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(54) **METHODES POUR DIAGNOSTIQUER LA MALADIE
D'ALZHEIMER**

(54) **METHODS FOR THE DETECTION OF ALZHEIMER'S DISEASE**

(57) L'invention concerne des méthodes pour diagnostiquer la maladie d'Alzheimer. En particulier, la présente invention concerne des anticorps contre la protéine bande 3, capables de distinguer entre un tissu atteint de la maladie d'Alzheimer et un tissu normal. En plus, l'invention concerne une méthode pour diagnostiquer la maladie d'Alzheimer en déterminant la phosphorylation différentielle de la bande 3.

(57) The present invention is directed to methods of detection of Alzheimer's disease. In particular, the present invention provides antibodies to the band 3 protein capable of differentiating Alzheimer's disease tissue from normal tissue. In addition, the invention provides a method of detection of Alzheimer's disease comprising determining differential band 3 phosphorylation.

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(54) Title: METHODS FOR THE DETECTION OF ALZHEIMER'S DISEASE (57) Abstract The present invention is directed to methods of detection of Alzheimer's disease. In particular, the present invention provides antibodies to the band 3 protein capable of differentiating Alzheimer's disease tissue from normal tissue. In addition, the invention provides a method of detection of Alzheimer's disease comprising determining differential band 3 phosphorylation.		

METHODS FOR THE DETECTION OF ALZHEIMER'S DISEASE

1 FIELD OF THE INVENTION

The present invention is directed to methods of detection of Alzheimer's disease. In particular, the present invention provides antibodies to the band
5 3 protein capable of differentiating Alzheimer's disease tissue from normal tissue. In addition, the invention provides a method of detection of Alzheimer's disease comprising determining differential band 3 phosphorylation.

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BACKGROUND OF THE INVENTION

Band 3 is a ubiquitous anion exchange protein. It is present in nuclear, Golgi, mitochondrial and cell membranes, and in all tissues
15 thus far examined. Band 3 proteins in nucleated cells participate in band 3 antibody-induced cell surface patching and capping. Band 3 maintains acid-base balance by mediating the exchange of anions, is the binding site for glycolytic enzymes, and is the major
20 structural protein linking the plasma membrane to the cytoskeleton. Because of its central role in CO₂ respiration, band 3 is the most heavily used ion transport system in vertebrate animals.

The human erythrocyte band 3 protein is the
25 prototype for all band 3 proteins. Based upon the translated sequence of cDNA, the human erythrocyte band 3 protein consists of 911 amino acids (Tanner, et al., 1988, Biochem. J. 256:703.) The N-terminal 397 amino acids are located within the cytoplasm of the
30 cell. Species and tissue-specific variation in band 3

and the few known band 3 polymorphisms are located primarily in this "cytoplasmic" segment, which, by convention based on enzymatic cleavage studies refers to amino acids from 1 to about 400. Two crucial properties of the band 3 molecule, anion transport and antigenicity of the senescent cell antigen (SCA), map to the membrane associated "domain", which consists of the C-terminal ~500 amino acids. Kay, et al. (1991) Trans. Med. Rev. 5:173-195.

SCA, a biomarker for aging and neurological disease, is generated by oxidation and the breakdown of band 3. SCA appears on old cells and acts as a specific signal for the termination of that cell by initiating the binding of IgG autoantibody and subsequent removal by phagocytes. Kay (1978) J. Supramol. Struct. 9:555-567. This appears to be a general physiologic process for removing senescent and damaged cells in mammals and other vertebrates. SCA has been found on all cells examined including neurons. Besides its central role in the removal of senescent and damaged cells, SCA is present in neurofibrillary tangles and senile plaques in Alzheimer's disease, is involved in the removal of neural cells and platelets, and the removal of erythrocytes (RBC) in clinical hemolytic anemias, sickle cell anemia, and malaria.

Band 3 performs the same structural and functional activities in adult brain as it does in erythrocytes and lymphocytes. Kay, et al. (1983) Proc. Natl. Acad. Sci. USA 80:6882-6886. Thus, it exhibits the same characteristics as that of band 3 found in erythrocytes and lymphocytes. Cover, et al.

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(1996) Life Sciences 58:655-664; Kay and Goodman
(1996) Gerontology 11, in press. Band 3 ages as cells
1 and tissues age. Initially, band 3 undergoes an, as
yet, uncharacterized structural change. Following
this structural change, band 3 undergoes cleavage in
the transmembrane anion transport region, resulting in
5 generation of SCA. The present inventor has developed
antibodies against this "aged band 3" that bind to
distinct regions of band 3 in old but not middle-aged
or young cells. These regions, called "aging
antigenic sites", on band 3 are located at residues
10 538-554 and 812-830 of the band 3 molecule. Aging of
band 3 is associated with functional changes including
decreased efficiency of anion transport, decreased
glucose transport, increased degradation to smaller
fragments, and increased binding of physiologic IgG
15 autoantibodies in situ.

Saitoh, et al. (1992) Ann. NY Acad. Sci.
674:180 and Kay, et al. (1994) Ann. NY Acad. Sci.
719:419 have suggested that degradation of band 3
occurs in brain membranes from patients with
20 Alzheimer's disease. By immunohistochemistry, an
antibody to a band 3 synthetic peptide (pep-COOH,
residues 812-827 as numbered by Tanner, et al.)
stained more neurons in frontal cortex from
Alzheimer's patients than from normal controls. Serum
25 autoantibodies were increased in Alzheimer's disease
patients to band 3 peptides 588-602 and 822-839
compared to controls. Kay (1991) Ann. NY Acad. Sci.
621:179 suggests that brain band 3 may be altered in
Alzheimer's disease.

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In accordance with the present invention, it has been surprisingly found that certain anti-band 3 antibodies can discriminate between normal red blood cells and red blood cells of Alzheimer's patients, thus allowing a convenient and reliable assay for the disease. Further, unexpected alterations in the phosphorylation state of band 3 in Alzheimer's patients provide another assay for the disease. Moreover, it has been discovered that posttranslational changes occur in RBC band 3 that parallel changes in brain band 3. These changes include decreased ^{32}P phosphate labeling of band 3 polypeptides in brain and RBC in vitro, increased degradation of band 3, alterations in band 3 recognized by polyclonal and monoclonal antibodies decreased anion and glucose transport by red blood cells and increased degradation of band 3 to smaller fragments.

SUMMARY OF THE INVENTION

The present invention provides a method for the detection of Alzheimer's disease comprising: contacting an antibody that differentiates Alzheimer's band 3 from normal band 3 with a tissue sample from a patient suspected of having Alzheimer's disease under conditions whereby an antibody-antigen complex is formed; detecting said complex; and comparing the quantity of said complex with the quantity of complex formed under the same conditions with a control sample from a healthy individual. In a preferred embodiment the tissue sample is cerebral cortex, blood or a

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fraction thereof, red blood cells, white blood cells or red blood cell membranes.

1 The present invention further provides a method for the detection of Alzheimer's disease comprising: contacting an antibody that
5 differentiates Alzheimer's band 3 from normal band 3 with a tissue sample from a patient suspected of having Alzheimer's disease under conditions whereby an antibody-antigen complex is formed and detecting said complex.

10 The present invention further provides a method for the detection of Alzheimer's disease comprising determining the amount of phosphorylation of band 3 protein or degradation products thereof in a first tissue sample of a patient suspected of having Alzheimer's disease, and comparing the quantity of
15 said phosphorylation of band 3 protein or degradation products thereof to the quantity of phosphorylation of band 3 protein or degradation products thereof in a second tissue sample of a healthy control individual, wherein decreased phosphorylation in said first sample
20 is indicative of Alzheimer's disease. In a preferred embodiment the tissue sample is cerebral cortex, blood or a fraction thereof, red blood cells, white blood cells or red blood cell membranes.

25 BRIEF DESCRIPTION OF THE DRAWINGS

 Figures 1A-1D are bar graphs depicting results of immunoblots of blood samples assayed with band 3 antibodies.

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Figure 2 demonstrates decreased band 3 phosphorylation in old mice as compared to middle-aged mice.

Figure 3 demonstrates decreased band 3 phosphorylation in Alzheimer's patients as compared to control temporal cortex samples.

Figure 4 demonstrates binding of 32 Phosphate to Alzheimer's disease (AD) and control RBC band 3. band 3 protein bands were identified by antibody binding as detected with alkaline phosphatase. Phosphorylation was quantitated by 32 P binding.

Figure 4A demonstrates binding of anti-phosphotyrosine antibodies to band 3 in frontal gyrus brain membranes or middle aged RBC membranes from AD and controls. A, Control frontal gyrus; B, AD frontal gyrus; C, AD middle-aged RBC membranes; D, Control middle-aged RBC membranes.

Figure 5 demonstrates binding of 980 antibody to AD but not control brain membranes. Lanes: A, E, G, H = control membranes; B, C, D, F = AD membranes. AB = Amido black stain for proteins; AR = autoradiograph.

Figure 6 demonstrates that antibody 980 recognizes an internal band 3 anion transport segment, ANION 2, residues 588-602 (LRKFKNSSYFPGKLR) which is an aging vulnerable site for damage to band 3. ANION 2 (AN 2), residues 588-602 (LRKFKNSSYFPGKLR), with trace binding to COOH. ANION 2 appears to be intracellular because the potential N-glycosylation site at ASN-593 is not glycosylated. RBC, red blood cell membrane; ANION 1 (AN 1) = position 538-554: (SKLIKIFQDHPLQKTYN); COOH = 812-827:

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(LFKPPKYHPDVPYVKR); COOH-6, 813-818: (FKPPKY);
Glycos, 630-648 (QKLSVPDGFKVSNSARGW); BR, brain.

1 Autoradiograph showing binding of antibodies to band 3
on the left and antibodies to aged band 3 on the
right. AB = Amido black stain for proteins, AR =
autoradiograph.

5 Figure 7A demonstrates antibodies AD-BD3 and
peptide COOH differentiate between AD and control
temporal cortex. Antibodies were tested against AD
and control temporal cortex in immunodots. Other
anti-band 3 peptide antibodies also distinguished AD
10 from normal tissue.

Figures 7B and 7C demonstrate antibodies to
band 3 peptides (AD-BD3 and pep-CYTO) differentiate
between AD and control frontal cortex and middle-aged
(MA) RBC in immunodots.

15 Figure 8 demonstrates examples of
heterohybridoma antibodies to erythroid synthetic SCA
that react more with AD tissue. Monoclonals from
passages 1-3 were tested in immunodots. Antibody
incubation was for 5h.

20 Figure 9 demonstrates examples of
heterohybridoma antibodies to erythroid synthetic SCA
that react more with control tissue. Monoclonals from
passage 6 were tested in immunodots.

25 Figure 10 demonstrates antibodies to band 3
and synthetic peptides of band 3 distinguish AD from
normal control brain in tissue sections. Electron
microscopy was performed on etched sections of AD and
control brain tissue reacted with antipeptide
antibodies. Antibody binding was visualized with
30 protein A attached to gold colloid. Antibodies to

pep-COOH which recognizes band 3 degradation products
(10A) and a glucose transporter channel protein (10B)
1 reacted with AD but not control brain tissue.
Antibodies did not react with control tissue (10C).

Figure 11 demonstrates heterohybridoma
antibodies to erythroid SCA that differentiate between
5 AD and control RBC.

Figure 12 demonstrates AD and control serum
autoantibodies to synthetic peptides of band 3 and SCA
quantitated by ELISA. Values shown are mean $\pm$ SEM.

10 DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for
the detection of Alzheimer's disease. In particular,
it has been discovered in accordance with the present
invention that certain antibodies against band 3 or
15 fragments thereof are capable of distinguishing
between Alzheimer's and normal tissue samples. The
antibodies are capable of distinguishing both blood
and brain samples.

In a preferred embodiment, the antibody
20 useful in the method of the present invention is an
antibody against a species of band 3 known as "aged
band 3". It is known in the art and described, for
example, by Kay, et al. (1994) Ann. NY Acad. Sci.
719:419 that the transport membrane molecule band 3
25 undergoes an undefined change associated with aging
which occurs prior to degradation of band 3 to SCA.
This intact but altered band 3 is known as "aged band
3." Antibodies against aged band 3 can be identified
by their ability to bind to band 3 in old but not
30 middle-aged or young cells. Red blood cells can be

easily separated into populations of different ages by well-known methods (Bennett, et al., 1981, Exp. Hematol. 9:297) to provide a source of cells to identify antibodies against aged band 3. A preferred antibody against aged band 3, antibody 980, is described by Kay, et al. (1989) Proc. Natl. Acad. Sci. USA 86:5834, which is incorporated herein by reference. Antibody 980 identifies aging changes in band 3 that occur prior to its formation of SCA. Kay (1991) Ann. NY Acad. Sci. 621:179-205. Another preferred antibody against aged band 3, antibody AD-BD3, is described by Kay, et al. (1990) Clin. Exper. Immunogenetics 7:181-199. AD-BD3 is made as an antiidiotype to senescent IgG (SC IgG), the autoantibody eluted from senescent erythrocytes. Antiidiotypic antibodies can be conventionally prepared by methods according to Kay (1985) Proc. Natl. Acad. Sci. USA 82:1731-1735, incorporated herein by reference. Pep-CYTO is an antibody against a cytoplasmic peptide of band 3, residues 122-144. Other antibodies against aged band 3 can be isolated as IgG autoantibodies from human serum or can be prepared by standard methods known to one of ordinary skill in the art. Both polyclonal and monoclonal antibodies are useful in the method of the present invention. Monoclonal antibodies, including rodent, rabbit, human, humanized, CDR-grafted and chimeric, are preferred. Human and humanized monoclonal antibodies against aged band 3 are particularly preferred. Stable, human antibody-secreting heterohybridomas were prepared by fusion of human B cells with a mouse myeloma cell line according to the

methods of Thompson, et al. (1986) J. Immunol. 94:7-12, incorporated herein by reference.

1 It has been discovered by the present inventor that antibody 980 recognizes band 3 peptide residues 588-602. Residues 588-602 are also known as ANION 2. ANION 2 is an "aging vulnerable site". An
5 "aging vulnerable site" as defined by the present invention is a band 3 region which contains a number of amino acids which are vulnerable to aging damage and initiate band 3 degradation. For example, Asparagine, at Position 593 deaminates and causes a
10 concomitant change in band 3 configuration. ANION 2 is also vulnerable to serine proteases.

Accordingly, antibodies against peptide residues 588-602 are also contemplated in accordance with the present invention. Residues 588-602 have the
15 sequence LRKFKNSSYFPGKLR.

In another preferred embodiment, the antibody useful in the method of the present invention is an antibody against a peptide having the residues 812-830 of band 3 as numbered by Tanner, et al.
20 Residues 812 to 830 have the sequence LFKPPKYHPDVPYVKRVKT.

In another preferred embodiment, the antibody useful in the method of the present invention is an antibody against a peptide having the residues
25 822-839 of band 3 (VPYVKRVKTWRMHLFTGI).

In another preferred embodiment, the antibody useful in the method of the present invention is an antibody against a peptide having the residues 538-554 of band 3 (SKLIKIFQDHPLQKTYN).
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1 In another preferred embodiment, the
antibody useful in the method of the present invention
is an antibody against a peptide having the residues
853-870 of band 3.

5 In still another preferred embodiment, the
antibody useful in the method of the present invention
is an antibody against a peptide having the residues
122-144 of band 3 (DLQETSLAGVANQLLDRFIFEDQ).

10 In still another preferred embodiment, the
antibody useful in the method of the present invention
is an antibody against a peptide having the residues
812-827 of band 3 (LFKPPKYHPDVPYVKR).

15 In still another preferred embodiment, the
antibody useful in the method of the present invention
is an antibody against a peptide having the residues
526-541 of band 3 (FLISLIFIYETFSKLI).

20 In still another preferred embodiment, the
antibody useful in the method of the present invention
is an antibody against a peptide having the residues
549-566 of band 3 (LQKTYNYNVLMPKQGP).

25 In still another preferred embodiment, the
antibody useful in the method of the present invention
is an antibody against a peptide having the residues
561-578 of band 3 (PKPQGPLENTALLSLVLM).

30 In still another preferred embodiment, the
antibody useful in the method of the present invention
is an antibody against a peptide having the residues
573-591 of band 3 (LSLVLMAGTFFFAMMLRKF).

35 In still another preferred embodiment, the
antibody useful in the method of the present invention
is an antibody against a peptide having the residues
597-614 of band 3 (FPGKLRRVIGDFGVPISI).

In still another preferred embodiment, the antibody useful in the method of the present invention
1 is an antibody against a peptide having the residues 818-827 of band 3 (YHPDVPYVKR).

In still another preferred embodiment, the antibody useful in the method of the present invention
5 is an antibody against a peptide having the residues 818-823 of band 3 (YHPDVP).

In still another preferred embodiment, the antibody useful in the method of the present invention
is an antibody against a peptide having the residues
10 822-827 of band 3 (VPYVKR).

In still another preferred embodiment, the antibody useful in the method of the present invention
is an antibody against a peptide having the residues
15 804-811 of band 3 (QLFDRILL).

In still another preferred embodiment, the antibody useful in the method of the present invention
is an antibody against a peptide having the residues
20 828-834 of band 3 (VKTWRMH).

In still another preferred embodiment, the antibody useful in the method of the present invention
is an antibody against a peptide having the residues
25 534-540 of band 3 (YETFSKL).

In still another preferred embodiment, the antibody useful in the method of the present invention
is an antibody against a peptide having the residues
30 538-544 of band 3 (SKLIKIF).

In still another preferred embodiment, the antibody useful in the method of the present invention
is an antibody against a peptide having the residues
35 543-549 of band 3 (IFQDHPL).

In still another preferred embodiment, the antibody useful in the method of the present invention is an antibody against a peptide having the residues 547-553 of band 3 (PLQKTYN).

Antibodies against e.g. peptides 122-144, 812-830, 812-827, 588-602, 822-839, 538-554 and 853-870 can be prepared by standard methods known in the art using the peptides made by standard methods of peptide synthesis. Polyclonal and monoclonal antibodies are useful in the present method. Monoclonal antibodies, including rodent, rabbit, human, humanized, CDR-grafted and chimeric antibodies are preferred and can be produced by methods well-known in the art. Antibody production is described, for example, by Burton, et al. (1994) Adv. Immunol. 57:191; Canerari, et al. (1993) Int. J. Biol. Markers 8:147; Zaccolo, et al. (1993) Int. J. Clin. Lab. Res. 23:192; Potter, et al. (1993) Intl. Rev. Immunol. 10:103; Kalsi, et al. (1992) Autoimmunity 13:249; and Harlow et al., eds. (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.

The above-identified antibodies are capable of differentiating band 3 in tissue samples from Alzheimer's patients from band 3 in tissue samples of normal age-matched subjects. Suitable sources for tissue samples include cerebral cortex, blood, blood fractions, red blood cells, red blood cell membranes and white blood cells. Blood samples are particularly preferred.

In accordance with the present invention, an antibody capable of discriminating the Alzheimer's

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band 3 from normal band 3 is contacted with the tissue sample from a patient suspected of having Alzheimer's under conditions whereby an antibody-antigen complex is formed. In a corresponding control sample, the same antibody is contacted with the age-matched control tissue sample under the same conditions. The resulting antibody-antigen complexes are then compared. A statistically significant increase in antibody reactivity in the sample of the patient suspected of having Alzheimer's disease relative to the control sample is indicative of the presence of the disease. Furthermore, if no antibody reactivity is observed in the control sample, then positive reactivity in the sample of the patient suspected of having Alzheimer's disease is indicative of the presence of the disease.

By "statistically significant" is meant a 10-60% difference in antibody reactivity in a sample from a patient suspected of having Alzheimer's disease relative to a control sample and/or positive reactivity in the sample from a patient having Alzheimer's disease and no antibody reactivity in the control sample.

In accordance with the present invention, an antibody capable of discriminating the Alzheimer's band 3 from normal band 3 is contacted with the tissue sample from a patient suspected of having Alzheimer's disease under conditions whereby an antibody-antigen complex is formed. Conventional methods known to the skilled artisan are then employed to detect the formation of an antigen-antibody complex.

Standard immunoassay methods known to one of ordinary skill in the art can be used for antibody
1 binding and detection and quantitation in accordance with the present method. Suitable immunoassays include solid phase and solution assays,
radioimmunoassays, western blots, enzyme-linked
5 immunosorbent assays, immunofluorescent assays, chemiluminescent assays, and bioluminescent assays. Assays, conditions, and detection methods are well-known to one of ordinary skill in the art.

The present invention further provides a
10 method for the detection of Alzheimer's disease comprising determining the amount of phosphorylation of band 3 protein or degradation products thereof in a first tissue sample of a patient suspected of having Alzheimer's disease, and comparing the quantity of
15 said phosphorylation of band 3 protein or degradation products thereof to the quantity of phosphorylation of band 3 protein or degradation products thereof in a second tissue sample of a healthy control individual, wherein statistically significant decreased
20 phosphorylation in said first sample relative to the control sample is indicative of Alzheimer's disease. Furthermore, if no phosphorylation is observed in the control sample, then positive reactivity in the sample of the patient suspected of having Alzheimer's disease
25 is indicative of the presence of the disease.

In a preferred embodiment the tissue sample is cerebral cortex, blood or a fraction thereof, red blood cells or red blood cell membranes.

It has been discovered in accordance with
30 the present invention that band 3 and its fragments

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exhibit decreased phosphorylation in Alzheimer's disease.

1 Detection and quantitation of
phosphorylation of band 3 and its fragments can be
assessed by methods well known to one of ordinary
skill in the art. For example, phosphorylation can be
5 detected by observing shifts in molecular weight on
SDS-PAGE gels, or by assessing reactivity with
antiphosphotyrosine antibodies. In a preferred
embodiment, phosphorylation is detected by incubating
a homogenized tissue sample with labeled phosphate,
10 preferably in the form of $\gamma^{32}\text{P}$ -ATP, under conditions
whereby the phosphate in band 3 is exchanged with ^{32}P ,
and detecting and quantitating the ^{32}P that becomes
incorporated into band 3 proteins. ^{32}P -labeled band 3
can be detected by immunoassay with anti-band 3
15 antibodies as described hereinabove. Detailed methods
for detection of phosphorylation are provided at
Example 2 hereinbelow. In accordance with the present
method, a statistically significant decrease in
phosphorylation of band 3 or its degradation products
20 in a tissue sample from a patient suspected of having
Alzheimer's relative to an age-matched control sample
is indicative of Alzheimer's disease.

It has been discovered in accordance with
the present invention that brain band 3 undergoes
25 distinct changes in Alzheimer's disease such as
differences in glucose and anion transport, and that
these changes are accompanied by changes in the
peripheral blood as shown hereinbelow in Example 7.

Besides band 3 proteins, it has also been
30 discovered that there are other proteins that also age

and can be used to differentiate between Alzheimer's
disease tissue and normal tissue. An example of such
1 a protein is the Glucose Transporter Protein. For
example, Hepatic Glucose Transporter Protein (HEPG2)
and Insulin Regulated Glucose Transporter Protein
(IRGT) and the like. In addition, it has also been
5 discovered that there are fragments or antigenic
determinants of said Glucose Transporter Proteins that
complex with the antibody that differentiate
Alzheimer's tissue from normal tissue, e.g. HEPG2
residues (QGGASQSDKTPPEELFHP) and IRGT residues
10 (MPSGFQQIGSEDG). As another example, an IRGT
antigenic determinant is represented by the sequence
SQQLSGINAVFYYST. Thus, any of these proteins can be
used in lieu of the band 3 proteins or fragments in
the methodologies described hereinabove to
15 differentiate between Alzheimer's tissue and normal
tissue, in accordance with the present invention.

The following examples further illustrate
the invention.

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EXAMPLE 1

1 Antigenic changes in band 3 in aging and
Alzheimer's diseased brain samples were detected by
antibodies to band 3 and its peptide and breakdown
products.

5 Polyclonal antibodies against the following
proteins or peptides were obtained or generated in
rabbits by standard methods:

 Band 3 and breakdown products (band 3 Brk);
 Cytoplasmic peptide of band 3 (Cyto);
10 residues 812-827 of the COOH terminus of
band 3 (R812-827);
 aged band 3 prior to generation of SCA
(980);
 channel peptide of the glucose transport
15 protein (Gluc) residues 469-485 (QGGASQSDKTPEELFHP);
and
 residues 812-830 of the COOH terminus of
band 3 (R812-827).

20 Middle-aged (7 months old) and old (12-13
months old) mice were sacrificed by cervical
dislocation. The brain was removed under aseptic
conditions and placed in ice-cold homogenization
buffer (5 mM Hepes, 0.32 M sucrose, 5.0 mM imidazole,
5.0 mM benzamidine, 0.3 mM EGTA, 0.5 mM MgSO₄, 0.5 mM
25 ZnSO₄, 5.0 mM glycerophosphate, 2.0 mM
mercaptoethanol, 0.1 mg/ml Aprotinin (Sigma), 0.1
mg/ml Leupeptin (Sigma), 0.05 mg/ml Peptstatin A
(Sigma), pH 8.0). The brain was homogenized for 5
min. with a "Tissue Tearor" (Brinkman) and subjected
30 to differential centrifugation for 8 minutes, 4°C. at

5,000 rpm in a Sorvall RC-5B ultracentrifuge. The supernatant was decanted and placed on ice while the
1 remaining pellet was further homogenized in homogenization buffer and centrifuged at the same settings. After repeating this 3 times, supernatants were then centrifuged for 30 min., 4°C., at 20,000
5 rpm. The supernatant was discarded and the pellet resuspended in homogenization buffer, 10 mM ATP, and 20% ultrapure glycerol, and stored at -70°C. for immunodot and phosphorylation assays.

The immunodot assay was used to compare band
10 3, altered band 3 and band 3 breakdown products in young and old mice and control and Alzheimer's disease patients. Controls in which either membranes or band 3 antibodies were omitted were determined, and were the same. The control value was subtracted from
15 experimental points. All samples to be compared were run at the same time. Nitrocellulose membranes were spotted with brain homogenate protein at concentrations ranging from 2 ng to 18 µg per well. The membranes were then dried for 45 min. at room
20 temperature and blocked for 60 min. in a Tris buffered saline (TBS)-Tween buffer containing 50 mM Trisma (pH 7.4), 0.1% Tween, and 6.0% nonfat dry milk (NFDM). Membranes were then washed 3 times, one minute each in TBS-Tween (Biorad) buffer, and incubated for 6 hours
25 in polyclonal antibody. Each membrane was then washed 4 times for 4 minutes with TBS. After a final wash with TBS-Tween for 10 min., dishes were changed and each membrane was incubated in TBS-Tween with 6.0% NFDM. After one hour, membranes were incubated in 140
30 µl ¹²⁵I-Protein A (Amersham) and 100 ml TBS-Tween for

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two hours. Membranes were washed 4 times for 5 min.
each in TBS-Tween and then 2 times for 30 min. Wet
1 membranes were cut along the grid marks for cpm
determination with a gamma counter (Beckman). All
determinations were performed in triplicate.

Results of the immunoblot assays are
5 presented in Table I.

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TABLE I

ANTIGENIC CHANGES IN AGING AND AD BRAIN DETECTED BY
 ANTIBODIES TO BAND 3 AND ITS BREAKDOWN PRODUCTS, AGED
 BAND 3, SYNTHETIC PEPTIDES OF BAND 3, SYNTHETIC SCA
 PEPTIDE, OR A GLUCOSE TRANSPORTER CHANNEL PEPTIDE
 (GLUC)

	<u>ANTIBODY TO</u>	<u>MOUSE</u>	<u>HUMAN</u>		
		<u>MA</u>	<u>OLD</u>	<u>FRONTAL CORTEX</u>	<u>C</u>
	MOUSE BRAIN	58498	62989	ND	ND
		(1726)	(3685)		
	BAND 3 BRK	45534	246006*	28150	31555
		(3006)	(40327)	(6296)	(2995)
	CYTO	27430	24537	4364	6705
		(2149)	(2160)	(815)	(514)
	R812-827	29875	33199	15027	6090
		(1422)	(4811)	(8835)	(1210)
	980	27813	24483	1948	5181*
		(2046)	(2180)	(764)	(770)
	GLUC	7423	7338	2046	2266
		(299)	(485)	(447)	(292)
	812-830	17578	8404+	2380	4406+
		(2041)	(575)	(647)	(300)

(TABLE I, continued)

MA, middle-aged; C, age-matched control; AD,

1 Alzheimer's disease; results are expressed as cpm;
*P $\leq$ 0.0001, +P $\leq$ 0.01, ND, not done. Antibodies to
synthetic peptides of band 3 were reacted with brain
tissue from young treated and untreated mice. CYTO,
5 antibody against a cytoplasmic peptide of band 3,
residues 122-144; BD3-BRK, antibody that recognizes
band 3 and its breakdown products; 980, antibody
against aged band 3 prior to generation of SCA (SCA);
812-827 and 812-830, antibodies against these peptide
10 residues from the carboxyl terminus of the transport
domain of band 3; GLUC, antibody against a channel
peptide of the glucose transport protein.

The data in Table I verify that an increase
in band 3 breakdown is associated with age. The data
15 further indicate that binding by antibodies 980 and
812-830 to Alzheimer's disease membranes is
significantly increased relative to binding to normal
brain membranes. Thus antibodies 980 and 812-830
distinguish Alzheimer's disease from normal brain
20 tissue.

Immunodots using antibodies Cyto, band 3
Brk, 980 and Gluc were also performed on red blood
cell membranes obtained from blood samples from
Alzheimer's patients and normal age-matched controls.
25 Results are graphed in Figures 1A-1D. As shown in
Figure 1C, antibody 980 is also capable of
distinguishing blood samples obtained from normal and
Alzheimer's patients.

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EXAMPLE 2

1 The following example demonstrates band 3
phosphorylation changes in Alzheimer's disease versus
normal age-matched controls. Phosphorylation was
compared by quantitating the amount of ^{32}P binding to
5 band 3 in membranes of lysed cells using SDS-PAGE.

The phosphorylation reaction was performed
by mixing 100 μg brain homogenate in 50 μl
homogenization buffer with 70 μl of 200 mM Tris-HCl,
pH 7.6, containing Protein kinase A Inhibitor at a
10 final conc of 100 $\mu\text{g}/\text{ml}$ (Sigma), 200 mM MgSO_4 , 2 mM
ethylenedinitrilo-tetra acetic acid disodium salt
(EDTA), 0.138 mM 2-mercaptoethanol, and $\gamma^{32}\text{P}$ ATP, 0.15
mM (37 GBq/mmol) (ICN), and incubating for 12 minutes
at 37°C. At the end of the phosphorylation reaction,
15 samples were diluted with a 1M Tris-HCl buffer (pH
6.8) containing 40% glycerol and 25 mM EDTA. Fifty μg
of each sample (75 μl) were loaded into a 6-23% sodium
dodecyl sulfate polyacrylamide gel electrophoresis
(SDS-PAGE) gel and allowed to run to within 2 cm from
20 the bottom of the gel. Band 3 was identified by
antibody/alkaline phosphatase staining of an
immunoblot (western blot). A western blot was
performed on polyvinylidene difluoride microporous
membrane (PVDF:Millipore) membrane at 28 amps for 22
25 hours. Upon completion of transfer, the membrane was
washed 3 times for 15 min each in a Tris Buffered
Saline (TBS) containing 50 mM Trizma base, 3% fish
gelatin, 0.1% Tween 20 then incubated for one hour in
a polyclonal antibody to band 3 peptide residue 812-
30 827 diluted in TBS. The PVDF membrane was then washed

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3 x 5 minutes in TBS and incubated for one hour in 50
1 μ l goat anti-rabbit alkaline phosphatase (Zymed), 0.1%
magnesium and zinc chloride, diluted in TBS. The
membrane was again washed 3 times for 5 minutes each
in 50 mM Tris pH 7.4), 0.1% Tween 20. Band 3 labeled
5 antibodies were identified by incubating the membrane
in 2.5% Diethanolamine, 0.3% 5-bromo-4-chloro-3-
indolyl-beta-D-galactoside, 0.7% p-nitro blue
tetrazolium chloride, until the color just began to
develop. Radioactively phosphorylated proteins were
10 identified by exposing the membrane in a lightning
plus cassette at -70° C. for 24 hrs.

The results of this experiment indicated
that phosphorylation was decreased in aged mice brains
as compared to middle-aged and young brains (Fig. 2).
Further, phosphorylation of band 3 and fragments was
15 decreased in Alzheimer's disease versus age-matched
controls (Fig. 3). The differential band 3
phosphorylation observed in Alzheimer's disease occurs
at tyrosine residues.

Phosphorylation of band 3 was also compared
20 by quantitating the amount of 32 P binding to band 3 in
the membranes of lysed red blood cells using SDS-PAGE
as described for brain tissue. The results of this
experiment indicated that phosphorylation was
decreased in Alzheimer's disease red blood cells
25 compared to age-matched controls (Figure 4).

As with the brain results, the decreased
phosphorylation of band 3/binding of 32 P to red cells
was due to increased phosphorylation in vivo (i.e.
within the molecule), thus leaving fewer sites for 32 P
30 labeling. Figure 4A shows binding of anti-

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phosphotyrosine antibodies to band 3 in frontal gyrus
brain membranes or middle-aged RBC membranes from AD
1 and controls.

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EXAMPLE 3

1 The following example demonstrates in vitro
studies with antibodies that distinguish between
Alzheimer's disease and control brain tissue.

Brain tissue was obtained from Dr. J.
5 Rogers, Sun City Research, Phoenix, Arizona and from
Dr. Bosman, Department of Biochemistry, Faculty of
Medicine, University of Nijmegen, The Netherlands.

The immunodot assay with antibodies
described above to band 3 was performed as illustrated
10 in the Examples above and as described by Cover, et
al. (1996) Life Sci. 58:655-664, incorporated herein
by reference. Nitrocellulose membranes were spotted
with brain homogenetic protein at concentrations
ranging from 2 ng and 18 μ g as well. Membranes were
15 dried, blocked and incubated for six hours with
polyclonal band 3 antibody. Membranes were washed and
incubated in 140 μ l (0.14 μ Ci/ml) 125 I-protein A
(specific activity, 37 MBq; 0.100 mCi/ml; Amersham) in
100 ml TGS-Tween for two hours. Washed membranes were
20 cut along grid lines for cpm determination using a
gamma counter (Beckman).

Results of the immunodot assays are
presented in Figures 5 and 6. Figure 5 illustrates
that antibody 980 distinguishes between Alzheimer's
25 disease and normal brain membranes. Figure 6
illustrates the binding of antibodies to band 3 and to
aged band 3. Antibody 980 recognizes a major anion
transport segment of band 3 (ANION 2; Kay (1990) FASEB
J. 5:109-115). ANION 2, residues 588-602, is an
30 internal segment of band 3 to which antibody 980 binds

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strongly. Figure 6 also illustrates trace binding of antibody 980 to COOH, but not to ANION 1. Antibody
1 980 recognition of ANION 2 suggests that the band 3 change that precedes generation of SCA occurs in or near ANION 2, residues 588-602.

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EXAMPLE 4

1 The following example demonstrates that
antibodies to band 3 differentiate between Alzheimer's
disease and control temporal cortex and between
Alzheimer's disease and control red blood cells.

5 Antibodies to synthetic peptides of band 3
differentiate band 3 proteins in brain membranes from
normal individuals from those from patients with AD.
One of these, an antibody to band 3 synthetic peptide
10 pep-COOH detects a triplet in the 60,000-70,000 dalton
range in brains from patients with AD but not from
age-matched controls (see Saitoh, et al. (1992) Ann.
NY Acad. Sci. 674:180-192). Antibodies to pep-COOH
were used for immunohistochemical analysis of AD and
normal frontal cortex. Pep-COOH antibodies were used
15 because COOH is a component of senescent cell antigen.
Pep-COOH antibodies only weakly stained neurons in
normal cortex. Only a few large neurons were strongly
reactive. In AD, the number of neurons labeling with
antibodies to pep-COOH was increased by 260% ($p \leq$
20 0.01). Because of a 50% neuronal loss in AD compared
to controls ($p \leq 0.002$), the proportion of neurons
remaining that were pep-COOH-positive was increased by
540%. In AD, antibodies to pep-COOH labeled glial
cells which were found around plaques. This suggests
25 that band 3 exists in normal neurons in,
predominately, an uncleaved state, and that
degradation of band 3 occurs in AD thus allowing
binding of antibodies to pep-COOH.

30 Figure 7A shows that there is a significant
difference in binding to AD and control brains by a

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number of antibodies to band 3 including those to AD-BD3 and COOH which recognizes peptide breakdown
1 products of band 3 and aged band 3 ($p \leq 0.001$).

In addition to differentiating AD from normal brain, several anti-peptide antibodies differentiate AD from normal RBC. Sera were screened
5 for antibodies to band 3 residues 538-554, 588-602, 812-827 using ELISA. These synthetic peptides duplicate key sequences of relevant portions of the band 3 molecule. Kudrycki & Shull (1989) J. Biol. Chem. 264:8185-8194, incorporated herein by reference.
10 ELISA was performed essentially as described by Bennett & Kay (1981) Exp. Hematol. 9:297-307, incorporated herein by reference. Peroxidase conjugated goat anti-mouse immunoglobulin was used as the second antibody. Peptide antigens were dissolved
15 in 0.2 M carbonate buffer at 1 mg/ml and 100 μ g per well was used to coat the plates. After drying down overnight at 37°C, plates were washed three times with phosphate buffered saline containing .5% Tween 20 (PBST) and then quenched with blocking buffer, 0.01%
20 gelatin (porcine skin) in PBST, for one hour on a rocker at room temperature. Plates were then washed three times with PBST and incubated for one hour with mouse serum serially diluted in blocking buffer with the initial dilution of 1:40 and final dilution of
25 1:5. Plates were washed and incubated for one hour with horseradish peroxidase conjugated goat anti-mouse IgG and IgM (heavy and light chain) (Caltag) at a 1:6400 dilution in blocking buffer. After washing with PBST, 0.03% 2-2' ANZO bis(3-Ethylbenzthiazoline-6-Sulfonic acid) diammonium salt in 0.1 M citrate
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buffer pH 4.0) with 0.01% H₂O₂ was added to the wells. The plates were read at 405 nm in a BioRad 3559 micro Plate Reader (BioRad Laboratories, Richmond CA) after 30 minutes.

The 'dot-blot'/immunodot assay was used to compare band 3, altered band 3, anti-peptide antibodies, and band 3 breakdown products in red cells from individuals with AD and age-matched controls. There was a significant difference between red cell membranes from patients with AD and the spouse who was used as a control when reacted with the following antibody: AD-BD3 ($p \leq 0.04$) (Figure 7A).

There were also significant differences between red cell membranes from patients with Alzheimer's disease and the spouse who was used as a control when reacted with the following antibodies: BD3-BRK ($P \leq 0.01$), a glucose channel protein ($P \leq 0.05$) and pep-CYTO. Thus, the same band 3 changes occur in erythrocytes and brain (Figures 7B-7C).

EXAMPLE 5

1 The following example demonstrates that
anti-SCA monoclonal antibodies can differentiate
between Alzheimer's disease and control brain tissue.

5 Naturally occurring B cells in peripheral
blood of normal donors that produce antibody to SCA
peptides ANION 1 and COOH were fused to the mouse
P3X63-AG8.653 myeloma cell line to produce
heterohybridomas. A number of heterohybridomas which
10 screened against erythrocyte SCA, distinguished AD
from control brain (Figures 8 and 9) and AD from
control erythrocytes (see below). Some monoclonals
exhibited increased binding to AD tissue while others
exhibited increased binding to control tissue.

15 This suggests that (a) AD tissue lacks some
antigenic determinants or combinations of determinants
that normal tissue contains, and (b) AD tissue has
acquired some antigenic determinants or combinations
that normal tissue lacks. This indicates that normal
human B cells produce antibodies to SCA. These
20 antibodies require T-cell help suggesting that the
age-related decline in immune function may contribute
to AD. In addition, the fact that B cells from normal
humans produce antibodies that distinguish AD band 3
from that of controls suggests that there are
25 alterations in AD that can initiate, and others that
can block, recognition by a healthy individual's
immune system. This supports an immunological and/or
autoimmune component to AD.

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EXAMPLE 6

1 This example demonstrates that antibodies to
band 3 and synthetic peptides of band 3 distinguish
Alzheimer's disease from normal control brain in
tissue sections.

5 Immunoelectron microscopy was performed on
etched sections of AD and control brain tissue reacted
with antipeptide antibodies or preimmune rabbit serum.
Antibody binding was visualized with protein A
conjugated to gold colloid (Figure 10). Antibodies to
10 pep-COOH and a glucose transporter channel protein
reacted with AD but not control brain tissue. Normal
rabbit serum did not react with AD or control tissue.

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EXAMPLE 7

1 This example demonstrates the differences
observed in glucose and anion transport between 10
individuals with Alzheimer's disease and 10 age
matched controls.

5 Anion and glucose transport differences can
be detected in peripheral blood by methods well known
to one of ordinary skill in the art. For example,
sulfate uptake measurements were performed on middle-
aged erythrocytes after separation on Percoll
10 gradients as described by Cover, et al. (1996) Life
Sciences 58:655-664. Erythrocytes were washed
extensively in influx buffer (300 mM sucrose, 10 mM
Trisma, pH 7.0) to remove external and intracellular
chloride. After cells were resuspended in influx
15 buffer, 2×10^8 cells were aliquoted into
borosilicated culture tubes for the assay to be
performed in triplicate at five sulfate (substrate)
concentrations. Prior to the addition of the
appropriate substrate concentration, cells were
20 incubated for 10 minutes at 37°C. At appropriate time
intervals, 100 μ l of substrate cocktail (400 mM K_2SO_4 ,
0.25 mCi $Na_2^{35}SO_4$) or one of its four subsequent serial
dilutions (200, 100, 50 and 25 mM K_2SO_4), was added to
the appropriate cell suspension tube so that the final
25 substrate concentrations were 40, 20, 10, 5 and 2.5
mM, respectively. After incubating at 37°C for
exactly 5 minutes, 0.5 ml of cell suspension was
extracted and added to 9.0 ml ice-cod wash buffer
(1.15 KCl, 10 mM MOPS, pH 7.4). Cells were then
30 washed three times with wash buffer, centrifuged and

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all supernatants discarded. Cell pellets were deproteinized with 0.8 ml 10% trichloroacetic acid, centrifuged, and 600 μ l of supernatant from each tube was added to 5.0 ml Opti-Fluor scintillation cocktail for determining β -emission. Sulfate uptake rates are expressed as nmol/ 10^8 cells/min.

D-glucose uptake measurements were performed on middle-aged peripheral erythrocytes separated on Percoll gradients. Erythrocytes were washed extensively in 20 mM phosphate buffer (pH 7.4) to remove external and intracellular glucose. After cells were resuspended in phosphate buffer, 2×10^8 cells were aliquoted into borosilicated culture tubes and put on ice for the assay to be performed in triplicate at five D-glucose (substrate) concentrations. At appropriate time intervals, 100 μ l of substrate cocktail (20 mM D-glucose, 0.3 mCi D- 3 H-glucose) or one of its four subsequent serial dilutions (10, 5, 2.5 and 1.25 mM D-glucose) was added to the appropriate cell suspension tube so that the final substrate concentrations were 2, 1, 0.5, 0.25 and 0.125 mM, respectively. After incubating at 0°C for exactly 5 minutes, 0.5 ml of cell suspension was extracted and added to 9.0 ml ice-cold stop buffer (20 mM phosphate buffer, 0.1 mM phloretin, pH 7.4). Cells were then washed three times with stop buffer, centrifuged and all supernatants discarded. Cell pellets were deproteinized with 0.8 ml 10% trichloroacetic acid, centrifuged and 600 μ l of supernatant from each tube was added to 5.0 ml Opti-Fluor scintillation cocktail for determination β -

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emission. D-glucose uptake rates are expressed as pmol/ 10^8 cells/min.

1 Anion and glucose transport by AD patient's erythrocytes was consistently less than that of the paired control run in the same experiment. Because of
5 the small sample size and the variability between individuals, the data was analyzed as the patient's transport relative to the control, which was usually the spouse. Differences in the $V^{\max}$ and K_m of glucose transport were statistically significant ($p \leq 0.04$ and
10 0.03, respectively; Table II). Anion transport differences were also statistically significant for $V^{\max}$ ($p \leq 0.001$) and K_m ($p \leq 0.04$; Table II).

 The rate of sulfate uptake decreased (i.e. anion transport decreased) with age in erythrocytes, brain and lymphocytes. Specifically, anion transport
15 by splenic lymphocytes decreased by 24% with age ($P \leq 0.005$ for middle-aged compared to old mice; 30% for young compared to old mice, $P < 0.001$). See Example 10.

 Thus, RBC from individuals with Alzheimer's disease exhibited decreased efficiency of anion and
20 glucose transport and a decrease in the number of anion and glucose binding sites (K_m) compared to their spouses and age-matched controls. Anion transport also decreased with age in lymphocytes.

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TABLE II

	Anion Transport ¹		Glucose Transport ²	
	V_{max}	K_m	V_{max}	K_m
Alzheimer's Controls	16.5 ± 2.2	3.46 ± 0.16	214 ± 35	1.47 ± 0.41
	20.4 ± 1.0	3.16 ± 0.18	269 ± 43	1.81 ± 0.56
	$p \leq 0.001^3$	$p \leq 0.04$	$p \leq 0.04$	$p \leq 0.03$

¹ V_{max} , mmol/10⁸ cells/min.; K_m , mM.

² V_{max} , pmol/10⁸ cells; K_m , mM.

³ Statistical analysis (p value) was based on comparison of the patient's transport to control transport for each experiment. The control was usually the spouse to compensate for environmental effects.

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EXAMPLE 8

1 This example demonstrates that anti-SCA
monoclonal antibodies can differentiate between
Alzheimer's disease and control red blood cells. A
number of the heterohybridomas described in Example 5
5 distinguished Alzheimer's disease from control
erythrocytes (Figure 11). Some monoclonal antibodies
reacted with Alzheimer's disease while others reacted
with control red blood cells.

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EXAMPLE 9

1 This example demonstrates that serum
autoantibodies to band 3 peptides 588-602 and 822-839
increased in Alzheimer's disease patients compared to
controls.

5 Naturally occurring autoantibodies were
quantitated to senescent cell antigen in human serum
with ELISA during aging to determine whether these
antibodies increased with AD. Naturally occurring
autoantibodies may also be detected or quantitated by
10 their reaction with known Alzheimer's disease tissues,
e.g. brain.

Serum autoantibodies were increased in AD
patients compared to controls to band 3 peptides 588-
602 and 822-839 (two-tailed t test $p \leq 0.06$; Figure
15 12). Significance is expected to increase as sample
size increases. These band 3 residues lie in anion
transport/binding regions. This is consistent with
alteration of this region in AD since it is recognized
as antigenically different by the patient's immune
20 system. This is consistent with the observed decrease
in anion transport (Example 7). Appearance of
autoantibodies to the 588-602 region are consistent
with data indicating that this is an "aging vulnerable
site".

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WHAT IS CLAIMED IS:

1. A method for detecting Alzheimer's disease comprising:

(a) contacting blood or a fraction thereof, red blood cells, red blood cell membranes or white blood cells containing band 3 from a patient suspected of having Alzheimer's disease with an antibody that differentiates Alzheimer's band 3 from normal band 3 under conditions effective to form a complex between said antibody and band 3;

(b) detecting said complex;

(c) contacting blood or a fraction thereof, red blood cells, red blood cell membranes or white blood cells containing band 3 from a healthy individual with an antibody that differentiates Alzheimer's disease band 3 from normal band 3 and incubating for sufficient time and under conditions effective for a complex, if any, to form between said antibody and band 3; and

(d) comparing the amount of complex formed in step (b) with the complex formed in step (c), a statistically increased quantity of complex formed in step (b) relative to step (c) indicating the presence of Alzheimer's disease.

2. A method for detecting Alzheimer's disease comprising:

(a) contacting blood or a fraction thereof, red blood cells, red blood cell membranes or white blood cells containing band 3 from a patient suspected of having Alzheimer's disease with an antibody that differentiates Alzheimer's band 3 from normal band 3 under conditions effective to form a complex between said antibody and band 3; and

(b) detecting said complex.

3. The method of Claim 1 or 2 wherein said antibody is antibody 980.

4. The method of Claim 1 or 2 wherein said antibody is AD-BD3.

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5. The method of Claim 1 or 2 wherein said antibody complexes with Peptides 812-827 of band 3.

6. The method of Claim 1 or 2 wherein said antibody complexes with Peptides 812-830 of band 3.

7. The method of Claim 1 or 2 wherein said antibody complexes with Peptides 822-839 of band 3.

8. The method of Claim 1 or 2 wherein said antibody complexes with Peptides 853-870 of band 3.

9. The method of Claim 1 or 2 wherein said antibody complexes with Peptides 538-554 of band 3.

10. The method of Claim 1 or 2 wherein said antibody complexes with Peptides 588-602 of band 3.

11. The method of Claim 1 or 2 wherein said antibody complexes with Peptides 469-485 of band 3.

12. The method of Claim 1 or 2 wherein said antibody complexes with a mixture of Peptides 538-554 and 812-827 of band 3.

13. The method of Claim 1 or 2 wherein said antibody complexes with a mixture of Peptides 588-602 and 812-827 of band 3.

14. A method for detecting Alzheimer's disease comprising:

(a) determining the amount of phosphorylation of band 3 protein or degradation products thereof in a first tissue sample of a patient suspected of having Alzheimer's disease; and

(b) comprising the quantity of said phosphorylation of band 3 protein or degradation products thereof to the quantity of phosphorylation of band 3 protein or degradation products thereof in a second tissue sample of a healthy control individual; wherein statistically decreased phosphorylation in said first sample is indicative of Alzheimer's disease.

15. The method of Claim 14 wherein said tissue sample is brain tissue, blood or a fraction thereof, red blood cells, red blood cell membranes or white blood cells.

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FIG. 1B

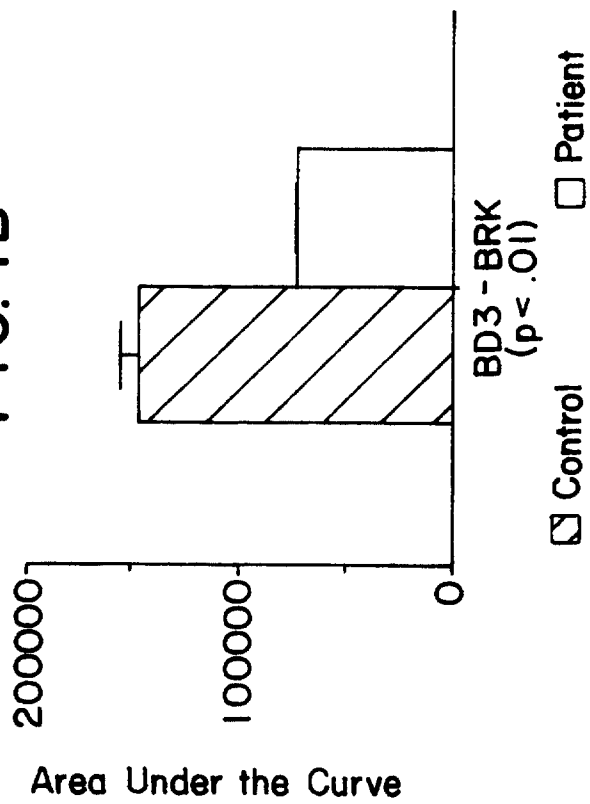


FIG. 1D

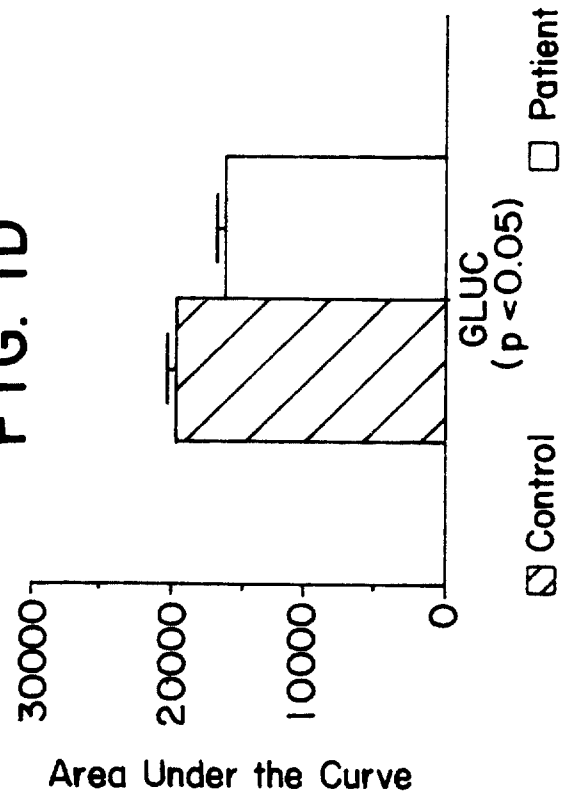


FIG. 1A

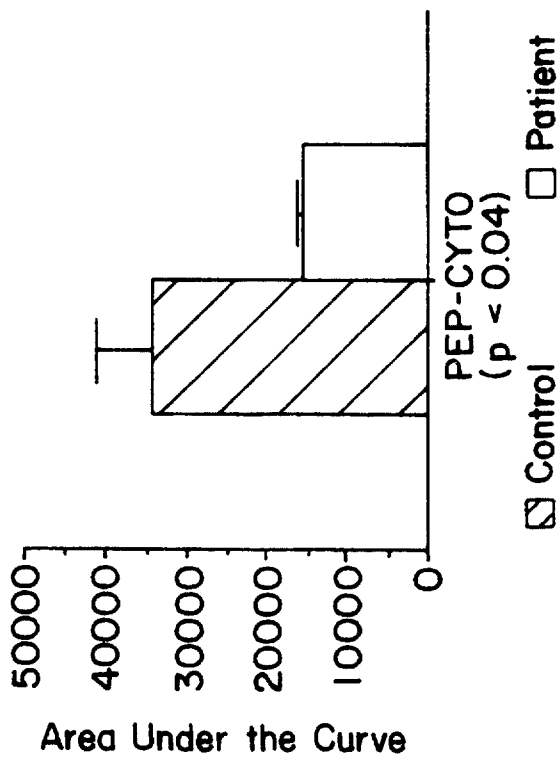
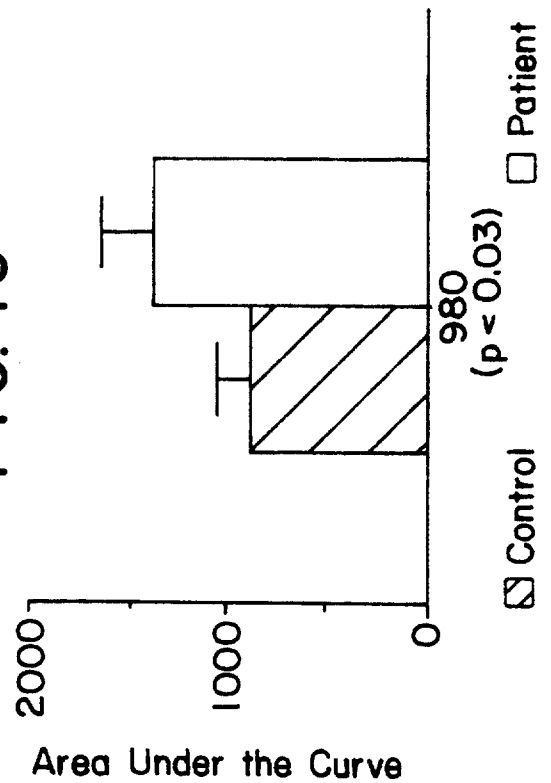
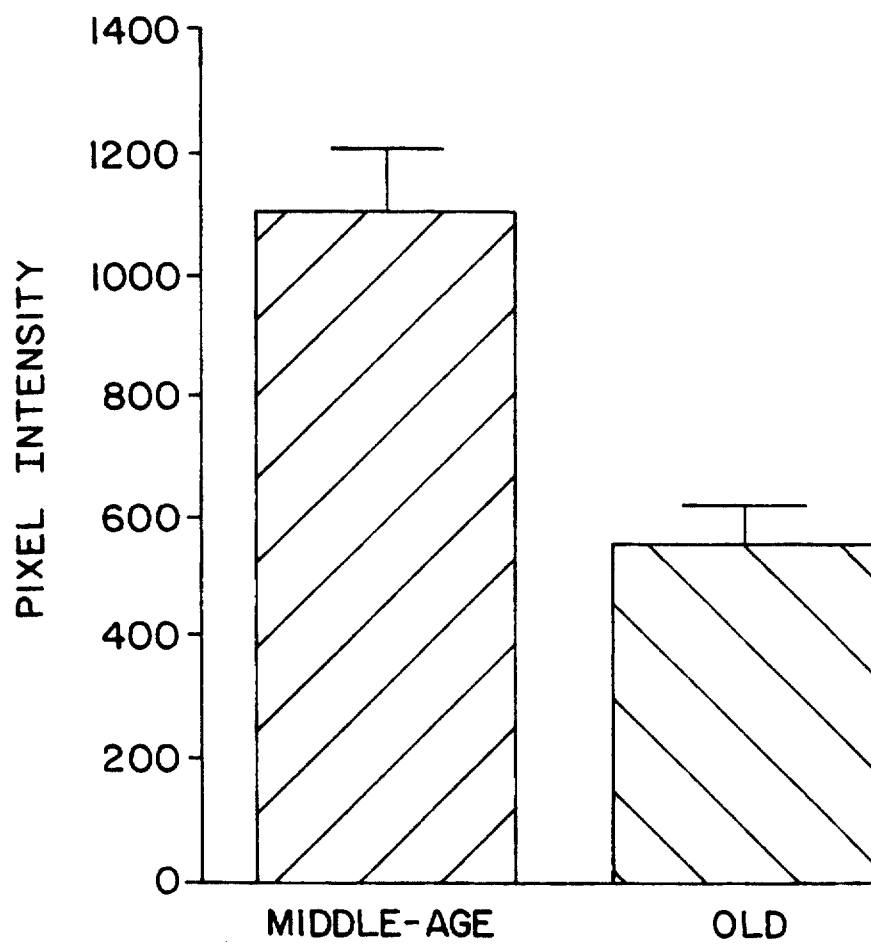


FIG. 1C



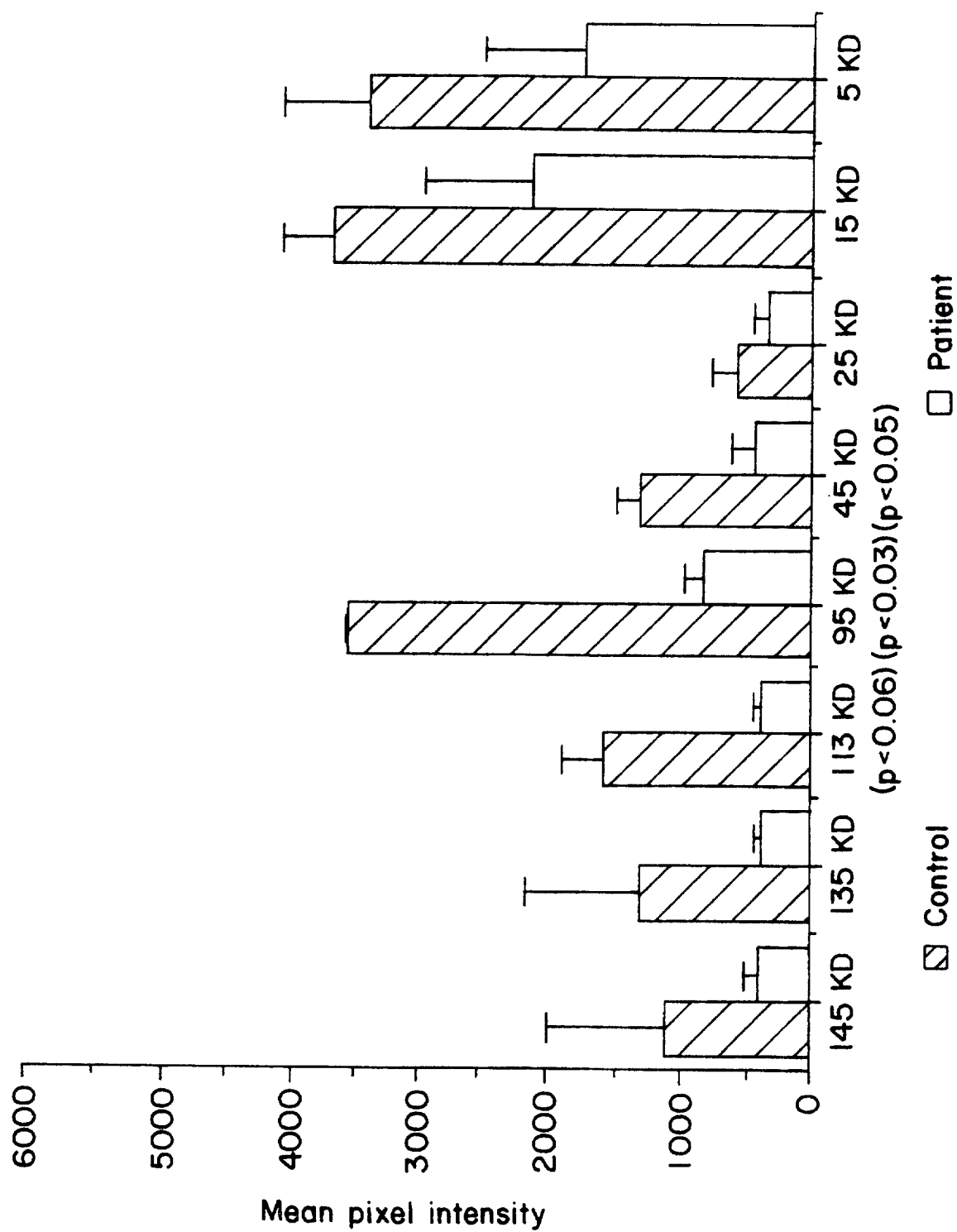
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FIG. 2



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FIG. 3



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FIG. 4

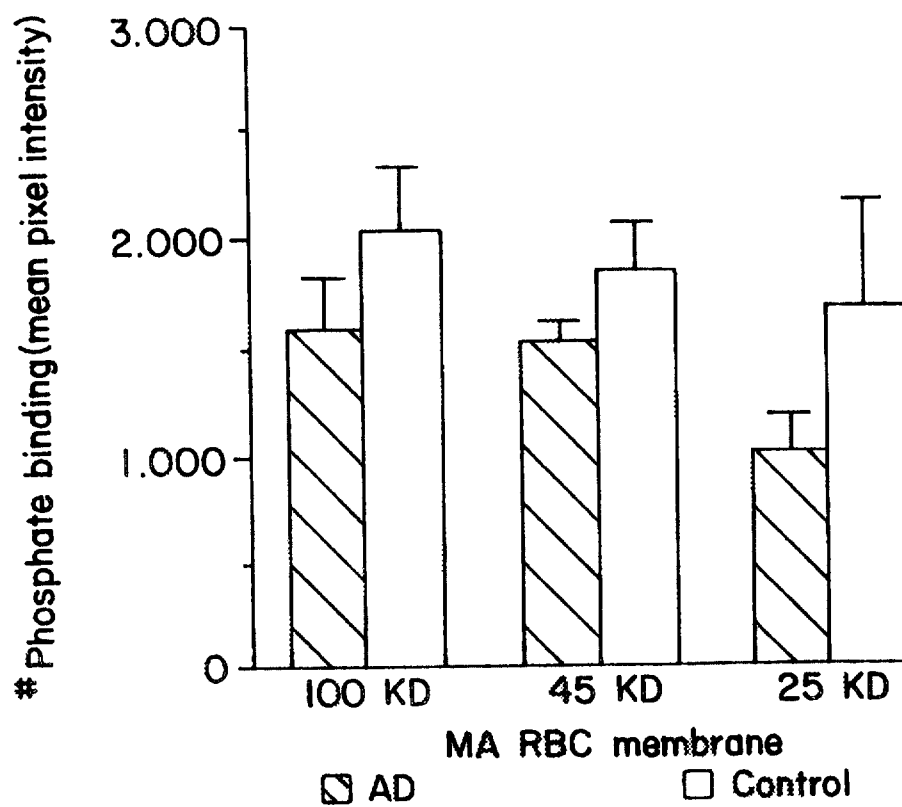
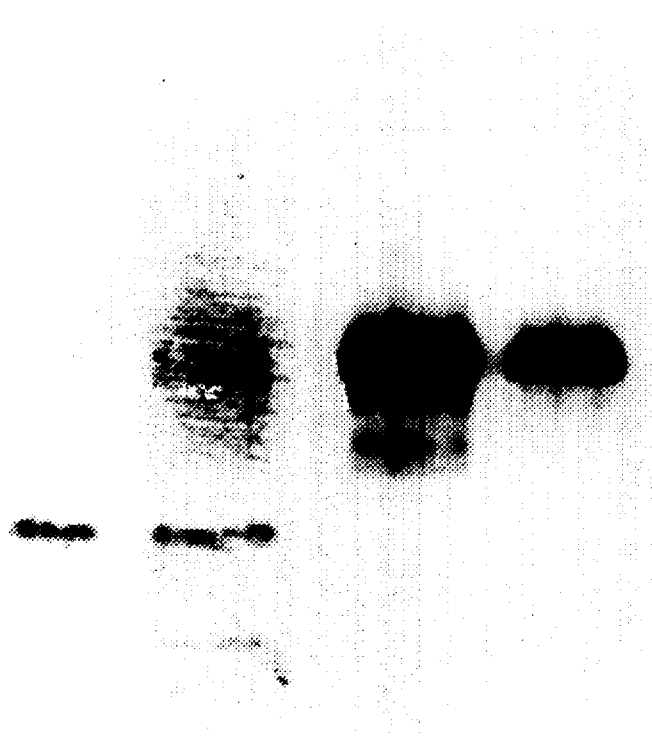


FIG. 4A

A B C D

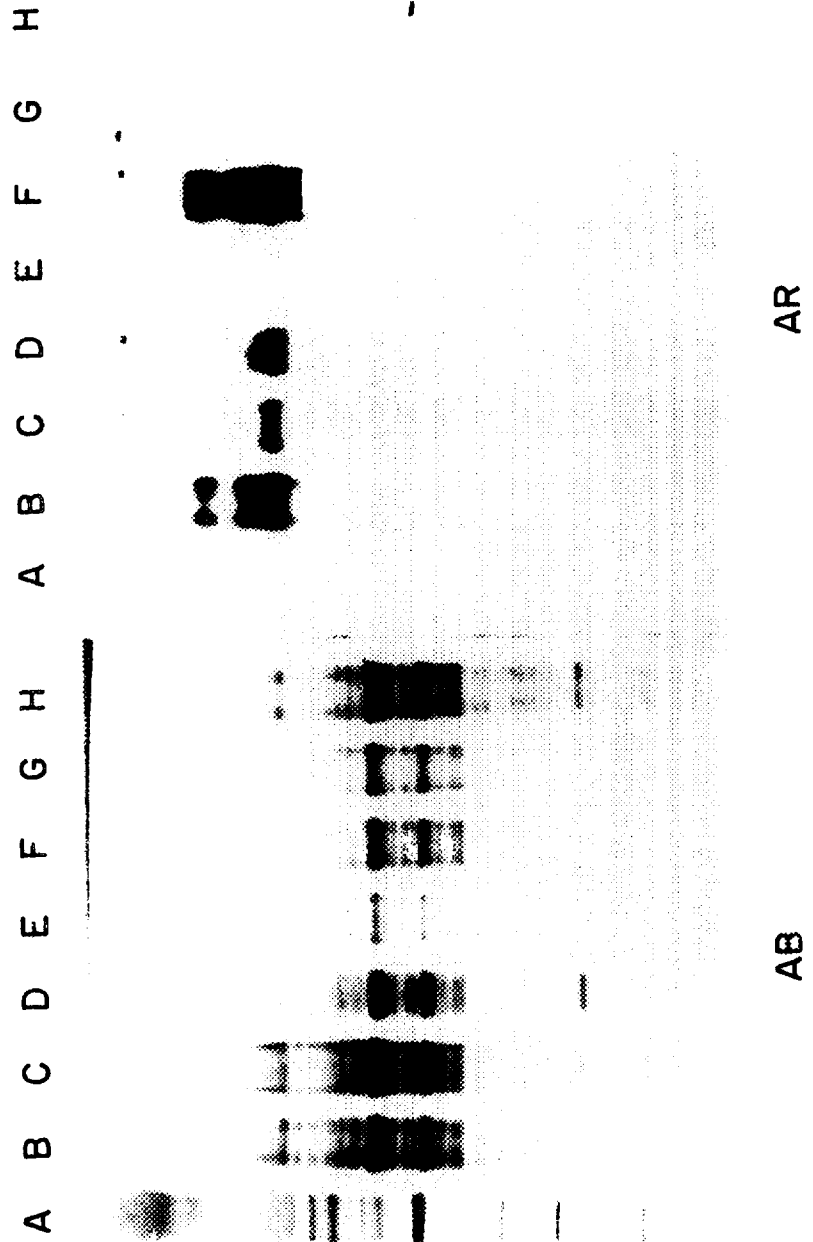


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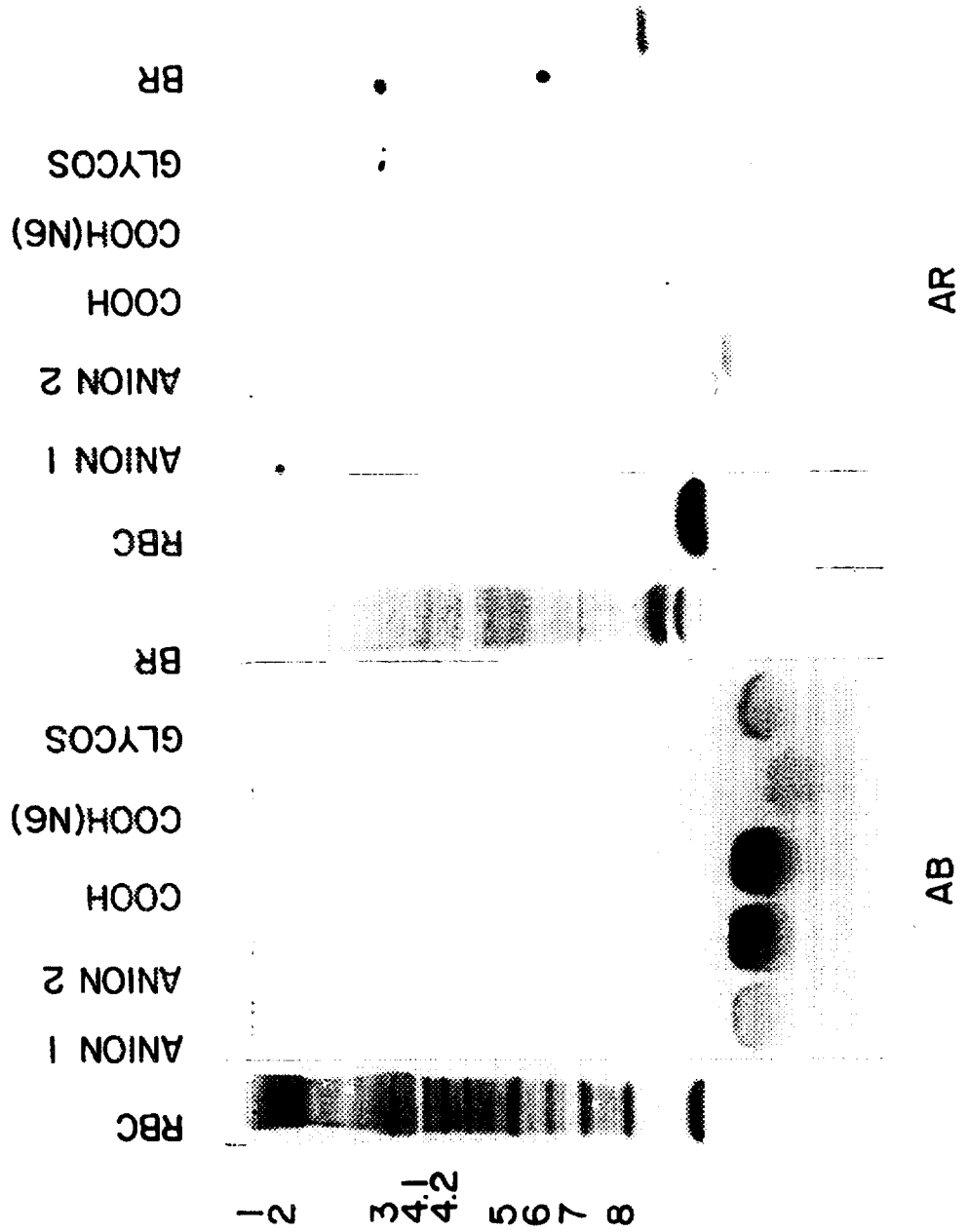
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FIG. 5



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FIG. 6



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FIG. 7A

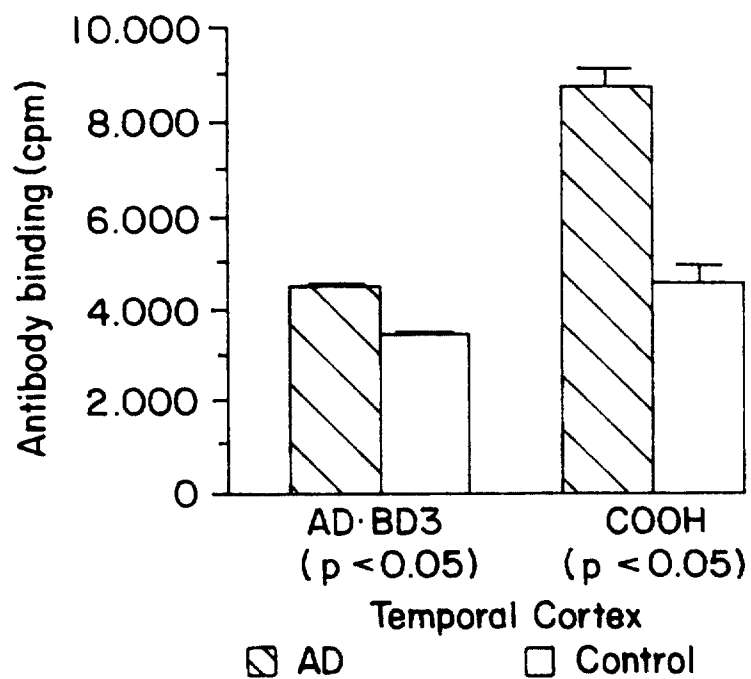
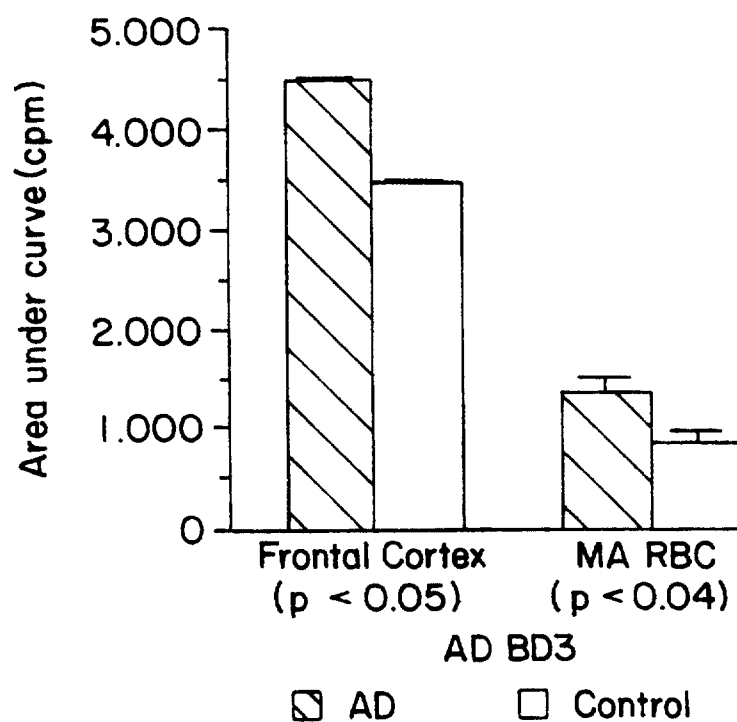
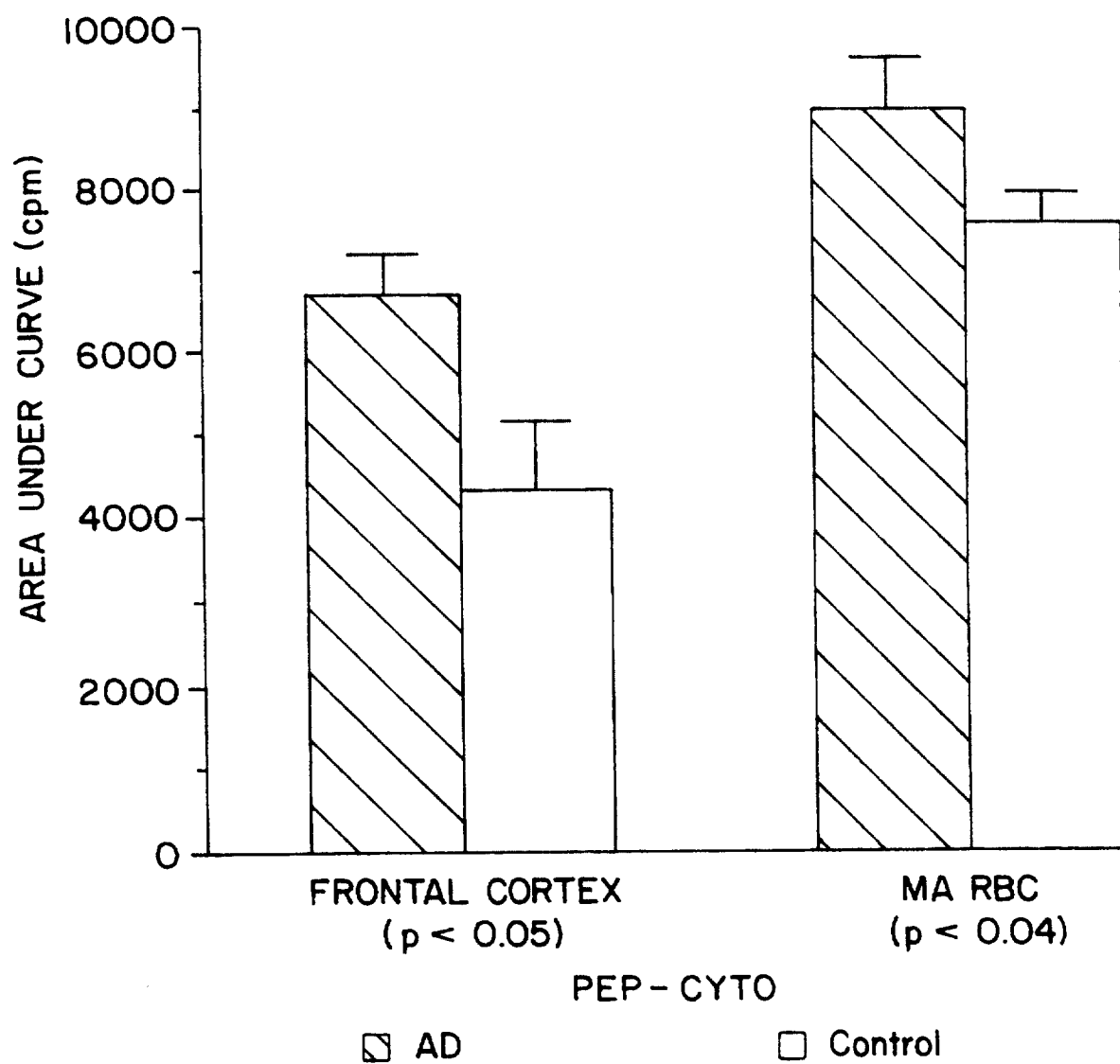


FIG. 7B



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FIG. 7C



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FIG. 8

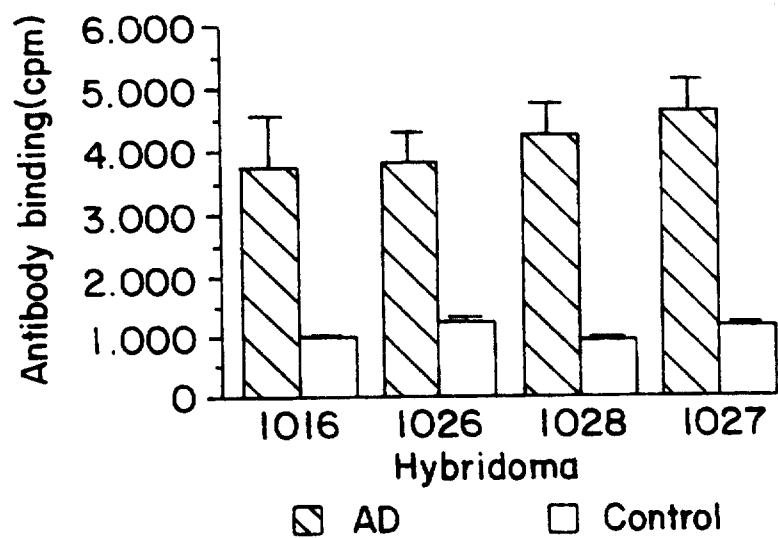
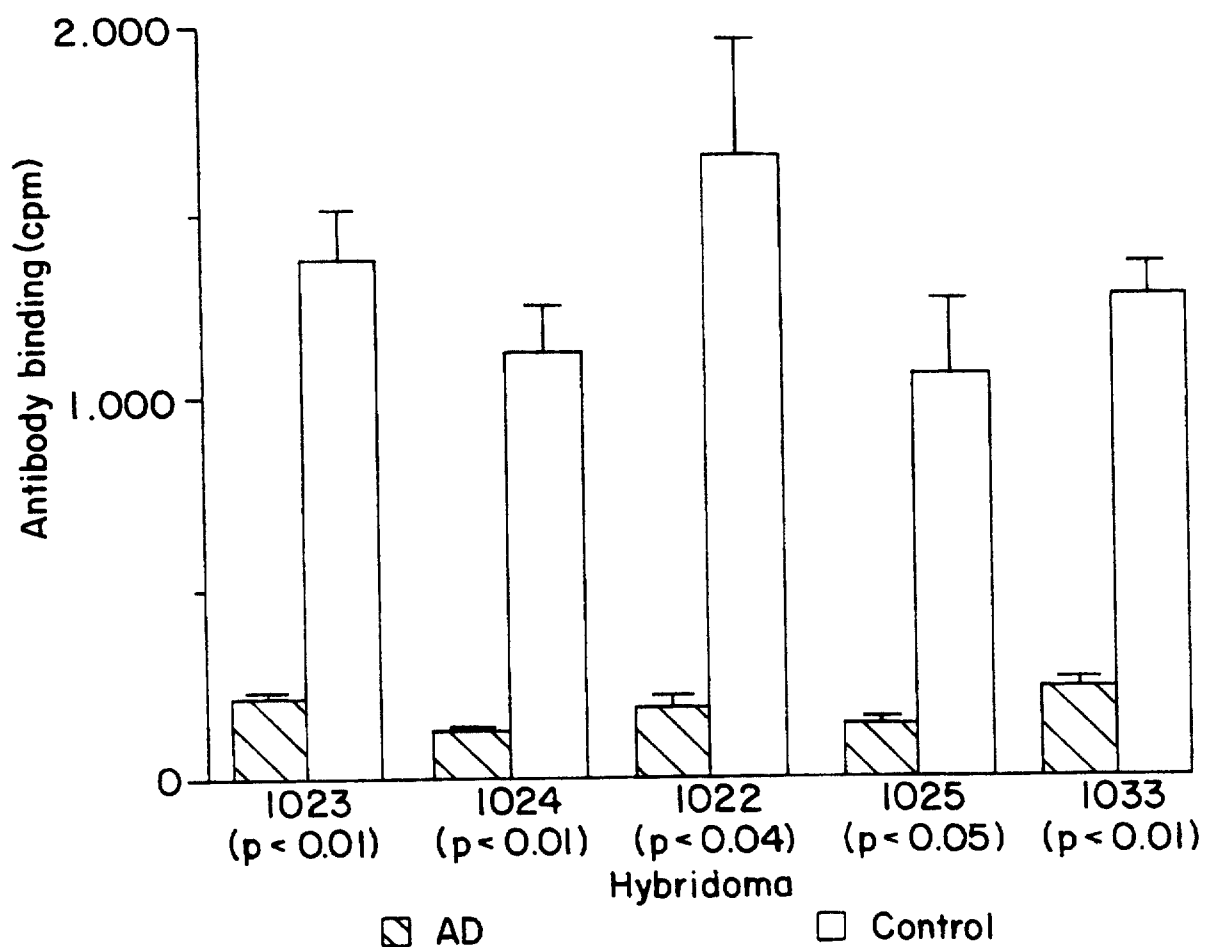


FIG. 9



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FIG. 10C



FIG. 10B

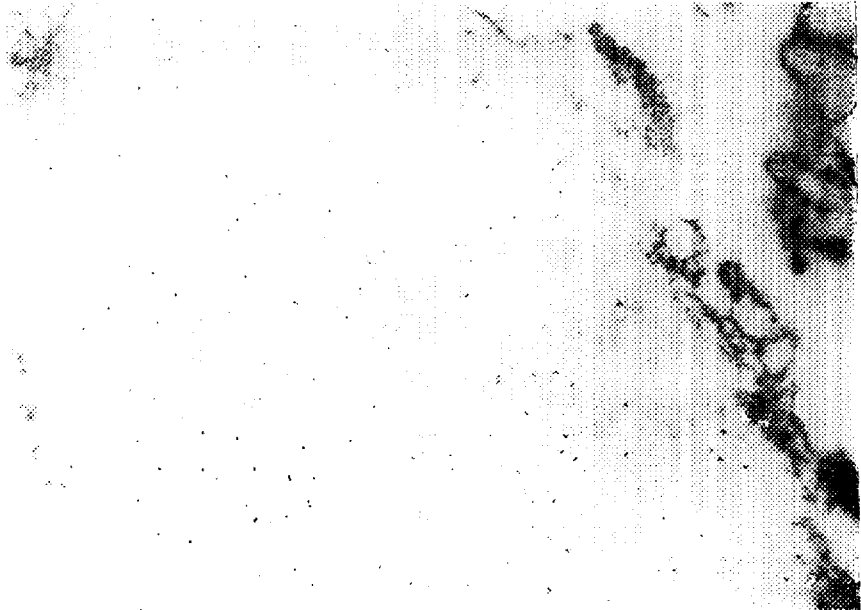
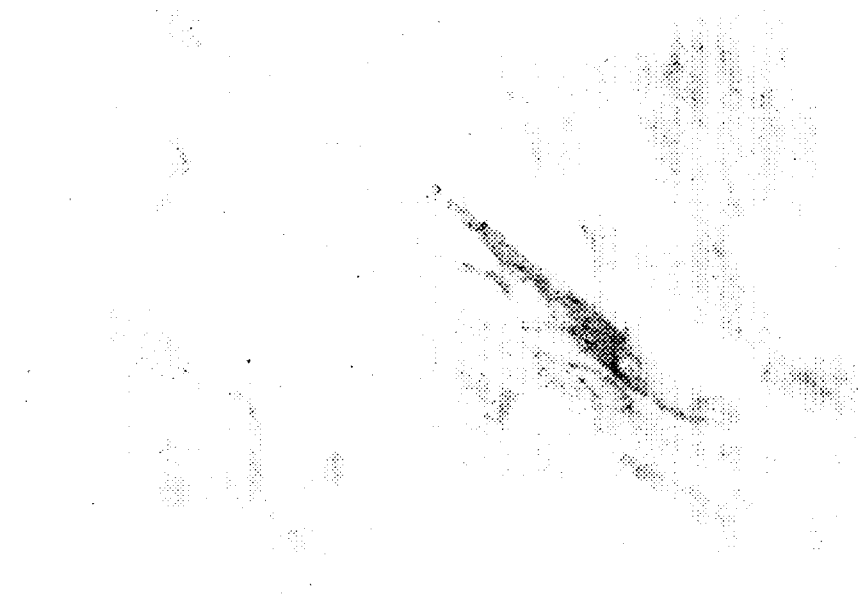


FIG. 10A



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FIG. 11

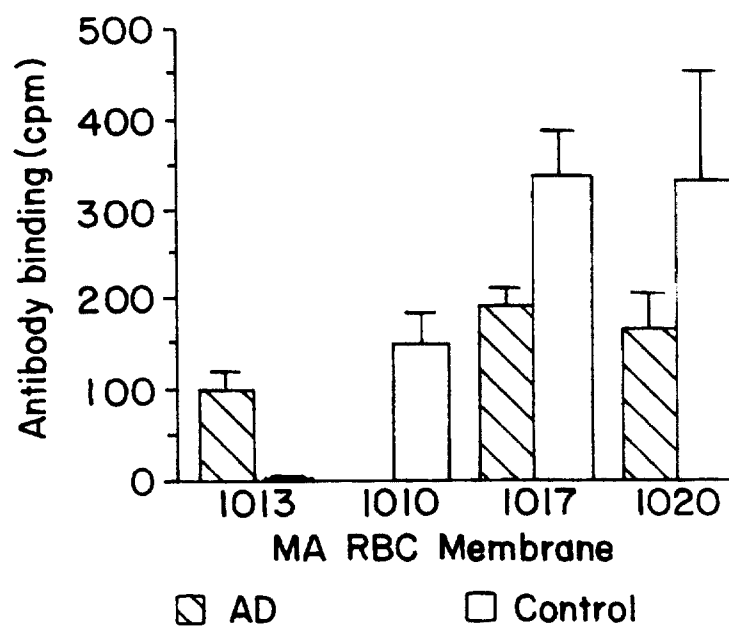


FIG. 12

