Purification of 10-5A9 Antigen in Alzheimer’s Disease

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Abstract

Alzheimer’s disease (AD) is a neurodegenerative disorder that results in memory loss and reduced cognitive function. Characteristic lesions include senile plaques and neurofibrillary tangles. Evidence for a previously unknown component, cockroach 10-5A9 antigen, within these lesions has recently been discovered. It is the purpose of this study to purify the 10-5A9 antigen for further analysis.

Immunohistochemistry was used to determine the antigen’s presence in AD lesions and the Lewy bodies of Parkinson’s disease. Although SDS-PAGE/Western blotting and immunoprecipitation techniques proved ineffective, the former indicated antigen presence in the particulate matter of AD brain homogenates. Negative results from ammonium sulfate experiments also correlated the belief that the antigen was present in the particulate matter. Since chromatographic techniques could not be incorporated due to the absence of an assay, direct immunostaining of AD particulate matter concluded the study. These results suggest that the 10-5A9 antigen is tightly associated with some constituent(s) of AD lesions that pellets at low-speed centrifugation. Immunostaining of purified plaque cores, known to reside within the particulate matter fraction, corroborated these conclusions.
Purification of 10-5A9 Antigen

In Alzheimer’s Disease

Background

Alzheimer’s disease, named after Alois Alzheimer for his clinical report of a 51 year old woman in 1906,\textsuperscript{1} is a disease of dementia with characteristic lesions in the brain. At autopsy, Alzheimer noted the presence of tangled fibrils within a significant portion of the neurons of the cerebral cortex along with “milliary foci” representing deposition of a particular substance.

These lesions are now known as neurofibrillary tangles (NFTs) and senile plaques (SPs). Diagnosis of AD is difficult since the best method is observation of NFTs and SPs present at autopsy. Since these lesions can occur in brains of normal, older individuals,\textsuperscript{23,60} the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD)\textsuperscript{87} suggests an age related tier system where the number of NFT and SP lesions are compared with the age of the patient in order to determine the likelihood of AD. The disease is considered multifactorial though its cause has still to be elucidated.

What is known about the disease, however, has resulted from the identification of molecules linked to AD biology/pathology. The isolation and purification of individual AD components has enhanced the understanding of AD by revealing a larger portion of the biological puzzle. The complex research surrounding AD, at present, began with the employment of basic purification techniques, usually immunological, by past researchers. In order to demonstrate the importance of specific proteins within Alzheimer’s disease, several are mentioned below with their individual roles. Each one of these molecules
was identified and purified in order to determine its potential role in the biology of Alzheimer’s disease. Since that time, a better portrait has been painted of the interactions involved in AD pathology. This present study attempts to reveal more of the AD story by isolating and identifying yet another component within the disease in the hopes of furthering the fight against AD.

**Tau.**

Tau functions to stabilize neuronal microtubules and promote neuronal outgrowth.\(^{24,64}\) This microtubule-associated protein (MAP) is normally concentrated in axons. Abnormal tau proteins, located in nerve cell bodies, axons and dendrites, were first purified in 1991 from AD brains.\(^{81}\) Since that time, its isolation has been referenced 35 times in other works. In humans, six isoforms exist ranging in size from 352-441 amino acids. The isoforms also differ from one another by the presence or absence of three inserts.\(^{37}\) All of the isoforms have three or four tandem repeats which show microtubule-binding properties. These phosphoproteins\(^ {11}\) can be modified by phosphorylation\(^ {31,56,70}\) and primarily compose an integral component of paired helical filaments (PHF) in AD.\(^ {38,40,56,65,69,70,71,74}\) Paired helical filaments, one of the most characteristic neuropathological lesions in AD\(^ {51}\), are present in neurofibrillary tangles (NFT), dystrophic neurites around senile plaques, and numerous abnormal neurites in the neuropils.\(^ {7,51,62}\) Although paired helical filaments can be formed with non-phosphorylated tau from bacteria,\(^ {19,140}\) tau from PHF appears as distinct bands on gels only after alkaline phosphatase treatment\(^ {36}\) suggesting PHF are composed of tau in a hyperphosphorylated state. The hyperphosphorylated tau has a greatly reduced ability to
bind and stabilize microtubules\textsuperscript{8,143} while PHF-tau has no ability to bind to microtubules.\textsuperscript{8}

There are reports, however, that the repeat domain forms the core of Alzheimer PHFs\textsuperscript{67,102} and that the abnormal tau is believed to self-assemble into the paired helical filaments possibly by means of the repeat domains,\textsuperscript{143} although this process is not fully understood. Since this does not require phosphorylation, more research is needed to explain the relationship between tau phosphorylation and PHF formation.

\textbf{β-Amyloid Precursor Protein (βPP).}

The amyloid precursor protein (βPP) is a 110-130 kDa protein that structurally resembles an integral transmembrane cell surface glycoprotein. The extracellular portion of βPP contains an amino terminal 17 residue signal sequence, a cysteine-rich domain, and an anionic domain of approximately 100 residues (reviewed in ref. 67). Certain exons are also present including one with homology to the Kunitz family of serine protease inhibitors and one with homology to the OX-2 surface antigen in leukocytes. The ectodomain can be glycosylated, sulfonated, and phosphorylated.\textsuperscript{53} The Aβ peptide is predominantly the intermembrane domain of βPP, although, it is slightly part of the ectodomain.

βPP includes binding sites for copper,\textsuperscript{8} zinc,\textsuperscript{10} heparan sulfate proteoglycan,\textsuperscript{94,95} other βPP molecules,\textsuperscript{10} collagen and laminin.\textsuperscript{96} The expression, formation, and degradation of βPP is regulated by molecules of the extracellular matrix (ECM) and by cytokines. These regulators are produced in response to tissue damage reflecting the role of βPP in the brain’s immune response.\textsuperscript{2,89-91} βPP also functions in synaptogenesis,\textsuperscript{92} neurite outgrowth,\textsuperscript{86} and cell adhesion.\textsuperscript{120}
The βPP can be metabolized by endocytosis via clathrin-coated vesicles. The vesicle is shuttled to the endosomal-lysosomal pathway where proteases cut the protein into a series of carboxy terminal fragments. The most abundant secreted fragment, βPPs, is approximately 100-110 kDa. This cleavage falls within the Aβ sequence and precludes Aβ formation. This is known as the α-secretase pathway. An alternate pathway producing the two proteolytic cleavages necessary for Aβ formation (i.e., β-secretase and γ-secretase cleavages) is thought to occur in an acidic vesicle near the cell membrane after which Aβ is quickly exported out of the cell. A reciprocal relationship exists between the formation of the α-secretase product APPs and Aβ. Although the α-, β-, and γ-secretases have never been isolated, their enzymatic activity has been experimentally detected.

Secreted fragments function as serine protease inhibitors, growth factors, enzyme effectors, and mediate cell adhesiveness via interactions with heparin, laminin, and basement membrane heparan sulfate proteoglycan. One interesting function is the activation of microtubule-associated protein kinase (MAPK) by βPPs. Although it has only been demonstrated indirectly, βPPs may be a player in the formation of PHF via the hyperphosphorylation of tau.

Amyloid

Amyloid fibrils associate characteristically to form β pleated sheet fibrous proteins, based on its x-ray diffraction pattern. Beta-amyloid (Aβ) has been isolated and purified using various techniques of centrifugation and solubility. As a result of Aβ
indentification, thousands of papers have referenced this material demonstrating the impact and usefulness of purification techniques.

Aβ is an insoluble peptide composed of 39-42 amino acids derived from the proteolytic cleavage of beta-amyloid precursor proteins (AβPP) encoded by a single gene on chromosome 21. The senile plaques and cerebral blood vessels in patients with AD contain predominantly the 42 amino acid isoform.

Some controversy exists concerning the pathology of Aβ. One school of thought suggests that amyloid is neurotoxic. In the cerebral amyloid angiopathy of AD, vascular smooth muscle cells show signs of degeneration attributed to amyloid. Also, neurons located with the Aβ deposits in senile plaques have been shown to degenerate. Still other studies have shown cultured neuron degeneration in the presence of Aβ, though the direct relationship is still questioned.

Not all research has shown Aβ to be neurotoxic. Synthetic Aβ and Aβ from plaque cores have been a substrate to which neurons adhere and develop normally hinting that other factors must cause neuronal death. One of these factors may be an astrocytic reaction to senile plaques involving chondroitin sulfate proteoglycan.

**Apolipoprotein E.**

Apolipoprotein E (apoE) is a protein that plays a major role in both the metabolism of cholesterol and triglycerides and in lipid transport in the central nervous system. This 34 kDa protein is the product of a single gene on chromosome 19 and exists in three isoforms (E2, E3, E4) and is located in the neuronal cytoplasm.
Apolipoprotein E has been observed to participate in various neurobiological roles. The mRNA encoding apoE is abundant in the brain where it is secreted by astrocytes. ApoE-containing lipoproteins are the major lipid transporters of the CNS. Also, apoE levels increase dramatically after injury and promote neurite extension in dorsal root ganglion cells when in the presence of cholesterol. ApoE scavenges the lipids produced from axon degradation and redistributes them to growing neurites for axon formation and myelination.

Recently the apolipoprotein E4 isoform has been linked to Alzheimer’s disease, particularly associated with late-onset familial and sporadic AD. Polymorphisms of the APOE gene affect the mean age of AD onset, with homozygous E4 individuals having the greatest risk. The apoE4 isoform readily bonds with Aβ to form a very stable complex, possibly resulting in reduced amyloid removal. This is the major difference between E4 and the other apoE isoforms (E3 and E2) thought to increase pathology of AD in individuals with at least one E4 allele.

It has been demonstrated that tau reacts with apoE3 in vitro, but not with apoE4. Results imply that the apoE3-tau interaction may prevent hyperphosphorylation and thus preclude PHF formation, while overproduction of apoE4 would enable tau to be vulnerable for hyperphosphorylation. This has yet to be proven directly.

Laminin.

Laminin was first isolated in 1983 using chromatography along with sodium dodecyl sulfate polyacrylimide gel electrophoresis (SDS-PAGE) and
immunoprecipitation methods. Since that time, this work has been referred to 114
times demonstrating the importance of its isolation and reflecting the magnitude of said
purification on future studies.

Located as a component of basement membrane, laminin is a promoter of
neurite outgrowth in the peripheral and central nervous systems. Although the
functions of laminin are largely unknown, it is associated with reactive glial cells in
response to CNS injury.

It is composed of three polypeptide chains (B1-A-B2) linked by disulfide bonds
and interacts with glycosaminoglycans. It also appears to be associated with
heparan sulfate proteoglycan (HSPG) which is another component of the basement
membrane as well as APP (cited earlier).

**Heparan sulfate.**

Heparan sulfate is a proteoglycan associated with the extracellular matrix,
especially the basement membrane (cited earlier). The less sulfonated analog of heparan
sulfate, heparin, has been shown to bind with high affinity to APP. It is this interaction
of heparin with the N-terminus domain of APP which provides the neurite outgrowth
promoting effect of APP. Not only do heparan sulfate proteoglycans (HSPGs) bind to
APP at two different domains, but they are also known to be constituents of amyloid
plaques. Although the exact function of HSPGs in Alzheimer’s pathology is uncertain,
binding of HSPGs to amyloid plaques is thought to interfere with APP in that region.
This results in a cascade effect of increased amyloidogenic processing.
It has been shown, for instance, that heparin and heparan sulfate both promote β-secretase cleavage of APP\textsuperscript{75} leading to an increase in the amyloidogenic pathway. The activities affecting the sulfonation of heparan sulfate are believed to be important in this shunt towards Aβ production. The increase of Aβ is thought to attract more HSPGs and thus affect more APP processing.

HSPGs are not only associated with amyloid plaques but also with the PHFs of neurofibrillary tangles and extracellular ghost tangles.\textsuperscript{103} Non-phosphorylated recombinant tau isoforms have formed PHFs in the presence of sulfonated glycosaminoglycans such as heparin or heparan sulphate.\textsuperscript{35} Since heparin is more sulfonated than heparan sulphate, the interaction between heparin and tau will be more potent.\textsuperscript{49} Heparin not only prevents tau from interacting with microtubules, but it also promotes the disassembly of existent microtubules. Heparan sulfate also coexists with hyperphosphorylated tau in nerve cells of Alzheimer’s disease brain\textsuperscript{35} supporting the findings that heparin stimulates tau phosphorylation by a number of protein kinases.\textsuperscript{9,85,141} These facts indicate that sulfated glycosaminoglycans might allow a cascade effect for neurofibrillary tangle production.

Other sulfonated proteoglycans, such as chondroitin sulfate, are also associated with senile plaques and neurofibrillary tangles of Alzheimer’s disease\textsuperscript{22} reflecting the relevance of proteoglycans within AD pathology.

**Calpain.**

Calpain is a calcium-activated neutral proteinase. Because calcium levels are good indicators of cell health, calpain is believed to be a key proteinase in neuronal
degradation. Calpain is involved in membrane protein degradation, membrane modification, enzyme activation and hormonal regulation. Inhibition of calpain resulted in attenuation of cytoskeletal protein loss indicating cytoskeletal proteins (microtubule-associated proteins, intermediate filament proteins, and microfilaments) as substrates for calpain proteolysis.

m-Calpain and \( \mu \)-calpain are the two isoforms of calpain in brain tissue requiring millimolar and micromolar levels of calcium for activation, respectively. An increase of activated \( \mu \)-calpain has been determined by Western blot and immunohistochemical analysis in AD brains. Although no increase of m-calpain activity in AD has been determined, studies indicate an accumulation of m-calpain with neuritic plaques and neurofibrillary tangles. Because of this localization, it seems probable that calpain plays a part in AD pathology.

**Presenilin-1.**

Encoded on chromosome 14, presenilin-1 (PS-1) is a transmembrane protein with at least seven transmembrane domains and predominantly found associated with the endoplasmic reticulum (ER) and Golgi complex. Mutations in presenilin genes have been implicated in the pathogenesis of the early-onset of familial Alzheimer's disease (FAD). Alterations in presenilin genes, mostly by missense mutations, result in elevated levels of the APP processing fragment \( \alpha \beta 42/43 \) as well as other \( \alpha \beta \) isoforms. The increase \( \alpha \beta \) was noted both intracellularly and extracellularly.
Although the function of PS-1 remains unclear, sequence homology to proteins in *C. elegans*\textsuperscript{76-78,124} suggests involvement in signalling and protein trafficking. Because PS-1 is localized to intracellular membranes,\textsuperscript{68} a regulatory role in biosynthesis/transport of membrane proteins would also be consistent.\textsuperscript{79} Based on their structure, these proteins could also function as, or part of, an ion channel or transporter.\textsuperscript{4,79}

**Purification Techniques**

Protein purification techniques are used to isolate or at least increase a target protein’s concentration versus total protein for the purpose of analysis. Several techniques exist, of which, only eight will be mentioned, as described by Rosenberg.\textsuperscript{112} Usually, each method takes advantage of a different characteristic of the target molecule. Because of this, one technique may be more useful than another in certain purifications depending on the characteristics of the target molecule(s). Not only can one technique be more specific than another, but sometimes multiple techniques are used to eliminate extraneous molecules of various shapes, sizes, and properties resulting in a net purification of the target molecule. This purification scheme, as it is called, may vary in form depending on the target molecule, and so, is only determined experimentally.

The partial biology of AD has been discussed to reflect the biochemical scope of the disease and the variety of molecules which interact in the processes of AD biology/pathology. Mentioned below are some of the techniques that were used to isolate the proteins in AD brain to determine their respective roles.
**Immunoprecipitation.**

Antibodies are very specific reagents used in protein detection and purification. Polyclonal antibodies recognize multiple epitopes on the same protein, while monoclonal antibodies react with a specific epitope. Both antibody types can be used. Immunoprecipitation takes advantage of this association in order to separate target proteins usually from cell lysates.

Antibodies bound to Sepharose beads through their constant region are added to the lysate. The variable antigen-specific region binds to the target antigen. Centrifugation precipitates the bead-antibody-antigen complex from the lysate solution. In this way, the antibody serves as a specific bridge between the heavy bead marker and the target protein. Denaturing the antibody-antigen complex is all that remains in order to extract the antigen in a relatively specific manner.

**Immunohistochemistry.**

In order to purify a certain protein, one must demonstrate its presence within a sample or tissue before further analysis proceeds. Immunohistochemistry is an antibody-dependent method which not only reveals the presence or absence of a particular protein, but also its relative location within the tissue.

Tissue slices are fixed on slides and blocked. The blocking process, involving peroxide, destroys the tissue’s natural peroxidase activity. The natural peroxidase activity would cause a misrepresentation of the peroxidase-dependent stain used later. Prepared slides must be subject to a series of xylene and decreasing alcohol baths in order to dissolve the parafin storage material and hydrate the tissue. The antigen-specific
antibody, known as the primary antibody, is added followed by the secondary antibody (an antibody which binds to the primary antibody). The secondary antibody contains peroxidase activity. Peroxidase anti-peroxidase (PAP) is then added to complete the complex. Diamino benzidene (DAB) reveals the existence and location of the complex due to the formation of its characteristic stain in the presence of peroxidase activity. Immunohistochemistry basically builds a complex on top of the antigen so that it can be detected by microscope analysis.

**Centrifugation.**

AD proteins, as would be expected, are extracted from brain tissue. Since a majority of the cellular components are unwanted, they must be removed. Centrifugation is the means in which large masses of unwanted cellular material and debris are removed from the stock sample. Heavier material collects at the bottom of the centrifuge tube allowing easy separation via aspiration of the liquid supernate. Higher speeds of centrifugation will precipitate smaller particles. Centrifugation is also employed between other separation methods as part of a wash process.

**Gel Electrophoresis.**

Gel electrophoresis has been pivotal for analyzing and characterizing macromolecules. Polyacrylamide gel electrophoresis (PAGE) is a type of gel electrophoresis which is beneficial for molecular weight determination, protein purity, and various other protein characteristics.

Acrylamide is the repetitive unit which composes the gel. It is used as a retardant to hinder the passage of macromolecules and allow the passage of smaller particles, thus
separating the molecules by size. Higher concentrations of acrylamide will retard smaller molecules more than lower concentrations of acrylamide. The acrylamide forms a cross-linked network with the addition of ammonium persulfate (APS), a polymerizing agent, and N,N,N’,N’-tetramethylene-diamine (TEMED), a catalytic agent. The percentage of acrylamide is inversely proportional to the pore sizes within the gel.

Two layers of acrylamide are used in discontinuous electrophoresis. The top layer is the stacking gel while the bottom layer is referred to as the separating or resolving gel. The bottom layer is poured first and allowed to polymerize. It is within this layer that protein separation will occur. The stacking gel, poured second, has a lower concentration of acrylamide and pH than the resolving gel. This allows all samples to enter the resolving gel in an even stack for efficient separation. A comb is placed in the stacking gel leaving sample wells after polymerization.

Sodium dodecylsulfate (SDS) can be used to coat sample proteins with a negative charge. The charge-to-weight ratio is the same for each protein allowing separation to be determined by molecular weight. When an electrical current is passed through the gel, proteins will migrate through the acrylamide pores with the smallest proteins travelling farthest.

Simple staining of the gel reveals the location of the protein band(s). Known markers are used as guides to plot migration vs. molecular weight in order to determine the molecular weight of the proteins based on their band location.
Western/dot blots.

When the molecular weight of a pure protein sample is needed, SDS-PAGE is sufficient, but what if the protein sample contains multiple proteins? How does one determine which band is the band of interest? Western blotting is an antibody-dependent method which normally follows SDS-PAGE and reveals the location of the target band. Once this has been determined, the molecular weight of a certain protein within a gel can be ascertained.

The bands within the gel are transferred to a special membrane called nitrocellulose paper which binds to all proteins. The membrane is blocked usually with 10% non-fat milk to stop additional substances from binding to the paper. Primary antibody is applied followed by the addition of the secondary antibody. This method follows that of immunohistochemistry except PAP is not used. DAB is the staining reactant causing the location of the target band to appear.

Dot blots follow the same procedure except the source of the proteins is not from a gel. Samples can be loaded directly to the nitrocellulose paper. This technique would only demonstrate the presence or absence of an antigen in a particular sample, while western blots demonstrate the location of antigen bands used in molecular weight determination.

Chromatography.

Chromatography is a purification technique which uses two phases, usually solid and liquid, to separate samples. Many types of chromatography exist, such as: gel filtration, ionic-exchange, hydrophobic interaction, and affinity chromatography.
One of the simplest, gel filtration, separates samples according to size. Chromatography occurs in a column packed with gel beads. The sample loaded at the top of the column is pulled through by gravity. Separation occurs when particles interact with the pores on the gel beads. In contrast to PAGE, smaller particles will travel the slowest in a column rather than faster. Since the larger particles cannot penetrate the pores, they simply elute straight out of the column. Smaller particles have a greater interaction with the pores on the beads; thus, they have a slower the rate of flow through the column. The separated sample is collected in fractions at the bottom of the column and is tested for purity and concentration.

Affinity chromatography is a very specific technique for purification. Because of this, affinity chromatography is usually the last method in a particular separation scheme. The mechanism behind affinity chromatography is similar to immunoprecipitation with the exception that ligands may be used instead of antibodies. The column is packed with antibody-bound beads. As the sample passes through the column, only the antigen which correlates with the packed antibody binds to the column matrix while other particles flow through the column. Collecting the antigen is then done by elution with basic, acidic, or saline buffers depending on empirical data.

Ammonium sulfate.

Techniques for separating substances according to their size have already been mentioned, but what if the purified sample contains material of similar molecular weight? Another characteristic of protein separation must be employed. Solubility is an important separation factor which varies between molecules. Salts, such as ammonium sulfate, can
be used to precipitate proteins from solution in a manner dependent on solubility. Ammonium sulfate can separate similar proteins in a way which centrifugation alone can not.

Dissolved material is actually material coated with a lattice of water molecules. By adding an extremely soluble salt, water molecules will entropically favor the solvation of the salt over the less soluble material. Water molecules bound to the sulfate ion reduce the water molecules available to keep a given protein in solution. This process is known as “salting out”. Increasing the salt concentration will precipitate proteins according to their solubility, with each precipitate being extracted by centrifugation.

10-5A9 Antigen Purification.

Introduction

This present study began with the development of an antibody from cockroach nervous system by Jefferey Denburg in 1993, known as 10-5A9. He discovered the presence of 10-5A9 antigen in developing and adult cockroach nervous system. Although Denburg focused on the developmental proteins of cockroaches, immunohistochemical research conducted by David DeWitt with AD brains demonstrated that the 10-5A9 antigen is also present in the lesions of Alzheimer’s disease. Immunohistochemistry of AD brains using 10-5A9 and controls revealed specific immunostaining of both senile plaques and neurofibrillary tangles. This posed the question as to the identity of the 10-5A9 antigen localized in these regions and its role in Alzheimer’s pathology.
The purpose of this study was to determine a separation scheme that would purify the 10-5A9 antigen for the purpose of further analysis and amino acid sequencing. A major drawback was the lack of information known about the 10-5A9 antigen. The only given about the antigen was its association with the 10-5A9 antibody and its immunolocalization to AD lesions.

Methods and Materials

Immunohistochemistry. Prepared slides of Alzheimer’s, Parkinson’s, and control brains were obtained from the Kathleen Price Bryan Brain Bank. Slide preparation followed the following rehydration: xylene (twice), 100% ethanol, 95% ethanol, bleaching solution, 70% ethanol, and Tris Buffered Saline (TBS: 150 mM NaCl, 50 mM Tris, pH 7.6). Bleaching consisted of a 30 minute soak in a 10% H₂O₂ /90% methanol solution made fresh each time. This blocking mixture destroyed the natural peroxidase activity of the brain tissue. The duration of use for the other solvents was 10 minutes. All slides were treated with 10% normal goat serum (NGS) solution in TBS for 30 min. and rinsed with a 1% NGS wash. The 10% NGS was a stringency solution used to absorb unbound antibodies. Three categories of slides were used: experimental, positive control, and negative control. With experimental slides, the primary antibody was 10-5A9 (dilution varied). Antibody against neurofilament (NF)(1/150 in 1% NGS-TBS) was the primary antibody with positive controls, while no primary antibody was used on the negative controls. Primary antibody, depending on slide category, was added (35-50 µl) and slides were left overnight at 4° C in a sealed moist container.
All slides were rinsed with 1% NGS-TBS followed by a 10 min. soak in 10% NGS-TBS. Once again the slides were rinsed with 1% NGS-TBS after which the secondary antibody was applied (35-50 µl) for 30 min. The secondary antibody was goat vs. mouse (1/50 in 1% NGS-TBS) since the primary antibodies were developed in mouse.

After slides were rinsed with 1% NGS-TBS, soaked in 10% NGS-TBS (10 min.), and rinsed again, they were subject to tertiary antibody (1/250 PAP in 1% NGS-TBS) for at least an hour at ambient temperature. A 1% NGS-TBS rinse and 10% NGS-TBS wash (10 min.) then preceded development.

Development of the slides began with a 10 min. wash in Tris buffer (50 mM, pH 7.6). Slides were stained simultaneously with diaminobenzidine solution (10 ml of 50 mM Tris, pH 7.6 and 5 µl H₂O₂ used to dissolve DAB to 0.75 mg/ml) and observed under light microscope. Slides were preserved by reversing the xylene/alcohol hydration steps and mounting with Paraffin.

*Lysate preparation.* AD brains were stored at -80°C while 1g sections were dissected at a time. After blood vessels and white matter were removed, the remaining gray matter was weighed, minced, and homogenized; the latter two over ice. 3g samples of gray matter were homogenized in 10ml RIPA buffer (50 mM Tris-HCl, pH 7.4-8.0, 150 mM NaCl, 1.0% Triton X-100, 0.5-1% Sodium deoxycholate, 0.1% SDS, 2 mM EDTA) with protease inhibitors (100 µM Leupeptin, 1 µM Pepstatin, 100 µM Aprotinin, 100 µM Chymostatin, 2 mM PMSF). The cell lysate was separated from the heavy particulate matter by centrifugation at 1500 x g.
**SDS-PAGE.** The 10% Acrylamide resolving gel was made by the addition of 2.5 ml 40% Acrylamide (800cc: 200g Acrylamide, 5.33g BIS), 3.33 ml running buffer 3x (6mM EDTA, 1.125 M Tris, 0.3% SDS, pH 8.8), 4.06 ml H₂O, 0.1 ml of 10% APS, and 10 µl TEMED. The stacking gel was then made using 0.5625 ml of 40% Acrylamide, 1.88 ml H₂O, 2.5 ml spacer buffer 2x (4 mM EDTA, 0.25 M Tris, 0.2% SDS, pH 6.8 and bromophenol blue indicator), 50 µl of 10% APS, and 5 µl TEMED. Electrode buffer consisted of 0.25 M Tris, 0.192 M Glycine, and 0.1% SDS, pH 8.2-8.3. Samples were prepared AD brain lysate and broad range markers in Laemmli sample buffer. The 20 µl samples were boiled for 5 min. and 2 µl of BME-Pyronin Y added before loading. Gels were run at 30-150 V, stained for 8 min. with Coomasie (0.25% w/v Coomassie Brilliant blue R-250, 10% conc. Acetic acid, 40% methanol, QS with deionized water) and destained (50% deionized water, 10% conc. Acetic acid, 40% methanol) for at least an hour. Silver staining was also employed with some gels according to the Bio-Rad Laboratories procedure.

**Western blotting.** Gels from SDS-PAGE were rinsed in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). The transfer occurred overnight at 30 V. A solution of %10 non-fat milk in TBS was used to block the membrane for an hour. After a TBS rinse, 50 µl of 10-5A9 antibody was added. Incubation occurred overnight at 4°C. The membrane was then washed five times with TBS and 50 µl of the secondary antibody, goat vs. mouse peroxidase (1/250), was added. Incubation at ambient
temperature for an hour followed. Four TBS washes and a 50 mM Tris pH 7.6 wash preceded DAB development.

***Immunoprecipitation.*** 800 µl of lysate obtained from AD homogenate was incubated at 4°C and shaken for an hour along with 40 µl of mouse-serum agarose and 40 µl of Anti-mouse IgG. The mixture was then centrifuged at 200 g's and the supernatant collected. Four samples were used for the following purposes: one served as the experimental sample, two served as negative controls, and the final sample was a positive control (Table 1).

<table>
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<tr>
<th>Sample</th>
<th>Label</th>
<th>Primary Anti-body used*</th>
<th>Function</th>
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<tr>
<td>AD Lysate</td>
<td>AB+B</td>
<td>10-5A9</td>
<td>Experimental</td>
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<tr>
<td>10-5A9</td>
<td>AB</td>
<td>10-5A9</td>
<td>Negative Control</td>
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<td>B</td>
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<tr>
<td>AD Lysate</td>
<td>NF</td>
<td>α-Neurofilament</td>
<td>Positive Control</td>
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*All four samples received the same secondary antibody.

100 µl of the 10-5A9 antibody was placed in the experimental tube as well as the AB negative control tube, while 100 µl of anti-neurofilament antibody was placed in the positive control tube. 200 µl of the prepared supernatant was placed in the experimental, the B negative control, and positive control tubes. All tubes were then brought to 1 ml with dilution buffer (1% Triton X-100 TBS, BSA 1 mg/ml) and incubated on ice for an hour. Each tube then received 50 µl of Anti-mouse IgG (50/50 slurry with dilution
buffer) before incubation at 4°C for an hour. The tubes were centrifuged and the supernatant discarded. The remaining pellets were washed four times, twice with Dilution buffer and then with TBS pH 8.0, and 50 mM Tris pH 8.0. Each wash consisted of resuspending the pellet, centrifuging, and discarding the supernatant. To the washed pellets was added 25 μl Laemmli sample buffer (2% SDS, 10% Glycerol, 50 mM Tris pH 6.8, Bromophenol blue). Tubes were boiled for 5 min. and centrifuged. The supernatant was collected. 1 μl and 2 μl of BME were added to molecular weight markers and the four samples, respectively. All samples were run in SDS-PAGE with the molecular weight markers located in the terminal wells.

The entire process was repeated with the following modifications: new homogenate was used, petroleum jelly used as PAGE sealant to prevent electrode buffer leakage, an extra sample labeled 2AB+B where 200 μl of 10-5A9 was used with 100 μl of brain, and the employment of longer PAGE run time.

*Dot blots.* New homogenate was made and the lysate obtained. The samples were labeled as follows: 1 ml of normal AD lysate (N series), .5 ml of 1% Triton X-100 lysate (T series), .5 ml of 0.1% Triton X-100 lysate (.T series), .5 ml of 1% SDS lysate (S series), and 0.1% SDS lysate (.S series). All samples were centrifuged at 4°C at the following speeds: 1000 rpms for 5 min., 4000 rpms for 5 min., 7000 rpms for 10 min., 12000 rpms for 30 min., and 14000 rpms for 30 min. After the first centrifugation speed, the supernate was removed from each sample and used for the next centrifugation speed. Labels changed by the addition of the centrifugation number along with the pre-existing
label (demonstrated in Table 2). The last centrifugation of the N series (N5) did not produce a pellet. Each of the beginning samples resulted in the collection of 5 pellets and a remaining lysate. Since 5 beginning samples were used (N, T, .T, S, .S), 30 samples were produced for dot blot assay.

Nitrocellulose paper was cut into 3 (1” x 1”) sections. Each section (labeled AB, NF, and B) contained 1 μl of the 30 samples described above. The AB was the

Table 2. Centrifugation of the N series beginning sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Centrifugation (rpm)</th>
<th>Time (min)</th>
<th>Pellet Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD lysate</td>
<td>1,000</td>
<td>5</td>
<td>N</td>
</tr>
<tr>
<td>N supernate</td>
<td>4,000</td>
<td>5</td>
<td>2N</td>
</tr>
<tr>
<td>2N supernate</td>
<td>7,000</td>
<td>10</td>
<td>3N</td>
</tr>
<tr>
<td>3N supernate</td>
<td>12,000</td>
<td>30</td>
<td>4N</td>
</tr>
<tr>
<td>4N supernate</td>
<td>14,000</td>
<td>30</td>
<td>5N</td>
</tr>
<tr>
<td>5N supernate*</td>
<td>none</td>
<td>-----</td>
<td>6N*</td>
</tr>
</tbody>
</table>

*N-5N were all pellets, while 6N was the 5N supernate.

experimental, while the NF and B were the positive and negative controls, respectively.

The paper was wet with methanol and then rinsed with 50 mM Tris pH 7.6. The nitrocellulose was allowed to dry to fix the samples. The paper was rewet with methanol, rinsed with 50 mM Tris pH 7.6, blocked with 10% dry non-fat milk in TBS for an hour while shaking, and then rinsed in TBS. The sections labeled AB, NF, and B, received 50 μl of 10-5A9 antibody, 50 μl of neurofilament antibody, and 50 μl of 1% goat serum respectively (Table 3). All three sections were immunostained with DAB following the previously described western blotting method.
Another immunoblot series, identical to the method above, was conducted using homogenate of the white matter in AD brain vs. controls. Finally, a third immunoblot series was run using multiple concentrations of the N sample vs. controls. The loaded dots ranged from 1-2 µl samples of 1:1, 1:2, 1:4, and 1:8 dilutions.

Table 3. Labels for dot blot sections with corresponding antibodies.

<table>
<thead>
<tr>
<th>Label</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>10-5A9</td>
<td>Goat vs. Mouse</td>
</tr>
<tr>
<td>NF</td>
<td>α-Neurofilament</td>
<td>Goat vs. Mouse</td>
</tr>
<tr>
<td>B</td>
<td>None*</td>
<td>Goat vs. Mouse</td>
</tr>
</tbody>
</table>

*1% NGS was used since it was used to dilute both 10-5A9 and α-NF.

*Ammonium sulfate.* New homogenate was made with 10g AD gray matter in 30ml RIPA buffer (50 mM Tris-HCl, pH 7.4-8.0, 150 mM NaCl, 1.0% Triton X-100, 0.5-1% Sodium deoxycholate, 0.1% SDS, 2 mM EDTA). The homogenate was centrifuged at 1600 x g’s for 20 min., 2500 x g’s for 10 min., and 3500 x g’s for 5 min. to remove particulate matter. This pellet was labeled P1 and placed in storage. Centrifugation at 10,000 x g’s (30 min.) produced a pellet labeled P2 and supernatant used for ammonium sulfate treatment. Samples were kept in ice to increase protein stability and decrease solubilities. Litmus paper was used to keep ammonium sulfate treated samples at neutral pH. The 20 ml of supernatant was transferred to a centrifuge tube labeled P3 and 2.88g of ammonium sulfate was added, bringing the solution to 25% saturation. Before centrifugation at 10,000 x g’s for 20 min., 27 drops of 1 M NaOH was added to neutralize the solution. Approximately 10ml of the 25% supernatant was transferred to a tube labeled P4 and
0.93g of ammonium sulfate was added, bringing the 25% solution to 40% saturation. Because 13 drops of 1 M NaOH was used to neutralize the solution, an additional 0.279g of ammonium sulfate was added to keep the solution at 40% saturation. Centrifugation at 12,500 x g’s for 10 min. produced the fourth pellet. Approximately 13ml of 40% supernatant was transferred to a tube labeled P5. The solution was brought to 60% saturation by the addition of 1.716g ammonium sulfate, and kept neutral by the addition of 10 drops of 10 M NaOH. Centrifugation at 16,000 x g’s for 10 min. produced pellet five. Approximately 13ml of 60% supernatant was transferred to a tube labeled P6. The solution was brought to 80% saturation by the addition of 1.86g ammonium sulfate, and kept neutral by addition of 6 drops of 10 M NaOH. Centrifugation at 25,000 x g’s produced the sixth pellet and the final supernatant labeled L7 (Table 4). All samples (P2, P3, P4, P5, P6, L7) were individually loaded into dialysis tubing. Samples P3, P4, P5, and P6 were slightly diluted with TBS for transfer purposes. The samples were placed in 1000ml of dialysis buffer (10 mM Tris-HCl pH 7.4 containing 10 mM MgSO4) for 24 hours.

Table 4. Ammonium sulfate fractionalization.

<table>
<thead>
<tr>
<th>Label</th>
<th>Centrifugation (rpm)</th>
<th>Duration (min)</th>
<th>Ammonium sulfate (%)</th>
<th>NaOH (drops)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>10,000</td>
<td>30</td>
<td>****</td>
<td>****</td>
</tr>
<tr>
<td>P3</td>
<td>10,000</td>
<td>20</td>
<td>25</td>
<td>27*</td>
</tr>
<tr>
<td>P4</td>
<td>12,500</td>
<td>10</td>
<td>40</td>
<td>13*</td>
</tr>
<tr>
<td>P5</td>
<td>16,000</td>
<td>10</td>
<td>60</td>
<td>10**</td>
</tr>
<tr>
<td>P6</td>
<td>25,000</td>
<td>30</td>
<td>80</td>
<td>6**</td>
</tr>
<tr>
<td>L7</td>
<td>****</td>
<td>****</td>
<td>80</td>
<td>****</td>
</tr>
</tbody>
</table>

*1M NaOH, **10M NaOH, † supernate from the P6 centrifugation.
hours. The buffer was replaced with new and the samples soaked for another 24 hours. The dialyzed samples were then loaded onto nitrocellulose for dot blot examination, using dilution gradient and NF, B controls described above.

*Pellet Immunostaining.* The low centrifugation, particulate matter collected from the AD brain homogenate was fixed to slides using ethanol and heat fixative techniques. Two slides, labeled HA and HC were heat fixed, while the other two, labeled EA and EC, were fixed with 95% ethanol (Table 5). Two prepared parafin slides were also used as controls. Both control slides were hydrated according to immunohistochemical procedure. Immunostaining of all slides followed: primary antibody was 40 μl of 10-5A9, while the secondary antibody was an equal volume of goat vs. mouse antibody. An equal amount of PAP was used prior to DAB development. The HC, EC, and CC slides went through the exact procedure except 40 μl of 1% goat serum was replaced as the primary antibody.

**Table 5. Pellet Immunostaining Samples.**

<table>
<thead>
<tr>
<th>Label</th>
<th>Fixative Method</th>
<th>Primary Antibody</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>Heat</td>
<td>10-5A9</td>
<td>Experimental</td>
</tr>
<tr>
<td>HC</td>
<td>Heat</td>
<td>1% NGS</td>
<td>Control</td>
</tr>
<tr>
<td>EA</td>
<td>95% Ethanol</td>
<td>10-5A9</td>
<td>Experimental</td>
</tr>
<tr>
<td>EC</td>
<td>95% Ethanol</td>
<td>1% NGS</td>
<td>Control</td>
</tr>
<tr>
<td>CA</td>
<td>*</td>
<td>10-5A9</td>
<td>Experimental</td>
</tr>
<tr>
<td>CC</td>
<td>*</td>
<td>10-5A9</td>
<td>Control</td>
</tr>
</tbody>
</table>

*Slides were obtained having been already fixed.*
Results

*Immunohistochemistry.* Staining of experimental slides revealed localization of the 10-5A9 antigen in the senile plaques as well as the neurofibrillary tangles associated with AD (Figure A-F). Stained regions also included the perimeter of blood vessels. Negative controls lacked immunostained structures. Staining of Lewy bodies in Parkinson’s disease and diffuse Lewy body disease was noted using the 10-5A9 antibody.

*SDS-PAGE/Western blotting.* The stained gels revealed a protein smear within each lane confirming the presence of proteins. Neurofilament controls revealed bands on nitrocellulose demonstrating the effectiveness of protein transfer and immunostaining methods. No bands were produced using the 10-5A9 antibody. Since this method proved ineffective, immunoprecipitation was next method of choice.

*Immunoprecipitation.* Ideally, the experimental lane would have one band while the other lanes would have none. Because this wasn’t the case (all lanes revealed multiple bands), the lanes were compared to determine if a band(s) was present in the experimental lane that was not present in the other lanes. There was no difference in the 10-5A9 samples and the controls. Neurofilament samples were negative resulting in inconclusive data. Because immunoprecipitation also proved ineffective, column chromatography was the next method to be used in purification attempts.

*Dot blots.* The purpose of the dot blots was to develop an assay for the 10-5A9 antigen.
This was critical for the use of chromatography since separated fractions would have to be tested for antigen presence. The neurofilament positive controls resulted in well defined blots indicating the effectiveness of the technique. Blots of experimental samples using 10-5A9, on the other hand, resembled the negative control blots. The development of a 10-5A9 assay was ineffective. It was thought that the antigen concentration was too low for assay detection. In order to detect the antigen, its concentration would need to be increased; thus, the incorporation of ammonium sulfate.

Ammonium sulfate. The results from the incorporation of ammonium sulfate paralleled that of the previous section. Control samples assayed with neurofilament antibody demonstrated positive results, indicating effective technique. Once again, the experimental 10-5A9 blots were indistinguishable from the negative control blots. This method proved ineffective in concentrating the antigen. Because purification and concentration of the AD lysate had not produced better results, it was thought that somewhere in the homogenate separation scheme the antigen had been lost or its concentration had been greatly reduced. This led to immunostaining of AD particulate matter.

Pellet Immunostaining. Immunostaining of the pellet collected from low-speed homogenate centrifugation was compared to control slides. Experimental slides stained darker than control slides indicating the presence of the 10-5A9 antigen within the pellet
Figure A. Immunostaining of tau protein within neurons of AD. (brown)

Figure B. Immunostaining of Aβ within AD plaque. (blue)

Figure C-E. Immunostaining of both neurofibrillary tangles and senile plaques of AD using 10-5A9 antibody. (compare with Fig. A and Fig. B)

Figure F. Immunostaining of the Lewy bodies of diffuse Lewy body disease using 10-5A9 antibody.

Figure G-H. 10-5A9 immunostaining of developing (H) and adult (G) cockroach nervous system.
fraction. Plaque cores, known to occupy this fraction, also demonstrated the presence of the 10-5A9 antigen when immunostained; thus supporting our previous finding.

Discussion

The data collected by immunohistochemistry demonstrates the presence and location of the 10-5A9 antigen in AD. This is the link which compels further research with 10-5A9. The specific staining of both senile plaques and neurofibrillary tangles by 10-5A9 confirms a link between the 10-5A9 antigen and the lesions of AD indicating an unknown role in AD pathology. Since other proteins found by immunostaining have been significant, 10-5A9 could be as well, especially in senile plaques and neurofibrillary tangles.

Slides from Parkinson’s disease brains revealed an interesting correlation between the 10-5A9 antigen and the Lewy bodies characteristic of the disease. Not only does the 10-5A9 antigen demonstrate association with Alzheimer’s disease, but it also seems linked to another neurodegenerative disease indicating similar pathological roles in both AD and Parkinson’s disease. 10-5A9 could play a role in neuronal inclusion formation.

These results also serve as an indicator of the functionality of the 10-5A9 antibody throughout the research process. Immunohistochemistry was done multiple times at various time intervals during the research period. Since it has now been determined that the antigen is present within AD brains, it should be possible to purify the antigen from AD brain tissue.

It was hoped that western blotting would produce a band from the protein smear and give the molecular weight. This was unsuccessful. At first it was believed that the
loaded gel sample was too dilute in respect to its 10-5A9 concentration. The ratio of target protein to total protein was too high as evidenced by the stained gel. Either the concentration of 10-5A9 antigen had to increase or a more specific method of extraction had to be utilized. The latter was used first in the form of immunoprecipitation. Because immunoprecipitation was also unsuccessful, separation was attempted using column chromatography.

Since chromatography was to be incorporated, blotting techniques had to be developed first. Dot blots were conducted for two main reasons. The first was to determine the lysate fraction which contained the highest concentration of the 10-5A9 antigen. If this could be determined, the concentrated sample could be used in chromatographic techniques which normally dilute the loaded sample. The concentrated fraction could also be used in additional immunoprecipitation and/or western blotting procedures in the hopes of a successful outcome.

The second purpose of the dot blots was to serve as a chromatographic assay of 10-5A9. Since experimental data mirrored that of control samples, this purpose was not fulfilled. The assay was ineffective, and so, chromatography could not be used.

It was then believed that if the antigen within the lysate could be concentrated, the dot blot assay might prove effective enabling chromatographic methods. Ammonium sulfate concentrated the lysate by separating it into fractions of proteins with similar solubilities. Even with the increased lysate purity, the dot blots of these samples failed to distinguish between the experimental and control samples. Since the lysate could not easily be separated further, frustration increased. All hope that the antigen was present
within the lysate fraction slowly diminished as each purification and concentration yielded negative data. Since immunohistochemical data indicated the antigen’s presence in the tissue, it was believed that the antigen precipitated out with the particulate matter and cellular debris of the low-speed centrifugation.

The immunostaining of this pellet with 10-5A9 indicated the presence of the antigen. This was positive data which explained the unsuccessful attempts to purify the antigen from AD lysate. Although most proteins are contained within the lysate fraction, some are known to associate with larger material and precipitate within an earlier fraction.

Plaque cores tested for antigen presence proved positive; directly supporting the notion of 10-5A9 within the initial pellet. This association must be strong since core purification is a harsh procedure involving high temperatures and SDS. The data collected from this study indicates that not only is the 10-5A9 antigen present in Alzheimer’s disease, Parkinson’s disease, and diffuse Lewy body disease, but it is also tightly associated with plaque cores and possibly other cellular material within the brain.

**Conclusion**

It is believed, at present, that the reason all other methods proved ineffective was because the antigen concentration was too low in the experimental samples. If the antigen is most concentrated in the first centrifuge fraction, all previous tests can be viewed as negative controls.

Normal purifications of cellular protein begin with the removal of the tissue particulate matter. It is known that some proteins, when associated with other structures,
precipitate at lower speeds. The staining pattern noted with SDS-PAGE seemed to show some material that did not enter the gel. Based on the pellet immunostaining evidence, it is believed that this may be some of the particulate matter. The structure or complex must be stable since it is believed to withstand SDS treatment.

Future study focuses on the purification of cores from AD particulate matter since it has been shown that neurofilament proteins have been associated with the core complex. Concentrated SDS treatment methods will be used to destroy all cellular material except the stable core complex. The purified cores can then be immunostained for antigen presence. All that remains, then, is to dissociate the core-antigen complex so that the antigen might be isolated in a relatively pure form.
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References


