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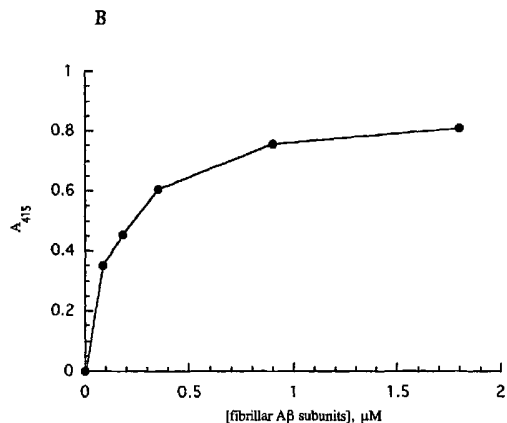
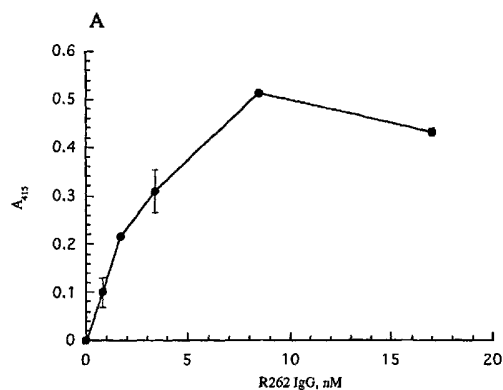
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- (71) Applicant (for all designated States except US): **RESEARCH FOUNDATION FOR MENTAL HYGIENE, INC.** [US/US]; New York State Department of Mental Hygiene, Inc., 44 Holland Avenue, Albany, NY 12229 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **MILLER, David L.**
- (74) Agent: **LOMBILLO, Sybil A.**; Morgan & Finnegan LLP, 3 World Financial Center, New York, NY 10281-2101 (US).
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(54) Title: ANTIBODIES SPECIFIC FOR FIBRILLAR AMYLOID AND A PROCEDURE TO DETECT FIBRILLAR AMYLOID DEPOSITS



(57) Abstract: The present invention provides novel antibodies, and antibody fragments, that specifically bind to fibrillar amyloid, and not to the non-fibrillar peptide. Also provided are methods for generating, identifying, and isolating the antibodies and antibody fragments of the invention. The antibodies described herein are useful in the study, diagnosis, and treatment of Alzheimer's disease.

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## **Antibodies Specific for Fibrillar Amyloid and a Procedure to Detect Fibrillar Amyloid Deposits**

### **FIELD OF THE INVENTION**

**[0001]** The present invention relates to antibodies, and antibody fragments, as well as to methods for generating, identifying, and isolating antibodies, and antibody fragments, that bind with greater affinity and specificity to fibrillar amyloid than to the non-fibrillar peptide. The antibodies prepared according to the methods described herein are useful for the study, diagnosis, and treatment of Alzheimer's disease.

### **BACKGROUND OF THE INVENTION**

**[0002]** Amyloid is the generic term applied to poorly soluble proteinaceous deposits, which possess similar tinctorial properties when treated with histochemical stains, and which occur in various tissues in response to disease (Glennner, G.G. (1980) *N. Engl J. Med* 302, 1283-92). Amyloid is frequently formed from a peptide cleaved from a larger precursor, whose amino acid sequence predisposes it to aggregate in fibrillar beta-sheets. As an abnormal substance composed of an endogenous peptide, amyloid presents a difficult problem for the humoral immune system. To eliminate the amyloid, the system must raise antibodies to the neo-epitopes in the aggregated peptide without provoking an autoimmune response, which would possibly destroy tissues that produce the amyloid peptide or its precursor. Such autoimmune processes account for some of the tissue damage occurring in multiple sclerosis, lupus erythematosus and arthritis. The  $\beta$ -amyloid peptide ( $A\beta$ ) linked to Alzheimer's disease forms deposits in the brain, which presents an additional problem in that the location of the deposits is, to a large extent, isolated from the systems of acquired immunity. This may limit their capacity either to generate or to react with a humoral immune response.  $A\beta$  is cleaved from a precursor, which is produced by nearly all tissues, and it is found in body fluids. The cell biology of  $A\beta$  and its postulated role in

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Alzheimer's disease have been reviewed (Huse, J.T., and Doms, R.W. (2001) *Traffic* 2, 75-81; Parvathy, S. et al. (1999) *Biochemistry* 38, 9728-34; Selkoe, D.J. (2001) *Physiol Rev* 81, 741-66).

**[0003]** One study reports that peripheral immunization with fibrillar human A $\beta$  lowered the level of  $\beta$ -amyloid deposits in transgenic mice expressing the human  $\beta$ -amyloid peptide precursor ( $\beta$ -APP) in their brains (Schenk, D. et al. (1999) *Nature* 400, 173-7). Although the direct application of this procedure to humans may not be feasible, the result has stimulated interest in the involvement of the immune system in Alzheimer's disease and in the utility of immunotherapy to treat the disease (Janus, C. et al. (2000) *Nature* 408, 979-82; DeMattos, R.B. et al. (2001) *Proc Natl Acad Sci USA* 98, 8850-5; Dodart, J.C. et al. (2002) *Nat Neurosci* 5, 452-7; Lemere, C.A. et al. (2000) *Ann N Y Acad Sci* 920, 328-21; Morgan, D. et al. (2000) *Nature* 408, 982-5). Nevertheless, this transgenic mouse Alzheimer's disease model, in which human  $\beta$ -APP mRNA expression is driven by the platelet-derived growth factor promoter, may not reproduce the humoral immune response of humans, because the transgenic human  $\beta$ -APP (whose A $\beta$  sequence differs from that of the mouse) is principally produced in the mouse brain, where it and the  $\beta$ -amyloid are virtually unseen by mouse antibody-forming cells (Games, D. et al. (1995) *Nature* 373, 523-7).

**[0004]** Another study reports that Alzheimer's disease patients immunized with aggregated amyloid peptide developed antibodies which bound to vascular amyloid deposits (Hock, et al. (2002) *Nature Medicine* 8, 1270-5). However, the antibodies have not been isolated or characterized. Moreover, the antibodies would be of limited use, since they were produced by patients afflicted with Alzheimer's disease, and could not be obtained in commercially significant amounts.

### **SUMMARY OF THE INVENTION**

**[0005]** In its broadest aspect, the present invention provides novel antibodies, and antibody fragments, that bind with a high degree of affinity and specificity to fibrillar amyloid, but not to the non-fibrillar peptide. The antibodies are useful in numerous research, diagnostic, and therapeutic applications relating to Alzheimer's disease. Moreover, the antibodies, and antibody fragments, may be produced in commercially significant amounts using the methods described herein.

**[0006]** In one aspect, the present invention relates to antibodies, and antibody fragments, that are specific for an epitope present in the amino terminal of the  $\beta$ -amyloid peptide, and that bind to fibrillar  $\beta$ -amyloid with an affinity constant over 1000-fold greater than the affinity constant for their binding to the monomeric sequence. Antibodies, and antibody fragments, specific for additional epitopes present in fibrillar  $\beta$ -amyloid are also contemplated by the present invention and may be developed and characterized using the methods described herein.

**[0007]** Accordingly, the present invention also relates to methods of generating, identifying, and isolating a diverse panel of antibodies and antibody fragments characterized by their ability to specifically bind to fibrillar  $\beta$ -amyloid. In particular, the method comprises the steps of immunizing a host, preferably a rabbit, with fibrillar  $\beta$ -amyloid or fibrils composed of truncated  $\beta$ -amyloid peptides, and isolating the host antibodies which are specific for fibrillar  $\beta$ -amyloid or truncated fibrillar forms.

**[0008]** Yet another aspect of the present invention is to provide methods of using the antibodies, or fragments thereof, of the invention for the detection, localization, or isolation of fibrillar  $\beta$ -amyloid and diagnosis of  $\beta$ -amyloid-related diseases in a subject. Accordingly, the antibodies, and/or fragments thereof, of the present invention can be utilized in a broad variety of assays known to those skilled in the art including, but not limited to, immunohistochemical assays, ELISA,

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Western Blot, dot blot, immunoprecipitation, sandwich and competition immunoassays, immunofluorescence protocols, immunoaffinity chromatography and/or radioimmunoassays.

**[0009]** Still a further aspect of the present invention is to provide methods of using the antibodies, or fragments thereof, of the invention for the study of the mechanism of  $\beta$ -amyloid fibril formation. The formation of  $\beta$ -amyloid fibrils can be followed using the antibodies and methods described herein. In another aspect, one of the pharmacological targets for the design of Alzheimer's disease therapeutic agents is the process of  $\beta$ -amyloid fibril formation; therefore, the antibodies of the present invention can be used to test the efficacy of therapeutic agents by following the inhibition of fibril formation using the antibodies and methods described herein.

**[0010]** It is another aspect of the present invention to provide antibodies having specificity for fibrillar  $\beta$ -amyloid, or binding fragments thereof, bound to a solid support, substrate, or matrix. Also, according to the present invention, the described antibodies, or binding fragments thereof, can be conjugated to a detectable label, such as a fluorophore, a chromophore, a radionuclide, or any other agent, for use in diagnostic, therapeutic, imaging, and screening compounds, for example.

**[0011]** In a further aspect, the present invention is directed to a method of localizing fibrillar  $\beta$ -amyloid in a subject. For example, diagnostic applications include, but are not limited to, the neuropathological localization of cerebrovascular amyloid deposits in tissue sections from brains of Alzheimer's disease patients. Through additional modifications, the antibodies may be used for *in vivo* diagnosis by administering one or more of the antibodies described herein, or binding fragments thereof, to bind fibrillar  $\beta$ -amyloid within the subject and determining the location of the one or more antibodies within the subject. For such methods, the antibodies, or binding fragments thereof, are also preferably

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labeled with a detectable and physiologically acceptable label, such as a radionuclide. In addition, according to the present invention, the stage of the disease, and/or effectiveness of treatment, can be monitored by determining the levels or changes over time of the specifically recognized fibrillar  $\beta$ -amyloid *in vivo*.

**[0012]** It is yet another aspect of the present invention to provide compositions comprising one or more of the antibodies, or binding fragments, and a physiologically acceptable carrier, diluent, or excipient.

**[0013]** It is another aspect of the present invention to provide kits for detecting fibrillar  $\beta$ -amyloid comprising one or more of the antibodies, or antibody fragments, of the invention, for the diagnosis, study or treatment of Alzheimer's disease.

**[0014]** Further aspects, features and advantages of the present invention will be better appreciated upon a reading of the detailed description of the invention when considered in connection with the accompanying figures/drawings.

#### **BRIEF DESCRIPTION OF THE FIGURES**

**[0015]** **Figure 1.** Sedimentation of IgG with fibrillar  $A\beta_{1-42}$ . Panel A: Fibrillar  $A\beta_{1-42}$ , 0.08  $\mu$ g (18 pmol of monomer), was incubated with increasing amounts of immunopurified R262 IgG in a volume of 10 $\mu$ L and the mixture was processed with anti-rabbit IgG-alkaline phosphatase conjugate as described in Example 1. One-tenth of the sedimented complex was assayed for nitrophenyl phosphatase activity. Absorbance at 415 nm was measured after 30 min. Data points represent averages of 2 independent measurements. Panel B: Immunopurified R262 IgG (0.17 pmol) was incubated with increasing concentrations of fibrillar  $A\beta_{1-42}$  in a volume of 10mL and was processed as in Panel A. The abscissa units indicate concentrations of fibrillar subunits. Error bars represent the deviation from the mean of 2 independent measurements.

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**[0016]** **Figure 2.** Electron micrograph of antibody R262 bound to fibrillar revealed by gold-labeled protein A. Magnification: 112,000 X.

**[0017]** **Figure 3** shows the inhibition of antibody R286 binding by peptides. Circles, squares,  $A\beta_{1-11}$ ; open triangles,  $A\beta_{1-16}$ ; open diamonds,  $A\beta_{1-8}$ ; filled triangles,  $A\beta_{1-8}$ , amino terminal Cys-aminohexanoyl derivative; filled diamonds,  $A\beta_{3-11}$ . ELISA plates were coated with  $A\beta_{1-28}$ .

**[0018]** **Figure 4.** ELISA of inhibition of antibody R286 binding to  $A\beta_{1-42}$  or  $A\beta_{1-28}$  by  $A\beta_{1-11}$ . Closed symbols: crude R286 antiserum; open symbols: affinity-purified R286. Circles:  $A\beta_{1-42}$ -coated plate; triangles:  $A\beta_{1-28}$ -coated plate.

**[0019]** **Figure 5.** ELISA of the inhibition of antibody R286 binding to an  $A\beta_{1-28}$ -coated plate by fibrillar  $A\beta_{1-42}$ , filled circles, and  $A\beta_{1-11}$ , open circles. Averages of 3 independent measurements.

**[0020]** **Figure 6.** ELISA of the inhibition of mAb 6E10 binding to an  $A\beta_{1-28}$ -coated plate by fibrillar  $A\beta_{1-42}$ , filled circles, and  $A\beta_{1-16}$ , open circles; averages of 3 independent measurements.

**[0021]** **Figure 7.** Comparative immunostaining of AD brain sections by affinity-purified R286 and mAb 6E10 with or without  $A\beta_{1-16}$  blocking peptide. Sections *a-d*: amygdala; sections *e-h*: cerebellum. Sections *a* and *c* were pre-treated with formic acid. Sections *a*, *c*, *e*, *g* stained with R286. Sections *b*, *d*, *f*, *h* stained with mAb 6E10. Sections *c*, *d*, *g*, *h*: 25mM  $A\beta_{1-16}$  added.

### **DETAILED DESCRIPTION OF THE INVENTION**

**[0022]** The  $\beta$ -amyloid peptide is a normal product of the proteolytic processing of its precursor ( $\beta$ -APP). Normally it elicits a very low humoral response; however, the aggregation of monomeric  $\beta$ -amyloid to form fibrillar  $\beta$ -amyloid creates neo-epitopes to which antibodies can be generated. The present invention is based upon the discovery that rabbits immunized with fibrillar  $\beta$ -



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amyloid produced antibodies that have a greater affinity for fibrillar  $\beta$ -amyloid than for the monomeric or precursor forms of  $\beta$ -amyloid. Because the  $\beta$ -amyloid sequences of rabbits and humans are identical, their immune systems should undergo similar processes of tolerance of  $\beta$ -amyloid during development. Thus, the methods described herein can be used to generate a panel of antibodies and antibody fragments characterized by their ability to specifically bind to fibrillar  $\beta$ -amyloid.

#### Anti-Fibrillar $\beta$ -Amyloid Antibodies

**[0023]** In one embodiment, the present invention relates to novel antibodies, and antibody fragments, that specifically bind to neo-epitopes present in fibrillar  $\beta$ -amyloid. In a specific embodiment, the present invention relates to antibodies, and antibody fragments, that bind to an epitope in the first eight (8) residues of the amino terminus of the  $\beta$ -amyloid peptide with about 1000-fold greater affinity for fibrillar  $\beta$ -amyloid than to the monomeric peptide. The present invention further relates to the development and characterization of additional antibodies using the methods described herein.

**[0024]** Accordingly, the antibodies, and/or fragments, of the invention may be specific for any region of the  $\beta$ -amyloid peptide. For example, antibodies may be generated by immunizing a host, preferably a rabbit, but also including mice, chickens, goats, or other species with a variety of types of fibrillar amyloid. In a preferred embodiment, the host is a rabbit. These amyloid fibrils can be formed from any number of peptide monomers including, but not limited to,  $A\beta_{1-40}$ ,  $A\beta_{2-40}$ ,  $A\beta_{2-42}$ ,  $A\beta_{3-40}$ ,  $A\beta_{3-42}$ ,  $A\beta_{1-38}$ ,  $A\beta_{[\text{pyroglu-3}]-42}$ ,  $A\beta_{11-42}$ ,  $A\beta_{17-42}$ ,  $A\beta_{25-35}$ , etc. An important consideration for purposes of the invention is that the amino acid sequence of the amyloid peptide of the host species should contain the amino acid sequence of the peptide used for immunization.

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**[0025]** The relative affinity of an antibody, or fragment thereof, for an epitope in the monomeric  $\beta$ -amyloid peptide to that of the  $\beta$ -amyloid fibril can be determined using a variety of techniques known in the art including, but not limited to, sedimentation assay, ELISA, radioimmunoassay, and immunohistochemistry. Example 1 illustrates a simple sedimentation assay that was developed to estimate binding constants. Preferred binding affinities can be calculated, for example, from the ratio of the dissociation constant of the fibril-antibody complex to that of the monomeric peptide-antibody complex as described in Example 1. Preferred relative binding affinities include those with dissociation constants  $K_{rel}$  ( $K_{monomer}/K_{fibril}$ ) of about 70, 150, or 300. More preferred binding affinities include those with a dissociation constant  $K_{rel}$  of at least about 500. Even more preferred antibody binding affinities include those with a dissociation constant  $K_{rel}$  of about 1000 or 2000. Even higher affinity antibodies may be generated and are encompassed by the present invention.

**[0026]** The antibodies of the present invention can be monospecific, bispecific, trispecific, or of greater multispecificity. Multispecific antibodies can be specific for different epitopes of the fibrillar  $\beta$ -amyloid peptide, or can be specific for both a  $\beta$ -amyloid peptide and a heterologous epitope, such as a heterologous polypeptide or a solid support material.

**[0027]** The present invention also encompasses antibodies that competitively inhibit the binding of the antibodies described herein to a fibrillar  $\beta$ -amyloid neo-epitope as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In a preferred embodiment, the antibody competitively inhibits the binding of an anti-fibrillar  $\beta$ -amyloid antibody to the neo-epitope by at least about 50%, more preferably at least about 80%, and even more preferably by at least about 90% to 95%.

### Generation of Anti-Fibrillar $\beta$ -Amyloid Antibodies

**[0028]** In a preferred embodiment, the antibodies of the present invention are prepared by administering fibrillar  $\beta$ -amyloid to a host. Fibrillar  $\beta$ -amyloid can be prepared by methods well known in the art and/or as described in Example 1. Briefly, sufficient quantities of fibrillar  $\beta$ -amyloid may be generated by preparing a solution of monomeric  $\beta$ -amyloid peptide at a concentration of about 0.04 mg/ml to about 2 mg/ml, and more preferably in the range of about 0.1 mg/ml to about 1 mg/ml. The solution may then be seeded with about 0.01  $\mu$ g to about 1  $\mu$ g, more preferably between about 0.1  $\mu$ g to about 0.2  $\mu$ g of fibrillar  $\beta$ -amyloid. Alternatively, one skilled in the art would understand that the amount of  $\beta$ -amyloid peptide may be adapted depending upon the specific preparation.

**[0029]** Full-length and truncated forms of the  $\beta$ -amyloid peptide may be used according to the invention to develop antibodies specific for other neo-epitopes found in fibrillar  $\beta$ -amyloid. Antibodies can be developed against the entire  $\beta$ -amyloid peptide, or portions thereof, such as, a truncated amino terminal domain. For example, antibodies can be generated to fibrillar amyloid formed from peptides such as those described above including, but not limited to,  $A\beta_{1-40}$ ,  $A\beta_{2-40}$ ,  $A\beta_{2-42}$ ,  $A\beta_{3-40}$ ,  $A\beta_{3-42}$ ,  $A\beta_{1-38}$ ,  $A\beta_{[\text{pyroglu-3}]-42}$ ,  $A\beta_{11-42}$ ,  $A\beta_{17-42}$ ,  $A\beta_{25-35}$ , etc. These peptides can be obtained from commercial suppliers, or they can be custom-synthesized and partially purified by established methods. The peptides can be dissolved at a concentration of 1-10 mg/ml in 50 mM ammonia. The peptide solutions are diluted to a concentration of 10  $\mu$ M to 200  $\mu$ M and adjusted to pH 7-7.5 in .01-.05 M phosphate or Tris buffer containing 0-0.15 M NaCl. The solutions are incubated at a temperature of 20° C to 37° C for 1-3 days to induce fibrillization. The fibrillar amyloid is then mixed with complete or incomplete Freund's adjuvant and injected into the desired host.

**[0030]** Once sufficient quantities of fibrillar  $\beta$ -amyloid are prepared, the fibrillar  $\beta$ -amyloid can be introduced into a host, preferably a rabbit, by

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intraperitoneal and/or intradermal injection. Other possible hosts include, but are not limited to, goats, mice, rats and chickens. As stated above, the important consideration for purposes of the invention is that the amino-acid sequence of the fibrillized amyloid peptide must be identical to an amino-acid sequence contained in the host's amyloid peptide. Injection material is typically an emulsion containing about 100 $\mu$ g of fibrillar peptide and complete Freund's adjuvant, or any other adjuvant known in the art for stimulating an immune response. Booster injections may be given in incomplete Freund's adjuvant until a useful titer of anti-fibrillar  $\beta$ -amyloid antibody can be detected, for example, by ELISA assay using fibrillar  $\beta$ -amyloid peptide adsorbed to a solid surface. Antisera raised in this manner may be used in both crude or purified preparations. The titre of anti-fibrillar  $\beta$ -amyloid antibodies in serum from an animal can be increased by selection of anti-fibrillar  $\beta$ -amyloid antibodies, e.g. by adsorption of the peptide onto a solid support and elution of the selected antibodies according to methods well known in the art.

**[0031]** In one embodiment, antibodies from the host are purified on a solid medium containing fibrillar  $\beta$ -amyloid. In a preferred embodiment, fibrillar  $\beta$ -amyloid-binding antibodies may be purified on agarose containing fibrillar  $\beta$ -amyloid peptide. The antibody is mixed with the  $\beta$ -amyloid-peptide containing agarose and incubated for a time sufficient to allow the fibrillar  $\beta$ -amyloid-specific antibodies to bind to the agarose. The unbound serum proteins are removed, and after thorough washing in an appropriate buffer, the bound antibodies may be eluted from the agarose with 4.0M to 4.5M MgCl<sub>2</sub>, for example, or 0.05M H<sub>3</sub>PO<sub>4</sub> or 0.2M HCl/lysine, pH 2.2, or 0.1M Li diiodosalicylate. The antibody containing fraction can be dialyzed in a Tris HCl buffer, pH 7.5, or other suitable buffer, and stored for later use. The purified antibodies can be stored for 1 month at 4°C in 50 mM Tris HCl buffer, pH 7.5, containing 10 mM NaN<sub>3</sub> and 50% glycerol. For long-term storage the preceding solution may be kept at -20°C.

**[0032]** Alternatively, antibodies may be purified on a beaded agarose matrix containing a covalently bound amyloid peptide. For instance, a peptide consisting

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of the first 11 residues of A $\beta$  is modified by the addition of a carboxyl-terminal cysteinyl residue. This peptide can be linked to epoxy-activated beaded agarose and provides a matrix with sufficient affinity to isolate the amyloid fibril binding antibodies. The antibodies can be eluted by the previously described solutions.

**[0033]** Antibody fragments that recognize specific epitopes can be generated by known techniques. For example, Fab and F(ab')<sub>2</sub> fragments of the invention can be produced as described in Example 1 by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')<sub>2</sub> fragments). Antibody fragments, as used herein, include, but are not limited to, Fab, Fab', F(ab')<sub>2</sub>. Fab fragments of anti-fibrillar amyloid antibodies retain the specificity for fibrillar amyloid.

**[0034]** The antibodies of the present invention may be modified for certain applications. For example, for *in vivo* use in humans, or for some *in vitro* detection assays, it may be preferable to use chimeric, hybrid, humanized, or human antibodies. For instance, human antibodies are potentially available from patients who were immunized with fibrillar  $\beta$ -amyloid. Methods for producing chimeric antibodies are known in the art (Riechmann L., Clark M, Waldmann H, Winter G. Reshaping human antibodies for therapy. Nature 1988; 332: 323-327). Antibodies can be humanized using a variety of techniques known in the art (Bruggemann, MS, Neuberger, MS. Strategies for producing human antibody repertoires in transgenic mice. Immunology Today 1996; 17:391-397).

#### Methods of Using Anti-Fibrillar $\beta$ -Amyloid Antibodies

**[0035]** Yet another embodiment of the present invention is to provide methods of using the antibodies of the invention, or fragments thereof, in immunoassays for the detection, localization, or isolation of fibrillar  $\beta$ -amyloid and/or diagnosis of fibrillar- $\beta$ -amyloid-related diseases in a subject. Accordingly, the antibodies and/or fragments thereof, of the present invention can be utilized in a variety of assays known to those skilled in the art, including but not limited to,

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immunohistochemical assays, Western Blot, dot blot, immunoprecipitation assays, amyloid sedimentation assays, sandwich and competition immunoassays, immunofluorescent protocols, radioimmunoassays, and/or immunoaffinity chromatography. Such assays are routine and well known in the art (see, Cell Biology, A Laboratory Handbook, J.Celis ed. 2nd edition, vol. 2, 1998, pp381-492).

**[0036]** The antibodies, or binding fragments thereof, according to the present invention, may be used to quantitatively or qualitatively detect the presence of fibrillar  $\beta$ -amyloid as described. This can be achieved, for example, by immunofluorescence techniques employing a fluorescently labeled antibody, coupled with light microscopic or fluorometric detection. The antibodies, or binding fragments thereof, according to the present invention may additionally be employed histologically, as in immunofluorescence, immunoelectron microscopy, or non-immuno assays, for the *in situ* detection of fibrillar amyloid, such as for use in monitoring, diagnosing, or detection assays. An example of such an application is demonstrated in Example 1, Figure 7a-g.

**[0037]** Immunoassay and non-immuno assays for fibrillar  $\beta$ -amyloid, or variants, or fragments thereof, typically comprise incubating a sample, such as a biological fluid, tissue extract, freshly harvested cells, or lysates of cells that have been incubated in cell culture, in the presence of a detectably-labeled antibody that recognizes a neo-epitope of fibrillar  $\beta$ -amyloid or fragments thereof, such as the antibodies, or binding fragments thereof, of the present invention. Thereafter, the bound antibody, or binding fragment thereof, is detected by a number of techniques well known in the art.

**[0038]** In a preferred embodiment, fibrillar  $\beta$ -amyloid is quantified by a competition ELISA method, which is described in Example 1. In brief, the antibody is incubated for 1-2 hr. with a sample that is thought to contain fibrillar amyloid. The mixture is transferred to a microtiter plate, whose wells are coated with  $A\beta_{1-16}$ .

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The antibody that did not bind to fibrillar amyloid in the sample then binds to the A $\beta$  peptide coating the well. The wells are then washed free of the sample-bound antibody, and the amount of antibody bound to the plate is determined. The amount of antibody bound to fibrillar amyloid in the sample is calculated by differences between the absorbances of wells containing no amyloid and those containing the amyloid sample, and the amount of fibrillar amyloid is determined by reference to a standard curve prepared from measurements on samples to which known amounts of fibrillar  $\beta$ -amyloid had been added.

**[0039]** In another embodiment, the biological sample may be brought into contact with, and immobilized onto, a solid phase support or carrier, such as nitrocellulose, or other solid support or matrix, which is capable of immobilizing cells, cell particles, membranes, or soluble proteins. The support may then be washed with suitable buffers, followed by treatment with the detectably-labeled antibodies or antibody fragments. The solid phase support may then be washed with buffer a second time to remove unbound antibody. The amount of bound label on the solid support may then be detected by conventional means. Accordingly, in another embodiment of the present invention, compositions are provided comprising the antibodies, or binding fragments thereof, bound to a solid phase support, such as described herein.

**[0040]** By solid phase support or carrier or matrix is meant any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, plastic, nylon wool, polystyrene, polyethylene, polypropylene, dextran, nylon, amylases, films, resins, natural and modified celluloses, polyacrylamides, agarose, alumina gels, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent, or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration as long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, cylindrical, as in the inside surface of a test tube, or the external surface of a

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rod. Alternatively, the surface may be flat, such as a sheet, film, test strip, stick, and the like. In addition, the solid support is preferably inert to the reaction conditions for binding and may have reactive groups, or activated groups, in order to attach the antibody, a binding fragment, or the binding partner of the antibody. The solid phase support may also be useful as a chromatographic support, such as the carbohydrate polymers Sepharose®, Sephadex®, or beaded agarose. Indeed, a large number of such supports for binding antibody or antigen are commercially available and known to those having skill in the art.

**[0041]** The binding activity for a given anti-fibrillar  $\beta$ -amyloid antibody may be determined by well-known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation. As mentioned previously, binding assays include, but are not limited to, sedimentation, ELISA, radioimmunoassays, and competition assays.

**[0042]** With respect to the anti-fibrillar  $\beta$ -amyloid antibodies, numerous ways to detectably label such protein molecules are known and practiced in the art. For example, one way the antibodies can be detectably labeled is by linking the antibody to an enzyme, e.g., for use in an enzyme immunoassay (EIA), (A. Voller et al., 1978, *The Enzyme Linked Immunosorbent Assay (ELISA)*, *Diagnostic Horizons*, 2:1-7; *Microbiological Associates Quarterly Publication*, Walkersville, MD; A. Voller et al., 1978, *J. Clin. Pathol.*, 31:507-520; J.E. Butler et al., 1981, *Meths. Enzymol.*, 73:482-523; *Enzyme Immunoassay*, 1980, (Ed.) E. Maggio, CRC Press, Boca Raton, FL; *Enzyme Immunoassay*, 1981, (Eds.) E. Ishikawa et al., Kigaku Shoin, Tokyo, Japan). The enzyme that is bound to the antibody reacts with an appropriate substrate, preferably a chromogenic substrate, so as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric, or by visual detection means. Nonlimiting examples of enzymes which can be used to detectably label the antibodies include malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast



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alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by calorimetric methods, which employ a chromogenic substrate for the enzyme, or by visual comparison of the extent of enzymatic reaction of a substrate compared with similarly prepared standards or controls.

**[0043]** A variety of other immunoassays may also be used for detection. For example, by labeling the antibodies, or binding fragments thereof, with a radioisotope, a radioimmunoassay (RIA) can be used to detect fibrillar  $\beta$ -amyloid (e.g., B. Weintraub, Principles of Radioimmunoassays, *Seventh Training Course on Radioligand Techniques*, The Endocrine Society, March, 1986). The radioactive isotope label can be detected by using a gamma counter or a scintillation counter or by radiography.

**[0044]** The antibodies, or their antigen-binding fragments can also be labeled using a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are, without limitation, fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. Detectably labeled fluorescence-emitting metals, such as  $^{152}\text{Eu}$ , or others of the lanthanide series, can be used to label the antibodies, or their binding fragments, for subsequent detection. The metals can be coupled to the antibodies via such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA), or ethylenediaminetetraacetic acid (EDTA).

**[0045]** The antibodies can also be detectably labeled by coupling them to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that develops during the course of a chemical reaction. Examples of particularly useful

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chemiluminescent labeling compounds include, without limitation, luminol, isoluminol, therromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Similarly, a bioluminescent compound may be used to label the antibodies of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Useful bioluminescent labeling compounds include luciferin, luciferase and aequorin.

**[0046]** In a preferred embodiment, the present invention relates to a method for studying the mechanism of  $\beta$ -amyloid fibril formation using the antibodies, and/or fragments of the invention. For example, without being bound by theory, the amyloid fibril is thought to be the final product in the process of amyloid peptide aggregation. Intermediate aggregation states, called oligomers and protofilaments, have been identified by electron microscopy. It is thought that oligomers and protofilaments are neurotoxic, but amyloid fibrils are non-toxic. The antibodies described herein can be used to observe the conversion of oligomers/protofilaments to fibrillar amyloid, and the conditions that promote or inhibit this conversion can be discovered. For instance, the binding of the antibodies to the growing fibrils can be followed by the sedimentation assay described in Example 1 of this application.

**[0047]** In a related embodiment, the invention provides a method for determining the effectiveness of a pharmacological agent or treatment for Alzheimer's disease by measuring the level of inhibition of fibril formation using the antibodies, and/or fragments of the invention. For example,  $A\beta$  monomers can be incubated with or without the pharmacological agent. At intervals, the antifibrillar amyloid antibody can be added and the amounts of fibril formation in the two experiments can be measured as described in Example 1. As an example of this application, 1 $\mu$ g portions of fibrillar  $A\beta$  are incubated with various concentrations of the pharmacological agent in a volume of 1mL of a physiological medium. At

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intervals aliquots containing 0.1 $\mu$ g (or less) of fibrillar amyloid are withdrawn and treated with 1 $\mu$ g of antifibrillar A $\beta$  IgG. After a 30 min. incubation the antibody-fibrillar A $\beta$  complexes are sedimented, and the amount of bound IgG is determined as described in Example 1. A control sample lacking the pharmacological agent is simultaneously run. The difference between the amount of antibody bound in the 2 samples indicates the amount of fibrillar A $\beta$  that was dissociated by the agent. The experiment can also be started with monomeric A $\beta$  to determine the effect of a pharmacological agent upon the rate of formation of fibrillar amyloid. The advantages of this technique over the current methods, which use dye-binding, are that it is about 1000-fold more sensitive, and it is not subject to interference by other substances such as serum proteins, which may bind dyes.

**[0048]** Another embodiment of the present invention provides diagnostics, diagnostic methods and imaging methods for Alzheimer's disease or other fibrillar  $\beta$ -amyloid-related diseases using the antibodies and binding fragments thereof, as described by the present invention. For example, the antibodies of the invention can be used for the neuropathological localization of cerebrovascular amyloid deposits in tissue sections of brains of Alzheimer disease patients. The methodology for performing this experiment is described in Example 1. An example of this application is depicted in Fig. 7 of Example 1. These anti-fibrillar A $\beta$  antibodies bind 1000-fold more tightly to fibrillar A $\beta_{1-40}$  or A $\beta_{1-42}$  than to non-fibrillar forms of A $\beta$ . Antibody concentrations and/or development times can be found, whereby fibrillar amyloid can be exclusively stained by the antibodies. As shown in Fig. 7, when the antibody concentration is lowered by the addition of non-fibrillar A $\beta_{1-16}$ , amyloid deposits in brain blood vessel walls are stained by the antibody, but parenchymal amyloid deposits are not stained. The latter amyloid deposits are not stained because they contain very low amounts of "full length" amyloid peptides, *ie*, those beginning with Asp-1.

**[0049]** The present invention is also directed to a method of localizing fibrillar  $\beta$ -amyloid in a subject by administering one or more of the antibodies

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described herein, or binding fragments thereof, to bind fibrillar  $\beta$ -amyloid within the subject and determining the location of the one or more antibodies within the subject. For such methods, the antibodies are preferably converted to Fab fragments. The Fab fragments may be labeled with a detectable and physiologically acceptable label, such as a radionuclide. In addition, according to the present invention, the stage of the disease, and/or effectiveness of treatment, can be monitored by determining the levels or changes over time of the specifically recognized fibrillar amyloid *in vivo*.

**[0050]** Another embodiment of the present invention relates to compositions comprising one or more of the antibodies, or binding fragments thereof, according to the invention, together with a physiologically- and/or pharmaceutically-acceptable carrier, excipient, or diluent. The antibodies, or binding fragments, specifically recognize a fibrillar  $\beta$ -amyloid neo-epitope. The fibrillar  $\beta$ -amyloid antigens are further characterized as described herein and above.

**[0051]** More specifically, formulations of the anti-fibrillar  $\beta$ -amyloid antibodies, or binding fragments thereof, are prepared for storage by mixing the antibodies or their binding fragments, having the desired degree of purity, with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 17th edition, (Ed.) A. Osol, Mack Publishing Company, Easton, PA., 1985), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols

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such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

**[0052]** Antibodies, or their binding fragments, to be used for *in vivo* administration must additionally be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The antibodies, or binding fragments thereof, ordinarily will be stored in lyophilized form or in solution.

**[0053]** Antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. The route of administration of the anti-fibrillar  $\beta$ -amyloid antibodies, or binding fragments thereof, in accordance with the present invention, is in accord with known methods, e.g., injection or infusion by intravenous, intraperitoneal, intramuscular, intraarterial, subcutaneous, intralesional routes, by aerosol or intranasal routes, or by sustained release systems as noted below. The antibodies, or binding fragments thereof, are administered continuously by infusion or by bolus injection. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer et al., 1981, *J. Biomed. Mater. Res.*, 15:167-277 and Langer, 1982, *Chem. Tech.*, 12:98-105), or poly(vinylalcohol)], polylactides (U.S. Patent No. 3,773,919; EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., 1983, *Biopolymers*, 22:547-556), non-degradable ethylene-vinyl acetate (Langer et al., *supra*), degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT.TM. (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

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**[0054]** While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in effectiveness. Rational strategies can be devised for antibody stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S--S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

**[0055]** In yet a further aspect of the invention, anti-fibrillar  $\beta$ -amyloid antibodies or binding fragments thereof, are provided labeled with a detectable moiety, such that they may be packaged and used, for example, in kits, to diagnose or identify fibrillar  $\beta$ -amyloid in diagnostic applications. The kits preferably contain an instruction manual for use of the kit. Non-limiting examples of such labels include fluorophores such as fluorescein isothiocyanate; chromophores, radionuclides, or enzymes. Such labeled antibodies or binding fragments may be used for the histological localization of the fibrillar  $\beta$ -amyloid, ELISA, cell sorting, as well as other immunological techniques for detecting or quantifying fibrillar  $\beta$ -amyloid, and cells bearing fibrillar  $\beta$ -amyloid, for example.

### **EXAMPLES**

**[0056]** The following example describes specific aspects of the invention to illustrate the invention and provide a description of the present methods for those of skill in the art. The example should not be construed as limiting the invention, as the example merely provides specific methodology useful in understanding and practice of the invention and its various aspects.

#### **Example 1**

### **The humoral immune response to fibrillar $\beta$ -amyloid peptide**

#### **Methods**

**[0057]** Human  $A\beta_{1-40}$  and  $A\beta_{1-42}$  were purchased from California Peptide Co. (Napa, CA) and from Bachem Bioscience, Inc. (King of Prussia, PA). Anaspec (San Jose, CA) supplied the  $A\beta$  subsequences 1-11, 1-16, 1-28, 10-20, 17-40, and 25-35. Invitrogen (Grand Island, NY) synthesized  $A\beta_{1-8}$  and  $N_1$ -Cys- $\epsilon$ -aminohexanoyl- $A\beta_{1-8}$  (Cys-ahx- $A\beta_{1-8}$ ). The following numbering denotes the subsequences:

$D^1AEFRHDSGY^{10}EVHHQKLVFF^{20}AEDVGSNKGA^{30}IIGLMVGGVV^{40}IA$ . The concentrations of  $A\beta$  and of peptides containing tyrosine were measured by UV spectrophotometry using an extinction coefficient at 280 nm of 1280 (Edelhoch, H. (1967) *Biochemistry* 6, 1948-54).

**[0058]** *Preparation and assay of fibrillar  $A\beta_{1-42}$ .* Fibrillar  $A\beta_{1-42}$  (f $A\beta$ ) was prepared by two different methods. In the first method, the peptide was dissolved and disaggregated in 0.05M  $NH_3$  at a concentration of about 1 mg/mL, and the solution was clarified by centrifugation for 3 min. at 12,000g. The solution was adjusted to pH 7.0 with  $NaH_2PO_4$  to give a final peptide concentration of 0.5 mg/mL in 0.05 M phosphate; to inhibit bacterial growth and oxidation, 3 mM  $NaN_3$  and 5 mM DTT were added. The solution was seeded with 1  $\mu$ g of fibrillar  $A\beta_{1-42}$  and was subjected to slow rotary mixing for 2 days, when aggregation, as measured by thioflavin T binding, was complete. In the second method the peptide was dissolved in hexafluoroisopropanol, dried, redissolved in  $H_2O$  and adjusted to pH 7.0, 0.05M sodium phosphate. Aggregation in these and other buffers (Dulbecco's phosphate-buffered saline or 50 mM Tris HCl, pH 7.5, 0.15 M NaCl) gave similar extents of thioflavin T binding. Thioflavin T binding was performed by a modification of the method of LeVine (LeVine, H., 3<sup>rd</sup> (1999) *Methods Enzymol* 309, 274-84). About 2  $\mu$ g of fibrillar  $A\beta_{1-42}$  was mixed with 2 mL of 5  $\mu$ M thioflavin T in 50 mM Tris HCl, pH 7.5, 0.15M NaCl. After 1 min., the fluorescence was read

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(450 nm excitation, 486 nm emission) in a Perkin-Elmer Model LS-5 spectrofluorometer. The fluorescence yield was about a factor of 10 greater than the background fluorescence.

**[0059]** *Generation of antisera.* Six rabbits (R261, R262, R285, R286, R333 and R334) were immunized with fibrillar A $\beta$ <sub>1-42</sub> according to the following schedule: 1) 100  $\mu$ g of FA $\beta$  and complete Freund's adjuvant (1:1), subcutaneously at day 1; 2) 100  $\mu$ g in incomplete Freund's adjuvant (IFA), subcutaneously on day 14; 3) 150  $\mu$ g of fA $\beta$  mixed with 4 mg of aluminum ammonium sulfate, intraperitoneally on day 30. Animals were bled at intervals of 3, 5 and 7 weeks after the first injection. The immunization was continued using IFA every two to three weeks until antibodies with high affinity were produced. The following additional rabbit antibodies were raised to peptides conjugated to keyhole limpet hemocyanin: R321 and R165, specific for the carboxyl terminus of A $\beta$ <sub>1-42</sub> (residues 35-42); and R287, to A $\beta$  residues 27-37.

**[0060]** *Other antibodies.* Monoclonal antibodies 4G8 (Kim, K.S. et al. (1988) *Neuroscience Research Communications* 2, 121-130), specific for A $\beta$  residues 16-24, and 6E10 (Kim, K.S. et al. (1990) *Neuroscience Research Communications* 7, 113-122.), which recognizes an epitope in residues 3-16, are available from Signet Laboratories. Alkaline phosphatase-conjugated secondary antibodies were obtained from Biosource International. The biotinylated secondary antibodies used for immunohistochemistry were products of Amersham Pharmacia Biotech.

**[0061]** *Antibody purification.* A $\beta$ -binding antibodies were purified on A $\beta$ <sub>1-16</sub>-agarose prepared by the reaction of A $\beta$ <sub>1-16</sub> modified by the addition of a C-terminal cysteinyl residue with epoxy-activated agarose (Sigma). One mL of antiserum was mixed with 0.3 mL of A $\beta$ <sub>1-42</sub> agarose for 2 hr. The unbound serum proteins were removed, and after thorough washing with 50 mM Tris HCl, pH 7.5, 0.25 M NaCl, the bound antibodies were eluted with 4.5 M MgCl<sub>2</sub>. The IgG-containing fraction was dialyzed against 50 mM Tris HCl, pH 7.5 and was stabilized with 1 mg/mL



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BSA and 3 mM  $\text{NaN}_3$ . Later work showed that all of the  $\text{A}\beta$ -specific antibodies could be eluted with 50 mM  $\text{H}_3\text{PO}_4$ .

**[0062]** *Fab fragment preparation.* The IgG fraction of the antiserum was prepared by Protein A-agarose chromatography using reagents from Pierce-Endogen. Fab fragments were prepared with the aid of a Pierce-Endogen kit according to supplier's directions. The papain digestion time was minimized to optimize the recovery of active Fab fragments. Undigested IgG was removed by Protein A chromatography, and the preparation was analyzed by PAGE to confirm the absence of undigested IgG. The Fab preparation was further purified on a Pharmacia Superose 12 HR gel filtration column. All of the fibrillar  $\text{A}\beta$ -binding activity emerged in an elution volume expected for a 50-kDa protein, *ie.*, slightly later than BSA.

**[0063]** *Quantitative immunoblotting procedures.* A previously described method (Potempska, A. et al. (1999) *Amyloid* 6, 14-21) was used to measure the titers and specificities of the antisera and to determine the stoichiometry of antibody-binding to  $\text{A}\beta$  fibrils. Two Protein A-purified rabbit IgG preparations were quantified by spectrophotometry, using an absorbance value of 1.40 for a 1-mg/mL solution (Miller, F., and Metzger, H. (1965) *J Biol Chem* 240, 4740-5). To measure the amounts of IgG bound to  $\text{A}\beta$  fibrils the samples and standards were dissociated in a sample buffer containing 10 mM dithiothreitol and were subjected to PAGE. The proteins were electroblotted to a nitrocellulose membrane, treated with goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Biosource), and developed with BCIP/NBT. The 55-kDa IgG heavy chains were quantified by photodensitometry using IPLab Gel software from Scanalytics. The  $\text{A}\beta_{1-42}$  contents of fibrils were measured on a duplicate blot developed with affinity-purified antibody R321, which is specific for the C-terminus of  $\text{A}\beta_{1-42}$ . Known amounts of fibrillar  $\text{A}\beta_{1-42}$  served as standards. Fibrillar  $\text{A}\beta_{1-42}$  samples were disaggregated by a brief treatment with 100  $\mu\text{l}$  of 98% formic acid, which was removed by centrifugal vacuum evaporation.

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**[0064]** *Fibril-binding assays.* The fibrillar A $\beta$ <sub>1-42</sub> preparation was initially sedimented for 3 min. at 12,000g to remove any unaggregated A $\beta$  and slowly sedimenting aggregates. The antibody preparations were clarified under the same conditions to remove any antibody aggregates. In a 1.5 mL microcentrifuge tube 0.1-0.5  $\mu$ g of fibrillar A $\beta$ <sub>1-42</sub> was incubated at ambient temperature with varying amounts of antibody in 100  $\mu$ L of binding buffer (25 mM sodium phosphate, pH 7.0, 250 mM NaCl, 3 mg/mL BSA, and 0.04% TWEEN 20). After 1 hr of gentle agitation on a vortex mixer the mixtures were diluted with 0.5 mL of wash buffer (100 mM Tris HCl, pH 7.5, 250 mM NaCl, 0.04% TWEEN), and the fibril-antibody complexes were sedimented at 12,000g for 3 min. About 95% of the supernatant liquid was aspirated, and the process was repeated twice. The sedimented fibrils were then suspended in 100  $\mu$ L of binding buffer containing 650 ng of goat anti-rabbit Ig coupled to alkaline phosphatase (from Tago Immunochemicals). After a 1 hr gentle incubation on the vortex mixer the fibrils were washed 3 times as previously described and suspended in 0.5 mL of phosphatase buffer (100mM Tris HCl, pH 8.8, 1 mM MgCl<sub>2</sub> ). An aliquot of the suspension was transferred to a fresh tube and was incubated with 1mM nitrophenyl phosphate (NPP) in phosphatase buffer. After about 1 hr, a period sufficient to yield an absorbance at 415 nm of about 0.6, the reaction was stopped by the addition of 10mM EDTA, and the absorbances were measured. Controls lacking fibrils or primary antibody exhibited absorbances less than 5% of those of samples containing immune antisera.

**[0065]** *Phage display epitope mapping.* The Ph.D.-7 phage display system (New England Biolabs) was used for epitope mapping of antibody R286. The panning was performed in a 96-well polystyrene microtiter plate at room temperatures following the protocol suggested by the supplier, with some modifications. Briefly, each well was coated with 100  $\mu$ L of antibody R286 (10  $\mu$ g/mL in 0.1 M sodium bicarbonate) for 60 minutes. The well was then blocked for 60 minutes before incubating with 100 l phage display library (2x10<sup>11</sup> PFU) for an

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additional 60 minutes to allow phage binding to coated antibody. After removing unbound phages by extensive washings, bound phages were then eluted by incubating each well with 100  $\mu$ L of 0.2M glycine (pH 2.5) for 10 minutes and immediately neutralized by mixing with 50  $\mu$ L 1M Tris (pH 8.0). The eluted phage was titered and amplified for the next round of panning. After four rounds of panning 10 random phage clones were selected for DNA sequencing.

**[0066]** *Rabbit antisera titer.* Wells of a microtiter plate were coated with 100  $\mu$ L of peptide (10  $\mu$ L of a 1mg /mL antigen preparation in 10  $\mu$ L 0.05M carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plate was washed with 0.01M PBS, pH 7.2, with 0.05% Tween 20 (PBST) and blocked with 10% normal sheep sera in PBS for 1 hr at room temperature. Rabbit antiserum were diluted from 1:1000 with 0.5% BSA in PBST, and 10 serial dilutions were made. The blocked plate was washed, and 100  $\mu$ L of diluted rabbit antisera were added to the wells and incubated for 2 hours at room temperature. The plate was washed, and 100  $\mu$ L of goat anti-rabbit IgG conjugated to alkaline phosphatase (BioSource), diluted 1:1000 with 0.5% BSA in PBST, were added and incubated for 2 hr at room temperature. The plate was washed and developed using a solution of p-nitrophenyl phosphate in 10% diethanolamine, pH 9.8. After 30 minutes the absorbance at 405nm was read using an automated microplate reader. The antibody titer was determined at a dilution showing an absorbance of about 1.0.

**[0067]** *Inhibition assay by ELISA.* Wells of a microtiter plate were coated with peptide and blocked as described earlier. The peptide used as an inhibitor was serially diluted, and the antibody was diluted in 0.5% BSA in PBST. The antibody control was a 1:2 dilution of the antibody in buffer. Equal volumes of inhibitor and antibody solutions were combined in a test tube, mixed and incubated at room temperature for 2 hours and then overnight at 4°C. The plate was washed, and 100  $\mu$ L of the different antigen-antibody solutions were added to the wells and incubated for 2 hours at room temperature. After washing, 100  $\mu$ L of goat anti-rabbit IgG conjugated to alkaline phosphatase diluted 1:1000 with 0.5%

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BSA in PBST was added and incubated for 2 hours at room temperature. The plate was washed and developed as above. When the antibody control wells reached an OD of approximately 1.0 at 405 nm, the plate was read using an automated microplate reader.

**[0068]** *Electron microscopy.* A drop of a solution containing fibrillar A $\beta$  (0.2-1  $\mu$ M as monomers) was applied to a 400-mesh formvar-coated grid. The grid was drained, washed with glass distilled deionized water, and treated with a solution of 1% BSA and 0.05% Tween-20 in PBS for 10 min. If the sample contained bound IgG, it was incubated with 1% protein A labeled with 10nm colloidal gold (Sigma) at a concentration of 0.05 A<sub>520</sub>/mL in BSA/PBS for 1 hr. All grids were rinsed with about 20 drops of BSA/PBS and 20 drops of distilled water. The grid was then negatively stained with 1% uranyl acetate, washed with 4 drops of water, drained and air dried. Samples were examined and photographed at a magnification of 10,000 x or 40,000 x in a Hitachi 7000 electron microscope.

**[0069]** *Immunohistochemistry.* Portions of brains from AD victims were fixed in 10% formalin for more than 1 month, dehydrated in ethanol, embedded in paraffin, and cut into 8-mm-thick serial sections. The endogenous peroxidase in the sections was blocked with 0.2% hydrogen peroxide in methanol. Some of the sections were treated with 90% formic acid for 30 min. The sections were then treated with 10% fetal bovine serum in PBS for 30 min to block non-specific binding. The antibodies were diluted in 10% fetal bovine serum in PBS and were incubated with the sections overnight at 4°C. The sections were washed and treated for 30 min. with either biotinylated sheep anti-mouse IgG antibody or biotinylated donkey anti-rabbit IgG antibody (each at a dilution of 1:200). The sections were treated with extravidin peroxidase conjugate (1:200) for 1 hr followed by diaminobenzidine (0.5 mg/mL) and 1.5% hydrogen peroxide in PBS. The sections were counterstained with cresyl violet.

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## **RESULTS**

**[0070]** *Fibril-binding assay.* Our initial objective was to determine whether the antibodies generated by immunization with fibrillar A $\beta$ <sub>1-42</sub> specifically bound to the fibrils. Due to the conceptual and experimental limitations of filtration and fibril immobilization methods to detect and quantify these antibodies, a sedimentation procedure was developed. Its advantages include the negligible level of non-specific binding and the certainty that the observed binding is not due to a low level of monomeric A $\beta$ . In addition the antibody-fibril complexes readily can be characterized by other techniques, such as EM and Western blotting. Its principal drawback is that it is tedious. Although the 0.1-mg aliquot of fibrillar amyloid used in the assay is invisible, about 80% of it can be recovered at the end of the assay. Loss of fibrillar A $\beta$ <sub>1-42</sub> due to dissolution is not a problem. Using the thioflavin T-binding assay we could detect no loss of A $\beta$ <sub>1-42</sub> kept for 70 hr at ambient temperature at a concentration of 2  $\mu$ g/mL in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl. The assay conditions were optimized for the amounts and incubation times of the primary and secondary antibodies. The amount of anti-fibrillar A $\beta$ <sub>1-42</sub> antibody was generally 10-20% of the binding capacity of the fibrils, and the amount of secondary antibody conjugate was about 5-10-fold greater than the amount of bound primary antibody. The half-times of the antibody binding reactions were 10-15 min. The 1-hr incubation periods allow both binding reactions to proceed nearly to completion. At subsaturating concentrations of primary antibody the rate of nitrophenolate production directly increases with the concentration of primary antibody (Fig. 1A). At higher concentrations of primary antibody the reaction rate reaches a plateau, where all of the fibril binding sites are filled. Similarly, at a fixed concentration of primary antibody the amount of sedimentable antibody increases as the amount of fibrillar A $\beta$ <sub>1-42</sub> is increased (Fig. 1B) until the entire amount of the fibril-specific antibody was bound.

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**[0071]** Identification of *fibril-binding antibodies*. After 2 injections of fibrillar A $\beta$ <sub>1-42</sub>, antibodies appeared, which were detectable by ELISA on A $\beta$ <sub>1-42</sub>-coated plates and by the fibril-binding assay (Table 1).

**Table 1. ELISA titers and fibril-binding activities of anti-fibrillar Ab antisera**

Antiserum	ELISA <sup>a</sup>	Fibril binding <sup>b</sup>
R262	45,000 (100)	0.63 (100)
R286	32,000 (71)	0.49 (78)
R333	11,000 (24)	0.29 (46)
R334	38,000 (84)	0.76 (120)
R286 preimmune	<100 (<0.3)	0.02 (<3)

<sup>a</sup>The ELISA was performed on a plate coated with A $\beta$ <sub>1-42</sub> as described in the text. <sup>b</sup> 0.5 $\mu$ L of each antiserum was incubated with 0.5 $\mu$ g fibrillar A $\beta$ <sub>1-42</sub> and processed by the sedimentation assay as described in the text. Results are  $\Delta A_{415}$ . Numbers in parentheses are % of the value for antiserum R262.

**[0072]** In contrast, these antisera did not react with A $\beta$ <sub>1-42</sub> on Western blots, at which concentrations other anti-A $\beta$  antisera reacted (result not shown.) This apparent lack of reactivity with monomeric A $\beta$  on Western blots suggested the possibility that the anti-fibrillar A $\beta$  antisera recognized an epitope that was unique to the fibrillar form of A $\beta$ . To confirm that the antibodies bound to A $\beta$  fibrils we examined the antibody-fibril complexes by electron microscopy after labeling with immunogold particles. The gold particles were predominantly associated the fibrils (Fig.2), which confirmed that the fibrils contained an epitope recognizable by the antibody. Further ELISA experiments (see Fig. 3) revealed that the antisera bound to wells coated with A $\beta$ <sub>1-28</sub>, a fragment with little fibril-forming tendency. This result suggested that the antiserum might also bind to a linear epitope; however, the antiserum did not bind to wells coated with A $\beta$ <sub>1-16</sub>, A $\beta$ <sub>10-20</sub>, or A $\beta$ <sub>17-42</sub>. To resolve this apparent paradox, we performed inhibition assays with the peptides (Fig. 3). In this assay A $\beta$ <sub>1-16</sub>, A $\beta$ <sub>1-11</sub> and A $\beta$ <sub>1-8</sub> completely blocked antibody-binding to wells coated with A $\beta$ <sub>1-42</sub>; whereas, Cys-ahx-A $\beta$ <sub>1-8</sub>, and A $\beta$ <sub>3-11</sub> did not inhibit antibody

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binding. We interpret these results to indicate that the antibodies are monospecific for an epitope within residues 1-8 and that binding requires an unmodified amino-terminal aspartyl residue. The lack of reactivity of the antisera with A $\beta$ <sub>1-16</sub> in the direct ELISA suggests that the epitope is masked when the peptide binds to the polystyrene plate. By an M13 phage display system the epitope also was determined to be located with residues 1-7 (Table 2).

**Table 2. Antibody R286 epitope identified by phage display**

Residue number	1 2 3 4 5 6 7
A $\beta$ sequence	<b>D A E F R H D</b>
Phage sequence A	<b>D A E F R N L</b>
B	<b>D A E F R T A</b>
C	<b>D A E S R T V</b>
D	<b>D A E I R N H</b>
E	<b>D A E P R R L</b>

**[0073]** Although the first 3 residues, aspartyl-alanyl-glutamyl, and arginyl-5 occur in all of the recognized sequences, there is some variability in the subsequent residues, which suggests that the antibodies are less specific for these residues. To determine whether the antibodies that bound to A $\beta$ <sub>1-8</sub> also bound to fibrillar A $\beta$ <sub>1-42</sub> we purified R262 and R286 by adsorption to an A $\beta$ <sub>1-16</sub> agarose affinity matrix. The antibodies adsorbed and recovered from the affinity matrix amounted to about 3% of the total IgG in the antisera. Both the purified antibodies and the depleted antisera were analyzed by ELISA and by the fibril-binding assay (Table 3).

**Table 3. ELISA and fibril-binding of affinity-purified antibody R286**

	ELISA titer <sup>a</sup>	Fibril-binding activity
Crude antiserum	34,000	0.30 $\pm$ 0.03
Unbound antiserum	70	0.020 $\pm$ 0.001
4 M MgCl <sub>2</sub> eluate <sup>b</sup>	7,500	0.13 $\pm$ 0.01

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<sup>a</sup>Assays were performed on a plate coated with A $\beta$ <sub>1-28</sub> as described in the text.

<sup>b</sup>Results were corrected by a factor of 2.5 for dilution during dialysis.

**[0074]** The ELISA revealed that virtually the entire amount of the A $\beta$ <sub>1-8</sub> binding antibody had been removed from the antiserum. Nearly all of the fibril-binding activity was also adsorbed to the affinity matrix, which indicated that the fibril-binding antibody did not necessarily recognize a topological epitope in the fibrils. Regardless of whether the wells were coated with A $\beta$ <sub>1-28</sub> or the full-length peptide, the affinity-purified antibody exhibited the same A $\beta$ <sub>1-11</sub>-binding properties as the crude antiserum (Fig. 4), which confirmed that the purified antibody contained the same A $\beta$ -binding activity as the crude antiserum.

**[0075]** *Detection and quantification of fibril epitopes.* The stoichiometry of antibody molecules bound per A $\beta$ <sub>1-42</sub> monomer was determined by measuring the amounts of antibody co-sedimenting with A $\beta$ <sub>1-42</sub> fibrils at saturating antibody concentrations. The ratio of antibody molecules to A $\beta$ <sub>1-42</sub> monomers was calculated to be 0.3 +/- 10% (n = 6). This ratio is about 6-fold higher than we expected from the presumed sizes of the IgG molecule and the fibril epitope (see Discussion). Other exposed peptide sequences in A $\beta$ <sub>1-42</sub> fibrils can be identified by the fibril-binding assay (Table 4).

**Table 4. Exposure of epitopes in fibrillar A $\beta$ <sub>1-42</sub>**

Antibody	Epitope residues	Binding activity <sup>a</sup>
R286	1-8	67 ± 4
R287	27-37	46 ± 16
R165	35-42	5 ± 1
6E10	3-16	44 ± 9
4G8	16-24	55 ± 4

<sup>a</sup>Binding activities were measured by the sedimentation assay. Similar amounts of antibodies (based upon ELISA titers) were used. The activities were expressed as the nitrophenolate absorbance of the sample relative to that of the non-immune control serum.



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**[0076]** Antibodies 6E10, 4G8 and R287 bound to the fibrils much more extensively than did non-immune control antibodies, but R165, which recognizes the C-terminal sequence of A $\beta$ <sub>1-42</sub>, bound scarcely more than the control. The determination of the binding stoichiometries of these antibodies would provide an accurate measure of the relative exposure of the epitopes in the fibrils.

**[0077]** *Relative affinities of the R286/R262 epitope in the A $\beta$  monomer and in the fibril.* The affinity of R262 or R286 for the epitope in monomeric A $\beta$  relative to that in the A $\beta$  fibril was studied by measuring the extent to which A $\beta$  peptides inhibited the binding of the affinity-purified antibody to A $\beta$ <sub>1-42</sub> fibrils. Two versions of the fibril-binding assay were performed. In the first version the antibody was preincubated with the monomer before adding the fibrils, and in the second version the peptide was added to the pre-formed antibody-fibril complex. In both protocols the extents of inhibition were similar (Table 5), which indicated that during the 1-hour incubation the ternary mixture reached equilibrium. The R262 and R286 antibodies bound much more strongly to the epitope in fibrils than in the monomeric peptides (Table 5).

**Table 5. Inhibition of R262 antibody binding to fibrillar A $\beta$ <sub>1-42</sub> (fAb)<sup>a</sup>**

[Inhibitor], $\mu$ M	[fA $\beta$ ], $\mu$ M	protocol <sup>b</sup>	%inhibition	K <sub>rel</sub> <sup>c</sup>
A $\beta$ 1-16, 25	0.16	1	36 $\pm$ 2	0.0045
82	0.16	1	55 $\pm$ 2	0.0033
82	0.16	2	49 $\pm$ 3	0.0026
250	0.16	1	64 $\pm$ 2	0.0017
A $\beta$ 1-40 <sup>d</sup> , 27	0.16	1	69 $\pm$ 2	0.014
A $\beta$ 1-42 <sup>d</sup> , 1.7	0.18	1	6.6 $\pm$ 1.3	0.006

<sup>a</sup>Measurements were performed by the sedimentation assay using 1.7 nM affinity-purified R262 antibody. <sup>b</sup>Protocol 1: antibody and inhibitor were preincubated for 30 min. at 37°C; protocol 2: antibody and fibrils were preincubated for 30 min at 37°C. <sup>c</sup>K<sub>rel</sub> = K<sub>f</sub>/K<sub>i</sub>, as defined in the text. <sup>d</sup>monomeric species.

**[0078]** The ratio of the dissociation constant of the fibril-antibody complex ( $K_B$ ) to that of the peptide-antibody complex ( $K_1$ ) could be calculated from the expression:  $K_{rel} = K_B/K_1 = ([B]/[I]) \times ([AI]/[AB])$  where [B], [I], [AI] and [AB] are,

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respectively, the concentrations of fibrillar amyloid (expressed as monomers), inhibitor, antibody-inhibitor complex and antibody-fibril complex. The ratio  $K_{rel}$  is much less than unity, which indicates that  $K_B$  is much smaller than  $K_I$ . The  $K_{rel}$  values in Table 5 were calculated using the concentrations of  $A\beta$  monomers in fibrillar  $A\beta$ . Since only a fraction of the  $A\beta$  monomers can bind the antibody, the concentration of fibrillar amyloid should be proportionately lowered, which would lower  $K_{rel}$ . The value of  $K_{rel}$  was not constant, but decreased as the concentration of inhibitor was increased. This trend may result from heterogeneity in the fibril binding sites such that the stronger binding sites are less readily displaced. The higher affinity of the  $A\beta$  fibrils for these antisera also could be demonstrated by ELISA (Fig.5). Fibrillar  $A\beta_{1-42}$  produced a 50% inhibition of the binding of R286 antibody to coated  $A\beta_{1-28}$  at a concentration over 1000-fold lower than the  $A\beta_{1-11}$  concentration needed to produce the same degree of inhibition. As in Table 5, the fibrillar  $A\beta$  concentration was expressed as monomers. The longer peptides,  $A\beta_{1-28}$ ,  $A\beta_{1-40}$  and  $A\beta_{1-42}$  appear to be better inhibitors (Table 5). It is possible that the secondary or tertiary structures of these peptides affect the conformation of the amino terminal epitope. Alternatively, there might be an unrecognized effect of anchoring the epitope to a larger structure, somewhat analogous to the equilibrium isotope effects observed in chemical reactions. To test this hypothesis  $A\beta_{1-16}$  was anchored to epoxy-activated agarose through a C-terminal cysteinyl residue, and its affinity for R262 antibody relative to that of soluble  $A\beta_{1-16}$  was determined (Table 6).

**Table 6. Inhibition of R286 antibody binding to agarose-linked  $A\beta_{1-16}$ <sup>a</sup>**

[ $A\beta_{1-16}$ inhibitor], $\mu$ M	[Matrix-bound $A\beta_{1-16}$ ], $\mu$ M	% inhibition
6	1	61 $\pm$ 2
17	1	80 $\pm$ 1
57	1	92 $\pm$ 1

<sup>a</sup>Measurements were performed as described in the text. The mixtures contained 23 nM affinity-purified R286 antibody.

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**[0079]** In this case the soluble peptide extensively displaced the antibody from the matrix, which indicated that the immobilization of the epitope on a large structure does not account for the higher affinity of fibrillar A $\beta$ . If the antibodies raised to fibrillar A $\beta$  were specifically selected for fibril binding, then antibodies raised to linear A $\beta$  epitopes might not selectively bind to fibrillar A $\beta$ . To test this hypothesis we measured the relative affinities of fibrillar A $\beta_{1-42}$  for mAb 6E10 (specific for residues 3-12) and mAb 4G8 (specific for residues 16-24). In contrast to its effect on antibodies R286 and R262, the monomeric peptide A $\beta_{1-28}$  strongly inhibited the binding of antibodies 6E10 and 4G8 to the fibrils (Table 7).

**Table 7. Inhibition of antibody binding to fibrillar A $\beta_{1-42}$  by A $\beta_{1-28}$ <sup>a</sup>**

[Antibody] nM	[Ab1-28] $\mu$ M	% inhibition
mAb 6E10, 9	6.1	78 $\pm$ 1
mAb 4G8, nd	6.1	89 $\pm$ 2

<sup>a</sup>Measurements were performed by the sedimentation assay using fibrillar A $\beta_{1-42}$  (2.2 $\mu$ M monomers). The concentration of mAb 4G8 was not determined; its ELISA titer was similar to that of mAb 6E10.

**[0080]** Similarly, by ELISA, A $\beta_{1-16}$  was about as effective as fibrillar A $\beta_{1-42}$  as an inhibitor of the binding of mAb 6E10 to A $\beta_{1-28}$ -coated plates (Fig.6). Thus some antibodies directed to A $\beta$  epitopes bind about as well to monomeric peptides as to fibrils.

**[0081]** *Relative affinities of IgG and the Fab fragment.* A possible explanation for the enhanced affinity of R262 and R286 is that their bivalent character allowed them simultaneously to bind 2 epitopes on the fibril, which would decrease the unfavorable entropy component of complex formation. To test this hypothesis, we prepared monovalent R286 Fab fragment and tested its relative affinity for fibrillar A $\beta_{1-42}$ . Contrary to this hypothesis, in the presence of excess A $\beta_{1-28}$ , the R286 Fab fragment preferentially bound to fibrillar A $\beta$  (Table 8).

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**Table 8. Inhibition by Ab 1-28 of R286 Fab fragment binding to fibrillar A $\beta$ <sub>1-42</sub><sup>a</sup>**

[Ab 1-28], $\mu$ M	% inhibition
A $\beta$ <sub>1-28</sub> , 25	42 $\pm$ 7
82	61 $\pm$ 4
250	71 $\pm$ 6

<sup>a</sup>Measurements were performed by the sedimentation assay using a Fab preparation estimated to contain 7 nM A $\beta$ -specific fragments. The fibrillar A $\beta$  concentration was 0.17  $\mu$ M monomers.

**[0082]** At similar concentrations of inhibitor peptide, the binding of R286 Fab (Table 8) and R262 IgG (Table 5) are inhibited to similar extents. Previous experiments had shown that the binding properties of R262 and R286 antibodies are indistinguishable.

**[0083]** *Immunoreactivity of R286 IgG with brain amyloid deposits.* A $\beta$ <sub>1-42</sub> fibrils generated *in vitro* may not have the same structure as  $\beta$ -amyloid fibrils that form in the AD brain parenchyma and vessels. Using AD brain sections we compared the immunoreactivities of affinity-purified R286 IgG and mAb 6E10 with and without A $\beta$ <sub>1-16</sub> added as a blocking peptide. R286 did not react well with parenchymal amyloid unless the slides were pre-treated with formic acid (Fig. 7a), an enhancer of brain amyloid immunoreactivity (Kitamoto, T. et al. (1987) *Lab Invest* 57, 230-6). In contrast, under our staining conditions 6E10 reacted with parenchymal amyloid even without formic acid pre-treatment (Fig. 7b). The binding of each antibody was blocked by added A $\beta$ <sub>1-16</sub> (Fig. 7c,d). These findings suggested that parenchymal amyloid does not possess the same structure as *in vitro* generated fibrillar A $\beta$ ; however, it might also be argued that formic acid treatment disaggregated the fibrillar structure, which allowed R286 IgG to bind to amino-terminal sequences. A strikingly different result was obtained from the immunostaining of cerebellar vascular amyloid. In this tissue both antibodies stained the amyloid without formic acid pre-treatment (Fig. 7e,f); however, the binding of mAb 6E10 was almost completely blocked by the addition of A $\beta$ <sub>1-16</sub> (Fig.7h); whereas, the binding of R286 IgG was scarcely diminished (Fig. 7g). We

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concluded that cerebellar vascular amyloid contains fibrils with binding properties similar to *in vitro* generated fibrillar amyloid. The methods and results reported above are further described in Miller, et al. (2003) Biochemistry 42, 11682-11692.

**[0084]** The contents of all issued and granted patents, patent applications, published PCT and U.S. applications, articles, books, references, reference manuals and abstracts as referenced or cited herein are hereby incorporated by reference in their entireties to more fully describe the state of the art to which the invention pertains.

**[0085]** As various changes can be made in the above-described subject matter without departing from the scope and spirit of the present invention, it is intended that all subject matter contained in the above description, or defined in the appended claims, be interpreted as descriptive and illustrative of the present invention. Many modifications and variations of the present invention are possible in light of the above teachings.

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**WHAT IS CLAIMED IS:**

1. An antibody, or binding fragment thereof, which binds specifically to fibrillar  $\beta$ -amyloid.
2. The antibody, or binding fragment thereof, according to claim 1, wherein the antibody, or binding fragment thereof, binds to fibrillar  $\beta$ -amyloid with at least about 1000-fold greater affinity for fibrillar  $\beta$ -amyloid than to the non-fibrillar peptide.
3. The antibody, or binding fragment thereof, according to claim 1 or 2, wherein the binding fragment is selected from the group consisting of Fab, Fab', F(ab')<sub>2</sub>.
4. The antibody according to claim 2, wherein the antibody is R286.
5. The antibody, or antibody fragment thereof, according to claim 1 or 2, bound to a solid matrix.
6. A method of producing an antibody specific for fibrillar  $\beta$ -amyloid, comprising:
  - a. administering fibrillar  $\beta$ -amyloid to a host; and
  - b. isolating from the host antibodies to fibrillar  $\beta$ -amyloid.
7. The method according to claim 6, wherein the host is a rabbit.
8. The method according to claim 6, wherein the fibrillar  $\beta$ -amyloid is formed from peptide monomers selected from the group consisting of  $A\beta_{1-40}$ ,  $A\beta_{2-40}$ ,  $A\beta_{2-42}$ ,  $A\beta_{3-40}$ ,  $A\beta_{3-42}$ ,  $A\beta_{1-38}$ ,  $A\beta_{[\text{pyroglu-3}]-42}$ ,  $A\beta_{11-42}$ ,  $A\beta_{17-42}$ , and  $A\beta_{25-35}$ .
9. An antibody, or antibody fragment thereof, prepared according to the method of claim 6.
10. A method of detecting fibrillar  $\beta$ -amyloid in a sample, comprising:

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- a. labeling an antibody, or antibody fragment thereof, according to claim 1, with a detectable label;
- b. allowing the detectably-labeled antibody, or binding fragment thereof, to bind fibrillar  $\beta$ -amyloid in the sample; and
- c. detecting the presence of the labeled antibody, or antibody fragment thereof, in the sample.

11. A method of studying  $\beta$ -amyloid fibril formation in a sample, comprising:

- a. labeling an antibody, or antibody fragment thereof, according to claim 1, with a detectable label;
- b. allowing the detectably-labeled antibody, or binding fragment thereof, to bind to fibrillar  $\beta$ -amyloid in sample containing  $\beta$ -amyloid oligomers and protofilaments; and
- c. detecting the conversion of  $\beta$ -amyloid oligomers and protofilaments to fibrillar amyloid by detecting the presence of the labeled antibody, or antibody fragment thereof, in the sample.

12. The method according to claim 11, wherein the binding of the antibody, or antibody fragments thereof, to fibrillar  $\beta$ -amyloid is determined by a sedimentation assay.

13. The method according to claim 11, wherein the conditions that promote or inhibit the conversion are determined.

14. A method of localizing fibrillar  $\beta$ -amyloid in a subject, comprising:

- a. administering to the subject a detectably-labeled antibody, or antibody fragment thereof, according to claim 1;
- b. allowing the detectably-labeled monoclonal antibody, or binding fragment thereof, to bind to the fibrillar  $\beta$ -amyloid within the subject; and

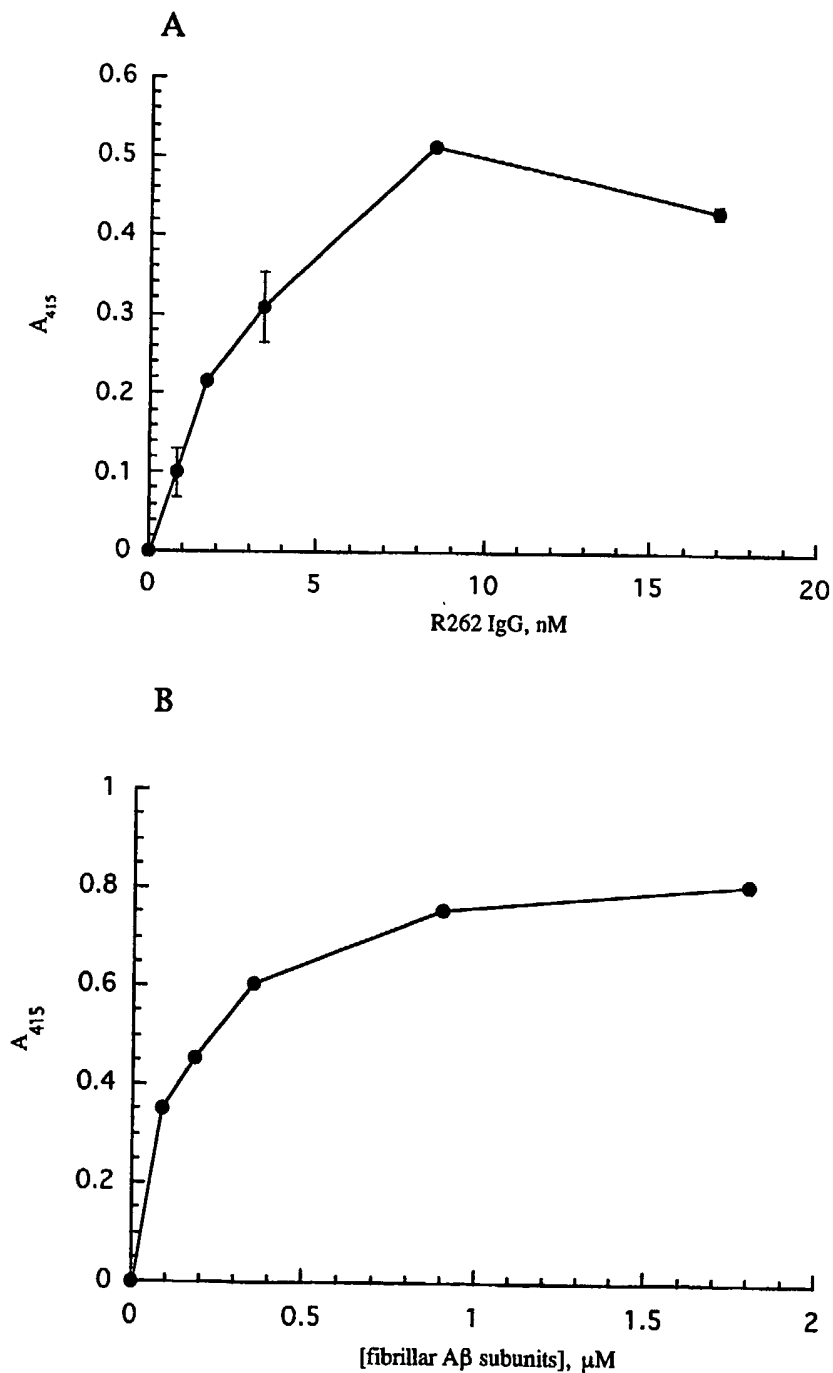
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- c. determining the location of the labeled monoclonal antibody or binding fragment thereof, within the subject.
15. A method of detecting the presence and extent of Alzheimer's Disease in a subject, comprising:
  - a. detecting the presence of fibrillar  $\beta$ -amyloid in a sample from the subject according to claim 10;
  - b. measuring the level of fibrillar  $\beta$ -amyloid in the sample; and
  - b. correlating the quantity of the fibrillar  $\beta$ -amyloid in the sample with the presence and extent of Alzheimer's Disease in the subject.
16. A method of diagnosing the presence of Alzheimer's Disease in a subject, comprising:
  - a. localizing fibrillar  $\beta$ -amyloid in the subject according to claim 14;
  - b. measuring the levels of fibrillar  $\beta$ -amyloid in the subject; and
  - c. comparing the measured levels of the fibrillar  $\beta$ -amyloid of (b) with levels of fibrillar  $\beta$ -amyloid from a normal human control, wherein an increase in the measured levels of the fibrillar  $\beta$ -amyloid in the subject versus the normal control is associated with the presence of Alzheimer's Disease.
17. A method of imaging fibrillar  $\beta$ -amyloid in a subject, comprising administering to the subject the antibody according to claim 1, wherein the antibody is detectably labeled.
18. The method according to claim 17, wherein the detectable label is a radioisotope.
19. A composition comprising the antibody, or antibody fragments thereof, according to claim 1.
20. A kit comprising the antibody, or antibody fragments thereof, according to claim 1.

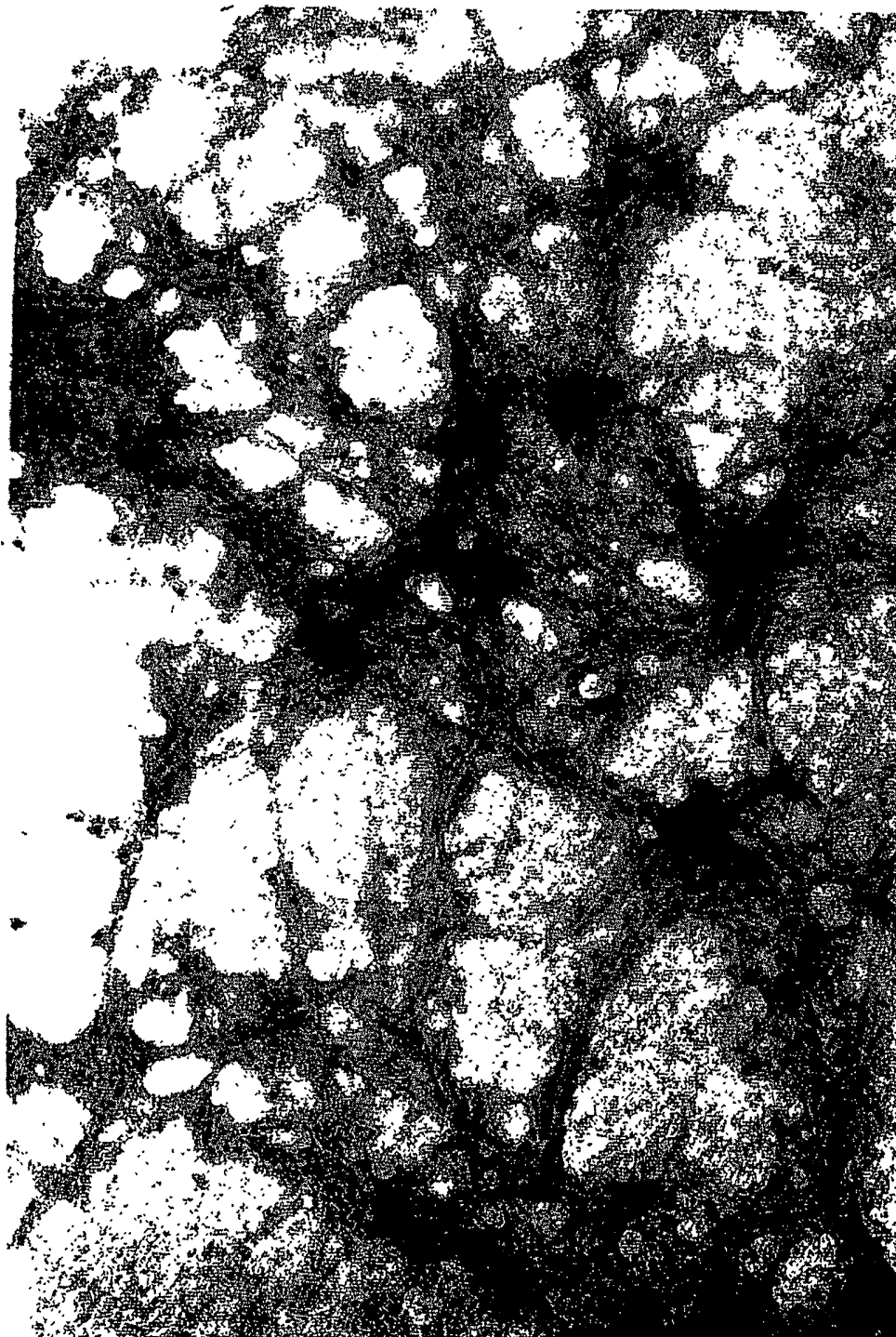


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21. An antibody, or binding fragment thereof, which binds to epitope residues 35-42 of A $\beta$ <sub>1-42</sub>.
22. The antibody according to claim 21, wherein the antibody is R165.



**Figure 1A and 1B**



**Figure 2**

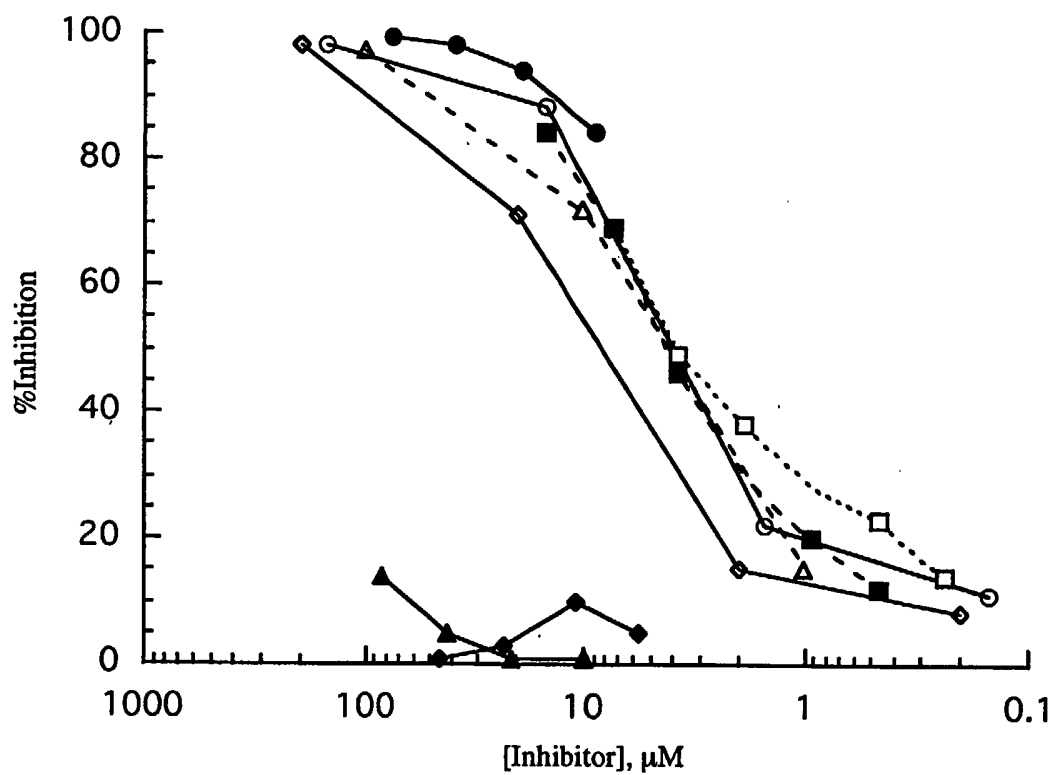
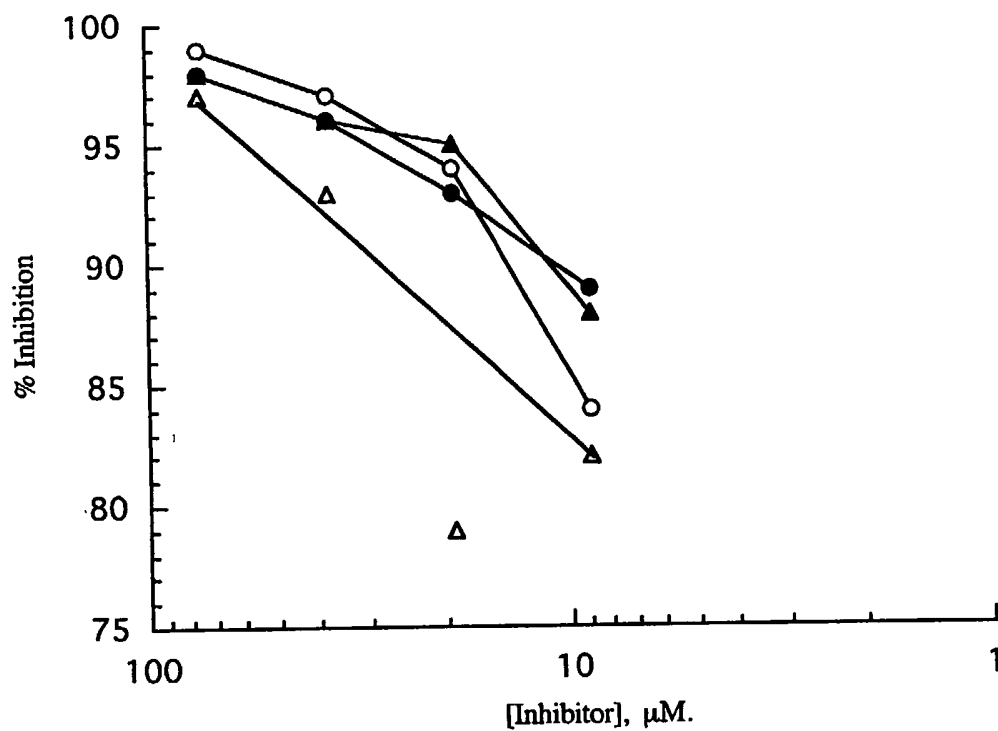
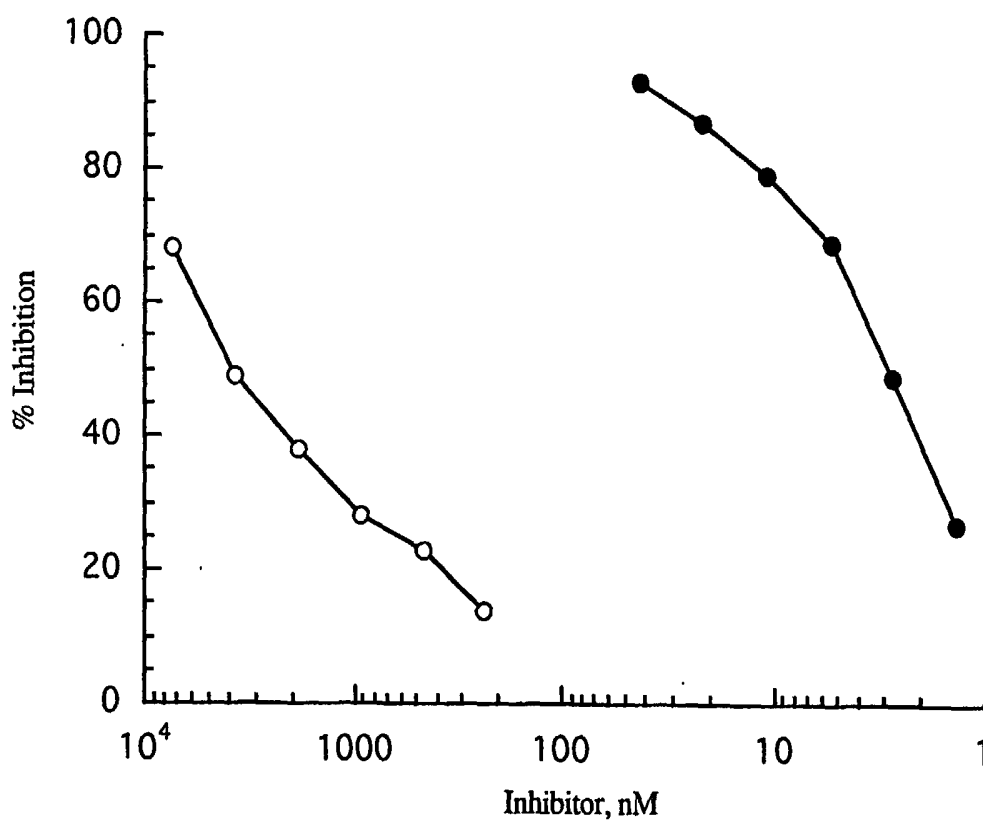


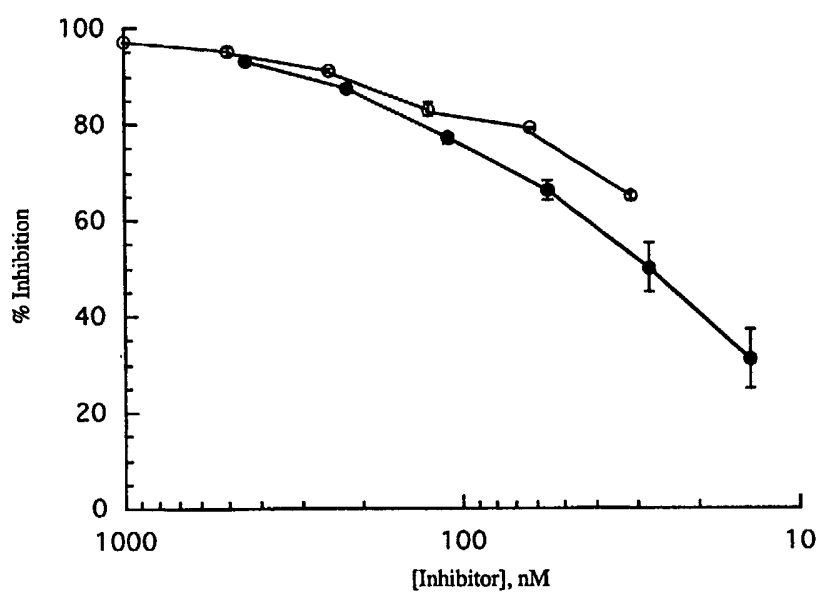
Figure 3

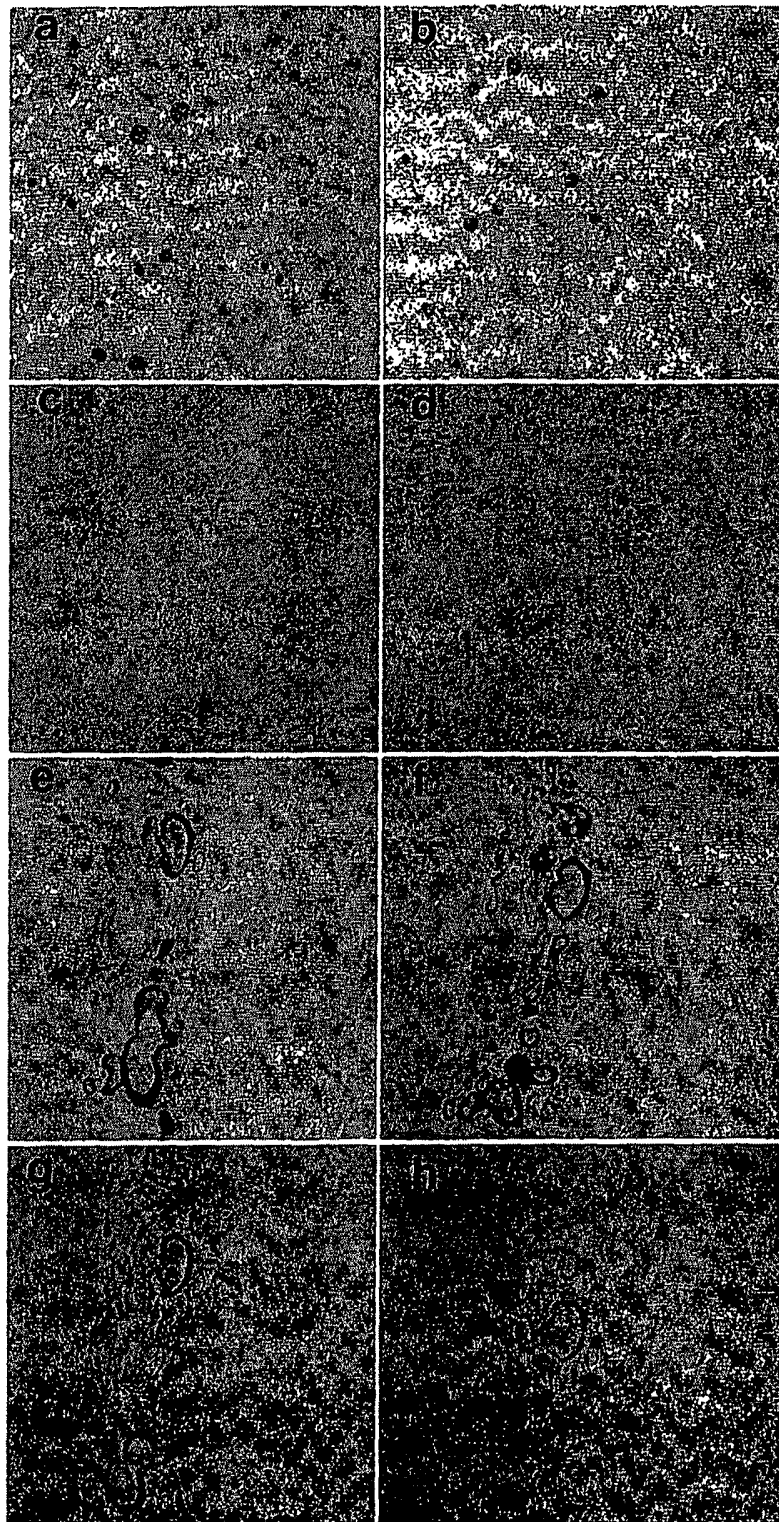


**Figure 4**



**Figure 5**

**Figure 6**



**Figure 7**