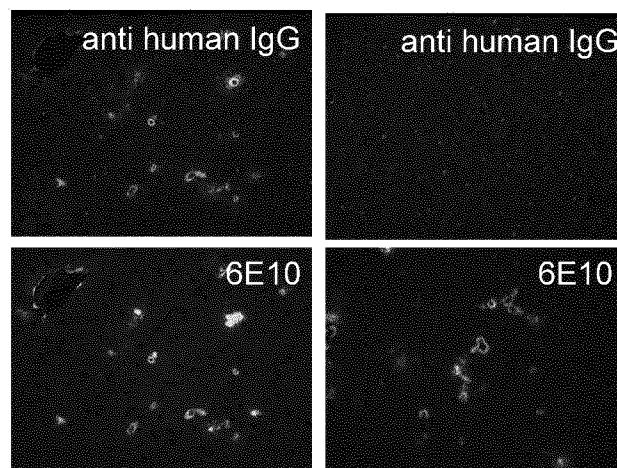


FIG. 17

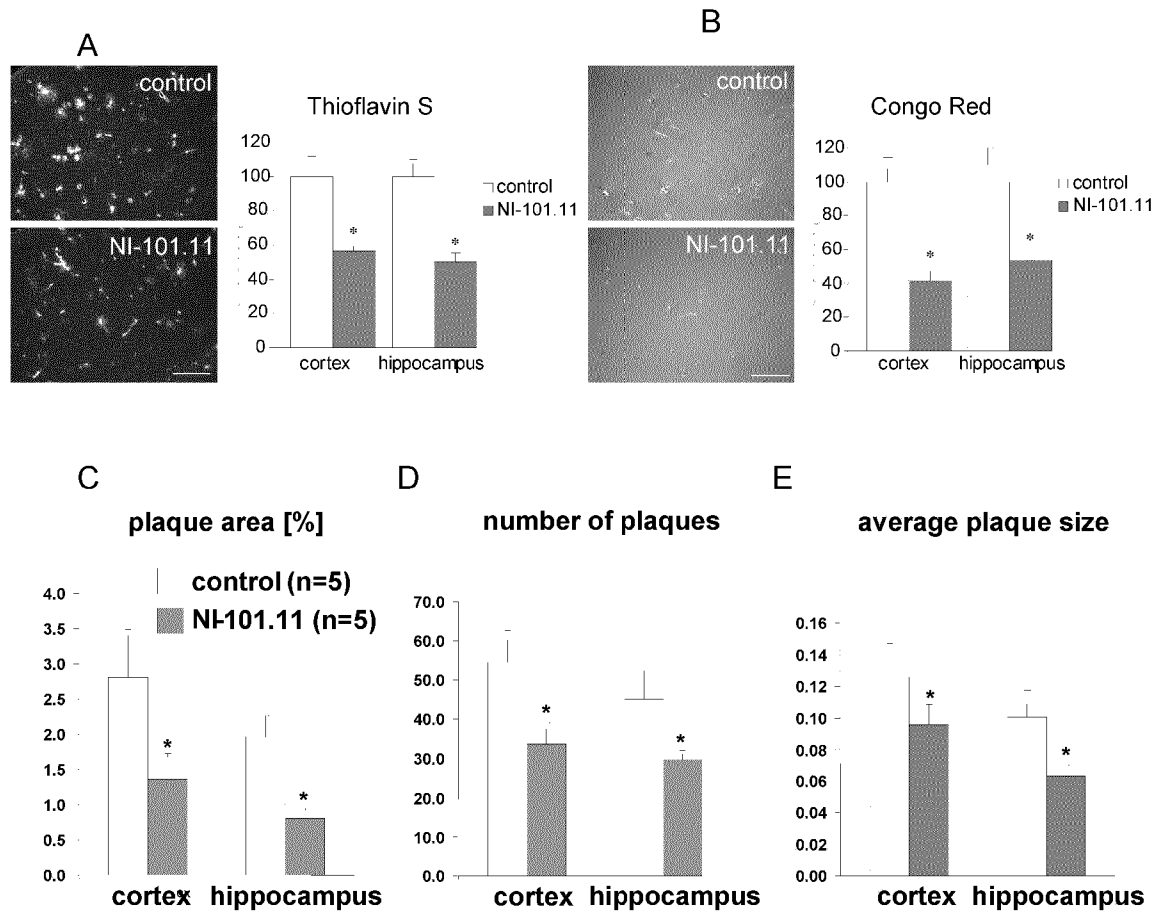


FIG. 18A-E

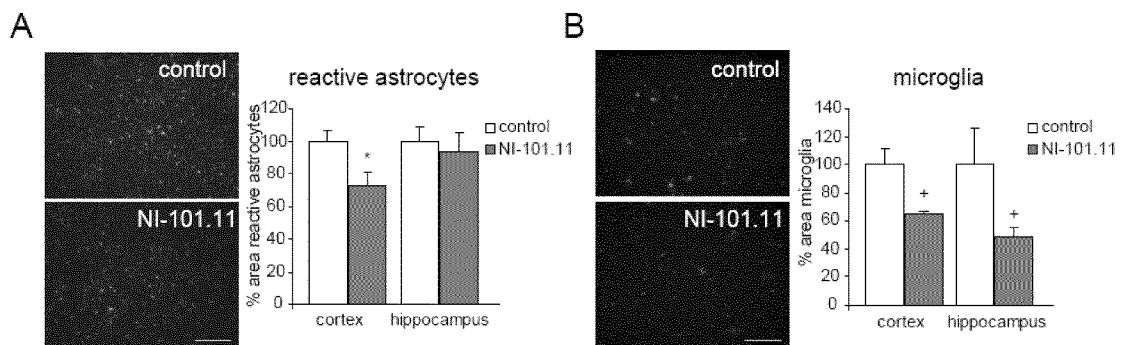


FIG. 19A-B

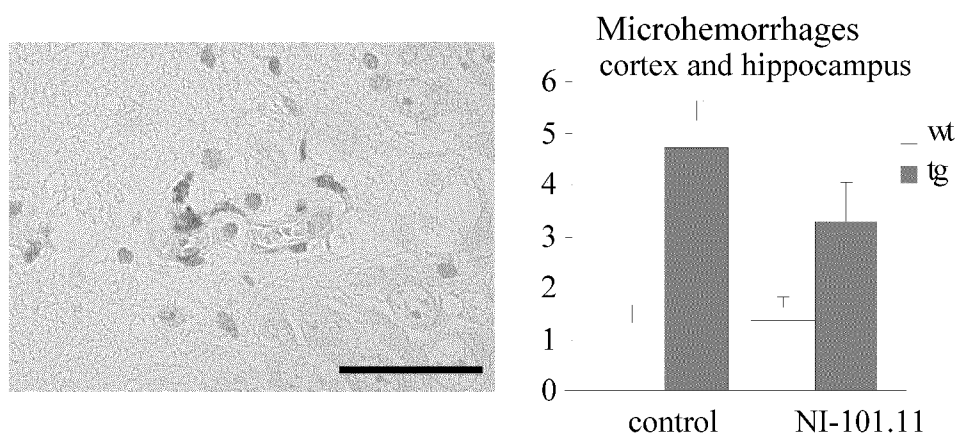


FIG. 20

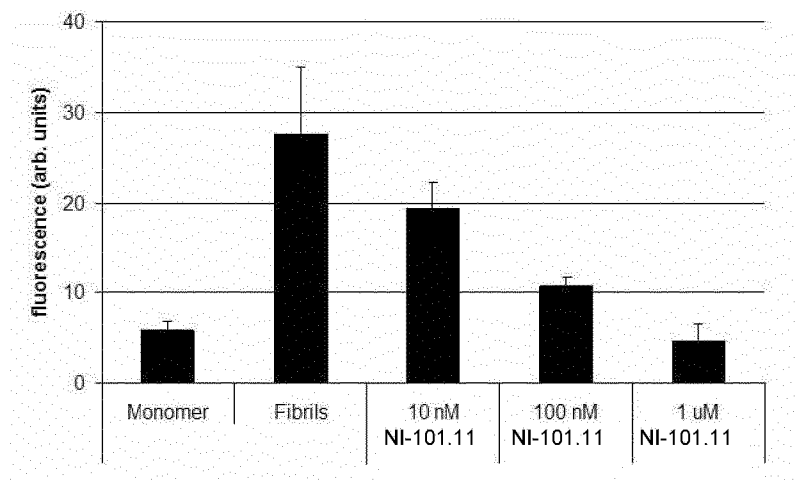


FIG. 21

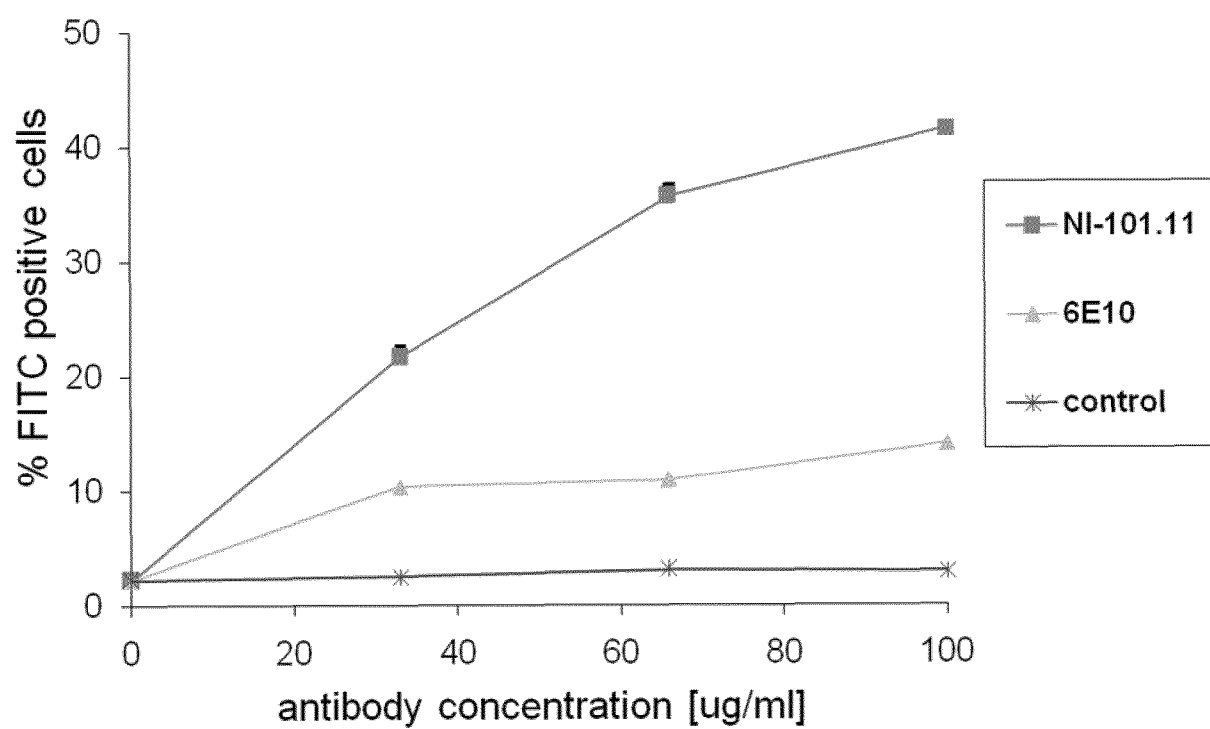


FIG. 22

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Eljárás betegségsspecifikus kötőmolekulák és célpontok biztosítására**Szabadalmi igénypontok**

1. Eljárás olyan monoklonális antitest izolálására, amely specifikus egy endogén fehérje rendellenességgel összefüggő patológiás variáns formájára, amely eljárás során:

(a) egy fenotipikusan egészséges, de az endogén fehérje patológiás variáns formájának patológiás fehérjestruktúráival jellemzett neurológiai rendellenesség által vagy annak kifejlődése kockázata által érintett alany, vagy a rendellenesség egy klinikailag szokatlanul stabil betegségfolyásával rendelkező páciens memória B-sejtjeinek tenyészetéből származó mintát kiteszünk olyan mintadarabbal, amely mutatja az endogén fehérje patológiás variáns formájának patológiás struktúráit;

(b) azonosítjuk egy olyan antitest jelenlétét a mintában, amely kötődik a mintadarabhoz, de nem kötődik egy egészséges alany ilyen patológiás struktúrák nélküli megfelelő szövetéhez;

(c) kinyerünk az olyan minta memória B-sejtjeiből antitestekre vonatkozóan immunglobulingén-repertoárt, amely mintáról azonosítottuk, hogy tartalmaz olyan antitesteket, amelyek kötődnek a mintadarabhoz, de nem kötődnek egy egészséges alany ilyen patológiás struktúrák nélküli megfelelő szövetéhez, a kinyerés során:

(i) kinyerünk mRNS-t a memória B-sejtekből;

(ii) az (i) lépésben kapott mRNS alapján cDNS-t nyerünk; és

(iii) primer extenziós reakciót alkalmazunk, hogy amplifikáljuk a cDNS-ből azokat a fragmenseket, amelyek megfelelnek az antitestek nehézláncainak (HC) és kappa/lambda könnyűláncainak (LC);



- (d) a (c) lépésben kapott HC és LC szekvenciák segítségével expresszáltatjuk az antitesteket; és
 - (e) izoláljuk az antitestet, amely specifikusan kötődik humán páciensből vagy a rendellenesség egy állatmodelljéből származó, patológiásan módosult szövethez, ahol a szövet tartalmazza a fehérje patológiás fehérjestruktúráit, de nem kötődik egy egészséges alany ilyen patológiás struktúrák nélküli szövetéhez.
2. Az 1. igénypont szerinti eljárás, ahol a patológiás szövet olyan humán páciensekből származik, akik neurodegeneratív betegségekben, hibás fehérjefeltekeredési betegségekben, szöveti amiloidózisban vagy patológiás lerakódásokkal kapcsolatos más betegségben szenvednek.
 3. Az 1. vagy 2. igénypont szerinti eljárás, ahol a páciens, illetve az alany ember.
 4. Az 1-3. igénypontok bármelyike szerinti eljárás, ahol a páciensről egy szurrogát marker jelenléte vagy hiánya alapján meg van állapítva, hogy érinti egy még nem manifesztálódott rendellenesség vagy fennáll nála a kockázata a rendellenesség kialakulásának, ahol a szurrogát markert előnyösen az idős kor, agyi amiloidfelhalmozódás, ApoE genotípus, APP genotípus, PS1 genotípus, az Abéta peptid szintje a testfolyadékokban, izoprosztánok, Tau, és foszfo-Tau által alkotott csoportból választjuk.
 5. Az 1-4. igénypontok bármelyike szerinti eljárás, ahol a rendellenesség Alzheimer-kór.
 6. Az 5. igénypont szerinti eljárás, ahol a mintát olyan páciensről nyerjük, aki megfelel a következő kritériumoknak:
 - (i) életkora legalább 65 év;
 - (ii) teljes kognitív kapacitással és jó egészséggel rendelkezik; és
 - (iii) nem mutatja demencia klinikai jeleit; vagy
 - (iv) szokatlanul lassú nála a betegség progressziója a valószínű Alzheimer-kór megalapozott klinikai diagnózisának létezése ellenére.