ISAS - INTERNATIONAL SCHOOL
FOR ADVANCED STUDIES

Looking for autoantibodies in Alzheimer's disease

Thesis submitted for the degree of "Doctor Philosophiae"

CANDIDATE
Roberta Bruno

SUPERVISOR
Dr. Andrew Bradbury

SISSA - SCUOLA INTERNAZIONALE SUPERIORE DI STUDI AVANZATI
TRIESTE
Via Beirut 2-4

TRIESTE

Declaration

The work described in this dissertation was carried out at the International School of Advanced Studies, Trieste, between November 1994 and November 1998. All work reported arise from my own experiments and this work has not been submitted in whole or in part to any other University.

Roberta Bruno

December 1998

TABLE OF CONTENTS

ABBREVIATIONS USED IN THE TEXT

Aβ = β-amyloid

ACH = amyloid cascade hypothesis

AChE = acetyl-cholinesterase

AD = Alzheimer's disease

AGE = advanced glycation end product

APOE = apolipoprotein E

APRP = amyloid precursor-related protein

βAPP = β-amyloid precursor glycoprotein

BBB = blood-brain barrier

BCB = blood-CSF barrier

BRA = brain-reactive antibody

CAD = coronary artery disease

ChE = cholinesterase

CIC = circulating immunocomplex

CMI = cell-mediated immunity

CNS = central nervous system

CSF = cerebrospinal fluid

CT = computed tomography

CVD = cerebrovascular dementia

DS = Down's syndrome

EGA = early Golgi apparatus

EM = electron microscopy

EMBL = European Molecular Biology Laboratory

ER =endoplasmic reticulum

ERT = estrogen replacement therapy

EST = expressed sequence tag

EVH = Enabled/VASP homology

FAD = familial Alzheimer's disease

FDG-PET = fluorodeoxyglucose - positron emission tomography

GA = Golgi apparatus

GAPDH = glyceraldehyde phosphate dehydrogenase

GFAP = glial fibrillary acidic protein

HSV1 = herpes simplex virus type 1

IEG = immediate early gene

IF = immunofluorescence

IgG = immunoglobulin

IL-6 = interleukin-6

IPTG = isopropylthio-β-D-galactoside

KPI = Kunitz family of protease inhibitor

LDL = low-density lipoprotein

LRP = low density lipoprotein receptor-related protein

LTP = long term potentiation

mAb = monoclonal antibody

MAGUK = membrane-associated guanylate kinases

MAP = microtubule-associated protein

mGluR = metabotropic glutamate receptor

MID = multi-infarct dementia

MRI = magnetic resonance imaging

NAI = neuroautoimmunity

NDC = non demented control

NFT = neurofibrillary tangles

NINCDS-ADRDA = National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association

NSAID = nonsteroidal anti-inflammatory drug

NT = neuropil threads

OND = other neurodegenerative disease

OFR = oxygen free radical

ORF = open reading frame

PCR = polymerase chain reaction

PD =Parkinson's disease

PHF = paired helical filaments

PS1 = presenilin 1

PS2 = presenilin 2

ROI = reactive oxygen intermediate

RT-PCR = reverse transcription PCR

SAD = sporadic Alzheimer's disease

SDAT = senile dementia of Alzheimer's type

SDS-PAGE = sodium dodecyl sulphate - polyacrylamide gel electrophoresis

SEREX = serological identification of antigens by recombinant expression cloning

SIB = Swiss Institute of Bioinformatics

SMC = smooth muscle cell

SP = senile plaques

VaD = vascular dementia

VASP = vasodilator-stimulated phosphoprotein

VSMC = vascular smooth muscle cell

Vesl = VASP/Ena-related gene up-regulated during seizure and LTP

WASP = Wiscott-Aldrich syndrome protein

WB = western blot

1. INTRODUCTION

1.1. DEMENTIA AND ALZHEIMER'S DISEASE

Dementia is a devastating and tragic syndrome highly prevalent in old age, which affects more than 10% of persons 65 years and older, and more than 30% of persons 80 years and over, often resulting in institutionalization and ultimately death. As we approach the twenty-first century, its public health importance is growing due to its marked occurrence with increasing age, particularly over 80, the age group with the greatest expansion in the next decade. Unfortunately, little is known about the etiology of dementia, particularly Alzheimer's disease (AD) which is, together with vascular dementia (VaD), the most common form of dementia.

1.2. ALZHEIMER'S DISEASE: CLINICAL PHENOTYPE

Although rare familial forms of AD exist, the majority of patients have no clear family history, and are classified as sporadic AD (SAD). Today, the diagnosis of SAD is based on clinical exclusion criteria, although a definite diagnosis is only possible at autopsy. The most commonly used criteria, the NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association) criteria (McKhann *et al.*, 1984), for 'probable AD', include: (1) the presence of progressive dementia; (2) no disturbance of consciousness; (3) onset between ages 40 and 90; and (4) absence of systemic disorders or other brain diseases that alone could account for the dementia, such as maniac-depressive disorder, Parkinson's disease (PD) and multi-infarct dementia (MID).

The patient with AD experiences gradually increasing forgetfulness, decreasing attention span, and alterations in mood, often with frustation and agitation, resulting in increasing difficulties in meeting the demands of daily living. These problems progress until the patient ultimately cannot attend to his (her) simplest needs and becomes bedridden, totally dependent on caregivers. The interval between initial diagnosis and death varies considerably, usually between 3 and 15 years.

1.3. ALZHEIMER'S DISEASE: NEUROPATHOLOGICAL PHENOTYPE

AD begins in the cerebral cortex and remains predominantly a disorder of this chief controlling entity of the brain. The destructive process underlying AD is by no means diffuse. On the contrary, the cortical changes exibit a characteristic area-specific, lamina-specific and even cell-type-specific distribution pattern (Fig. 1) (1.3.1.).

Independent of etiology, whether genetic or nongenetic, AD is characterized histopathologically by an increased number of senile plaques (SP) consisting chiefly of β-amyloid (Aβ) proteins (1.3.2. and 1.3.2.2.) and of neurofibrillary tangles (NFT) dominated by abnormally phosphorylated tau proteins (1.3.3. and 1.3.3.2.) (Neve *et al.*, 1990; Selkoe, 1991; Hardy and Higgins, 1992), and a degeneration of the cholinergic neuronal systems, in particular those projecting from the basal forebrain to the hippocampus and cerebral cortex (1.3.4.) (Bartus *et al.*, 1982; Whitehouse *et al.*, 1982; Mash *et al.*, 1985; Lehericy *et al.*, 1993). These neuropathological phenomena are phenotypic manifestations associated with the expression of the disease, but their role in the pathogenesis of the disease is unknown.

According to defined neuropathological criteria (Khachaturian, 1985) based on the presence of SP and NFT (1.3.2. and 1.3.3.), a diagnosis of definite AD can be made only by microscopic examination of brain tissue, either at biopsy or, more commonly, autopsy.

1.3.1. Pattern Of Cortical Lesions In Alzheimer's Disease

As mentioned above (1.3.) and well studied and illustrated by Braak (Figs. 1 and 2), the destructive cortical changes underlying AD exibit a characteristic area-specific, lamina-specific and even cell-type-specific distribution pattern with the following characteristics.

1.3.1.1. β-Amyloid Deposits

The Aβ deposits are initially seen in poorly myelinated areas of the basal neocortex (stage A). From here, they spread into adjoining neocortical areas and the

hippocampus (stage B) and eventually infiltrate all cortical areas, including densely myelinated primary fields of the neocortex (stage C). Evaluation of numerous non-selected autopsy brains reveals that a large proportion of normal subjects exhibit no Aβ deposits, even at an advanced age. A few cases develop initial deposits in young adulthood. The age distribution from stage A to stage C shows a shift towards higher age categories. The prevalence of stage C cases increases with age (Fig. 2a).

1.3.1.2. Neurofibrillary Changes

NFT and neuropil threads (NT) (1.3.3. and 1.3.3.1) develop selectively in specific sites within the cerebral cortex (Hyman *et al.*, 1990; Van Hoesen *et al.*, 1991; Braak and Braak, 1991; Hyman and Gomez-Isla, 1994; Braak and Braak, 1994; Braak and Braak, 1996). The changes then spread in a predictable, non-random manner across other cortical areas with little inter-individual variation, and thus provides a basis for staging the evolution of the changes (Figs. 1 and 2). Early stages occur predominantly in relatively young individuals, while the more advanced stages gradually appear with increasing age (Ohm *et al.*, 1995) (Fig. 2b). Many normal subjects exhibit no neurofibrillary changes at all, even at an advanced age. A few individuals show the lesions early in life.

The first neurons to show the development of NFT and NT are specific projection cells in the transentorhinal region (stage I). In stage II, additional NFT/NT occur in the entorhinal region. Stage I and II represent the pre-clinical phase of AD because no obvious impairments of intellectual capacities are present. Further advance of the disease leads to stages III and IV severely involving the entorhinal territory, the amygdala and the hippocampal formation (Fig. 1). Stage III and IV cases are considered to represent incipient AD for the presence of initial symptoms and the characteristic brain lesions. The final stages show large numbers of NFT/NT in virtually every subdivision of the cerebral cortex. Stages V and VI correspond to the conventional criteria for neuropathological diagnosis of AD.

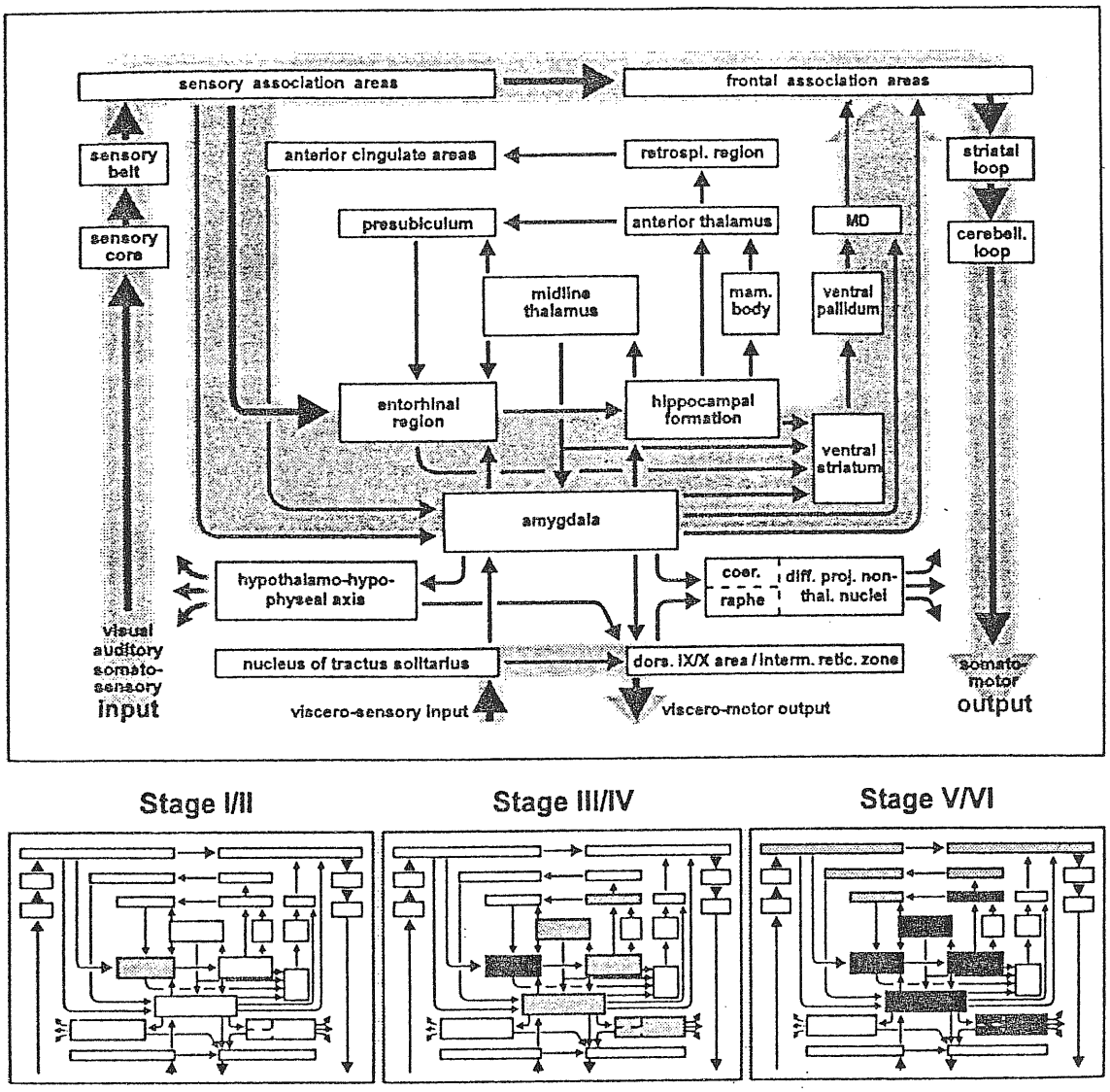


Figure 1. Upper half: schematic representation of neocortical and limbic imput to the entorhinal region, data transfer between the entorhinal region, amygdala and hippocampal formation, and organization of major limbic circuits. Abbreviations: cerebell., cerebellar; coer., locus coeruleus; diff. proj. non-thal. nuclei, non-thalamic nuclei diffusely projecting to the cerebral cortex; dors., dorsal; interm. retic. zone, intermediate reticular zone; mam., mamillary; MD, mediodorsal nuclei of the thalamus; retrospl., retrosplenial. Lower half: order and pattern of intraneuronal AD-related pathology. In stages I/II alterations are confined to the entorhinal territory; in stages III/IV this region, hippocampus and amygdala are severely involved, while stages V/VI are marked by destruction of virtually all neocortical association areas. Increasing density of shading indicates increasing severity of the changes (from Braak and and Braak, 1997).

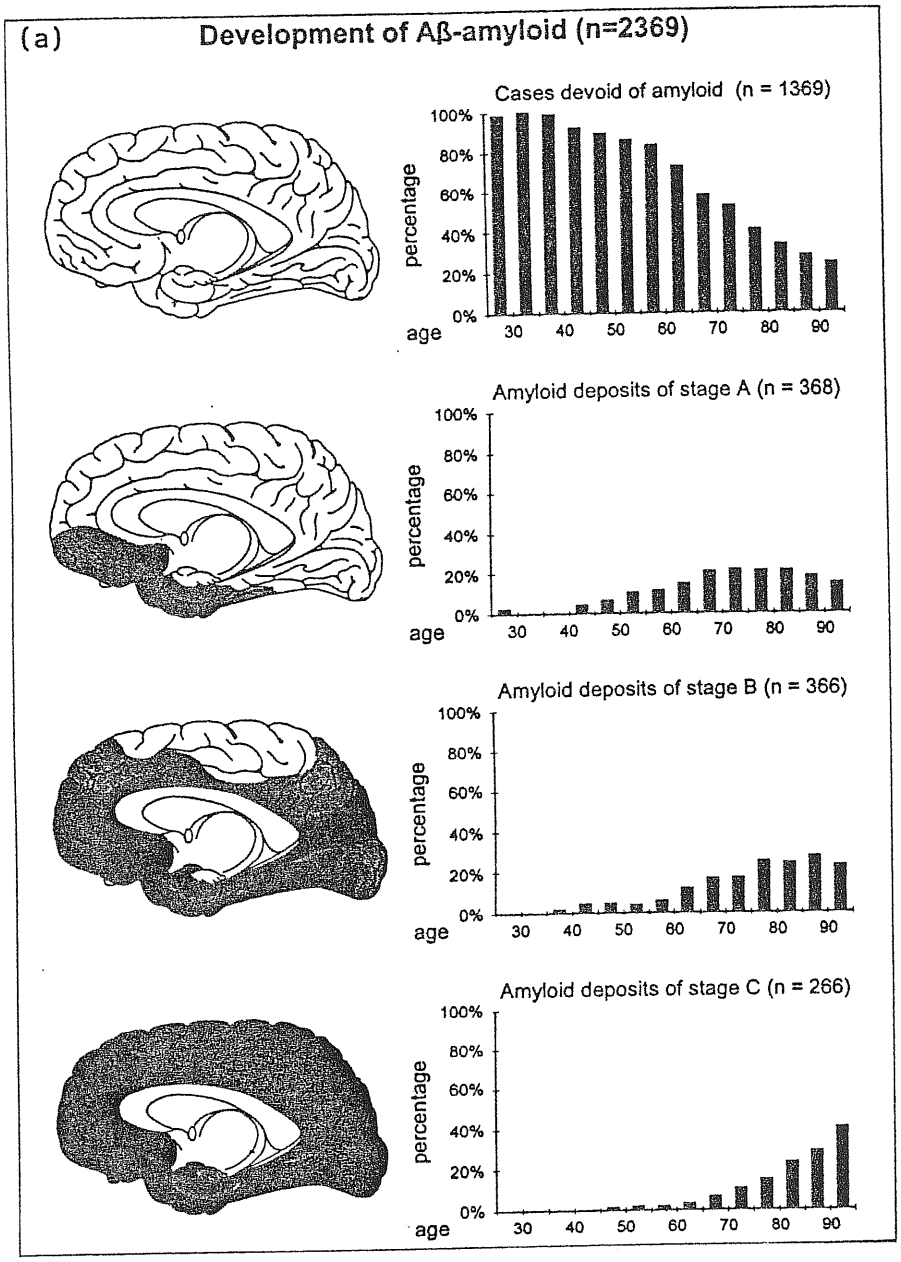


Figure 2. Development of Aβ deposits (a) and neurofibrillary changes (b) in a total number of 2369 non-selected autopsy cases. The first line displays the relative frequency of cases devoid of changes in the various age categories. The second, third and fourth lines are similarly designed, and show the evolution of the AD-related changes (from Braak and and Braak, 1997)

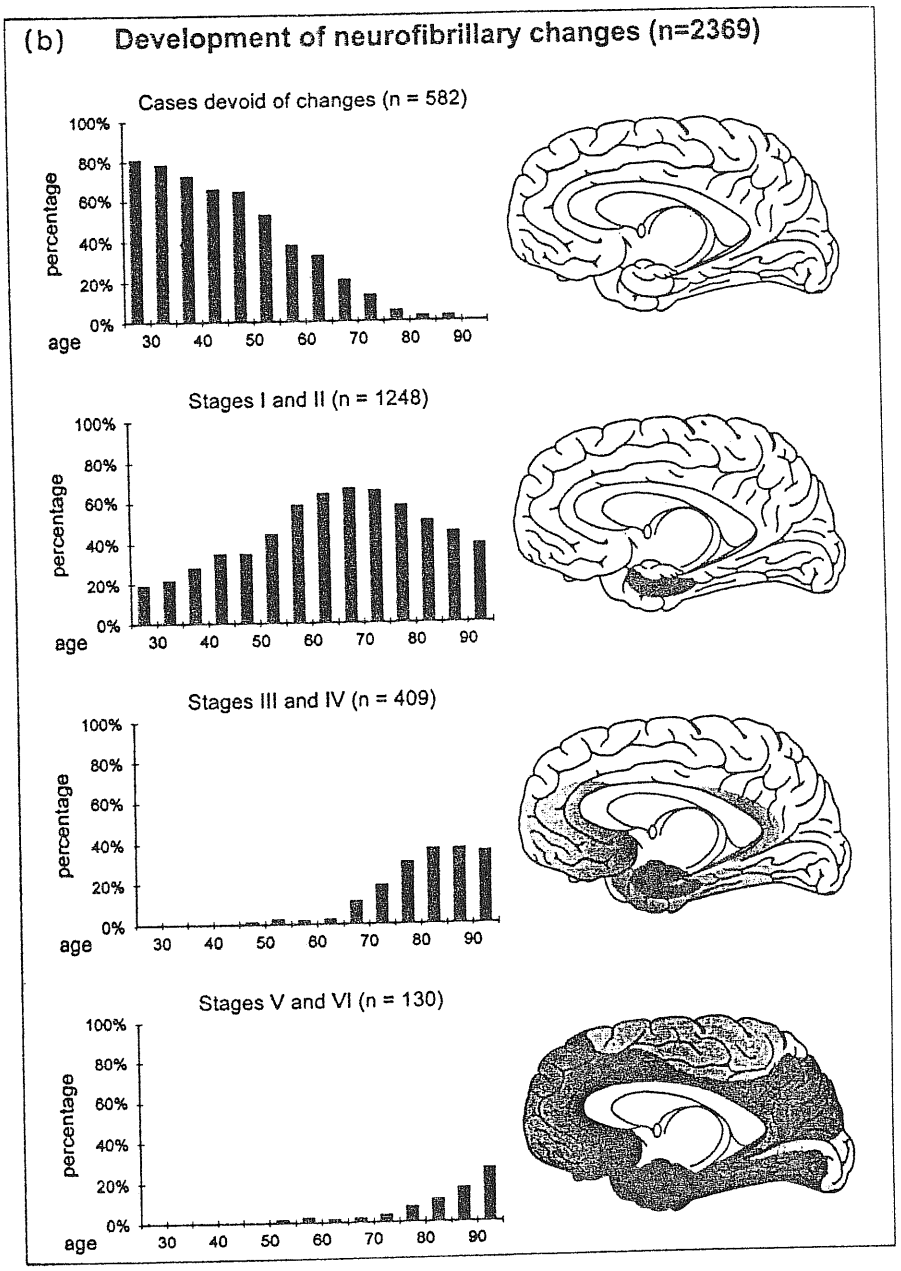


Figure 2. Continued (from Braak and and Braak, 1997).

1.3.2. Senile Plaques

SP are structures with complex and incompletely characterized molecular and cellular constituents. The major component of SP is a 39-43-residue peptide termed β-amyloid protein (Aβ) (Haass and Selkoe, 1993) (1.3.2.2.), proteolytically generated from the widely distributed β-amyloid precursor glycoprotein (βAPP) (1.3.2.1.) (Goldgaber *et al.*, 1987; Tanzi *et al.*, 1987) under normal metabolic conditions (for review, see Selkoe, 1994a). Other proteins found in SP include apolipoprotein E (APOE) (Namba *et al.*, 1991), βAPP (Arai *et al.*, 1990; Takahashi *et al.*, 1990), α_1-antichymotrypsin (Abraham *et al.*, 1988), acetyl-cholinesterase (AChE) (Moran *et al.*, 1993; Selkoe, 1994b), IgGs (Ishii and Haga, 1976; Ishii and Haga, 1984), several complement proteins (Eikelenboom and Stam, 1982; Rogers *et al.*, 1992), serum amyloid P component (Kalaria, 1992), cytokines (Griffin *et al.*, 1989), glycosaminoglycans (Benditt, 1989) and Sp40,40 (Ghiso *et al.*, 1993).

In SP characteristic central deposits of extracellular Aβ fibrils (the core) are surrounded by less densely packed Aβ. Neuritic plaques, which occur abundantly in AD and Down's syndrome (DS), are more complex lesions of the cortical neuropil with Aβ core infiltrated and surrounded by dystrophic neurites, fibrillary astrocytes and activated microglia. Noncompacted deposits of Aβ that contain very few or no surrounding dystrophic neurites and glia (diffuse plaques) and senile plaques without neuritic component are often found during normal brain ageing (Yamaguchi *et al.*, 1988). Diffuse plaques do occur both in the brain and outside the brain, in the skin and the gastrointestinal tract (Joachim *et al.*, 1989; Wen *et al.*, 1994).

In AD, in addition to plaque cores, Aβ deposits occur in the walls of cerebral and leptomeningeal blood vessels .

1.3.2.1. β-Amyloid Precursor Protein

The single copy βAPP gene located on chromosome 21 (Tanzi *et al.*, 1987) gives rise by alternative mRNA splicing to a related family of isoforms of 365, 563, 695, 714, 751, and 770 aminoacids, respectively (Kang *et al.*, 1987; Kitaguchi *et al.*,

1988; Ponte *et al.*, 1988; Tanzi *et al.*, 1988; de Sauvage and Octave, 1989; Golde *et al.*, 1990; Kang and Muller-Hill, 1990; Jacobsen *et al.*, 1991). These products represent the βAPP group of membrane-anchored polypeptides, 100-140 kDa in molecular mass, that undergo both N- and O-linked glycosylation, phosphorylation, sulfation and proteolytic cleavage and secretion of the large membranous region (Weidemann *et al.*, 1989; Oltersdorf *et al.*, 1990; Selkoe, 1993; Hung and Selkoe, 1994). The 365 and 563 amino acid long products of the βAPP group do not contain the Aβ region which is encoded within exons 16 and 17 (Lemaire *et al.*, 1989; Yoshikai *et al.*, 1990) and in order to distinguish them from βAPPs which include Aβ sequence they are dubbed as amyloid precursor-related proteins (APRP).

The shortest of the major isoforms, βAPP695, is expressed almost exclusively in neurons, whereas the other two common forms, βAPP770 and βAPP751, are expressed both in neural and non-neural cells and contain a domain with close similarity to the Kunitz family of protease inhibitors (KPI), which is lacking in the brain isoform. In normal brain they show a predominance in neuronal cell bodies and processes (Arai *et al.*, 1992) where βAPP immunoreactivity is found in vesicular compartments. They exist in different subcellular pools and can be axonally transported to nerve endings (Allinquant *et al.*, 1994).

The normal cellular function of βAPP is unknown. A possible role of βAPP in establishment of synaptic contacts during synaptogenesis is postulated from the transient increase of βAPP levels during rat brain development (Loffler and Huber, 1992) and their immunohistochemical localization at synapses (Shigematsu *et al.*, 1992). In addition, work with different cell lines has shown that the secreted or membrane-associated forms of βAPP regulate cell growth, neurite length and participate in cell-cell and cell-matrix adhesion (Shivers *et al.*, 1988; Saitoh *et al.*, 1989; Milward *et al.*, 1992; Roch *et al.*, 1992; Monning *et al.*, 1995). This idea is supported by the multi-ligand binding activity of βAPP to components of the extracellular matrix such as collagen, laminin and heparan sulphate side chains of proteoglycans (Breen *et al.*, 1991; Narindrasorasak *et al.*, 1992; Small *et al.*, 1994; Multhaup *et al.*, 1994). βAPP

isoforms containing a region homologous to the KPI also form complexes with extracellular proteases which are internalized by the APOE receptor LRP (low density lipoprotein receptor-related protein) (Kounnas *et al.*, 1995).

Amyloid plaques are thought to form when the amount of Aβ is increased as a result of either overexpression or altered processing of βAPP (Cai *et al.*, 1993; Selkoe, 1994a; Selkoe, 1994b).

1.3.2.2. β-Amyloid

Haass *et al.* (1992) showed that Aβ is generated as a soluble peptide during normal cellular metabolism of βAPP and circulates in human cerebrospinal fluid (CSF) and blood (Selkoe, 1993).

βAPP is processed by cellular proteases known as α, β and γ-secretases. The α-secretase cleaves within the Aβ peptide sequence in βAPP preventing the generation of Aβ. In contrast the β-secretase cleaves at the N-terminus and leads to the generation of the Aβ peptide, after cleavage by a γ-secretase, as shown in Fig. 3.

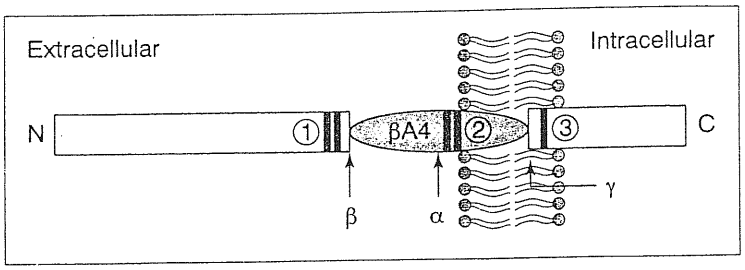


Figure 3. Proteolytic generation of β-amyloid (Aβ) from the β-amyloid precursor protein (βAPP).
The major component of SP in AD, the Aβ peptide, is generated from the transmembranous amyloid precursor protein (βAPP) by the action of two protease activities (β- and γ-secretases), although the bulk of normal constitutive processing of βAPP occurs by α-secretase cleavage, which yields nonamyloidogenic products.
In familial AD, mutations in the βAPP gene have been found to cluster around the cleavage sites (vertical bands: 1, Swedish mutation; 2, Dutch or AD mutation; 3, London mutation) and have been shown to interfere with βAPP processing, so causing increases in the amount of Aβ and/or in the more amyloidogenic, longer forms of Aβ generated (1.5. and 1.5.1.) (from Theuring *et al.*, 1997).

The site of cleavage by the γ-secretase enzyme(s) is C-terminal of residue 712. If cleavage occurs at residue 712-713, short Aβ, Aβ40, results; if it is after residue 714, long Aβ, Aβ42(43), is generated.

The finding that Aβ is generated during normal cellular metabolism of βAPP raised the possibility that it has a physiological function throughout life and that it is its chronically enhanced production and/or decreased clearance which leads to the gradual precipitation of aggregated non-diffusible Aβ in the form of SP and vascular deposits in AD and DS. To support this hypothesis studies of cultured neurons *in vitro* have shown that aggregated Aβ fibrils are toxic, while non-fibrillar Aβ is not (Yankner, 1996). When synthetic Aβ is placed in physiological saline it generates free radical peptides that can directly damage enzymes in a way that is directly correlated with aggregation kinetics (Hensley *et al.*, 1994), and induce accumulation of reactive oxygen species in cultured neurons (Behl *et al.*, 1994; Goodman and Mattson, 1994) and synaptosomes (Butterfield *et al.*, 1994). Studies of DS brains showed that diffuse deposits of Aβ could be detected as early as the mid-teens (Mann, 1989) and can remain for 10-20 years in a non-fibrillized form (Wen *et al.*, 1994). It thus appears that fibrillization *per se* is an important element of AD pathogenesis.

Recent work has shown that Aβ42(43), rather than Aβ40, is critically important in the pathogenesis of AD: Aβ42(43) is highly aggregatable (Hilbich *et al.*, 1991), is a major component of early diffuse and mature plaques in both DS and AD brains (Gravina *et al.*, 1995; Iwatsubo *et al.*, 1995; Tamaoka *et al.*, 1995) and soluble Aβ42(43) is present in DS brains long before the appearance of Aβ plaques while it is generally absent in age-matched controls affected by a variety of nonneurological and neurological conditions other than AD and DS (Teller *et al.*, 1996).

1.3.3. Neurofibrillary Tangles

NFT are dense bundles of long unbranched filaments in the cytoplasm of some neurons. These filamentous structures have been termed paired helical filaments (PHF) on the basis of their appearance in initial electron microscopy studies (1.3.3.1.) (Kidd,

1963; Wisniewski *et al.*, 1976). Neurons with these neurofibrillary changes degenerate, leaving behind what are called tombstones or 'ghost' tangles in the extracellular space; the degree of this degeneration directly correlates with the degree of dementia in the affected individuals (Tomlinson *et al.*, 1970; Alafuzoff *et al.*, 1987). Early stage NFT and a variable number of 'ghost' tangles are usually seen in cases with intraneuronal pathology. Just 'ghost' tangles alone were never found. The presence of early stage NFT in every case indicates that the pathological process continued up to the time of death.

The topology and abundance of NFT in the brains of patients who meet neuritic plaque criteria for AD relate better to the dementia than do the plaques (McKee *et al.*, 1991; Arriagada *et al.*, 1992): in fact, topographical distribution of NFT has been used for staging the progression of the disease (Braak and Braak, 1991). Although NFT and PHF are also seen in a few other human neurodegenerative diseases and in small numbers in normal aged humans, these lesions are not seen in aged animals or in any experimental induced conditions (Iqbal *et al.*, 1994).

1.3.3.1. Paired Helical Filaments

The accumulations of PHF occur as NFT in neuronal cell bodies, as NT in the dystrophic neurites of the affected neurons in the neuropil, and as dystrophic and degenerating neurites of neuritic plaques, surrounding a dense core or several wisps of extracellular amyloid (Braak *et al.*, 1986).

PHF are composed primarily of full length (Grundke-Iqbal *et al.*, 1986; Iqbal *et al.*, 1989; Lee *et al.*, 1991) and truncated (Novak *et al.*, 1993) forms of the microtubule-associated protein tau, which is also abnormally hyperphosphorylated (Grundke-Iqbal *et al.*, 1986; Iqbal *et al.*, 1986; Iqbal *et al.*, 1989; Lee *et al.*, 1991) and partly ubiquitinated (Grundke-Iqbal *et al.*, 1988; Morishima-Kawashima *et al.*, 1993), glycated (Ledesma *et al.*, 1994; Yan *et al.*, 1994) and glycosylated (Wang *et al.*, 1996).

1.3.3.2. Tau Protein

Tau is one of the major microtubule-associated proteins of a normal mature neuron in the brain. Tau is a family of closely related polypeptides with a molecular weight range of 50,000-68,000 on SDS-PAGE (Cleveland *et al.*, 1977a; Cleveland *et al.*, 1977b). The cDNA-derived sequences have revealed that in human brain there are at least six isoforms of tau (Goedert and Jakes, 1990). All of these isoforms are produced by alternative mRNA splicing from a single gene; their expression is developmentally regulated, with the shortest form being the only species in fetal brain (Goedert *et al.*, 1988; Goedert *et al.*, 1989; Andreadis *et al.*, 1992). They vary in length between 352 and 441 amino acids and differ from each other in whether they contain three or four microtubule binding domains (repeats) near the C-terminal and zero, one or two N-terminal inserts (29 amino acids) (Goedert *et al.*, 1988; Goedert *et al.*, 1989). The six human isoforms of tau are shown in Fig. 4.

Tau is a phosphoprotein which, in a normal neuron, promote microtubule assembly and stabilizes the structure of microtubules (Drubin and Kirschner, 1986); the degree of phosphorylation regulates its biological activity. Tau phosphorylation is developmentally regulated; to a certain extent, it is phosphorylated in normal adult brain, and even more so in fetal brain at identical sites to those of AD. Normal tau contains 2-3 mol of phosphate per mol of the protein. In AD brain tau is abnormally hyperphosphorylated both in PHF as well as in the cytosol (Grundke-Iqbal *et al.*, 1986; Iqbal *et al.*, 1986; Iqbal *et al.*, 1989; Kopke *et al.*, 1993). All six tau isoforms have been reported to be present in a hyperphosphorylated state in PHF (Grundke-Iqbal *et al.*, 1986; Lee *et al.*, 1991; Goedert *et al.*, 1992). Although examination of freshly isolated normal brain from biopsies (Matsuo *et al.*, 1994; Garver *et al.*, 1996) have revealed normal human brain tau to be phosphorylated at some of the same sites on AD tau, the degree of phosphorylation is less than 5% of that in the AD hyperphosphorylated tau. The abnormal tau from AD brain, which contains 5-9 mol of phosphate per mol of the protein (Kopke *et al.*, 1993), does not promote microtubule assembly (Iqbal *et al.*, 1986;

Alonso *et al.*, 1994; Iqbal *et al.*, 1994) leading to neurofibrillary degeneration and ultimately cell death (Alonso *et al.*, 1994; Alonso *et al.*, 1997) (1.6.2.).

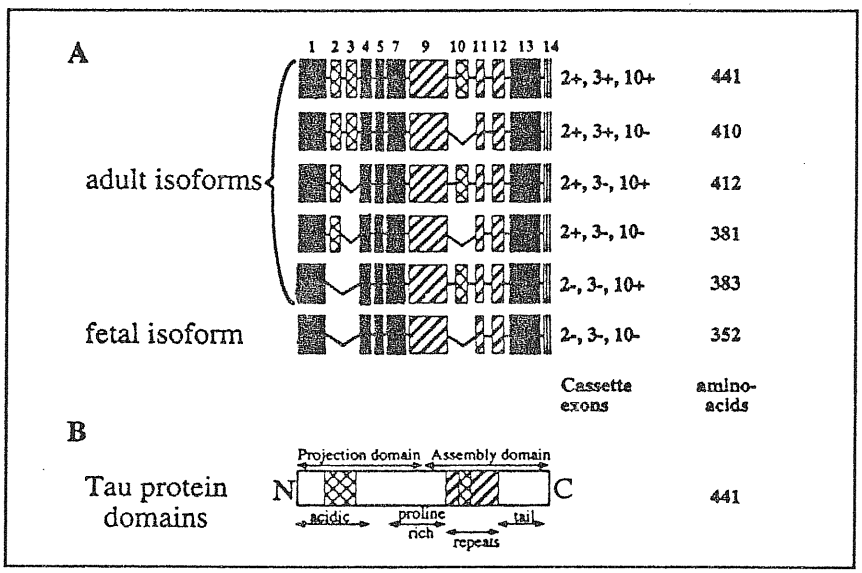


Figure 4. Schematic representation of the six tau isoforms.
A. The six tau isoforms differ from each other by the addition of one or two inserts corresponding to exons 2 and/or 3 in the amino terminus in combination with both three and four (addition of the exon 10) microtubule binding domains. Multiple splice acceptor sites between exons 13 and 14 lead to a striped carboxy terminus. The shortest tau isoform (without exons 2,3, and 10) is made of 352 amino acids and only found in fetal brain. It is referred to as fetal isoform. Adult Tau isoforms include the 383-amino acid form (2-, 3-, 10+), the 381-amino acid form (2+, 3-, 10-), the 412-amino acid form (2+, 3-, 10+), the 410-amino acid form (2+, 3+, 10-), and the 441-amino acid form (2+, 3+, 10+). With the exception of exon 14, the relative sizes of exons are correct.
B. Protein domains of the longest tau isoform. Tau proteins are organised into several regions that have different chemical and physiological properties. The amino terminus part (N) is highly acidic and corresponds to the projection domain, whereas the carboxy-terminus part (C) is highly basic and possesses microtubule binding activity and is referred to as the assembly domain. The central region is proline rich and contains target sites for different types of kinases (from Delacourte and Buee, 1997).

1.3.4. Degeneration Of The Cholinergic Neuronal Systems

Cholinergic neuronal systems, in particular those projecting from the basal forebrain to the hippocampus and cerebral cortex, play an intrinsic role in memory processing and learning (Bartus *et al.*, 1982; Whitehouse *et al.*, 1982; Mash *et al.*, 1985; Lehericy *et al.*, 1993) and their degree of degeneration has been shown to correlate with the loss of cognitive function (Terry *et al.*, 1991; Lehericy *et al.*, 1993), in particular with the loss of memory associated with AD (Davies and Maloney, 1976; Geula, 1994). This system is also vulnerable in a number of other neurodegenerative diseases which afflict the elderly, such as Pick's disease, PD and progressive supranuclear palsy (Whitehouse *et al.*, 1982; Tagliavini *et al.*, 1983; Uhl *et al.*, 1983; Whitehouse *et al.*, 1983; Rogers *et al.*, 1985).

A pronounced decline in hippocampal volume measured on magnetic resonance imaging (MRI) scans is a sensitive and early sign of AD, and hippocampal atrophy has been reported to correlate with severity of memory loss in AD (Lehtovirta *et al.*, 1995).

1.4. ALZHEIMER'S DISEASE: ETIOLOGY AND PATHOGENESIS

It is now a common view to state that AD must no longer be considered as a real disease, i.e. a disorder with one cause, one pathology, and the corresponding symptoms and signs. Rather, it should be viewed as a syndrome because several distinct events can trigger a common or similar pathological cascade which leads to neuronal atrophy and death, loss of synaptic contacts and disorganization of specific neuronal networks. This induces dementia and, according to the predominant distribution of pathological circuits, various other symptoms and signs.

Recently, most investigators have focused on the etiology of familial Alzheimer's disease (FAD) because known mutations that cause AD are valuable tools for dissecting molecular causes of the disease even if FAD represents a minority of all AD cases: fewer than 2% of AD patients are thought to carry one of the highly penetrant mutations associated with the disease (Farrer *et al.*, 1997). However, general principles underlying the etiology of SAD might emerge from such studies.

1.5. FAMILIAL ALZHEIMER'S DISEASE

To date, three genes have been identified that, when mutated, cause a rare early-onset (<60 years old) form of AD. In chronological order of their discovery, these loci include the βAPP gene on chromosome 21, the PS1 (Presenilin 1) gene on chromosome 14, and the PS2 (Presenilin 2) gene on chromosome 1. Pathogenic mutations in βAPP, PS1 and PS2 can directly cause AD with 100% penetrance and are thus considered to be 'deterministic' gene defects. However, the existence of early-onset FAD kindreds that do not appear to possess mutations in the coding regions of βAPP, PS1 or PS2 (Pericak-Vance *et al.*, 1997) suggests that at least one more early-onset FAD gene remains unknown.

APOE, a gene located on chromosome 19, was identified as an inherited 'risk factor' for late-onset (>60 years old) AD: a variant of the APOE gene (APOEε4) appears to confer increased risk for AD when one or two ε4 alleles are inherited. However, approximately one-third of late-onset families carry no APOEε4 allele, suggesting that additional genetic effects exist.

A strong association with the APOEε4 allele was identified also in sporadic late-onset cases (Corder *et al.*, 1993; Saunders *et al.*, 1993; Strittmatter *et al.*, 1993a), and extended to early-onset sporadic cases (Scott *et al.*, 1997; Roses, 1998). It has been estimated that APOE explains approximately 50% of all AD (Roses, 1995) and approximately 65-75% of SAD cases (Rogaev *et al.*, 1995; Sherrington *et al.*, 1995). However, not all people with the APOEε4 allele develop AD.

A common consequence of all known FAD-associated genetic alterations is the increased deposition of Aβ in SP and cerebral blood vessels in the brain (Table 1).

Table 1. Genetic factors predisposing to Alzheimer's Disease: relationships to the Aβ phenotype. Additional chromosomal loci exist but are not yet specifically identified (from Selkoe, 1997).

Chromosome	Gene defect	Age of onset	Aβ phenotype
21	βAPP mutations	50s	Production of total Aβ peptides or of Aβ42(43) peptides
1	Presenilin 2 mutations	50s	Production of Aβ42(43) peptides
14	Presenilin 1 mutations	40s and 50s	Production of Aβ42(43) peptides
19	ApoE polymorphism	60s and older	Density of Aβ plaques and vascular deposits

1.5.1. Mutations In β-Amyloid Precursor Protein Gene

Mutations in βAPP gene are found at codon 670, 671 or 717 that are either within or flanking the Aβ sequence and all (that result in an AD phenotype) affect the processing of βAPP to Aβ such that either more total Aβ is generated or the more amyloidogenic Aβ42(43) species alone. Mutations in βAPP at other sites are associated with other diseases of amyloidosis and/or dementia but not with AD.

An increased production of Aβ is observed also in middle-aged DS (trisomy 21) patients who inevitably develop AD-related neuropathology, most likely due to the presence of three copies of the βAPP gene on chromosome 21 (Tanzi *et al.*, 1987). However, βAPP mutations have been shown to be responsible for only a small proportion (2-3%) of reported cases of FAD (Tanzi *et al.*, 1992).

1.5.2. Mutations In Presenilin 1 And Presenilin 2 Genes

The majority of early-onset FAD is associated with mutations in the two genes PS1, on chromosome 14, and PS2, on chromosome 1, which encode for highly homologous, multi-transmembrane proteins (Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995; Sherrington *et al.*, 1995) with unclear physiological function (1.5.2.1.). More than 30 mutations in PS1 and in PS2 have been identified to date. Plasma and fibroblasts from patients and at-risk carriers with these mutations have been shown to contain

increased amounts of a longer, more amyloidogenic form of the Aβ peptide (Aβ x-42) (Scheuner *et al.*, 1996).

1.5.2.1. Presenilin Proteins

PS1 and PS2 are integral membrane proteins with six to nine predicted transmembrane domains along with one large and various smaller hydrophilic loops (Levy-Lahad *et al.*, 1995; Sherrington *et al.*, 1995). While 67% amino acid identity and the similar structure of the presenilins predict common function, two non-homologous regions at the N-terminus and the large hydrophilic loop most likely impart specificity of function and/or subcellular localization.

Both proteins are significantly homologous to two *C.elegans* gene products, *sel-12* (approximately 50% identity) and *spe-4* (approximately 26% identity) (L'Hernault and Arduengo, 1992). PS1 and PS2 both appear to be ubiquitously expressed (Levy-Lahad *et al.*, 1995; Sherrington *et al.*, 1995); in brain, messages for both PS genes are expressed primarily in neurons and their regional distributions are virtually indistiguishable (Kovacs *et al.*, 1996); however, PS2 transcripts appear to be less abudant than PS1 (Huynh *et al.*, 1997; Takami *et al.*, 1997). Immunocytochemical analyses have revealed that the presenilins are primarily localized intracellularly to the endoplasmic reticulum (ER) and early Golgi apparatus (EGA); this localization together with the similarity with the two *C.elegans* proteins supports a potential role for presenilins in protein trafficking and processing (Cook *et al.*, 1996; Kovacs *et al.*, 1996). The presenilins were also localized to the nuclear membrane, interphase kinetochores and centrosomes, indicating a role in chromosome segregation (Li *et al.*, 1997). They are proteolytically processed to produce stable amino- and carboxy-terminal fragments (shown in Fig. 5) (Haass, 1997) which form a heterodimeric complex that seems to be biologically relevant (Capell *et al.*, 1998).

PS1 and PS2 have been linked with programmed cell death (Vito *et al.*, 1996; Wolozin *et al.*, 1996; Kim *et al.*, 1997). Overexpression of PS2 in PC12 cells differentiated by nerve growth factor increases apoptosis initiated by trophic factor

withdrawal (Wolozin *et al.*, 1996). Furthermore, PS2 mutations can induce apoptosis, even without trophic factor withdrawal. During development, skeletal and central nervous system (CNS) defects occur in mice deficient for PS1 (Shen *et al.*, 1997) perhaps owing to increased apoptosis. Moreover, PS1 is required for Notch1 and Dll1 expression in paraxial mesoderm development (Wong *et al.*, 1997). The presenilin proteins seem to undergo complex alternative cleavage during apoptosis (Kim *et al.*, 1997). More recently, De Strooper *et al.* (1998) showed that PS1 is involved in γ-secretase-mediated proteolytic cleavage of the C-terminal transmembrane fragments of βAPP after their generation by α- and β-secretase(s) (1.3.1.2.). Although it is unlikely that PS1 itself is (one of) the elusive γ-secretase(s), given the absence of sequence identity and lack of structural homology between PS1 and any proteinase domain known (Sherrington *et al.*, 1995), it may be a regulatory factor in this processing step. An alternative explanation is that PS1 is involved in the transport of the membrane-anchored C-terminal βAPP fragments towards the γ-secretase(s)-containing subcellular compartment.

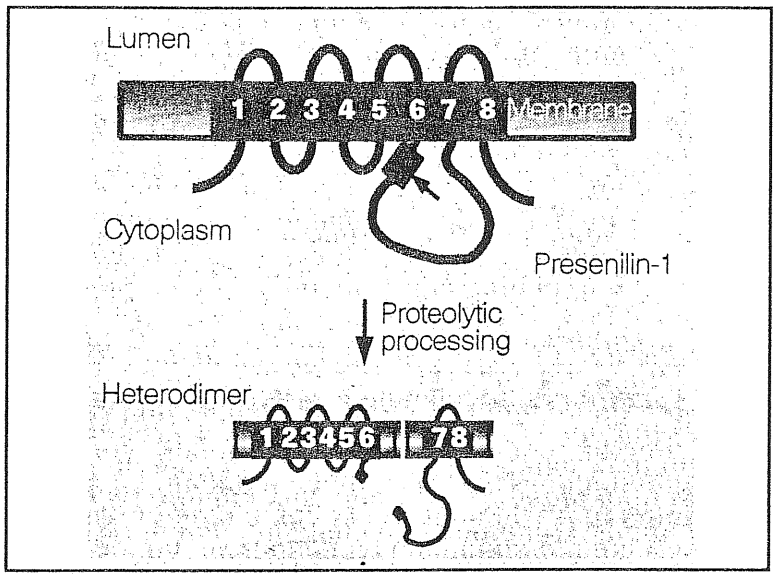


Figure 5. Proteolytic processing of presenilin proteins.
Presenilin proteins are proteolitically processed to produce stable amino- and carboxy-terminal fragments, which form a heterodimeric complex that seems to be biologically relevant (from Haass, 1998).

1.5.3. Variants Of Apolipoprotein E Gene

The most common allele of APOE, on chromosome 19, is APOEε3 representing ≈78% of all chromosomes; there are two common variants in most populations: APOEε4, 15%, and APOEε2, 7%. The proportion of different APOE alleles varies between racial and ethnic groups, particularly with regard to the relative proportions of APOEε2 and APOEε4 (Crews *et al.*, 1993; Mayeux *et al.*, 1993; Ueki *et al.*, 1993; Tsuda *et al.*, 1994).

The age of onset distribution of AD varies as a function of APOE genotype (Bird, 1995; Roses, 1995). Examination of the median age of onset for each genotype illustrates more than a 20-year difference in the age of onset between ε4/ε4 and ε2/ε3 individuals. Only 2-3% of the population carries the ε4/ε4 genotype, but their risk of AD is approximately nine times that of individuals without a single ε4 allele. The ε3/ε4 genotype is present in 21% of individuals and their risk of AD is more than three times that of individuals without an ε4 allele. The ε2/ε3 genotype is present in 12-14% of people and they have the least risk (Levy-Lahad *et al.*, 1995).

The ε4 allele increases the risk and lowers the age of onset distribution but does not change the rate of AD progression; so patients with an ε4 allele, on average, develop AD at an earlier age (Farrer *et al.*, 1995; Seshadri *et al.*, 1995); the ε2 allele appears to lower risk and increase the age of onset distribution (Saunders *et al.*, 1993). However, not all people with the APOEε4 allele develop AD.

1.5.3.1. Apolipoprotein E

The mature form of human APOE present in plasma, tissue and CSF is a single glycosylated 37 kDa protein (Rall *et al.*, 1982), with two major functional domains: an N-terminal domain that interacts with the family of the low-density lipoprotein (LDL) receptor family cell surface receptors, and a C-terminal domain that interacts with lipoproteins (Mahley, 1988). Three major isoforms of APOE (ε4, ε3 and ε2) differing by a single unit of net charge can be easily detected by isoelectrofocusing (Utermann *et al.*, 1979). These isoforms are expressed from multiple alleles at a single APOE genetic locus (Zannis and Breslow, 1981). The multiplicity in polymorphism is unique to

human as all other mammals examined so far exhibit only one APOE allele, ε4. APOEε2 differs from APOEε3 in its ability to bind to the LDL receptor (Weisgraber *et al.*, 1982), and APOEε2 and APOEε4 differ in their binding to lipoproteins of different compositions (Gregg and Brewer, 1986).

By *in situ* hybridization studies, it has been shown that in the brain APOE is expressed solely in glial cells, predominantly astrocytes. Due to the considerable amounts of APOE found in the CSF, it has been suggested that astrocytes secrete APOE into the extracellular space. Cortical neurons do not express APOE; however, they express at least two APOE receptors which mediate internalization of APOE-containing lipoproteins: the low-density lipoprotein receptor-related protein (Fagan *et al.*, 1996), and the very low-density lipoprotein receptor (Christie *et al.*, 1996). APOE is implicated in the growth and regeneration of nerves during development or following injury by transporting cholesterol and phospholipids to neurons undergoing dendritic and synaptic remodeling (Poirier, 1994). More interestingly, the role of APOE in the CNS is particularly important in relation to the cholinergic system which, in contrast to other amino acid-based neurotrasmitter systems, has to rely heavily on lipids to sustain cholinergic activity in neurons. Several hypotheses have been formulated to account for the genetic association of APOE with AD and they are summarised in Table 2.

Table 2. Hypotheses regarding the role of APOE in AD (from Crutcher, 1997).

Putative APOE function	Proposed mechanism	REFERENCES
Amyloid deposition/ clearance/fibrillogenesis	Binding to amyloid	(Schmechel *et al.*, 1993; Strittmatter *et al.*, 1993a; Strittmatter *et al.*, 1993b; Ma *et al.*, 1994; Sanan *et al.*, 1994; Wisniewski *et al.*, 1994)
Microtubule stability	Binding to tau	(Roses *et al.*, 1996; Strittmatter *et al.*, 1994a; Strittmatter *et al.*, 1994b)
Neurite outgrowth	Lipid uptake	(Nathan *et al.*, 1994; Holtzman *et al.*, 1995; Nathan *et al.*, 1995)
Neurotoxicity	?	(Clay *et al.*, 1995; Crutcher *et al.*, 1994; Marques *et al.*, 1996)

On the basis of APOE interaction with Aβ (Schmechel *et al.*, 1993; Strittmatter *et al.*, 1993a; Ma *et al.*, 1994; Sanan *et al.*, 1994; Wisniewski *et al.*, 1994) and its colocalization with SP and cerebrovascular amyloid in AD brains (Namba *et al.*, 1991; Strittmatter *et al.*, 1993a; Strittmatter *et al.*, 1993b) it was suggested that APOE could influence AD pathogenesis by directly modulating the process of Aβ fibrillogenesis (Wisniewski *et al.*, 1994) and/or by partecipating in the resolution of Aβ (Rebeck *et al.*, 1995).

On the basis of APOE interaction with tau, its colocalization with NFT and *in vitro* studies showing that APOEε3 binds to tau with high avidity, while APOEε4 does not bind tau, it was suggested that APOEε3, but not APOEε4, protects tau from posttranslational modifications slowing its self-assembly into PHF (Strittmatter *et al.*, 1994a; Strittmatter *et al.*, 1994b; Roses *et al.*, 1996).

An additional hypothesis is based on the putative role of APOE in promoting regeneration following neuronal injury (Ignatius *et al.*, 1986; Ignatius *et al.*, 1987; Boyles *et al.*, 1989). In fact, the ε3 isoform has been reported to promote neurite outgrowth whereas the ε4 isoform appears to have no effect or to inhibit neurite outgrowth in tissue culture (Nathan *et al.*, 1994; Nathan *et al.*, 1995; Holtzman *et al.*, 1995). Thus, APOEε4 has been proposed to play a role in AD through its failure to promote axonal sprouting, which might otherwise compensate for neuronal injury.

The neurotoxic hypothesis is based on the biological effect of APOE to inhibit the proliferation of certain cell types (Hui *et al.*, 1980; Curtiss *et al.*, 1984). Synthetic peptides derived from the receptor binding domain of APOE exhibit cytotoxic activity (Clay *et al.*, 1995) and cause neurite degeneration in culture (Crutcher *et al.*, 1994). Proteolytic fragments of APOE containing the same domain are present in human brain and CSF and are toxic to neurons. More importantly, the fragment derived from the ε4 isoform was significantly more toxic than the fragment derived from ε3, a difference in toxicity that may explain the APOE isoform-associated risk of the disease (Crutcher, 1997).

1.6. SPORADIC ALZHEIMER'S DISEASE

As described above (1.5.), understanding of the pathogenesis of AD comes primarily from the study of rare inherited forms of the disease. We saw that mutations that cause FAD appear to act by a common mechanism: that of increasing production of Aβ or the more amyloidogenic Aβ42(43) species alone. However, increased Aβ production has not been demostrated to occur in most cases of SAD, suggesting that genetic and environmental factors acting at other stages of the disease process can modify the risk for disease. Genetic factors are already discussed: both late-onset FAD and SAD have a strong association with APOEε4 allele (1.5., 1.5.3.), and there are several proposals for the role of APOE in AD (1.5.3.1.).

Researchers in laboratories around the world have suggested contributing factors such as environmental factors (virus, microorganism or aluminium), biochemical factors (Aβ, tau, neurotrophic factors, neurotransmitters, and neuropeptides), immune factors (cellular and autoimmunity), inflammatory factors (complement proteins), apoptosis, and yet other unknown factors. However, it should be emphasized that these factors are only suspected of contributing to the disease, but none is pathognomonic of AD.

1.6.1. The Controversial "Amyloid Cascade Hypothesis"

The major debate in AD research is about the role of SP and NFT (1.3.2. and 1.3.3.), and their molecular counterparts - Aβ and tau (1.3.2.2. and 1.3.3.2.) - in the pathogenesis of the disease. The intensity with which the different hypotheses are argued has led one critical observer (Dr. Robert Terry) to suggest that religious groups are formed, i.e., the baptists (who believe that Aβ and plaques are fundamental) and the tauists (who believe that tau and tangles are key), each of them with their dogma.

For the baptists the "amyloid cascade hypothesis" (ACH), originally propounded by Glenner, Masters, Beyreuther and others, has become the dominant hypothesis seeking to explain the etiology and pathogenesis of the disease. In its broadest form, the

hypothesis postulates that Aβ deposition is the causative agent in AD and that neurofibrillary pathology, cell loss and dementia follow as a result of Aβ.

To sustain the ACH, the well documented neurotoxicity of Aβ (1.3.2.2.) and the genetic studies indicating as a common consequence of all known FAD-associated genetic alterations the increased deposition of Aβ in SP and cerebral blood vessels in the brain (1.5.). A similarly abnormal deposition of Aβ was also observed in mice transgenic for the βAPP gene; however, these mice do not show the degree of neuronal loss and tau phosphorylation found in AD (Quon *et al.*, 1991; Games *et al.*, 1995; Irizarry *et al.*, 1997; Sturchler-Pierrat *et al.*, 1997). More recently, Geula *et al.* (1998) have shown that microinjection of plaque-equivalent concentrations of fibrillar, but not soluble, Aβ in the aged monkey, but not in young adult monkey or aged rat, cerebral cortex resulted in profound neuronal loss, tau phosphorylation and microglial proliferation. These findings indicate that the structural state of Aβ, as well as age and species, are critical determinants of Aβ neurotoxicity *in vivo* suggesting that longevity may contribute to the unique susceptibility of humans to AD.

1.6.2. Tau Hypothesis

For the tauists the most common argument against the ACH is that Aβ pathology does not correlate with clinical dementia as strongly as neurofibrillary pathology: tangle and neuritic plaque counts but not those of senile plaques correlate with dementia (McKee *et al.*, 1991; Arriagada *et al.*, 1992). Furthermore they do correlate with the deficits in major neurotransmitter systems (Mountjoy *et al.*, 1984), and also with the decline in number of large cortical neurons which accompanies early-onset dementia (Mountjoy *et al.*, 1983). Recent data garnered from a very large population indicated that neurofibrillary pathology precedes Aβ deposition by more than a decade (Braak and Braak, 1997).

So the question is whether the intraneuronal degeneration is able to explain the derangement of mental functions observed in AD.

Neurons with neurofibrillary changes undergo degeneration (Tomlinson *et al.*, 1970; Alafuzoff *et al.*, 1987) due to the progressive disruption and displacement of microtubules by PHF, which are composed mainly of hyperphosphorylated forms of tau protein (1.3.3.1.). The breakdown of microtubules leads to a compromised axoplasmic transport, consequent loss of synapses, and neurons degeneration resulting ultimately in cell death (Alonso *et al.*, 1994; Alonso *et al.*, 1997).

The abnormal hyperphosphorylation of tau is thought to be an important initial step in the neurofibrillary degeneration in AD brain: in fact, abnormally hyperphosphorylated tau not only inhibits microtubule assembly, competing with tubulin for the association with normal tau, microtubule-associated protein 1 (MAP1) and 2 (MAP2) (Alonso *et al.*, 1994) but also disassembles microtubules by sequestering normal tau, MAP1 and MAP2 (Alonso *et al.*, 1996; Alonso *et al.*, 1997). That it is the hyperphosphorylation of tau which is thought to play the major role is confirmed by the fact that the dephosphorylation of tau from AD brain restores its ability to promote the assembly of microtubules *in vitro* (Alonso *et al.*, 1994; Iqbal *et al.*, 1994; Wang *et al.*, 1995). The aggregates of hyperphosphorylated tau and normal tau work as "seeds" for the polymerization of tau into neurofibrillary tangles in which PHF are formed by glycosylation, which is not a normal physiological posttranslational modification of tau (Wang *et al.*, 1996). However, the processes which lead to hyperphosphorylation of tau are still unclear.

A decrease in the tau phosphatase activity in AD brains has been shown (Gong *et al.*, 1993; Gong *et al.*, 1995), suggesting that tau is abnormally hyperphosphorylated in AD brains, probably due to an imbalance in the protein phosphorylation/dephosphorylation system (Matsuo *et al.*, 1994; Garver *et al.*, 1996).

1.6.3. Immunological Hypothesis

Even though the etiology of the disease is obscure, a growing body of evidence suggests that elements of the immune system may be critical factors in the pathogenesis

of the characteristic AD lesions (Kalaria, 1993) and that both cellular and humoral immune mechanisms are activated in AD (reviewed by McGeer *et al.*, 1994).

1.6.3.1. Cell-Mediated Immune Response

Numerous markers of inflammation are seen in AD brains (McGeer *et al.*, 1994). Histopathologically, activated microglia and reactive astrocytes, activated complement proteins and increased expression of cytokines and other acute-phase proteins are all associated with SP but not with diffuse plaques (1.3.2.).

Most studies have found elevated levels of the acute-phase protein α_1-antichymotrypsin in the serum and CSF of AD patients (Matsubara *et al.*, 1990; Brugge *et al.*, 1992; Hinds *et al.*, 1994; Altstiel *et al.*, 1995), and of complement activation product C3a in the CSF of AD patients (Loeffler *et al.*, 1997).

Although initially thought to be a consequence of neurodegenerative changes, these inflammatory processes may be seminal to the expression and/or progression of AD dementia. Epidemiologic evidence strongly support this hypothesis: AD and rheumatoid arthritis co-exist less often than expected; this may suggests that anti-inflammatory drugs used to treat rheumatoid arthritis protect against the expression of AD (McGeer *et al.*, 1990). Retrospective studies support this notion: the use of nonsteroidal anti-inflammatory drugs (NSAIDs) appears to delay the expression of AD (Lucca *et al.*, 1994; Andersen *et al.*, 1995; Rich *et al.*, 1995). Among twins (Breitner *et al.*, 1994) and siblings (Breitner *et al.*, 1995), there is a negative association between previous use of anti-inflammatory drugs and clinical AD. The analysis of Baltimore Longitudinal Study of Aging more recently reported, which used data collected prospectively, so minimizing bias, confirmed the neuroprotective effect of NSAIDs (Stewart *et al.*, 1997).

In response to neuronal damage activated microglia, the immune cells of the brain, generate oxygen radicals which could, as they do *in vitro*, induce the oxidation of C-terminal residues of βAPP (Shigematsu *et al.*, 1992), causing the formation of the aggregating Aβ. Reactive astrocytes may act together with microglia in the

transformation of diffuse plaques into senile plaques. It has been demonstrated that Aβ causes astrocytes to become both activated and to release proteoglycans which binds to Aβ (Frederickson, 1992). This combination inhibits the proteolytic degradation of Aβ which could lead to plaque build-up. The intruder Aβ plaque, being resistant to degradation, could 'frustate' microglia which are no longer able to perform their normal scavenger role and respond by secreting complement, cytokines and neurotoxins. As this process continues, more neurons come under attack and the symptoms of AD could develop. This microglial reaction might be viewed as a chronic inflammatory state. A method to determine the presence of such responses during the disorder, or before clinical manifestations appear, would be very useful. Anti-brain antibodies have been considered as possible candidates for the detection of immunological events in AD. The findings of McRae *et al.* (1996a) suggest that anti-microglial antibodies may be signals for early phase immune mechanisms. If this is the case, then administration of anti-inflammatory drugs on finding CSF anti-microglial antibodies might slow down the progression of AD.

1.6.3.2. Humoral Immune Response

Brain-reactive antibodies (BRA) have been reported to occur in the serum of patients with different neurological disorders. Antibodies against cytoskeleton proteins have been found in spongiform encephalopaties, such as kuru and Creutzfeldt-Jacob Disease (Aoki *et al.*, 1982; Toh *et al.*, 1985) and PD (Karcher *et al.*, 1986).

There is also a growing body of evidence suggesting the existence of autoantibodies against brain-specific antigens in AD both in patients' sera (Chapman, 1986; Singh and Fudenberg, 1986; Franceschi *et al.*, 1989) and CSFs (McRae-Degueurce *et al.*, 1987; Dahlstrom *et al.*, 1990; McRae *et al.*, 1996a). For instance, in sera of AD patients the presence of antibodies which immunoreact to neuronal elements in the rodent central nervous system (Singh and Fudenberg, 1986), to vascular structures in rat and bovine brain (Fillit *et al.*, 1987; Foley *et al.*, 1988) and to cholinergic neurons in *Torpedo* (Chapman *et al.*, 1988; Chapman *et al.*, 1989) has been

described. Other authors have also reported the existence of antibodies in AD CSFs which recognize cholinergic neurons and amoeboid microglial cells in the rat nervous system (McRae-Degueurce *et al.*, 1987; McRae, 1990; McRae *et al.*, 1991). Several reports indicate that antibrain antigens of AD include cholinergic elements (Fillit, 1985; Chapman, 1986; McRae-Degueurce *et al.*, 1987), neurofilaments (Gaskin *et al.*, 1987), thyroglobuline (McRae-Degueurce *et al.*, 1987), vascular proteoglycans (Fillit *et al.*, 1987), and spectrin (Vazquez *et al.*, 1996). It has also been shown that lymphocytes from AD patients produce antibodies against Aβ and NFT (Gaskin and Fu, 1992; Gaskin *et al.*, 1993). All of these antigens have a relationship to AD and autoantibodies produced could be considered detrimental. In fact, AD serum antibodies have a deleterious action toward cholinergic neuronal elements (Foley *et al.*, 1988).
All the results found in literature are summarized in Table 3.

From the table it appears clearly that:

1. in older persons, both in healthy individuals as well as in various pathological conditions, there are more autoantibodies directed against autologous organs than in young; between these, there are autoantibodies directed against neuronal tissues;

2. among the autoantibodies recognising neuronal tissues, some are shared by AD, other neurodegenerative diseased (OND) patients and healthy non demented controls (NDC) but there are also autoantibodies which appear to be specifically present in AD;

3. in the most of published reports, which are immunofluorescence studies, the identity of the antigens recognized by the antibodies is not known, and they are described as just neuronal regions stained by patients' sera or CSFs;

4. exact antigens are identified only in some studies in which immunofluorescence experiments perfomed with patients' sera or CSFs are supported by:

a) immunofluorescence experiments performed with monoclonal antibodies giving similar staining patterns,

b) immunoblots or ELISA experiments performed with single antigens which show recognition of the antigen by the sera or CFSs.

Table 3. Autoantibodies found in the sera and in the CSFs of AD patients and controls.

ANTIGEN	LOCATION	SCREENED MATERIAL	SCREENING MATERIAL	METHOD	DISEASE SPECIFICITY	REFERENCE
immunoglobulins	senile plaques: amyloid fibrils mielin sheats vessel walls	AD frontal cortex	FITC antisera against human Ig chains HRPO rabbit antihuman IgG	IF and EM	AD	Ishii and Haga, 1975; Ishii and Haga, 1976
	brain	mouse brain	serum	IF	AD and senile dementia	Nandy, 1978
	oligodendrocytes fibroblasts neurons	rat cerebellum cultures	serum/CSF	IF	AD, OND and NDC	Watts *et al.*, 1981
	nuclei	rat and human brain	serum	IF	AD, OND and NDC	Watts *et al.*, 1981
	Schwann cells and fibroblasts	human dorsal root ganglion cultures	serum	IF	AD, OND and NDC	Watts *et al.*, 1981
C1q, C4 and C3	amyloid fibrils	AD frontal cortex	mAbs	IF and EM	AD	Ishii and Haga, 1984
AcChoEase AcChoATase	cholinergic cells caudate putamen ventral globus pallidus horizontal and vertical nucleus of diagonal band hippocampus dentate gyrus cerebral cortex	rat and human brain	serum	IF	AD	Fillit, 1985
doublet at 68-70 kDa		bovine choline acetyltrans-ferase	serum	WB	AD	Fillit, 1985
CRF receptor (?)	human pituitary prolactin cells (cytoplasm)	human pituitaries	serum/CSF	IF	AD	Pouplard, 1985
	gastric parietal cells islet cells	rat	serum	IF	AD and DS	Pouplard, 1985
	antithyroid endocrine cells (pancreas and gut)		serum		AD and DS	Pouplard, 1985
neurofilament (200K and 160K)		AD Hammon's corn cortex	serum	IF	AD and DS	D'Angelo, 1986
	cholinergic cell bodies and nerve terminals	Torpedo synaptoso-mes and cholinergic cell bodies	serum	ELISA	SDAT	Chapman, 1986
	nerve cell cytoplasm cerebellum neurons cerebral cortex hippocampus	young and adult rat thymus	serum	IF	AD	Singh and Fudenberg, 1986
vascular proteoglycan	vascular pattern in basal forebrain and hippoc.cortex	rat and bovine brain	serum	IF	SDAT	Fillit *et al.*, 1987

Table 3. Continued.

ANTIGEN	LOCATION	SCREENED MATERIAL	SCREENING MATERIAL	METHOD	DISEASE SPECIFICITY	REFERENCE
vascular heparan sulfate proteoglycan			serum	ELISA	SDAT	Fillit *et al.*, 1987
HLA-DR GFAP	reactive microglia	SDAT and normal human brain	mAbs	IF	SDAT	McGeer *et al.*, 1987
AcCho AcChoEAse	cholinergic neurons in MS and VDBcholinergic spinal cord motor neurons	rat brain	CSF (IgG3)	IF	AD	McRae-Degueurce *et al.*, 1987
	neurofibrillary tangles, brain tissue, astrocytes	(EBV)-transformed B-cell lines	peripheral blood	ELISA	AD	Gaskin *et al.*, 1987; Kingsley *et al.*, 1988
neurofilament AD: 200K+150 K (or 70 or 62K) DS: 70K+200K		rat spinal cord proteins	serum	WB	AD and DS	Kumar *et al.*, 1988
C1q, C3d, C4d, C7, C9, C5b-9	plaques, tangles, diffuse deposits, neuropil threads, dystrophic neurites		mAbs	IF	AD	McGeer *et al.*, 1989
HLA-A, B, C	capillaries		mAbs	IF	AD	McGeer *et al.*, 1989
HLA-DR, FcRgI, II receptors, leukocyte common antigen, CD4, CD8, GFAP	reactive microglia, leukocytes, T4 and T8 lymphocytes, reactive astrocytes	AD and NDC brain	mAbs	IF	AD	McGeer *et al.*, 1989
anti-TG (?)	neurons in MS	adult rat brain	CSF	IF	AD and VaD	McRae, 1990
anti-NF 200 (?)	fibers	E18 rat brain	CSF	IF	AD	McRae, 1990
HSV-1, CMV, VZV, EBV, RSV, Measles virus, Rubella			serum	ELISA, IF, hemagglu-tination	AD and NDC	Ounanian *et al.*, 1990
actin,tubulin, myosin, spectrin, trinitrobenzene-sulfonic.acid, albumina, peroxidase, myoglobin, transferrin, thyroglobulin, keratin			serum	EIA	AD and NDC	Ounanian *et al.*, 1990
	nuclei, mitochondria, thyroid, smooth muscle, gastric parietal cells, antibrain	tissue sections	serum	IF	AD and NDC	Ounanian *et al.*, 1990
S-100 NSE			serum	ELISA	AD and senile dementia	Jankovic and Djordjijevic, 1991

Table 3. Continued.

ANTIGEN	LOCATION	SCREENED MATERIAL	SCREENING MATERIAL	METHOD	DISEASE SPECIFICITY	REFERENCE
	nerve fibers ameboid microglial cells in CSP and CC	rat brain (from E18 to P5)	CSF	IF	AD and risk relatives	McRae *et al.*, 1991
thyroglobulin thyroid microsomal Ag			serum	ELISA	FAD and DS	Ewins *et al.*, 1991
thyroglobulin thyroid microsomal Ag				indirect hemagglu-tination	AD, possible AD with CVD and VaD, NDC	Lopez *et al.*, 1991; Lopez *et al.*, 1992
	nucleus smooth muscle mitochondria gastric parietal cells	mouse stomach and kidney		IF	AD, possible AD with CVD and VD, NDC	Lopez *et al.*, 1991; Lopez *et al.*, 1992
rheumatoid factor				fluore-scence immunoas say	AD, possible AD with CVD and VaD, non demented controls	Lopez *et al.*, 1991; Lopez *et al.*, 1992
cardiolipin				ELISA	possible AD with CVD and VaD	Lopez *et al.*, 1992
	neuronal			ELISA	possible AD with CVD and VaD	Lopez *et al.*, 1992
immunocomplexes				qualit. electro-phoretic assay	possible AD with CVD and VaD	Lopez *et al.*, 1992
	choroid plexus	rat and fetal brain	serum	IF	SDAT	Serot *et al.*, 1992
rabbit myelin basic protein			serum	WB	AD	Singh, 1992
GFAP			serum	ELISA	AD, SDAT, VaD and NDC	Mecocci *et al.*, 1992
	activated microglia neural macrophages	rat brain	CSF	IF	AD	McRae *et al.*, 1993
	ramified and giant-sized microglia in the cortex	AD cortical biopsies	CSF	IF	AD	McRae *et al.*, 1993
CIC			serum	C1q-binding/ congluti-nin-binding ELISA	DS, AD and MID	Heinonen *et al.*, 1993
histones			serum	ELISA	SDAT	Mecocci *et al.*, 1993
IgG IgM C1q fibrinogen fibronectin	basement membrane	autopsied AD brain (choroid plexus)	antisera to: IgG, IgM, IgA, C1q, C3, fibrinogen, fibronectin	IF	AD	Serot *et al.*, 1994

Table 3. Continued.

ANTIGEN	LOCATION	SCREENED MATERIAL	SCREENING MATERIAL	METHOD	DISEASE SPECIFICITY	REFERENCE
β-amyloid	plaque-reactive; plaque-nonreactive (poly-reactive): neurons, filaments, astrocytes	(EBV)-transformed B-cell lines	peripheral blood	ELISA and IF	AD	Fang *et al.*, 1995; Gaskin *et al.*, 1993; Xu and Gaskin, 1997
spectrin		human and rat brain, PC12 cells	serum and anti-brain spectrin polyclonal antibody	WB	AD	Fernandez-Shaw *et al.*, 1997; Vazquez *et al.*, 1996
tubulin			serum and CSF		AD, PD, ALS, MS, ON, GBS,VD	Terryberry *et al.*, 1998

In this examination of the literature, the most interesting result was that of McRae et al. (1991):

ANTIGEN	LOCATION	SCREENED MATERIAL	SCREENING MATERIAL	METHOD	DISEASE SPECIFICITY	REFERENCE
	nerve fibers ameboid microglial cells in CSP and CC	rat brain (from E18 to P5)	CSF	IF	AD and risk relatives	McRae *et al.*, 1991

in which the same CSF antibodies (identified by immunofluorescence on rat brains) were found in both AD patients and their risk relatives, some of these relatives later developed the disease. However, in this case, too, the exact antigen is not known.

Such antibodies or antibodies like these, specifically present in AD CSFs and/or sera could be employed as biological markers for monitoring the progression of the disease or as diagnostic tools for early detection of the disease.

1.6.4. Others

1.6.4.1. Oxidative Stress

A growing body of evidence implicates a contribution by oxidative stress damage to the pathogenesis of AD.

Markers for oxidative damage including tyrosine nitration, hydrazine-detectable carbonyls, advanced glycation end products (AGEs), and oxidative stress response factors such as heme oxigenase-1, have been noted at early stages of SP and NFT formation (Pappolla *et al.*, 1992; Smith *et al.*, 1994; Furuta *et al.*, 1995; Pappolla *et al.*, 1996).

Yan *et al.* (1995) have demonstrated that PHF-tau is modified by glycation to an AGE. Once AGEs modify accumulated tau, their generation of reactive oxygen intermediates (ROIs) induces oxidant stress. Two consequential short-term results of this are enhanced production of interleukin-6 (IL-6), which further accelerates formation of SP by increasing production of α_1-antichymotrypsin and increased release of Aβ.

Other studies showing AGEs in deposits of Aβ (Smith *et al.*, 1994; Vitek *et al.*, 1994) suggest that plaques and vascular Aβ could also be sites for induction of oxidant stress. Several investigators have demonstrated that the cytotoxic effects of Aβ are related to the production of oxygen free radicals (OFRs) and cells are protected by a number of antioxidants (Behl *et al.*, 1992; Harris *et al.*, 1995).

1.6.4.2. Abnormalities In Glucose Metabolism

In senile dementia of Alzheimer's type (SDAT), abnormalities in glucose metabolism have been found *in vivo* (Friedland *et al.*, 1989; Kumar *et al.*, 1991). Correspondingly , the activities of a number of enzymes involved in glycolytic and oxidative glucose breakdown were reduced in post-mortem brain tissue of SDAT (Bowen *et al.*, 1979; Iwangoff *et al.*, 1980; Gibson *et al.*, 1988). Such neuroglucopenia is due to a severe disturbance in the insulin/insulin receptor signal transduction. As metabolic consequences, both acetylcholine and ATP concentration become deficient. A cascade of abnormal events follows:

- neuroglucopenia raises the expression of mRNA-βAPP and decreases βAPPs formation what also happens due to acetylcholine deficit;
- ATP deficit reduces the passage of proteins across the ER and GA, and supports the formation of amyloidogenic derivatives;
- deficits of both ATP and insulin forward hyperphosphorylation of tau protein;
- maintained disturbances in glucose metabolism favor the formation of AGEs which contribute to the formation of SP and NFT;
- ammonia inhibits acetylcholine binding to synaptosomes.

These reductions appeared to be more severe than the 'non-specific' reductions in a number of biochemical constituents that had been related to brain atrophy (Bowen *et al.*, 1979). Thus, the hypothesis has been forwarded that primary abnormalities in glucose/energy metabolism might be an early contributing event to the secondary formation of SP and NFT in SDAT (Hoyer, 1998).

1.6.5. Risk Factors

To clarify the pathogenesis of SAD, environmental factors could be more important than genetic. Several environmental risk factors have been studied for their possible involvement in the evolution of AD; some of them are listed in Table 4.

Table 4. Risk factors for AD identified in case-control studies (from Takeda, 1997).

Risk factor	Reference
Family history of dementia	Heyman *et al.*, 1984
History of head trauma	Mortimer *et al.*, 1985
History of thyroid disease	Heyman *et al.*, 1984
History of depression	French *et al.*, 1985
Advanced age of mother at birth	Amaducci *et al.*, 1986
Family history of Parkinson's disease	Hofman *et al.*, 1989
Aluminium-containing antiperspirants	Graves *et al.*, 1990
Physical and social inactivity	Kondo, 1990

Beside conflicting results on the role of aluminium in AD (Doll, 1993), a number of other metal ions have been proposed as potential environmental risk factors for AD because they may interacts with βAPP, or its processing, at various levels; the ectodomain of βAPP has copper and zinc binding sites, and has the capacity of reducing Cu(II) to Cu(I) (Bush et al., 1993; Multhaup et al., 1996). Aβ has also been shown to bind zinc specifically and saturably, with zinc accelerating *in vitro* Aβ deposition (Bush *et al.*, 1994a; Bush *et al.*, 1994b). Zinc has also been shown to alter the degradation of βAPP and its carboxyl-terminal fragment (Li *et al.*, 1995).

Herpes simplex virus type 1 (HSV1) has been proposed as another risk factor in AD. The involvement of HSV1 in AD could be another way to explain the findings that anti-inflammatory drugs may prevent or delay the onset of AD symptoms (Breitner *et al.*, 1994). Inflammation can lead to reactivation of HSV1 (Beyer *et al.*, 1989), so such drugs could reduce the extent of reactivation and the damage caused. Findings on this issue are controversial: Itzhaki and colleagues (Itzhaki *et al.*, 1997) have shown that the

combination of HSV1 in the brain and the presence of at least one APOEε4 allele confer an even greater risk for AD when compared with individuals carrying the APOEε4 allele alone. In contrast, Beffert and colleagues' (1998) findings suggest that HSV1 does not confer increased risk for AD when combined with the presence of an APOEε4 , that HSV1 alone is not an independent risk factor for AD, and that APOEε4 allele carriers are not more susceptible to HSV1 infection than non-carriers.

Another suggested risk factor on the role of which conflicting results have been reported is smoking.

Smoking is a risk factor for vascular diseases, including atherosclerosis and thrombosis, and increases the risk of vascular dementia. Vascular involvement is probably more important than previously thought in the pathogenesis of AD. Nonetheless, an inverse association has been reported between smoking and AD (Lee, 1994). Van Duijn and colleagues (van Duijn *et al.*, 1995) restricted the possible protective effect of smoking in early-onset AD to carriers of the APOEε4 allele. More recently, Ott and colleagues (Ott *et al.*, 1998) in their large prospective population-based study have found that in general smokers are at increased risk of dementia and AD, nevertheless, this increased relative risk of dementia is greatest in smokers without the APOEε4 allele. By contrast, smokers with this allele do not have an increased risk of dementia. Smoking could have some effects on the cholinergic system that might counterbalance the selective impairment associated with APOEε4, for example increasing the density of nicotine receptors or facilitating the release of acetylcholine (Brenner *et al.*, 1993; van Duijn *et al.*, 1995). An alternative hypothesis is that smoking and the ApoEε4 allele may act similarly in predisposing to AD.

1.7. BIOCHEMICAL MARKERS

An understanding of AD pathogenesis also has implications for improving the diagnosis of AD (Geldmacher and Whitehouse, 1996) and developing biological markers to track the effects of therapeutic interventions. It is difficult to determine if a

drug has a fundamental effect on biology without *in vivo* markers of neural dysfunction. Biochemical markers for AD would be of great value both to help in diagnosis early in the course of the disease when patients will not fulfil the criteria for dementia and to follow the effects of therapeutic treatments: in fact, given the absence of treatment, it is not clear what patients can be offered, except NSAIDs.

1.7.1. In Cerebrospinal Fluid

Since the intercellular space in the brain is in direct contact with the CSF, biochemical changes in the brain may be reflected in the CSF. Thus, neuronal and synaptic proteins in CSF may function as biochemical markers for the neuronal degeneration in AD. Markers related to processes implicated in AD such as inflammation, oxidative damage, neuronal death and lipid changes have not shown major differences between living AD patients and controls. At present the most data support CSF tau and Aβ42 as biomarkers.

CSF tau is increased in AD (Vandermeeren *et al.*, 1993; Blennow *et al.*, 1995; Jensen *et al.*, 1995; Skoog *et al.*, 1995; Vigo-Pelfrey *et al.*, 1995), even very early in the disease course. Levels stay elevated longitudinally, suggesting that CSF tau reflects ongoing neuronal (possibly axonal) damage. Increased levels of CSF tau are found in acute stroke, Creutzfeld-Jakob disease and fronto-temporal dementia, and in a minority of patients with other neurological conditions. For diagnostic purposes, tau may be used to distinguish between very early AD and cognitive changes due to aging, but not in the differential diagnosis of dementia syndromes.

CSF Aβ42 is decreased in AD, even early in the course of the disease. CSF levels may inversely depend on the extent of Aβ42 deposition in the brain; they continue to decrease as AD progresses, and are directly related to the APOEε4 gene dose. Combined use of Aβ42 and tau together provide more accurate classification of AD than either alone.

Synaptic pathology is a central pathogenic process in AD, and CSF levels of specific synaptic vesicle proteins are reduced during the course of the disease.

Preliminary results showed reduced levels of synaptotagmin in AD (Davidsson *et al.*, 1996). In addition, the use of a new ELISA assay has confirmed that the CSF levels of rab3a are reduced in AD (Blennow, 1998).

The complex chain of neurochemical and pathologic events in AD suggests that multiple CSF biomarkers will be needed to monitor different aspects of the disease.

1.7.2. In serum

Several attempts have been made to develop a diagnostic peripheral marker of AD. Investigations of blood components have focused on lymphocytes, platelets and erytrocytes (Tavolato and Argentiero, 1980; Miller *et al.*, 1981; Tilvis *et al.*, 1987; Ershler, 1993; Chao *et al.*, 1994). Lymphocyte receptor binding studies (Bongioanni *et al.*, 1997; Bongioanni *et al.*, 1998), changes in specific proteins such as actin (Mattila and Frey, 1995) and GFAP (Laping *et al.*, 1994), and levels of acetylcholine-related enzymes (Miller *et al.*, 1991) have been examined as potential peripheral markers of AD. Other blood-based tests have included: investigation of the presence and importance of endocrinological markers and certain antibodies, e.g. to GFAP (Mecocci *et al.*, 1995), amino-acid concentrations (Mochizuki *et al.*, 1996; McCaddon *et al.*, 1998); presence of biochemical markers, such as α_1-antichymotrypsin (Matsubara *et al.*, 1990); levels of acetylcholinesterase isoforms (Yamamoto *et al.*, 1990; Oishi *et al.*, 1996). The diagnostic value of qualitative or quantitative changes in proteins that specifically relate to AD, such βAPP, and the deposition of Aβ-related proteins in the skin have also been examined as potential diagnostic tools for AD (Adler *et al.*, 1991; Soininen *et al.*, 1992). As yet there is no indication that any of these potential diagnostic markers will have sufficient sensitivity for use in current practice.

One interesting protein which is being investigating as a potential biomarker of AD is melanotransferrin (p97), a metalloprotein expressed by activated microglia associated with SP (Jefferies *et al.*, 1996), because in previous studies serum p97 levels have been able to distinguish highly probable AD with longitudinal clinical confirmation from cognitively normal controls (Feldman, 1997).

1.7.3. Others

Structural neuroimaging, including computed tomography (CT) and MRI, provides a marker of neurodegeneration in AD. Neuronal loss at a cellular level results in atrophy at a macroscopic level, which may be visualized and measured *in vivo* using CT or MRI. Several measures have shown statistically significant differences between moderately severe AD and normal ageing (Foundas *et al.*, 1996; Iyo *et al.*, 1997; Muller *et al.*, 1997). However, in mild cases there is considerable overlap between patients and age-matched controls so CT or MRI are relatively insensitive in assessing early AD and quantitatively measuring its progression. In contrast, *in vivo* metabolic measures with FDG-PET (^{18}F-FluoroDeoxyGlucose - Positron Emission Tomography) can demonstrate hypometabolism in parieto-temporal, frontal and hippocampal areas during the early stages of AD so they are potentially useful in measuring the progression of AD and the effects of treatment (Kondoh *et al.*, 1997; Turjanski and Brooks, 1997).

Biochemical tests and neuroimaging techniques can be used in conjunction with clinical assessments to confirm diagnosis, monitor progression and demonstrate efficacy of drug treatment in AD.

1.8. THERAPEUTIC STRATEGIES

At present, primarily because the pathogenesis of AD is still unknown (with the exception of the rarer forms of familial AD), treatment strategies are aimed at preventing or slowing down the mental decline characteristic of AD patients rather than the causes of the disease.

Many approaches have and are being undertaken to treat AD but, as yet, no therapy is available with any established efficacy. Given the heterogeneity of the etiological factors involved in AD and the difficulties encountered in the clinical diagnosis, the lack of pharmacological success is not surprising. Furthermore, the lack of an adequate animal model of AD has delayed the development of novel therapeutic strategies. Long-term studies making use of repetitive clinical examinations and

imaging techniques are required to study the effect of any drug on the disease. A sensitive group of markers which reflect the ongoing neuronal degeneration could therefore significantly reduce the time/cost factor for clinical trials.

Cholinesterase (ChE) inhibitors are at present the most advanced class of drugs for the treatment of AD. Two members of this class are already on the market: donepezil (Aricept; Eisai Company, Tokyo) and tacrine hydrochloride (Cognex; Parke-Davis, Ann Arbor, MI), the first therapy approved (in 1993) for Alzheimer's by the US Food and Drug Administration (FDA; Rockville, MD). Both are thought to act by raising levels of the neurotransmitter acetylcholine, which is profoundly depleted in the brains of AD sufferers. However, their low bioavailability after oral administration, the high propensity of gastro-intestinal side effects and liver function abnormalities, and a relatively low number of clinical responders limit their therapeutic value (Weinstock, 1995). Concurrently with the successful search for cholinergic augmentation therapy, new potential disease-modifying strategies (Table 5) should derive from neuroscientists' progress in understanding neurodegenerative mechanisms involved in AD.

Table 5. Disease-modifying strategies for Alzheimer's disease.
(from Aisen and Davis, 1997)

Neurodegenerative processes	Pharmacologic intervention
Apoptosis	Neurotrophic factors
Oxidative stress	Antioxidant vitamins and drugs
Inflammation: complement, acute-phase response	Glucocorticoids
Inflammation: microglia	Hydroxychloroquine, colchicine
Inflammation: cyclooxygenase	NSAIDs, glucocorticoids
Amyloid deposition	Colchicine, antiaggregants, kinase/phosphatase-modulating agents, cholinergic drugs
Hyperphosphorylation	Kinase/phosphatase-modulating agents
Excytotoxicity	Glutamate antagonists
Calcium influx	Calcium channel blockers
Heavy metal toxicity	Chelators

Results from a growing number of studies provide evidence that estrogen replacement therapy (ERT) can impact both the occurrence and the course of AD (Honjo *et al.*, 1989; Fillit, 1994; Henderson *et al.*, 1994; Paganini-Hill and Henderson, 1994; Schneider *et al.*, 1996) particularly among patients who do not have an APOEε4 allele. Potential mechanisms might include estrogen's effect on the expression of APOE, its effect on lowering serum lipoprotein levels (Sherwin and Gelfand, 1989), and its direct or indirect cholinergic effects. The anti-inflammatory effects of estrogen may also play a role (Josefsson *et al.*, 1992). Very recently, Xu *et al.* (1998) have provided evidence that one relevant neuropharmacological activity of ERT, contributing to its ability to protect against AD, is to reduce Aβ generation.

1.9. AUTOANTIBODIES IN DISEASES

Autoimmunity generally refers to an inappropriate immune response against host-self allowing antibodies or cells to bind to self components. Diseases are commonly labelled as autoimmune when the etiology and pathogenesis is not known. However, only some diseases are 'classical' autoimmune diseases while others are only suspected of having an autoimmune etiology. The clinical expression of autoimmune diseases is largely multifactorial: (i) environmental factors such as infectious agents; (ii) genetic linkage especially of genes for the immune response, haplotype, and Gm allotype; (iii) immune abnormalities in particular of thymus-derived immunoregulatory T lymphocytes, and (iv) gender and other hormonal factors. Furthemore, autoimmune diseases are generally characterized by certain criteria: circulating autoantibodies preferably organ-specific; definition of a specific autoantigen; specific cell-mediated immunity to autoantigen; production of the disease in an experimental animal by immunization of the autoantigen or passive transfer of the autoantibodies and/or self-reactive cells; and production of autoantibodies *in vivo* or *in vitro*.

In auto-immune diseases, autoantibodies may be the actual pathogenetic agents of the disease, for example, autoimmune hemolytic anemia; they may arise as a consequence of another disease process, for example, some antibodies that react with

nerve tissue or heart muscle after damage to those organs; or they may merely mark, like footprints, the presence of the etiologic agent while not themselves causing damage. This last role is suspected for many of the best known autoantibodies of human disease. In general, patients with autoimmune diseases have high titers of specific serum autoantibodies skewing in subclass distribution for particular autoantibodies.

Also in human tumours, cancer-specific antigens which are not expressed on most normal tissues have been found (Boon *et al.*, 1994; Srivastava, 1996): these observations have given rise to a view that human cancers share a number of antigens, which can form the basis for their immunotherapy (Rosenberg, 1997) or help their early diagnosis, the major problem in the management of cancer as well as in AD. Since it was not known how many antigens are expressed by a given tumour and how often such antigens elicit an immune response in the tumour-bearing patient, Sahin *et al.* (1995) established a novel strategy, the so called SEREX approach (1.9.1.) for the identification of tumour antigens using the antibody repertoire of cancer patients.

1.9.1. Experimental Method: SEREX Approach

SEREX is an acronym that stands for the SErological identification of antigens by Recombinant EXpression cloning (Sahin *et al.*, 1995). It allowed an unbiased search for an antibody response and the direct molecular definition of immunogenic tumour proteins based on their reactivity with autologous patients' sera.

In the SEREX approach, a cDNA library is constructed from fresh tumour specimens, packaged into λ-phage vectors and expressed recombinantly in *E.coli* (Fig. 6). Recombinant proteins expressed during lytic infection of the bacteria are transferred onto nitrocellulose membranes, which are then incubated with diluted serum from tumour patients. Clones reactive with high-titer IgG antibodies are identified using an enzyme-conjugated secondary antibody specific for human IgG. Positive clones are subcloned to monoclonality, and the nucleotide sequence of the inserted cDNA is determined. Although SEREX allows an unbiased search for antigenic proteins, it cannot detect the complete spectrum of antigens: glycosylated epitopes and epitopes

that undergo conformational changes when expressed in bacteria will be missed. The tissue-expression spectrum of the antigen can be subsequently determined by the analysis of the mRNA expression pattern by Northern blot and/or reverse transcription-PCR (RT-PCR).

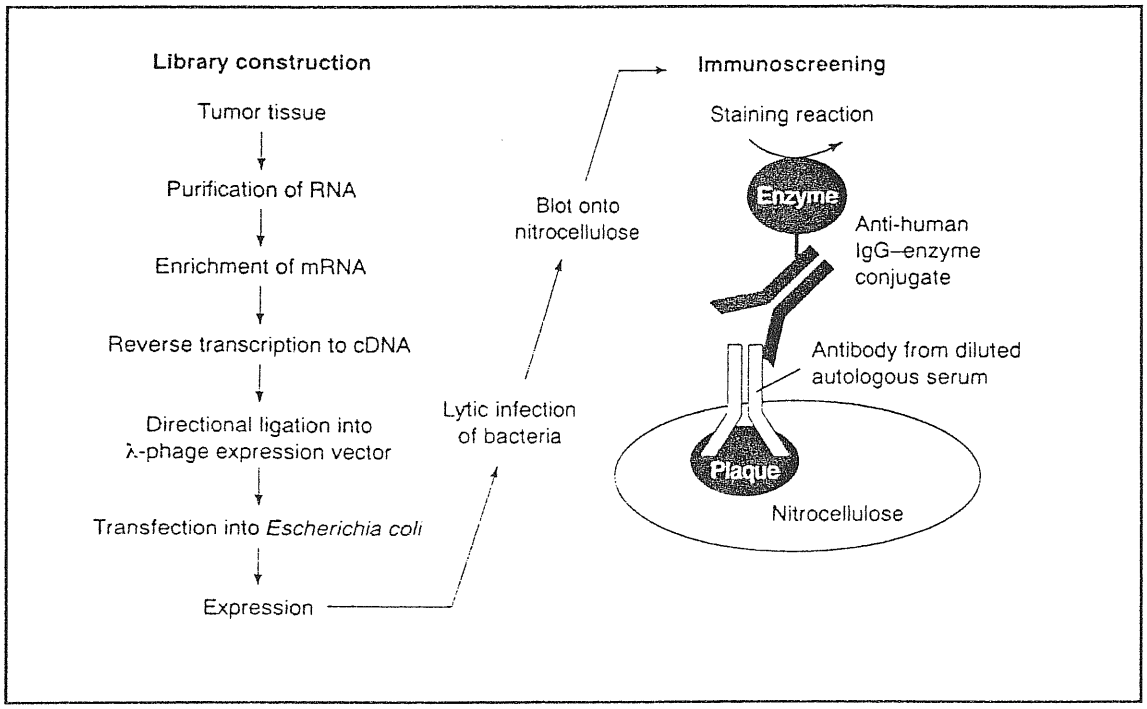


Figure 6. Molecular definition of tumour antigens by the SEREX technique.
A cDNA library is constructed from fresh tumour tissue and expressed in *E.coli* using a λ-phage expression vector. The bacteria are grown in colonies and the lytic plaques are transferred to a nitrocellulose membrane. The diluted autologous serum is then incubated with the membrane and those antibodies that bind to tumour-derived antigens expressed in lytic plaques are made visible by enzyme-conjugated goat anti-human IgGs (from Tureci *et al.*,1997).

In their first study, Sahin *et al.* (1995) applied the SEREX technique to the analysis of tumour tissue from human melanoma, renal cell cancer, astrocytoma and Hodgkin's disease. Screening a minimum of 10^6 clones from each tumour, at least four different antigens were identified in each tumour, demonstrating that many human tumours express multiple antigens eliciting an immune response in the autologous host. Extending their analysis to other types of human tumours, so far they have identified more than 100 different antigens. Antibodies against a given antigen are detected in only a small proportion (10-30%) of patients with a tumour expressing the respective antigen. On the basis of sequence data, in addition to the results of Northern blot and RT-PCR analyses, different types of antigen specificity have emerged (Table 6).

Table 6. Specificity of tumour antigens detected by SEREX (from Sahin *et al.*, 1997).

Class	Antigen	Homology/ identity	Source	Reference
'Shared' tumour antigens	HOM-MEL-40	SSX-2	Melanoma	Tureci *et al.*, 1996
Differentiation antigens	HOM-MEL-55	Tyrosinase	Melanoma	Sahin *et al.*, 1995; Tureci *et al.*, 1996
Mutated genes	NY-COL-2	p53	Colorectal carcinoma	Mathew *et al.*, 1995
Splice variants	HOM-HD-397	Restin	Hodgkin's disease	Sahin *et al.*, 1995; Sahin *et al.*, unpublished data
Viral antigens	HOM-RCC-1.14	HERV-K10	Renal cell cancer	Sahin *et al.*; unpublished data
Overexpressed antigens	HOM-HD-21	Galectin 9	Hodgkin's disease	Tureci *et al.*, 1997
Gene amplification	HOM-NSCLC-11	EIF-4g	Lung cancer	Brass *et al.*, 1997
Cancer-related autoantigens	HOM-MEL-2.4	CEBP-g	Melanoma	Sahin *et al.*; unpublished data
Cancer-independent autoantigens	HOM-TES-11	PCM-1	Testis	Sahin *et al.*; unpublished data

The majority of genes detected by SEREX are overexpressed genes; they are not strictly tumour specific, but are overexpressed in tumours compared with normal tissues. The clinical significance of anti-tumour antibodies in cancer patients is unclear. While it cannot be excluded that they function as no more than useful indicators for tumour antigens, so far no anti-tumour cytotoxicity of such antibodies has been demonstrated, and they have not been shown to induce any kind of autoimmunity to normal cells.

1.10. THE AIM OF THE RESEARCH

The aim of the research undertaken in this thesis was to find any protein which is recognized specifically by antibodies present in AD sera.

Any protein with these characteristics could be useful in exploring the etiology and pathogenesis of the disease; antibodies recognizing it could be employed as biological markers of AD if any correlation exists between their presence in AD sera and the progression of the disease.

Theoretical supports to this research came from findings reported in Table 3 (1.6.3.2.). So far, the interpretation of those experimental results was difficult for several intrinsic limits linked to the detection method used:

1) immunocytochemical staining is semi-quantitative, more sensitive techniques such as ELISA which may reveal specifically increased concentrations of anti-neuronal antibodies in AD serum and/or CSF require the identification of the specific antigen(s);

2) results can vary according to the fixation method used, even if using the same starting material (McRae-Degueurce and Geffard, 1986), because the antigen(s) recognized by serum and/or CSF immunoglobulins may be modified differently by different fixation conditions;

3) statistically significant results highly depend on parameters introduced in the statistical analysis: if gradations of staining (negative, weak, positive, or unspecific) are included, differences between groups are statistically significant (McRae, 1990); whereas if staining is simply considered as positive or negative (Loeffler *et al.*, 1997) they are not;

4) many brain samples used in AD research suffer from post-mortem delays which can be longer than 24 hours. This can affect the interpretation of the results: as Hardy said, the brain cells affected by AD are so damaged that the antibodies could be staining many things.

With this in mind, the SEREX approach developed by Sahin *et al.* (1995) for studying the presence of autoantibodies to tumour antigens (1.9.1.) was applied to Alzheimer's disease.

So, the aims of this research were:

1. the construction of a cDNA library from Alzheimer's brain

2. the screening of the cDNA library with patients' sera

3. the identification of the cDNAs isolated

4. the study of the expression patterns of the cDNAs isolated and

5. the production of recombinant proteins for testing the serum response of individuals with and without AD to these proteins.

2. RESULTS

2.1. ATTEMPT TO FIND PROTEINS SPECIFICALLY RECOGNIZED BY AD SERA

As already mentioned (see 1.10. of the Introduction), the aim of the research undertaken in this thesis was to find any protein which is specifically recognized by antibodies present in AD sera.

Since the results found in the literature on this subject were often contradictory and for this reason not exhaustive essentially for intrinsic limits of chosen experimental methods, I addressed the question by using a strategy which was set up and used successfully for the systematic identification of disease-specific antigens, the so-called SEREX approach (see 1.9.1. of the Introduction).

The research described in this thesis involved the following successive steps:

1. the construction of a cDNA library from Alzheimer's brain
2. the screening of the cDNA library with patients' sera
3. the identification of the cDNAs isolated
4. the study of the expression patterns of the cDNAs isolated and
5. the production of recombinant proteins for testing the serum response of individuals with and without AD to these proteins.

2.2. CONSTRUCTION OF A cDNA LIBRARY FROM AD HIPPOCAMPUS

Since very few AD cDNA libraries have been reported (Goedert, 1987; Octave *et al.*, 1988; Salim *et al.*, 1988; Vitek *et al.*, 1988) and all of these were constructed from patients' cerebral cortices except the commercial one from Clontech, constructed from AD hippocampus but no longer available, the construction of an AD hippocampal cDNA library was undertaken.

AD hippocampus was chosen as the starting material for the construction of the cDNA library, this being one of the first areas, together with temporal cortex, affected by the specific pathological lesions of the disease. This tissue also contains the greatest number of different cell types, and so should represent the greatest antigenic diversity. Besides this, to increase the diversity of the library, three pieces of hippocampi from three different AD patients were used (see 4.4.1. of the Materials and Methods) .

The unidirectional cDNA library was constructed using the ZAP Express^{TM} cDNA Synthesis and Gigapack^{R} II Gold Cloning Kits (Stratagene), as described in Materials and Methods (4.4.4.). A quality control and evaluation procedure was performed at each step of the cDNA library construction, to obtain a cDNA library of high quality, with the following characteristics:

1. representative, containing all sequences present in the initial poly(A)$^{+}$RNA population in the same relative frequencies;
2. unidirectionally cloned so that the orientation of each cDNA is known, facilitating subsequent sequence analysis and expression;
3. composed of a high proportion of long or full-length inserts;
4. not contaminated with genomic, mitochondrial or ribosomial RNA inserts; and
5. composed of a large proportions of inserts with short poly(A)$^{+}$ tails.

RNA extracted from the AD hippocampus was quantified by measuring the optical density at 260 nm. The quality of the RNA was checked spectrophotometrically by measuring the OD_{260}/OD_{280} ratio and confirmed electrophoretically on denaturing formaldehyde agarose gel (Fig. 7) and by Northern blot, using as probes β-actin (Fig. 8a) and glyceraldehyde phosphate dehydrogenase (GAPDH) (Fig. 8b). To ensure that the purification procedure was not introducing any degradation, RNA was extracted in parallel from fresh rat brain and checked in the same way (lanes 1 and 2 of Fig. 7; lanes 1 of Fig. 8).

Poly(A)$^+$RNA from AD hippocampus was used for cDNA synthesis, whose efficiency was monitored by incorporation of radioactive nucleotides and by performing the same reaction on a control sample of RNA of known length (1.8 kb). A portion (1/10) of each reaction product was separated electrophoretically on alkaline agarose gel and exposed to autoradiographic film, to determine the size range of the first- and second-strand cDNA (Fig. 9). After synthesis double-stranded cDNA from AD hippocampi was size-selected and excess adapters were removed by passage through a Sephacryl-S 500 spin column. 1/10 volume of each fraction collected was analysed electrophoretically on a 5% nondenaturing polyacrylamide gel and exposed to autoradiographic film to check sizes of cDNA in each fraction. cDNAs greater than 500 bp in size were pooled, precipitated, quantitated and ligated to the ZAP Express vector arms and subsequently packaged with Gigapack II Gold packaging extract (Stratagene). After construction, the quality of the cDNA library was evaluated by determining the ratio of recombinants to non-recombinants and the average size of the cDNA inserts using PCR analysis of approximately 50 individual clones (Fig. 10).

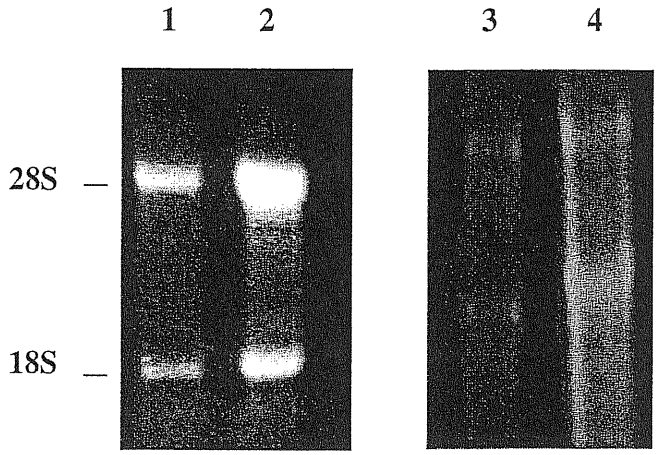


Figure 7. Electrophoresis on denaturing formaldehyde agarose gel of total and poly(A)$^+$RNA. Lane 1: 1 μg of poly(A)$^+$RNA purified from rat brain total RNA; lane 2: 10 μg of total RNA extracted from rat brain; lane 3: 1 μg of poly(A)$^+$RNA purified from AD hippocampi total RNA; lane 4: 10 μg of total RNA extracted from AD hippocampi. Total and poly(A)$^+$RNAs from rat brain were loaded as controls for checking the quality of human brain RNA. Comparing the relative intensities of 28S and 18S rRNA bands in total RNAs from rat and human brain, traces of degradation can be observed in the RNA extracted from human brain. Nevertheless, the traces of 28S and 18S rRNA (6333 bp and 2366 bp long) in both samples of poly(A)$^+$RNA indicate the presence of long poly(A)$^+$RNA molecules.

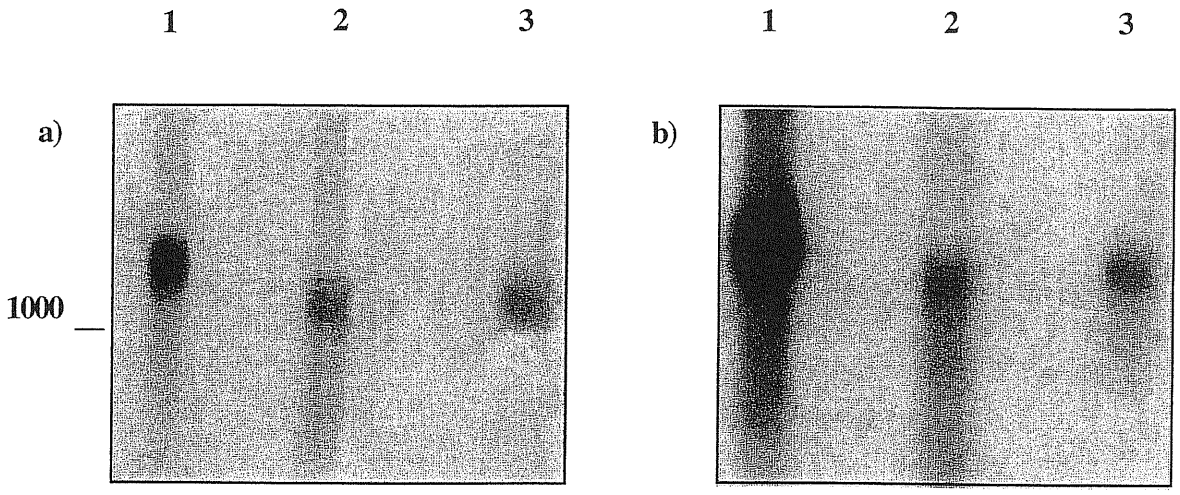


Figure 8. Northern blot.
Lane 1: 10 μg of total RNA extracted from rat brain; lane 2: 10 μg of total RNA extracted from AD hippocampi; lane 3: 1 μg of poly(A)$^+$ RNA purified from AD hippocampi total RNA. In a) blot was hybridized with a radiolabeled full length human actin; in b) with a radiolabeled fragment of 400 bp from rat GAPDH.
Results of Northern blot analysis confirmed those of electrophoresis: in particular, it can be observed the reduced signal intensity in poly(A)$^+$ RNA from AD hippocampi indicating a reduced yield of poly(A)$^+$ RNA (see 3.1.1.1. of Discussion)

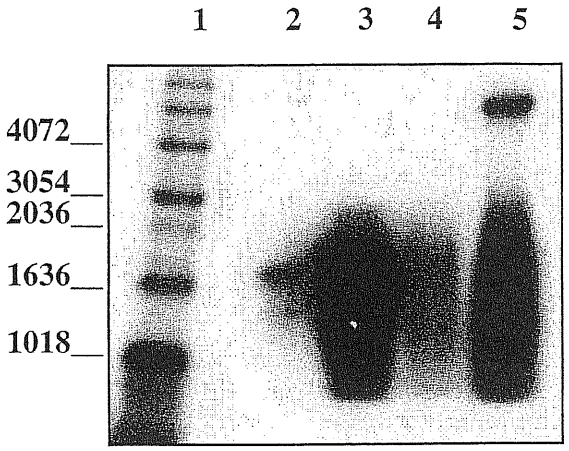


Figure 9. Autoradiography of first- and second-strand cDNA.
Lane 1: molecular weight markers; lane 2: second-strand cDNA of the control RNA (1.8 kb); lane 3: first-strand cDNA of the control RNA; lane 4: second-strand cDNA of AD hippocampal RNA; lane 5: first-strand cDNA of AD hippocampal RNA.
The average size of the first- and second-strand cDNA is 1.5 kb.

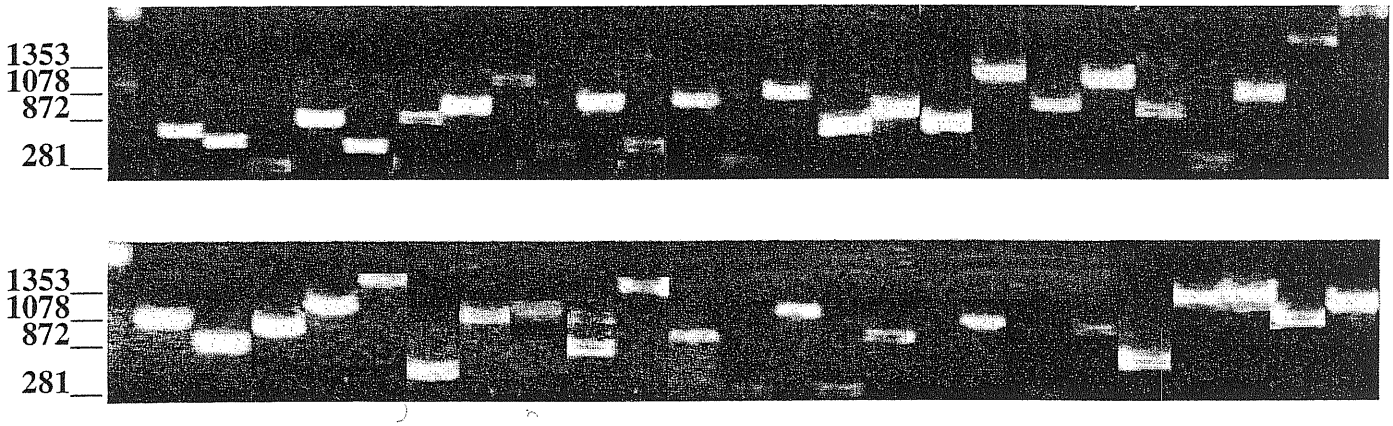


Figure 10. Electrophoresis on TBE agarose gel of 50 amplified plaques from AD hippocampal cDNA.
50 randomly chosen plaques from AD hippocampal cDNA library were picked up and subjected to PCR analysis using T3 and T7 commercial primers to detect the cDNA insert size. The same results were obtained performing PCR on purified clones after *in vivo* excision. cDNA inserts ranged from 300 bp to 2.5 kb.

In conclusion, from 3.18 g of AD hippocampi about 5 mg of total RNA were extracted, from these 6 μg of poly(A)$^+$RNA were purified; this poly(A)$^+$RNA was used for the construction of the AD hippocampal cDNA library which contained approximately $1.7x10^5$ pfu/ml and was amplified to a titer of approximately $1x10^8$ pfu/ml; the inserts, ranging in size from 0.3 to more than 2.5 kb, had an average size of 0.8 kb (see Table 7).

This library was used for the following immunological screening with patients' sera.

Table 7. Characteristics of AD hippocampal cDNA library.

Library mRNA Source	hippocampus from: 61-yr-old female with AD 97-yr-old female with AD 84-yr-old female with AD
Priming Method	oligo(dT)-primed
Vector	λZAPII
Cloning Site	EcoRI-XhoI (cloned unidirectionally)
Independent Clones	$1.7x10^5$
Insert Size Range (Avg.)	0.3 - 2.8 kb (0.8 kb)
% of Recombinants	90%
Estimated Titer	10^8 pfu/ml

2.3. IMMUNOLOGICAL SCREENING OF THE cDNA LIBRARY

In order to investigate the presence of proteins which are recognized specifically by antibodies present in AD sera, the AD cDNA hippocampal library was screened with patients' sera using the SEREX approach (1.9.1.). Immunological screening was performed as described in Materials and Methods (4.5.) and schematized in Fig. 11: approximately one half of the $1.7x10^5$ pfu from the AD hippocampal cDNA library were plated, blotted onto nitrocellulose membranes and screened with 1:100 diluted sera from AD patients and healthy age-matched controls.

Sera from AD patients used for primary immunoscreenings (Table 8) were from individuals (with one exception) who are positive for at least one APOEε4 allele (see 1.5. and 1.5.3. of the Introduction) because it has been shown that AD patients who have at least one APOEε4 allele with a probability greater than 90% will have a definite diagnosis of AD at autopsy (Welsh-Bohmer *et al.*, 1997).

Despite the problems encountered in sera preabsorption (2.3.1.), the immunological screening of AD hippocampal cDNA library resulted in the isolation of two putative AD-specific antigens (2.3.2.) which were further analyzed (2.4 and 2.5.).

On the basis of the immunoscreening results (2.3.2.), antibodies recognising these antigens are specifically present in AD patients: in fact, they are present in 20% of AD patients and not in healthy age-matched controls (see Table 11).

In order to extend these results to a larger number of individuals an ELISA experiment was set up for testing human sera on the single antigens purified (2.6.1.).

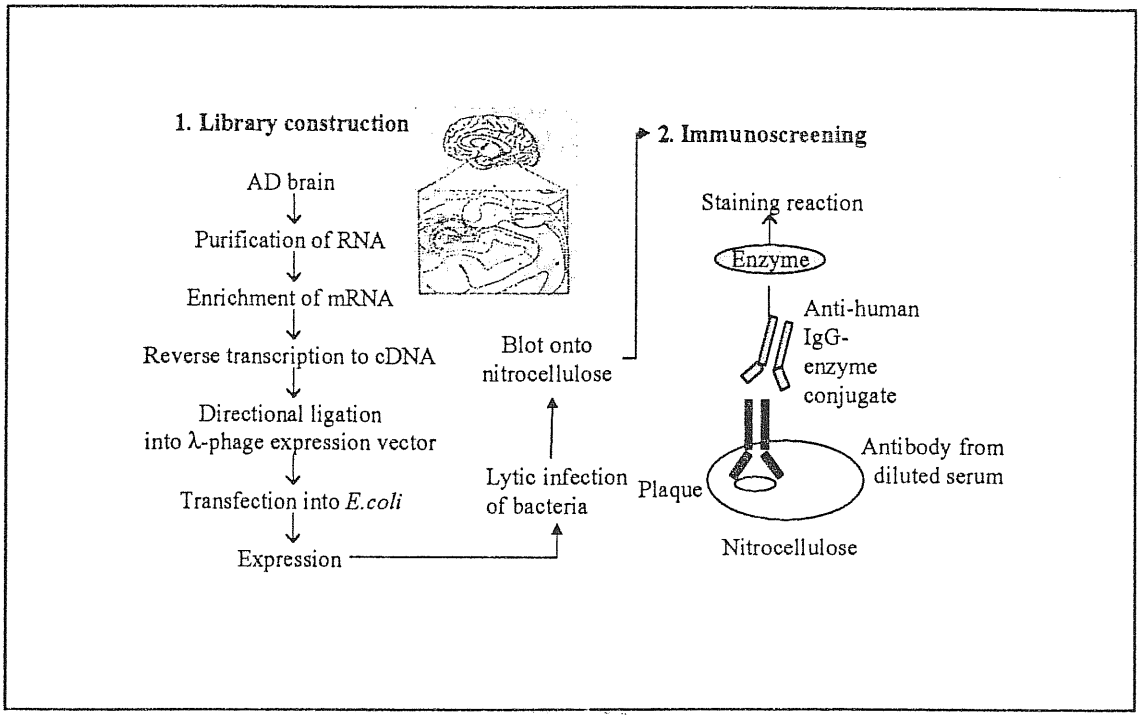


Figure 11. Molecular definition of AD antigens by the SEREX technique.
A cDNA library was constructed from AD hippocampi and expressed in *E.coli* using the λZAPII vector. The bacteria were grown and the lytic plaques were blotted to nitrocellulose membranes. Diluted sera were then incubated with the membranes and those antibodies that bind to AD antigens expressed in lytic plaques were made visible by alkaline phosphatase-conjugated anti-human IgGs (adapted from Tureci *et al.*, 1997).

Table 8. Sera used in primary immunoscreenings of AD cDNA library.

CODE NUMBER	CLINICAL DIAGNOSIS	AGE	SEX	APOE GENOTYPE	ORIGIN
8006	AD	72	F	ε3ε4	ITALY (BS)
8011	AD	78	F	ε3ε4	ITALY (BS)
9013	SDAT		F	ε3ε3	ITALY (PV)
9014	SDAT		F	ε4ε4	ITALY (PV)
9016	SDAT		M	ε4ε4	ITALY (PV)
V	MODERATE AD	79	M	ε4ε4	HOLLAND

2.3.1. Sera Preadsorption

In immunological screenings of cDNA libraries polyclonal sera cannot be used as such since they contain a lot of antibodies directed against bacterial and phage proteins. These antibodies must be removed before performing screening because they hamper the detection of positive clones causing all lytic plaques to be positive. So the crucial step in immunological screenings is the preabsorption of sera.

Different protocols were attempted for the removal of antibodies directed against bacterial, phage and phage-induced bacterial proteins but all were ineffective for almost all the AD sera: whatever the protocol followed for preabsorption, almost all lytic plaques on the plates are detected. After these preliminary attempts the protocol set up by Sahin *et al.* to preabsorb tumour patients' sera was used as described in Materials and Methods (4.5.2.) repeating the whole (or some steps of the) procedure more times. This method too, was inefficient for most of the AD sera preabsorbed as documented in Fig. 12.

In Fig. 12 are shown the preabsorption results obtained by using Sahin's protocol on ten different AD sera together with one serum from an healthy young individual. Out of the AD sera only sera n. 4 and 11 were suitable for immunoscreening after one round of preabsorption. Sera n. 2, 7 and 10 were subjected to a second round of preabsorption after which only serum n. 2 was suitable for immunoscreening. Sera n. 3 and 8 remained so cross-reactive that even after several rounds of preabsorption they reacted with all plaques on the plate. Serum n. 5 was from the healthy young control.

This difficulty, not encountered with sera from healthy age-matched individuals nor tumour patients which usually require no more than two-three rounds of preclearing, could mean that AD sera are particularly rich in antibodies directed against bacterial, phage and phage-induced bacterial proteins or these sera are unusually sticky for some other reason which could be interesting to further investigate (as discussed in 3.1.2.1.).

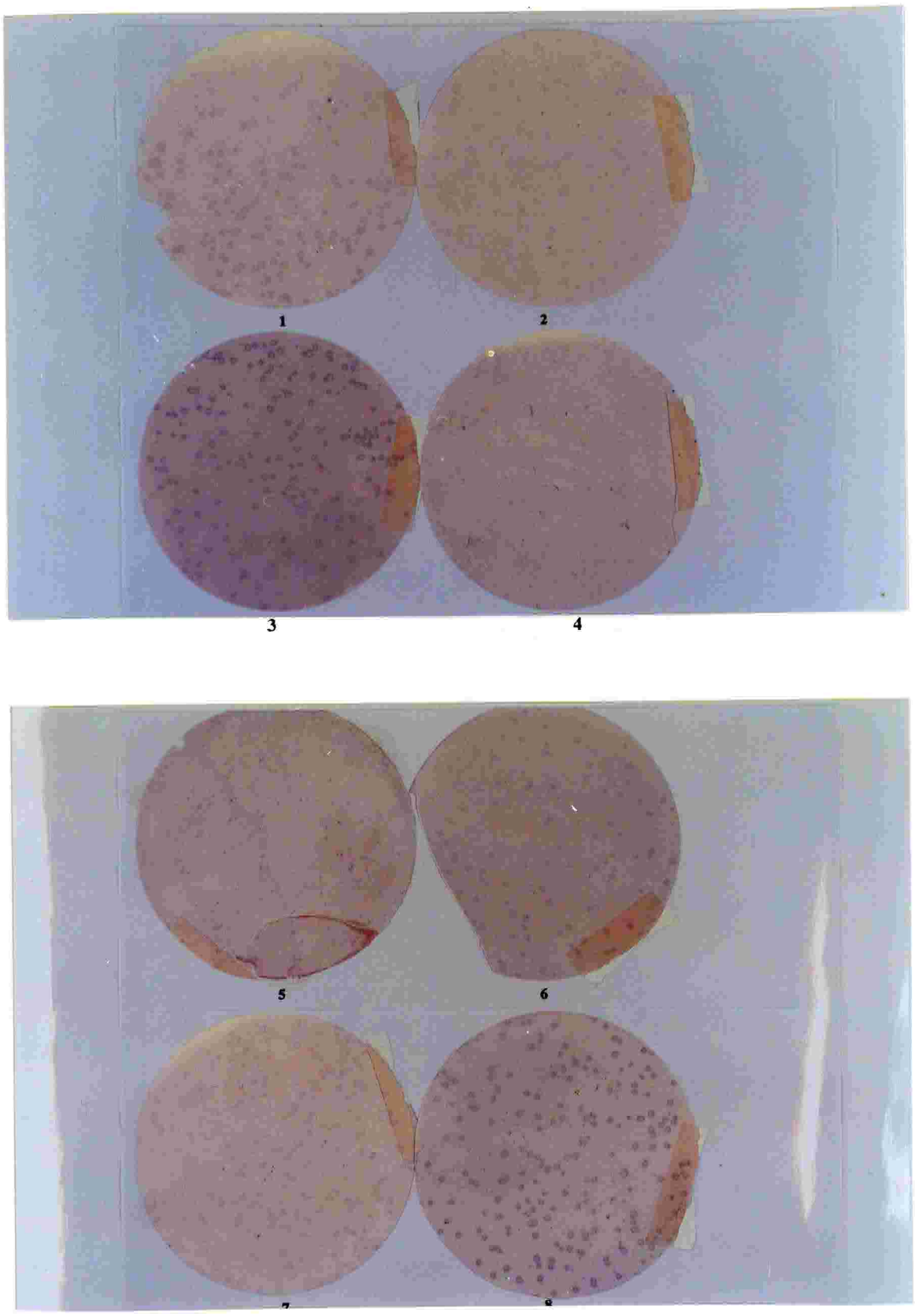


Figure 12. Preabsorption results obtained with eleven different sera by using Sahin's protocol.
Ten AD sera (1-4; 6-11) and one from an healthy young control (5) were preabsorbed by using Sahin's protocol (as described in 4.5.2. of Materials and Methods). In Table 9 are described the characteristics of the eleven sera whose preabsorption results are shown.

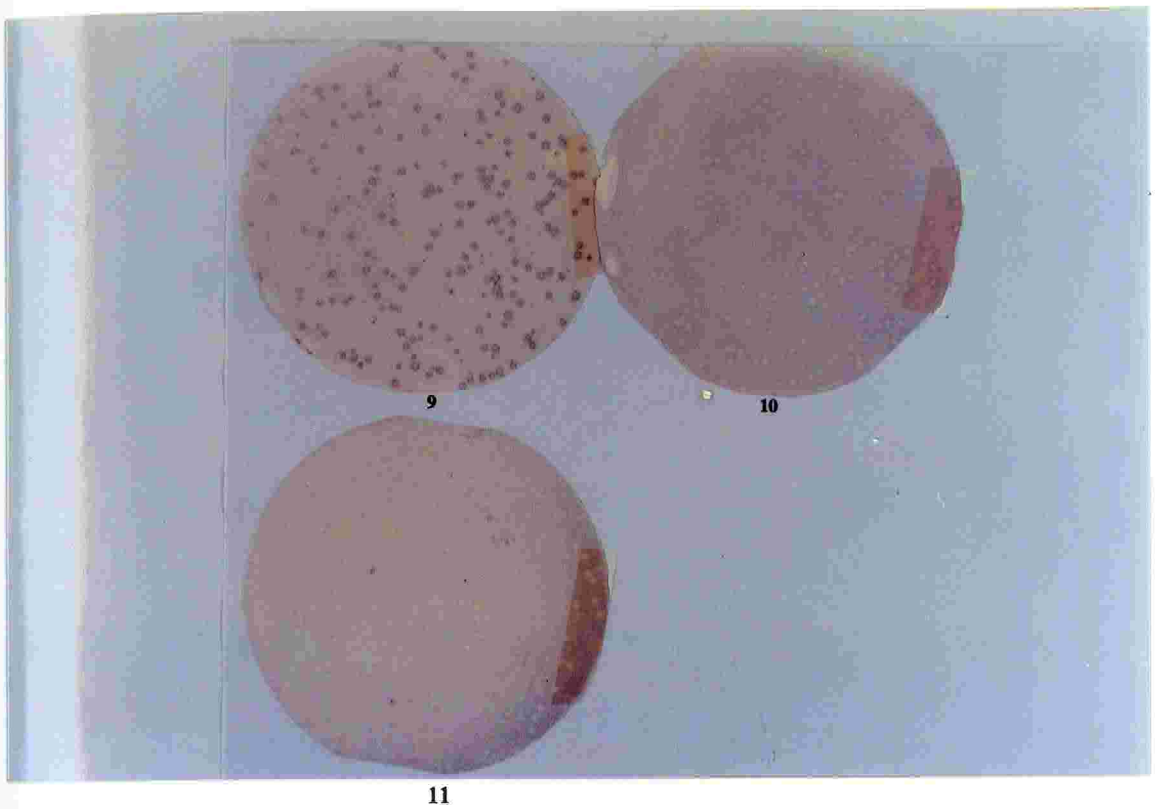


Figure 12. Continued.

The presence of high background/stickiness was not clearly associated with the state and/or progression of the disease (see Table 9). High background was seen in sera from patients with both mild and severe AD, and one patient with moderate AD showed low background. However, the number of patients in this sample is small and a definite conclusion will require further analysis.

Table 9. Characteristics of preabsorbed AD sera and relative preabsorption results.

FILTER NUMBER	CODE NUMBER	CLINICAL DIAGNOSIS	AGE	SEX	APOE GENOTYPE	ORIGIN	PREABSORPTION RESULTS
1	8012	AD			ε3ε4	ITALY (BS)	- -
2	8011	AD			ε3ε4	ITALY (BS)	+
3	8010	AD			ε3ε4	ITALY (BS)	- - -
4	8006	AD	72	F	ε3ε4	ITALY (BS)	++
5	RB	NDC	28	F		ITALY	++
6	8013	AD			ε3ε4	ITALY (BS)	- -
7	8014	AD			ε3ε4	ITALY (BS)	-
8	K	SEVERE AD	73	F	ε4ε4	HOLLAND	- - -
9	J	SEVERE AD	66	F	ε4ε4	HOLLAND	- -
10	W	MILD AD	76	M	ε3ε4	HOLLAND	-
11	V	MODERATE AD	79	M	ε4ε4	HOLLAND	++

++: suitable for immunoscreening after one cycle of preclearing
+: suitable after two cycles
- : not suitable after two cycles
- -: not suitable (not subjected to other cycles)
- - - : not suitable for immunoscreening even after 11 cycles of preclearing

2.3.2. Immunoscreening Results

Given the preabsorption results described above (2.3.1.), for primary immunoscreenings both the well preabsorbed AD sera (n. 2. 4 and 11, see Fig. 12) as well as dirtier sera (9013, 9014 and 9016, not shown in Fig. 12) were utilised and the screenings results are shown in Table 10. The high number of putative primary positive clones reported in the table, not confirmed by secondary screening, could be explained by the different results obtained in the preabsorption procedure.

Putative primary positive clones found with each AD serum tested (Fig. 13, upper panels) were isolated and submitted to further analysis: they were confirmed by secondary and, if it was necessary, by tertiary screening with the same serum (Fig. 13, lower panels) and, when confirmed, they were screened with sera from other AD patients and healthy age-matched controls (Fig. 14).

Secondary positive clones were also screened only with the secondary antibody to exclude the possibility they coded for human IgGs (the first filter on the upper left corner of Fig.14a).

TABLE 10. Antibody reactivity of AD sera with recombinant clones derived from AD brain cDNA.

		No. of positive clones	
Serum code	**No. of clones tested**	**after primary screening**	**after secondary screening**
8006	10^5	16	**1 (AD1)**
8011	$4x10^4$	5	?
9013	$9x10^4$	17	-
9014(G)	$1.5x10^4$	14	-
9016(G)	$1.8x10^5$	10	**1 (AD2)**
9016(I)	$3.5x10^4$	2	-
V	$9.5x10^4$	17	?

9016(G) and 9016(I) codes refer to the same serum, preabsorbed with two different procedures.
The question marks in the column named 'after secondary screening' refer to experiments still in progress.

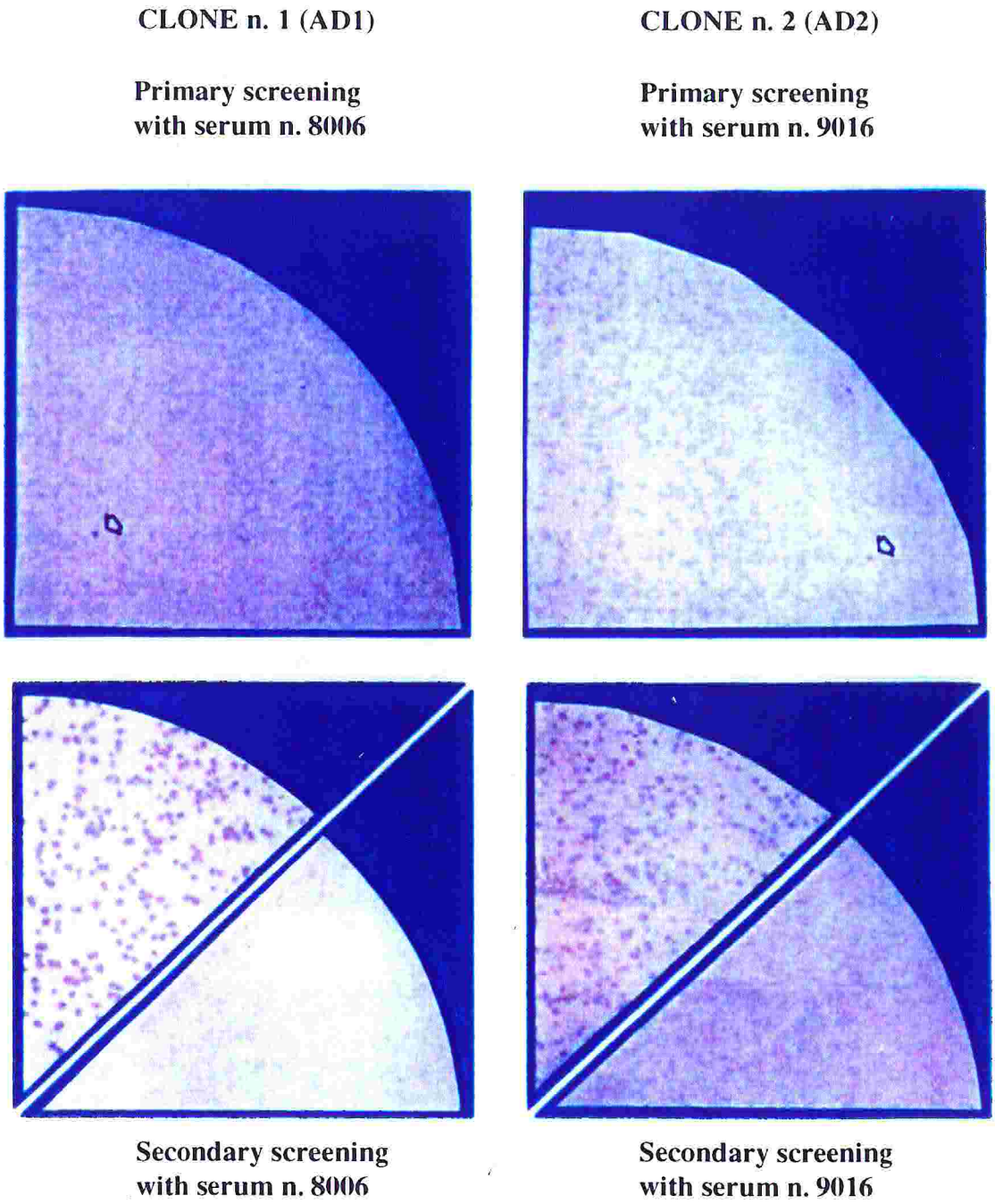


Figure 13. Immunoscreening of AD hippocampal cDNA library using patients' sera.
Membranes with bacteriophage plaques were incubated with preadsorbed patients' sera. Characterization of the two positive clones are shown in the left-hand and in the right-hand row.
Upper panels: primary screening. Phage recombinants were plated at a density of 5000 plaques per 140 mm plate. Complexes of antigens with antibodies in the serum were detected by secondary antibody. Positive clones are indicated by arrows.
Lower panels: secondary screening. Primary positive clones were isolated and subjected to a second round of screening plated at a density of 500-1000 plaques per 90 mm plate. The enrichment is demonstrated in the upper quarter and the library background in the lower quarter of the figure.

As shown in Table 10 and in Fig. 13, screening a total of $5.5x10^5$, two clones, named AD1 and AD2, remained positive after secondary and tertiary screening: AD1 was isolated using 8006 serum; AD2 using 9016(G) serum. Interestingly, 9016(G) serum was one of the dirtiest sera; nevertheless, it reacted with a strong putative AD-specific antigen (see Fig. 13, right hand row).

These two clones were screened with other 8 AD sera and 4 age-matched controls and each of them was recognized by another AD serum as shown in Fig. 14 and schematized in Table 11. Note that the two sera which recognized AD1 were different from the two sera which recognized AD2.

Table 11. Humoral immune responses against putative AD-specific antigens.

Subject	AD1	AD2
AD patients	2/10	2/10
Age-matched controls	0/4	0/4

The values given are the number of subjects with an immune response/total number of subjects tested.

After all these screenings, the two positive clones (Tables 10 and 11, Figs. 13 and 14) were submitted to *in vivo* excision and sequenced (2.4.).

a)

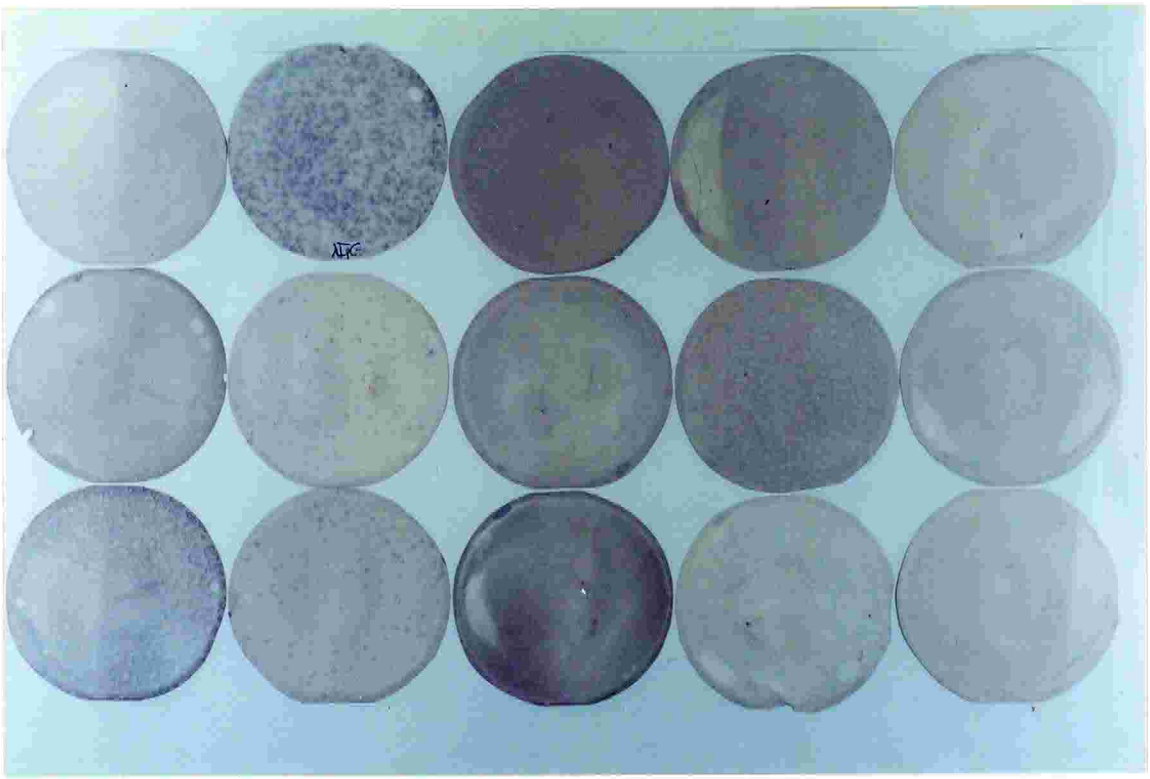


Figure 14. Immunoscreening results with other preabsorbed patients' and age-matched individuals' sera.
The two clones positive after secondary screening were plated at a density of 500-1000 plaques per 90 mm plate and incubated with age-matched individuals' sera (filters on the last column on the right), with the secondary antibody alone (the first filter on the upper left corner) and with the other preabsorbed AD sera (all the other filters except that one named λIgG). The filter named λIgG came from a plate on which are plated phage coding for an human IgG and was used as a positive control for the whole experiment.
The results shown are those obtained for the clone AD1. In a) all filters incubated with human sera; in b) the two positive filters at higher magnification.
The same pattern was observed with the other clone, AD2. Each clone was recognized by another AD serum. Note that the two AD sera which recognized AD1 were different from the two sera which recognized AD2.

b)

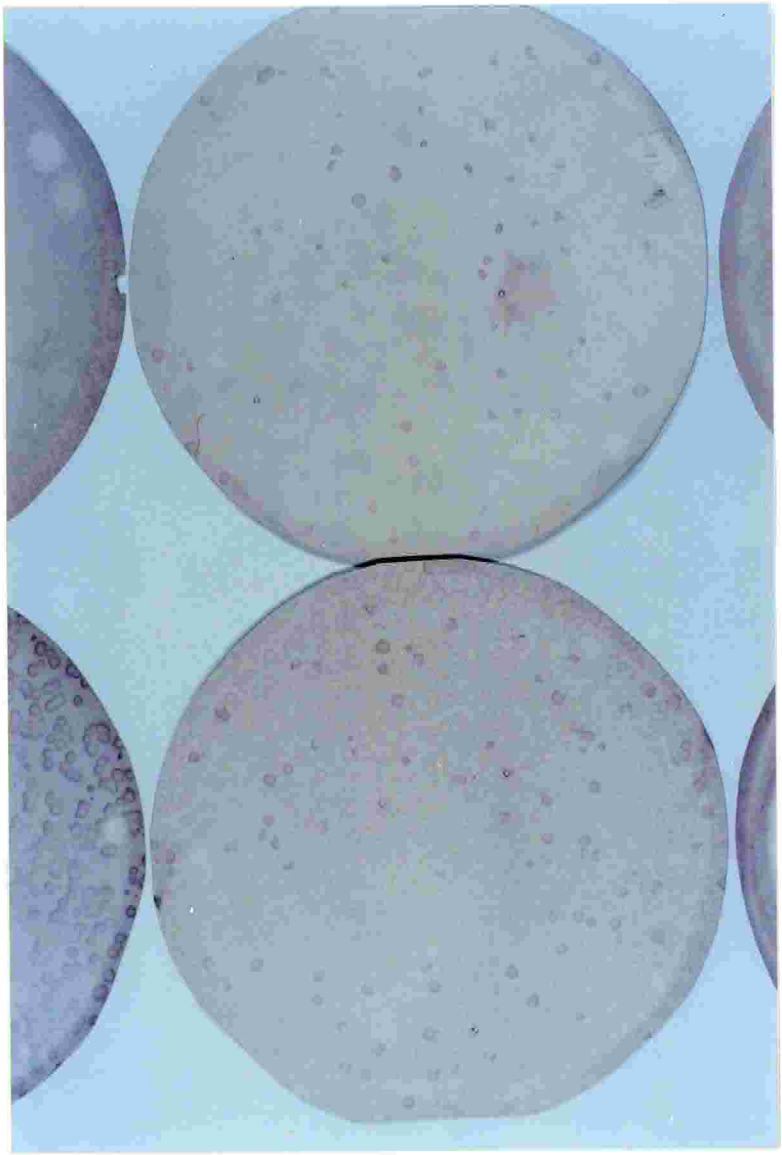


Figure 14. Continued.

2.4. IDENTIFICATION AND COMPUTER ANALYSIS OF THE cDNAs ISOLATED

The immunological screening of AD hippocampal cDNA library resulted in the isolation of two putative AD-specific antigens (2.3.2.): they were present in 20% of AD patients and not in healthy age-matched controls (see Table 11).

In order to identify these AD-specific antigens, the two positive clones were subcloned to monoclonality, *in vivo* excised and the entire nucleotide sequence of cDNA inserts determined. PCR analysis performed on purified phage stocks as well as on single colonies after excision revealed that the two clones contained inserts respectively 800 and 1300 bp long; these results were confirmed by restriction analysis with *EcoRI* and *XhoI* of the two clones after *in vivo* excision.

Sequence data were analyzed with BLAST (Altschul *et al.*, 1997) software on European Molecular Biology Laboratory (EMBL) and PROSITE software (Bucher and Bairoch, 1994; Bairoch *et al.*, 1997) on Swiss Institute of Bioinformatics (SIB). Results of sequence analysis were described in 2.4.1. and 2.4.2.

2.4.1. Sequencing Results For The Clone AD1

AD1 cDNA was 794 bp long and contained a putative open reading frame (ORF) of 176 amino acids corresponding to a protein with theoretical molecular mass of 19,556.53 kDa and isoelectric point (pI) of 4.71 followed by a 3'-untranslated region of 239 bp.

BLAST search for DNA sequence revealed that 783 bp of AD1 cDNA were identical to 783 of a 1125 bp long putative ORF contained in a human genomic sequence already published (Gen Bank accession number AC002985) as illustrated in Fig. 15. The whole ORF is 1125 bp long and codes for a hypothetical human protein of 374 amino acids (Gen Bank accession number 2443871) with theoretical molecular mass of 41,221.93 kDa and isoelectric point (pI) of 5.39.

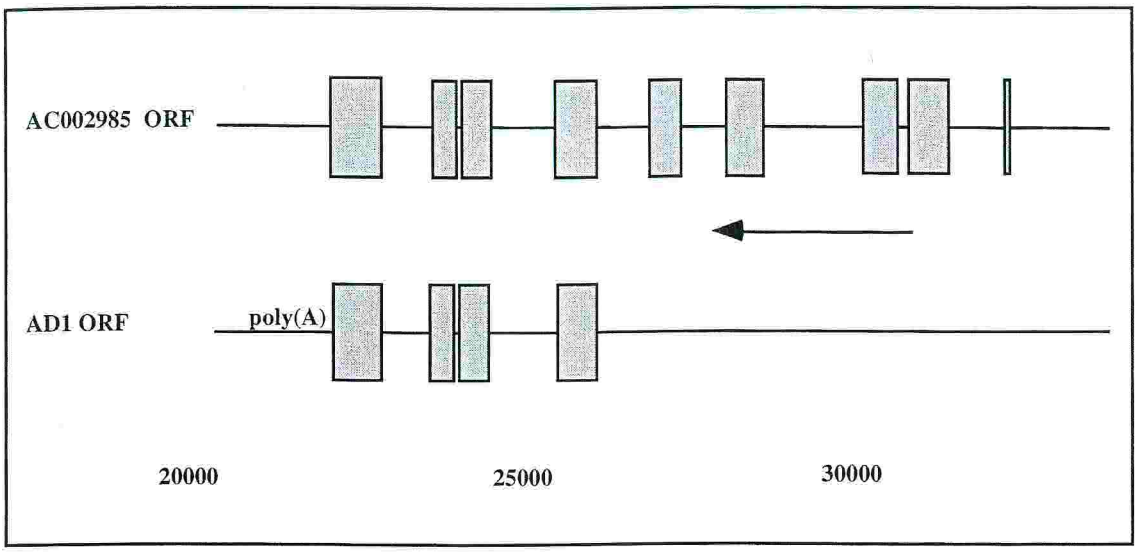


Figure 15. Alignment of the putative ORF in the genomic sequence (AC002985) and AD1 ORF.
In the genomic sequence from chromosome 19 the putative ORF is located on the complementary strand. It is constituted by 9 exons located between nt 22685 and 32224 (22685-22876; 24567-24653; 24751-24867; 25218-25374; 26166-26287; 27506-27652; 31595-31726). AD1 cDNA contained the last four exons.

In Fig. 16 the nucleotide sequence and the deduced amino acid sequence of the whole AC002985 ORF and the included AD1 cDNA sequence are shown. In addition to AC002985, a number of ESTs from man (see Fig. 29, 2.5.1.), mouse and Drosophila, showing different degrees of similarity to that ORF were also identified. The isolation of AD1 cDNA confirmed that this putative gene is indeed transcribed.

A BLAST search for protein similarity revealed that the AD1 protein is most similar to the Vesl family of rat proteins (3.2.1.1. of the Discussion) with an homology of 87% with Vesl-1S/Homer, the shortest rat isoform, and 66% and 64% with Vesl-1L and Vesl-2, respectively.

In Fig. 17 is reported the structure of the Vesl family of rat proteins compared to the new human protein (indicated as 'human Vesl') and the portion of it coded by the AD1 clone: the regions of homology are differently shaded and their functions indicated. In Fig. 18 the percentages of homology are detailed subdividing the proteins in two portions: an N- and a C-terminal portion, being the length of the N-terminal portion corresponding to the shortest rat isoform, Vesl-1S. In Fig. 19 the multiple sequence alignment of Vesl proteins made by CLUSTALW (Higgins, 1994) was reported.

AC002985 ORF

```
ATG TCC ACA GCC AGG GAG CAG CCA ATC TTC AGC ACA CGG GCG CAC GTG TTC CAA ATT GAC      60
 M   S   T   A   R   E   Q   P   I   F   S   T   R   A   H   V   F   Q   I   D      20

CCA GCC ACC AAG CGA AAC TGG ATC CCA GCG GGC AAG CAC GCA CTC ACT GTC TCC TAT TTC     120
 P   A   T   K   R   N   W   I   P   A   G   K   H   A   L   T   V   S   Y   F      40

TAC GAT GCC ACC CGC AAT GTG TAC CGC ATC ATC AGC ATC GGA GGC GCC AAG GCC ATC ATC     180
 Y   D   A   T   R   N   V   Y   R   I   I   S   I   G   G   A   K   A   I   I      60

AAC AGC ACT GTC ACT CCC AAC ATG ACC TTC ACC AAA ACT TCC CAG AAG TTC GGG CAG TGG     240
 N   S   T   V   T   P   N   M   T   F   T   K   T   S   Q   K   F   G   Q   W      80

GCC GAC AGT CGC GCC AAC ACA GTC TAC GGC CTG GGC TTT GCC TCT GAA CAG CAT CTG ACA     300
 A   D   S   R   A   N   T   V   Y   G   L   G   F   A   S   E   Q   H   L   T     100

CAG TTT GCC GAG AAG TTC CAG GAA GTG AAG GAA GCA GCC AGG CTG GCC AGG GAG AAA TCT     360
 Q   F   A   E   K   F   Q   E   V   K   E   A   A   R   L   A   R   E   K   S     120

CAG GAT GGC GGG GAG CTC ACC AGT CCA GCC CTG GGG CTC GCC TCC CAC CAG GTC AGC ACT     420
 Q   D   G   G   E   L   T   S   P   A   L   G   L   A   S   H   Q   V   S   T     140

CCC TAC TCC CCT ATG CCT GCC TGG GCA CCT GTG CCC CCG AGC CCT CTC GTC AGT GCC AAC     480
 P   Y   S   P   M   P   A   W   A   P   V   P   P   S   P   L   V   S   A   N     160

GGC CCC GGC GAG GAA AAA CTG TTC CGC AGC CAG AGC GCT GAT GCC CCC GGC CCC ACA GAG     540
 G   P   G   E   E   K   L   F   R   S   Q   S   A   D   A   P   G   P   T   E     180
                                                                        AD1 ORF
CGC GAG CGG CTA AAG AAG ATG TTG TCT GAG GGC TCC GTG GGC GAG GTA CAG TGG GAG GCC     600
 R   E   R   L   K   K   M   L   S   E   G   S   V   G   E   V   Q   W   E   A     200

GAG TTT TTC GCA CTG CAG GAC AGC AAC AAC AAG CTG GCA GGC GCC CTG CGA GAG GCC AAC     660
 E   F   F   A   L   Q   D   S   N   N   K   L   A   G   A   L   R   E   A   N     220

GCC GCC GCA GCC CAG TGG AGG CAG CAG CTG GAG GCT CAG CGT GCA GAG GCC GAG CGG CTG     720
 A   A   A   A   Q   W   R   Q   Q   L   E   A   Q   R   A   E   A   E   R   L     240

CGG CAG CGG GTG GCT GAG CTG GAG GCT CAG GCA GCT TCA GAG GTG ACC CCC ACC GGT GAG     780
 R   Q   R   V   A   E   L   E   A   Q   A   A   S   E   V   T   P   T   G   E     260

AAG GAG GGG CTG GGC CAG GGC CAG TCG CTG GAA CAG CTG GAA GCT CTG GTG CAA ACC AAG     840
 K   E   G   L   G   Q   G   Q   S   L   E   Q   L   E   A   L   V   Q   T   K     280

GAC CAG GAG ATT CAG ACC CTG AAG AGT CAG ACT GGG GGG CCC CGC GAG GCC CTG GAG GCT     900
 D   Q   E   I   Q   T   L   K   S   Q   T   G   G   P   R   E   A   L   E   A     300

GCC GAG CGT GAG GAG ACT CAG CAG AAG GTG CAG GAC CTG GAG ACC CGC AAT GCG GAG TTG     960
 A   E   R   E   E   T   Q   Q   K   V   Q   D   L   E   T   R   N   A   E   L     320

GAG CAC CAG CTG CGG GCG ATG GAG CGC AGC CTG GAG GAG GCA CGG GCA GAG CGG GAG CGG    1040
 E   H   Q   L   R   A   M   E   R   S   L   E   E   A   R   A   E   R   E   R     340

GCG CGG GCT GAG GTG GGC CGG GCA GCG CAG CTG CTG GAC GTC AGC CTG TTT GAG CTG AGT    1100
 A   R   A   E   V   G   R   A   A   Q   L   L   D   V   S   L   F   E   L   S     360

GAG CTG CGT GAG GGC CTG GCC CGC CTG GCT GAG GCT GCG CCC TGA GCCGGGGCTGGTTTTCTAT    1164
 E   L   R   E   G   L   A   R   L   A   E   A   A   P   *                        374

GAACGATTCCGGCCTGGGATGCGGGCCAGGCTGCAGGCGGCATAGTTGGGCCCATTCGTCCTGGAAAGGGACTGGGGGG    1243
TCCCAACTTAGCCCTGGGTGGGCCGGGCCGGGCTGGGCTGGGGTGGGCCCCAGTCGGCTCTGGTTGTTGGCAGCTTTGG    1322
GGCTGTTTTTGAGCTTCTCATTGTGTAGAATTTCTAGATCCCCCGATTACATTTCTAAGCGTGAAAAAAAAAAAAAAAA    1401
```

Figure 16. Nucleotide and amino acid sequence of the putative AC002985 ORF and AD1 clone.
The predicted ORF in the genomic nucleotide sequence was translated into the amino acid sequence below. The stop codon is marked by an asterisk. The *boldface* triplet (**GAG/E**) represents the first codon found in the AD1 cDNA clone. In the amino acid sequence potential N-glycosylation sites (X), protein kinase C (**X**) and casein kinase II phosphorylation sites (X) and N-myristoylation sites (*X*) are indicated. PEST sequence is underlined.

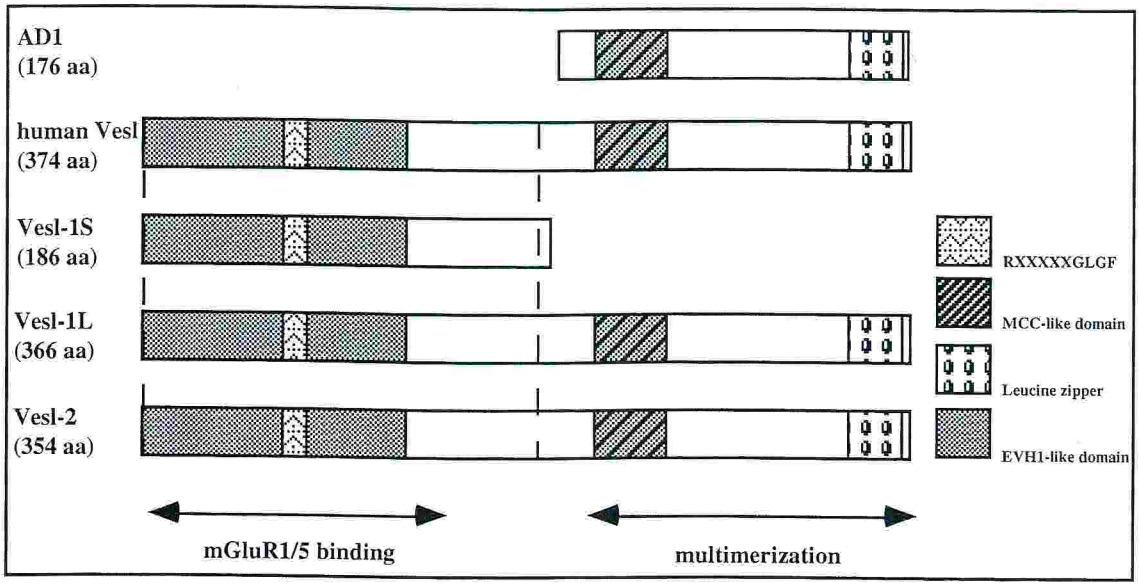


Figure 17. Schematic diagram of the structure of the Vesl family of proteins.
Graphical comparison between 'human Vesl', AD1 protein and the Vesl family of rat proteins. The differently shaded regions indicate the different homologous regions.

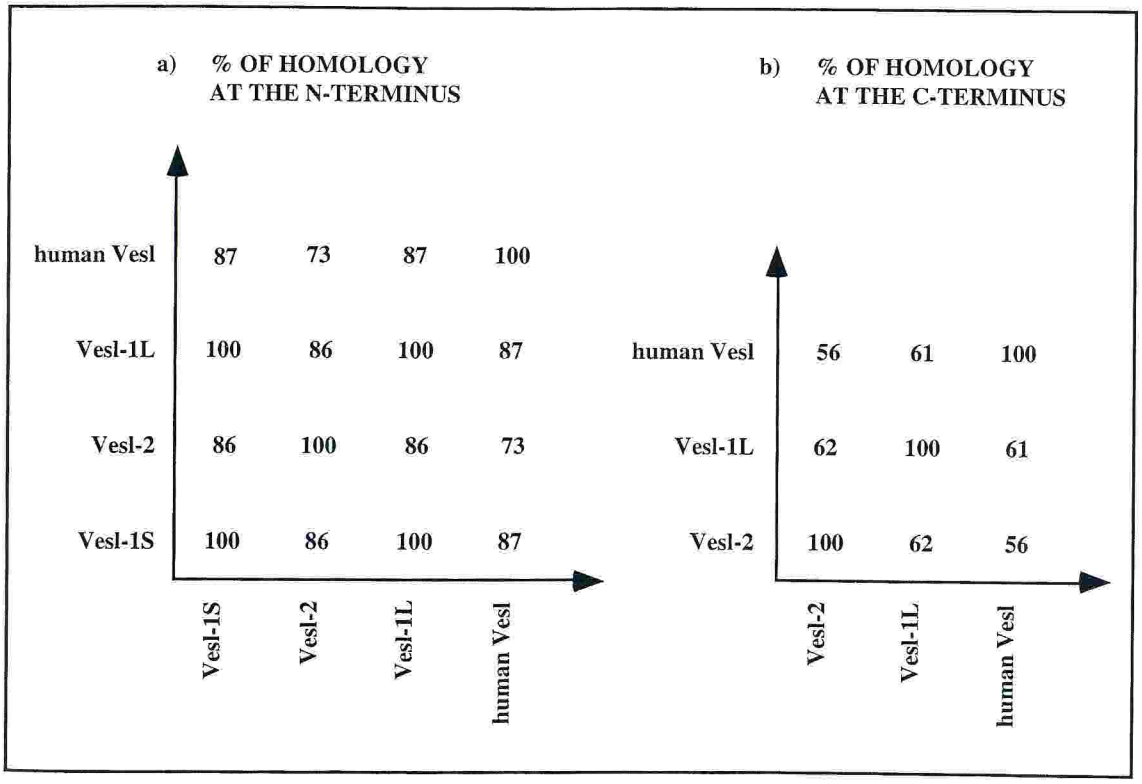


Figure 18. Percentages of homology between rat and human Vesl proteins.
In a) the N-terminal halves of the proteins are aligned by using the BLAST software (Altschul *et al.*, 1997), assuming as length that of Vesl-1S (the shortest rat isoform), 186 aa; in b) the remaining C-terminal portions of the proteins are compared.

```
rat Vesl        MG---EQPIFSTRAHVFQIDPNTKKNWVPTSKHAVTVSYFYDSTRNVYRIISLDGSKAII 57
rat Vesl 1L     MG---EQPIFSTRAHVFQIDPNTKKNWVPTSKHAVTVSYFYDSTRNVYRIISLDGSKAII 57
rat Vesl 2      MG---EQPIFTTRAHVFQIDPSTKKNWVPASKQAVTVSYFYDVTRNSYRIISVDGAKVII 57
human Vesl      MSTAREQPIFSTRAHVFQIDPATKRNWIPAGKHALTVSYFYDATRNVYRIISIGGAKAII 60
                *.   *****:********** **:**:*:.*:*:******* *** *****:.*:*.**

rat Vesl        NSTITPNMTFTKTSQKFGQWADSRANTVYGLGFSSEHHLSKFAEKFQEFKEAARLAKEKS 117
rat Vesl 1L     NSTITPNMTFTKTSQKFGQWADSRANTVYGLGFSSEHHLSKFAEKFQEFKEAARLAKEKS 117
rat Vesl 2      NSTITPNMTFTKTSQKFGQWADSRANTVFGLGFSSEQQLTKFAEKFQEVREAARLARDKS 117
human Vesl      NSTVTPNMTFTKTSQKFGQWADSRANTVYGLGFASEQHLTQFAEKFQEVKEAARLAREKS 120
                ***:************************:****:**::*::*******.:******::**

rat Vesl        QEKMELTS------TPSQESAGGDLQS--PLTPE---SINGTDDERTPDVTQNSEPRAEP 166
rat Vesl 1L     QEKMELTS------TPSQESAGGDLQS--PLTPE---SINGTDDERTPDVTQNSEPRAEP 166
rat Vesl 2      QEKIETSS------NHSQES-GCETPS--STQAS---SVNGTDDEK-----AS---HASP 157
human Vesl      QDGGELTSPALGLASHQVSTPYSPMPAWAPVPPSPLVSANGPGEEK--------LFRSQS 172
                *:  * :*      . . .:      :  .  ..   * **..:*:          ::..

rat Vesl        AQNALPFSHRYTFNSAIMIK---------------------------------------- 186
rat Vesl 1L     AQNALPFSHSAGDRTQGLSHASSAISKHWEAELATLKGNNAKLTAALLESTANVKQWKQQ 226
rat Vesl 2      ADTHLK---SENDKLKIALTQSAANVKKWEIELQTLRESNARLTTALQESAASVEQWKRQ 214
human Vesl      ADAPGP---TERERLKKMLSEGSVGEVQWEAEFFALQDSNNKLAGALREANAAAAQWRQQ 229
                *:       :  :: :     .:.   :** *: :*: .* :*: ** *: * . **::*

rat Vesl 1L     LAAYQEEAERLHKRVTELECVSS-QANAVHSHK-TELSQTVQEREETLKVKEEEIERLKQ 284
rat Vesl 2      FSICRDENDRLRSKIEELEEQCG-EINREKEKN-TQLKRRIEELESEVREKEMELKDLRK 272
human Vesl      LEAQRAEAERLRQRVAELEAQAASEVTPTGEKEGLGQGQSLEQLEALVQTKDQEIQTLKS 289
                :   : * :**:.:: ***  .. : .   .::     : ::: *  :: *: *:: *:.

rat Vesl 1L     EIDNARELQE--QRDSLTQKLQEVEIRNKDLEGQLSELEQRLEKSQSEQDAFRSNLKTLL 342
rat Vesl 2      QSEIIPQLMS--ECEYVSEKLEAAERDNQNLEDKVRSLKTDIEESKYRQRHLKGELKSFL 330
human Vesl      QTGGPREALEAAEREETQQKVQDLETRNAELEHQLRAMERSLEEARAERERARAEVGRAA 349
                :     :  .  : :   :*::  *  * :** ::  ::  :*::: .:   :.::

rat Vesl 1L     EILDGKIFELTELRDNLAKLLECS- 366
rat Vesl 2      EVLDGKIDDLHDFRRGLSKLGTDN- 354
human Vesl      QLLDVSLFELSELREGLARLAEAAP 374
                ::** .: :* ::* .*::*
```

Figure 19. Multiple sequence alignment of Vesl proteins made by CLUSTALW (Higgins, 1994).
The alignment was performed between all members of the Vesl family of proteins, rat and human.
In the consensus line: "*" indicates identical or conserved residues in all sequences in the alignment; ":" conserved substitutions and "." semi-conserved substitutions.

As schematized in Fig. 17 and detailed in Figs. 18 and 19 the sequence similarity between the 'human Vesl' and the Vesl family of rat proteins is highest in the first portion of the molecule. Even if in the C-terminal portion of the proteins the homology is lower, it remains high in two regions: the region homologous to the MCC protein, a putative colorectal tumour suppressor (Kinzler *et al.*, 1991), and the C-terminal leucine zipper domain.

Up to 44% similarity of amino acid sequences was found with other reported sequences, the Mena/VASP (VAsodilator-Stimulated Phosphoprotein) family of mouse proteins (see 3.2.1.1. of the Discussion) as shown in Figs. 20 and 21 where are indicated respectively the region and the relative percentages of homology of the proteins.
In Fig. 22 the relative sequence alignments made by CLUSTALW (Higgins, 1994) were reported.

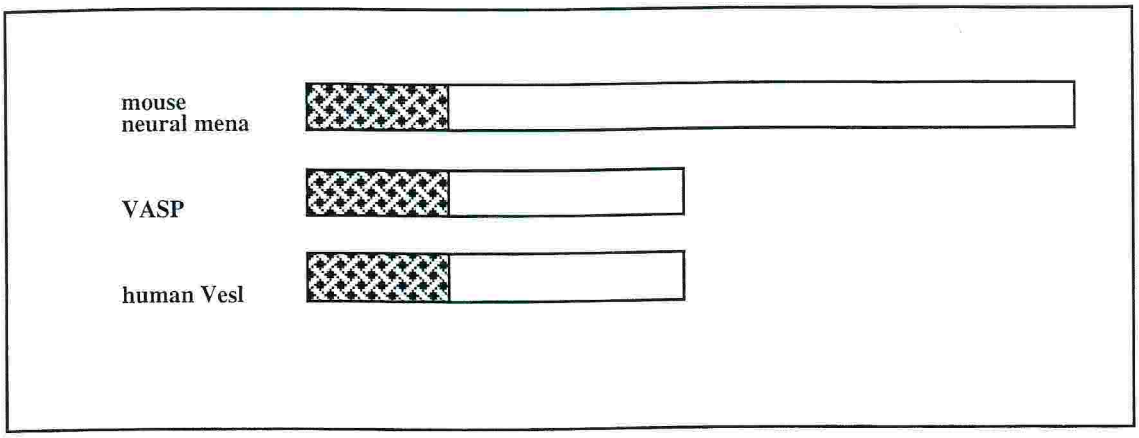


Figure 20. Schematic diagram of the homology between human Vesl and the Mena/VASP family of proteins. The homology between human Vesl and the Mena/VASP family of proteins involves a region of approximately 150 amino acids (shaded region). In the graph is reported only one member (the longest neural variant) of the Mena family of mouse proteins because all members are identical except in the length. VASP proteins are present in mouse, dog and human: only the human protein is reported because the homology between human Vesl and the different VASP proteins is approximately the same.

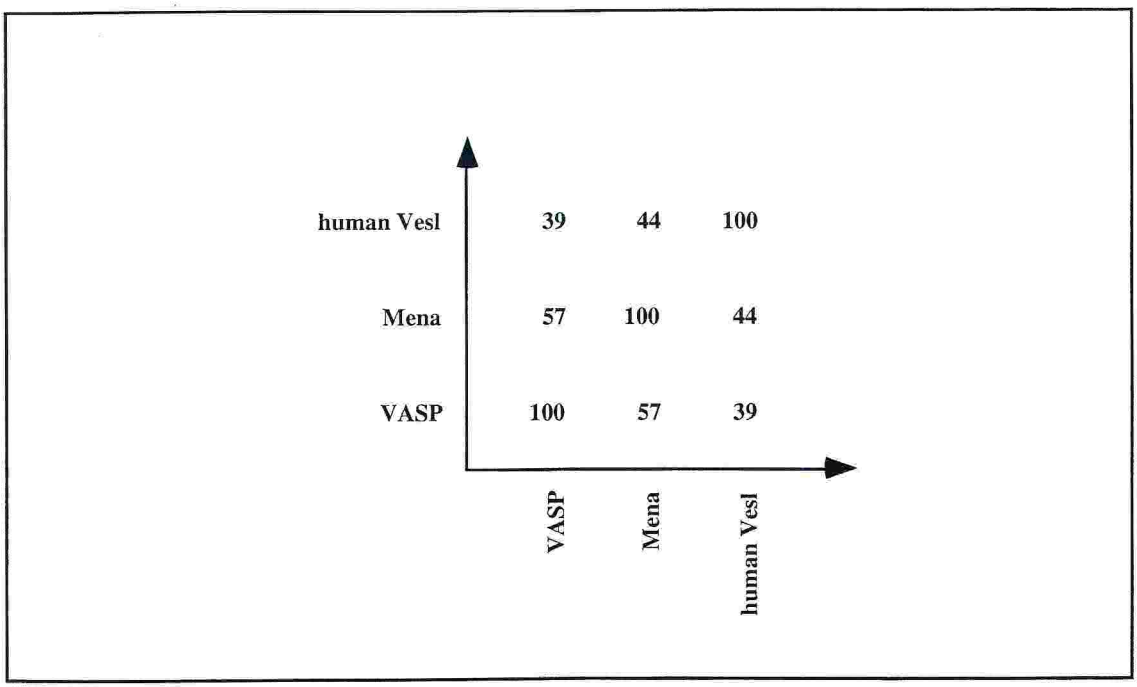


Figure 21. Percentages of homology between human Vesl and the Mena/VASP family of proteins. Protein sequences are aligned by using the BLAST software (Altschul *et al.*, 1997).

```
Mena            ---MSEQSICQARAAVMVYDDANKK-WVPAG-GSTGFSRVHIYHHTGNNTFRVVGRKIQ- 54
VASP            ---MSETVICSSRATVMLYDDGNKR-WLPAGTGPQAFSRVQIYHNPTANSFRVVGRKMQP 56
human Vesl      MSTAREQPIFSTRAHVFQIDPATKRNWIPAG--KHALT-VSYFYDATRNVYRIISIGGA- 56
                     *  * .:** *:  * ..*: *:***    .:: *  ::..  * :*::.

Mena            DHQVVINCAIPKGLKYNQATQTFHQWRDAR--QVYGLNFGSKEDANVFASAMMHALEVLN 112
VASP            DQQVVINCAIVRGVKYNQATPNFHQWRDAR--QVWGLNFGSKEDAAQFAAGMASALEALE 114
human Vesl      --KAIINSTVTPNMTFTKTSQKFGQWADSRANTVYGLGFASEQHLTQFAEKFQEVKEAAR 114
                  :.:**.::  .:.:.::: .* ** *:*   *:**.*.*::.   **  :  . *. .

Mena            SQEAAQSKVTATQDSTNLRCIFCGPTLPRQNSQLPAQVQNGPSQEELEIQRRQLQEQQRQ 172
VASP            G-----GGPPP--P----------PALPTWS------VPNGPSPEEVEQQKR-------- 143
human Vesl      L-----AREKS--------------QDGGE-------LTSPALGLASHQVS-------- 139
                      .   .                .        ..*:    . *

Mena            KELERERMERERLERERLERERLERERLEQEQLERQRQEREHVERLERERLERLERERQE 232
VASP            ----------------------------Q----------------------QPGPSE 150
human Vesl      ------------------------------------------------------TPYSPMP 146

Mena            RERERLEQLEREQVEWERERRMSNAAPSSDSSLSSAPLPEYSSCQPPSAPPPSYAKVISA 292
VASP            --------------HIE--RRVSNAG---------------------------------- 160
human Vesl      --------------AWAP------------------------------------------ 150

Mena            PVSDATPDYAVVTALPPTSTPPTPPLRHAATRFATSLGSAFHPVLPHYATVPRPLNKNSR 352
VASP            ------------------------------------------------------------
human Vesl      ------------------------------------------------------------

Mena            PSSPVNTPSSQPPAAKSCAWPTSNFSPLPPSPPIMISSPPGKATGPRPVLPVCVSSPVPQ 412
VASP            ------------------------------------------------------------
human Vesl      ------------------------------------------------------------

Mena            MPPSPTAPNGSLDSVTYPVSPPPTSGPAAPPPPPPPPPPPPPPLPPPPLPPLASLSHCG 472
VASP            -------------------------GPPAPPAGGPPPPPGP----------PP-------- 178
human Vesl      -------------------------VPPSPLVSANGPGEEK-------LFRS-------- 170
                                          *.:*      *              .

Mena            SQASPPPGTPLASTPSSKPSVLPSPSAGAPASAETPLNPELGDSSASEPGLQAASQPAES 532
VASP            ------------------------------------------------------------
human Vesl      ------------------------------------------------------------

Mena            PTPQGLVLGPPAPPPPPPLPSGPAYASALPPPPGPPPPPPLPSTGPPPPPPPPPPLPNQA 592
VASP            ---------PPGPPPPPGLPPSGVPAAAHGAGGGPPPAPPLPAAQ----GP--------- 216
human Vesl      ---------QSADAPGPTERERLKKMLSEGSVGEVQWEAEFFALQ--------------- 206
                         .. .* *         :  .       . : :

Mena            PPPPPPPPAPPLPASGIFSGSTSEDNRPLTGLAAAIAGAKLRKVSRVEDGSFPGGGNTGS 652
VASP            -----------GGGG--A-GAP--------GLAAAIAGAKLRKVSKQEE---ASGGPT-- 249
human Vesl      ----------DSNNK--LAGALR------EANAAAAQWRQQLEAQRAEAERLRQRVAELE 248
                           *:         . ***    :  :..: *

Mena            VSLASSKADAGRGNGPLPLGGSGLMEEMSALLARRRRIAEKGSTIETEQKEDRNEDAEPI 712
VASP            ----APKAESGRS------GGGGLMEEMNAMLARRRKATQVG----EKTPKDESANQEEP 295
```

```
human Vesl      AQAASEVTPTGEK------EGLGQGQSLEQLEALVQTKDQEI-----QTLKSQTGGPREA 297
                    :  : :*.        * *  :.:. : *  :   :       :  :... . .
Mena            TAKAPSTSTPEPTRKPWERTNTMNGSKSPVISRPKSTPSSQPSANGVQTEGLDYDRLKQD 772
VASP            EARVPAQS--ESVRRPWEKNSTTLPRMK--SSSSVTTSETQPCTP-SSSDYSDLQRVKQE 350
human Vesl      LEAAEREETQQKVQDLETRNAELEHQLR---AMERSLEEARAERE--R-ARAEVGRAAQ- 350
                   .   .  : .:    :.          :   :  .::.         :  *  *


Mena            ILDEMRKELAKLKEELIDAIRQELSKSNTA 802
VASP            LLEEVKKELQKVKEEIIEAFVQELRKRGSP 380
human Vesl      LLDVSLFELSELREGLARLAEAAP------ 374
                :*:    ** :::* :
```

Figure 22. Multiple sequence alignment of human Vesl and the Mena/VASP family of proteins made by CLUSTALW (Higgins, 1994).
The alignment was performed between human Vesl, the longest neural variant of the mouse Mena family and the human VASP.
In the consensus line: "*" indicates identical or conserved residues in all sequences in the alignment; ":" conserved substitutions and "." semi-conserved substitutions.

Analysis of the protein sequence of AD1 for possible post-translational modifications was made by scanning the whole protein against PROSITE (Bucher and Bairoch, 1994; Bairoch *et al.*, 1997). This revealed two putative N-glycosylation sites (aa 61-64, aa 67-70); six putative protein kinase C phosphorylation sites (aa 3-5, aa 11-13, aa 23-25, aa 74-76, aa 179-181, aa 286-288); five putative casein kinase II phosphorylation sites (aa 3-6, aa 179-182, aa 192-195, aa 330-333, aa 355-358); and five putative N-myristoylation sites (aa 54-59, aa 90-95, aa 123-128, aa 263-268, aa 265-270). All these sites are indicated in Fig. 16.

Analysis of the amino acid sequence of AD1 made by Expasy (Appel *et al.*, 1994) predicted that this protein is soluble: in fact, it contains no N-terminal signal peptides, no transmembrane domains and results of the k-NN Prediction (Horton and Nakai, 1997) are for a nuclear localization. The instability index calculated according to Guruprasad et al. (Guruprasad *et al.*, 1990) classified the protein as unstable, result which is in agreement with a potential PEST sequence found (aa 169-181). Proteins with intracellular half-lives of less than two hours are found to contain regions rich in proline, glutamic acid, serine and threonine (P, E, S and T). These so called PEST regions are generally flanked by clusters of positively charged amino acids and have been postulated to be involved in protein degradation (Rogers *et al.*, 1986; Rechsteiner and Rogers, 1996).

Secondary structure analysis of the deduced amino acid sequence predicted that AD1 protein had primarily an α-helical structure with some β-sheet regions only at the N-terminus (Fig. 23). These and other calculated features of AD1 are shown in Fig. 23.

In conclusion, AD1 is likely to be a short lived nuclear (on the basis of the predictions reported above) or cytoplasmic (on the basis of the homology with the Vesl family of rat proteins) protein, with the potential to be N-glycosylated and phosphorylated, although the former is unlikely in a cytoplasmic protein.

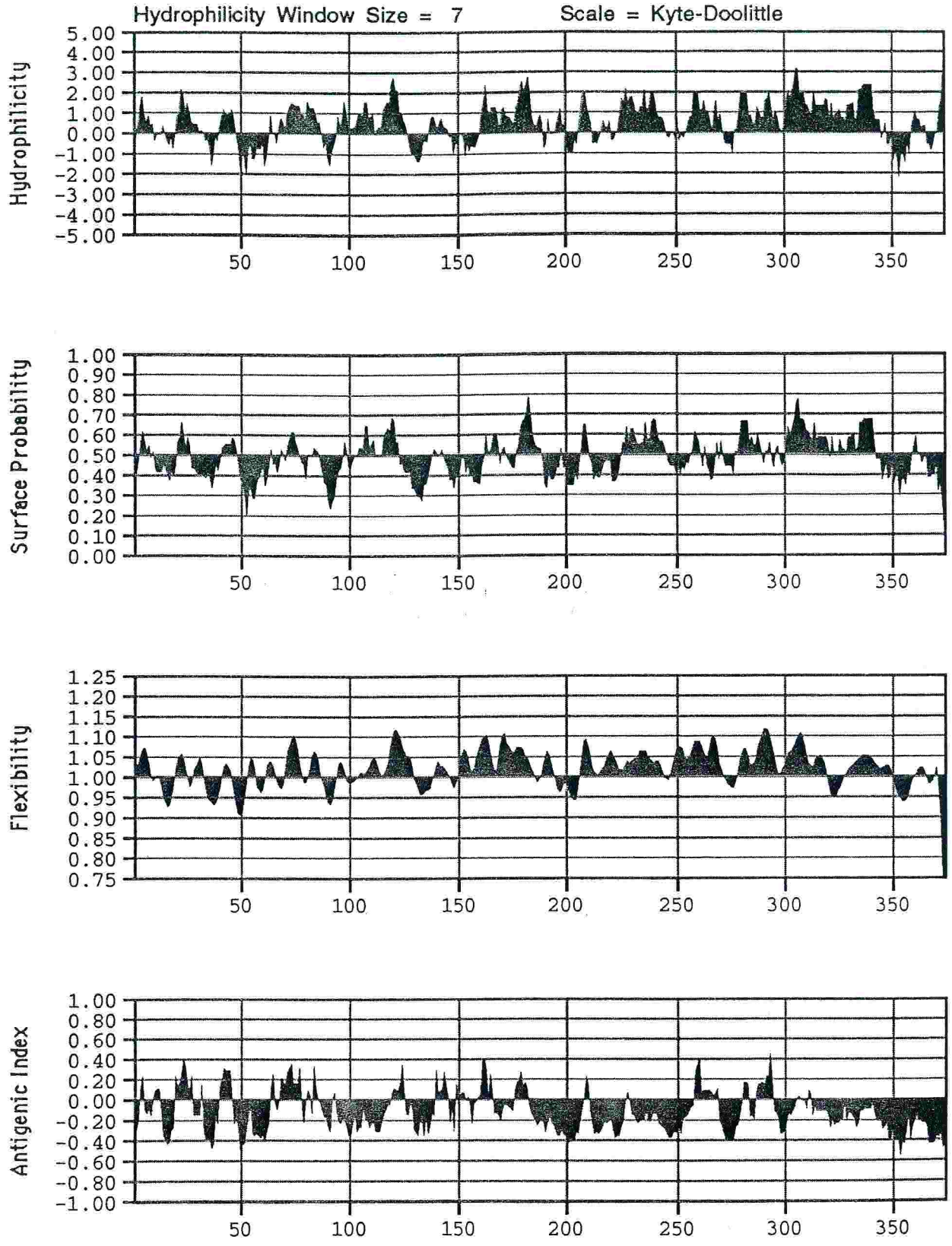


Figure 23. Results of the protein analysis made on human Vesl by MacVector software.

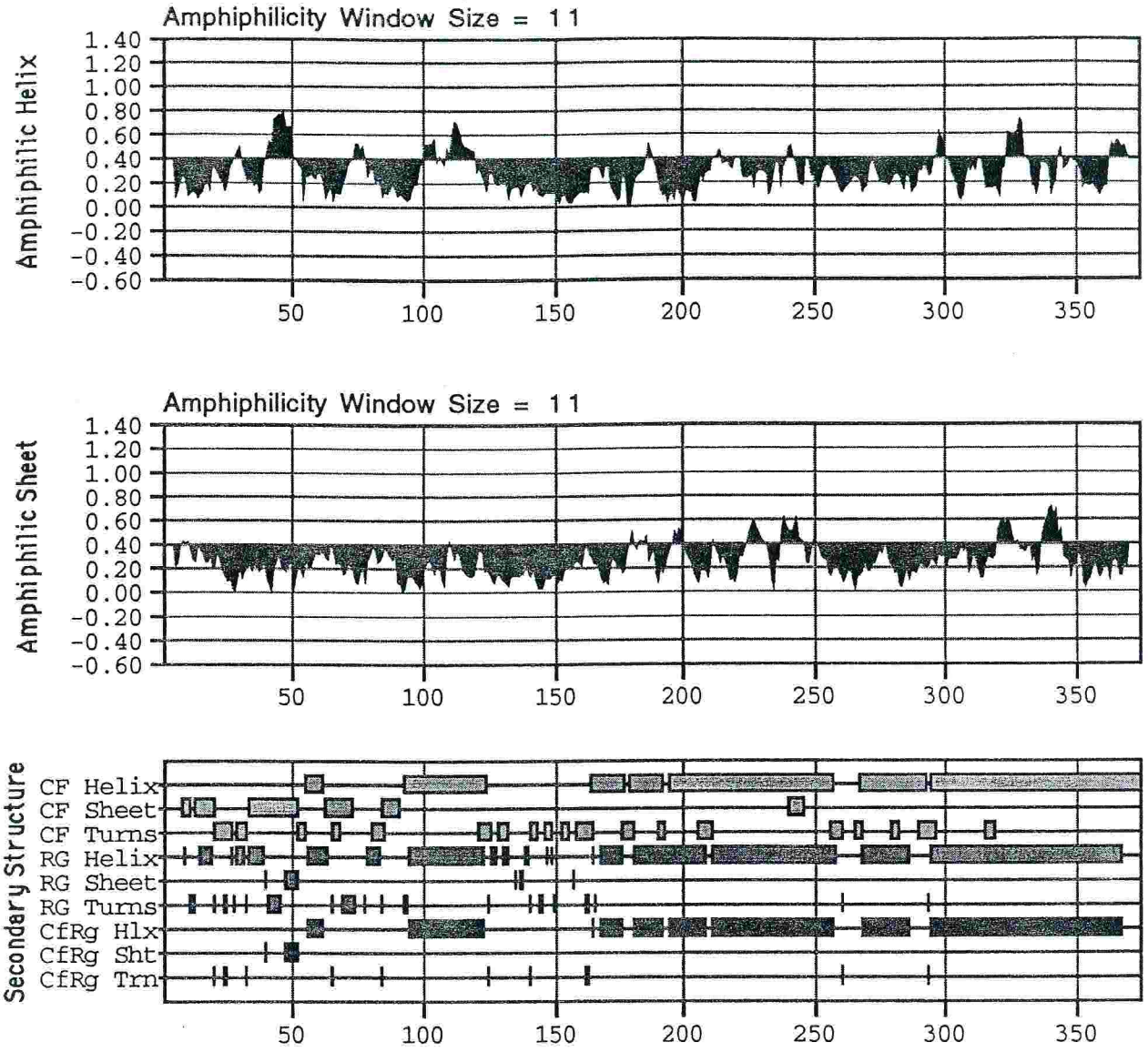


Figure 23. Continued.

2.4.2. Sequencing Results For The Clone AD2

AD2 cDNA was approximately 1300 bp long and contained a putative ORF of 252 amino acids corresponding to a protein with theoretical molecular mass of 27,962.98 kDa and isoelectric point (pI) of 9.08. The nucleotide sequence of AD2 cDNA and the deduced amino acid sequence are shown in Fig. 24.

BLAST search for DNA sequence revealed that AD2 cDNA had an homology of 86% with a rat sequence already published (Gen Bank accession number U06713) involving 149 of the 756 bp long ORF. The rat homologue sequence is the 2825 bp long mRNA coding for SM-20 (1067 bp long ORF), a growth factor-responsive gene in vascular smooth muscle cells (VSMCs) (described in 3.2.1.2. of the Discussion).

BLAST search for protein similarity confirmed the homology between AD2 protein and the rat growth factor-responsive protein which is of 78% and involves 170 out of 252 aminoacids. A 59% similarity involving 131 out of 252 aminoacids was found with another reported sequence, a *C. elegans* protein predicted from a genomic sequence already published (Gen Bank accession numbers Z71180 and AL021475). This putative protein shows a weak similarity with a rat protein, RP-8 (Gen Bank accession number 507906), involved in apoptosis. However, AD2 protein shows no significant similarity neither with the rat protein, RP-8, nor with the human homolog of RP-8, PDCD2 (Gen Bank accession number S78085).

Protein homologies described above are detailed in Figs. 25 and 26 and the relative sequence alignments are shown in Fig. 27.

AD2 ORF

```
CAT AGC AGT GGC GAG GCA AGT TCT AGG CTG AGG GAG GAA GCC CAG CCC TCT GCA CCT GAG      60
 H   S   S   G   E   A   S   S   R   L   R   E   E   A   Q   P   S   A   P   E      20

CGC CTG GCC TTG GAC TAT ATT GTG CCT TGC ATG CGG TAC TAT GGT ATC TGC GTC AAG GAC     120
 R   L   A   L   D   Y   I   V   P   C   M   R   Y   Y   G   I   C   V   K   D      40

AAC TTC TTG GGG GCA GTA CTG GGT GGC CGT GTG CTG GCT GAG GTG GAA GCC CTG AAG TGG     180
 N   F   L   G   A   V   L   G   G   R   V   L   A   E   V   E   A   L   K   W      60

GGC GGG CGT CTA CGT GAT GGG CAA CTA GTG AGC CAG CGG GCG ATC CCA CCG CGC ACG ATT     240
 G   G   R   L   R   D   G   Q   L   V   S   Q   R   A   I   P   P   R   T   I      80

CGT GGG GAC CAG ATT GCC TGG GTA GAA GGC CAC GAG CCA GGC TGC CGG AGC ATT GGT GCC     300
 R   G   D   Q   I   A   W   V   E   G   H   E   P   G   C   R   S   I   G   A     100

CTC ATG GCT CAT GTG GAC GCA GTA ATC CGC CAC TGT GCA GGG CGG CTG GGC AAC TAC GTC     360
 L   M   A   H   V   D   A   V   I   R   H   C   A   G   R   L   G   N   Y   V     120

CCA GGC ATC AAT GGG CGC ACC AAG GCC ATG GTG GCG TGT TAC AAT GGG CTC GGG TAC GTG     420
 I   N   G   R   T   K   A   M   V   A   C   Y   P   G   N   G   L   G   Y   V     140

AGG CAT GTT GAC AAT CCC CAC GGC GAT GGG CGC TGC ATC ACC TGT ATC TAT TAC CTG AAT     480
 R   H   V   D   N   P   H   G   D   G   R   C   I   T   C   I   Y   Y   L   N     160

CAG AAC TGG GAT GTT AAG GTG CAT GGC GGC CTC CTG CAG ATC TTC CCT GAG GGT CGG CCA     540
 Q   N   W   D   V   K   V   H   G   G   L   L   Q   I   F   P   E   G   R   P     180

GTG GTA GCC AAC ATC GAG CCA CTC TTT GAC CGG TTG CTC ATT TTC TGG TCT GAC CGA CGG     600
 V   V   A   N   I   E   P   L   F   D   R   L   L   I   F   W   S   D   R   R     200

AAT CCA CAT GAG GTG AAG CCA GCC TAT GCC ACC AGG TAC GCC ATC ACT GTC TGG TAT TTT     660
 N   P   H   E   V   K   P   A   Y   A   T   R   Y   A   I   T   V   W   Y   F     220

GAT GCC AAG GAG CGG GCA GCA GCC AGA GAC AAG TAT CAG CTA GCA TCG GGA CAG AAA GGT     720
 D   A   K   E   R   A   A   A   R   D   K   Y   Q   L   A   S   G   Q   K   G     240

GTT CAA GTA CCC GTG TCA CAG CCA GCT ACA CCT ACC TAA TGGCCAGCCCCAGAGCTGCATGGGCCA     786
 V   Q   V   P   V   S   Q   P   A   T   P   T   *                               252

GCAGCTCCTNTCCTTGAGGGGAGCCTCAGTGCCCGCCCTTCCGTGACACTGCTGCTGCTTCTGGCTTGCCTGGATGTGT     865
GGTGTGGAGGGCACTGAGTGTCACTNAGGGCAACAAGNAGANGCCTCTTCTTGCCCCGTGAGCAGTGGCCAATAT        940
```

Figure 24. Nucleotide and amino acid sequence of AD2 cDNA.
The predicted ORF was translated into the amino acid sequence below. The stop codon is marked by an asterisk. In the amino acid sequence are indicated potential protein kinase C (**X**) and casein kinase II (X) phosphorylation sites, N-myristoylation sites (***X***) and a cell attachment sequence (**X**).

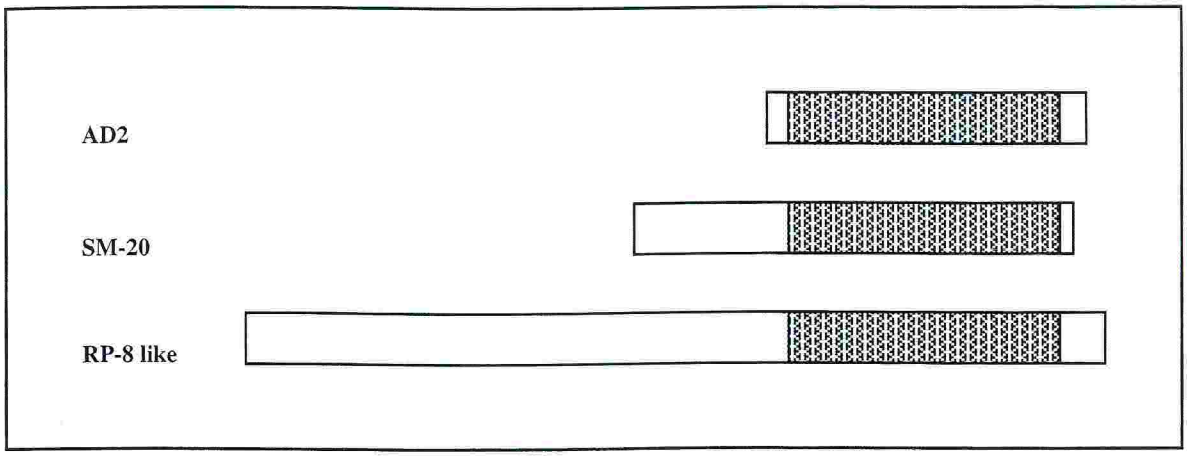


Figure 25. Schematic diagram of the homologies between AD2, rat SM-20 and *C.elegans* RP-8 like proteins.
The three proteins are 252, 352 and 792 amino acids long respectively and the homology involves a region of approximately 200 amino acids (shaded area).

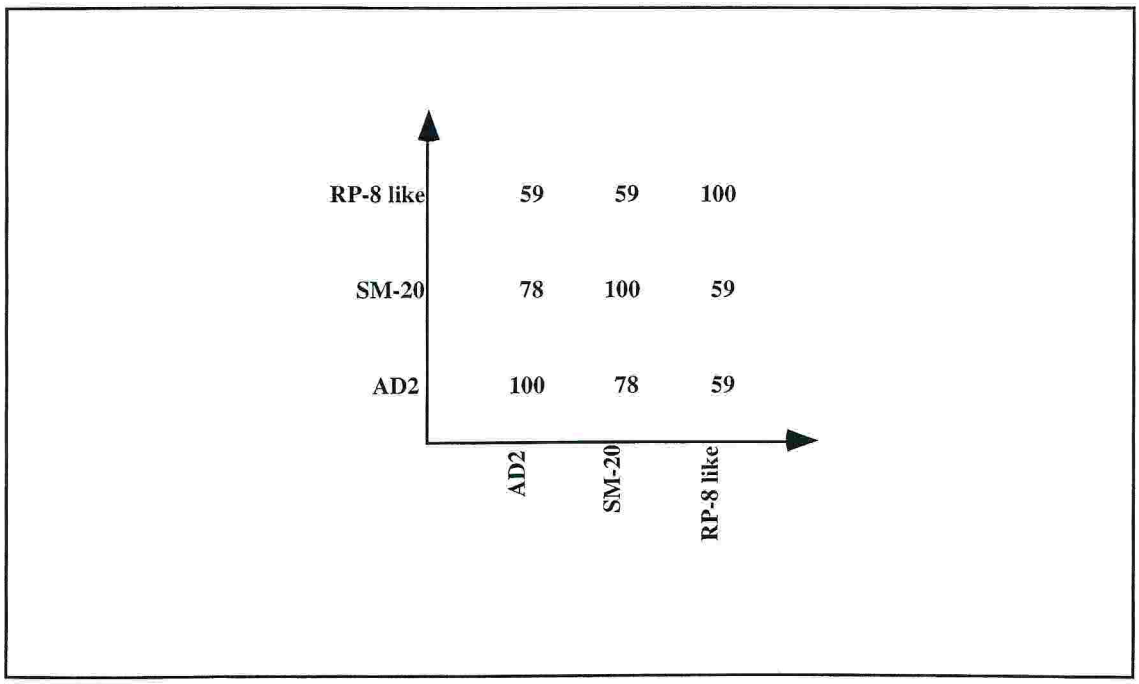


Figure 26. Percentages of homology between AD2, rat SM-20 and *C.elegans* RP-8 like proteins.
The three proteins are aligned by using the BLAST software (Altschul *et al.*, 1997).

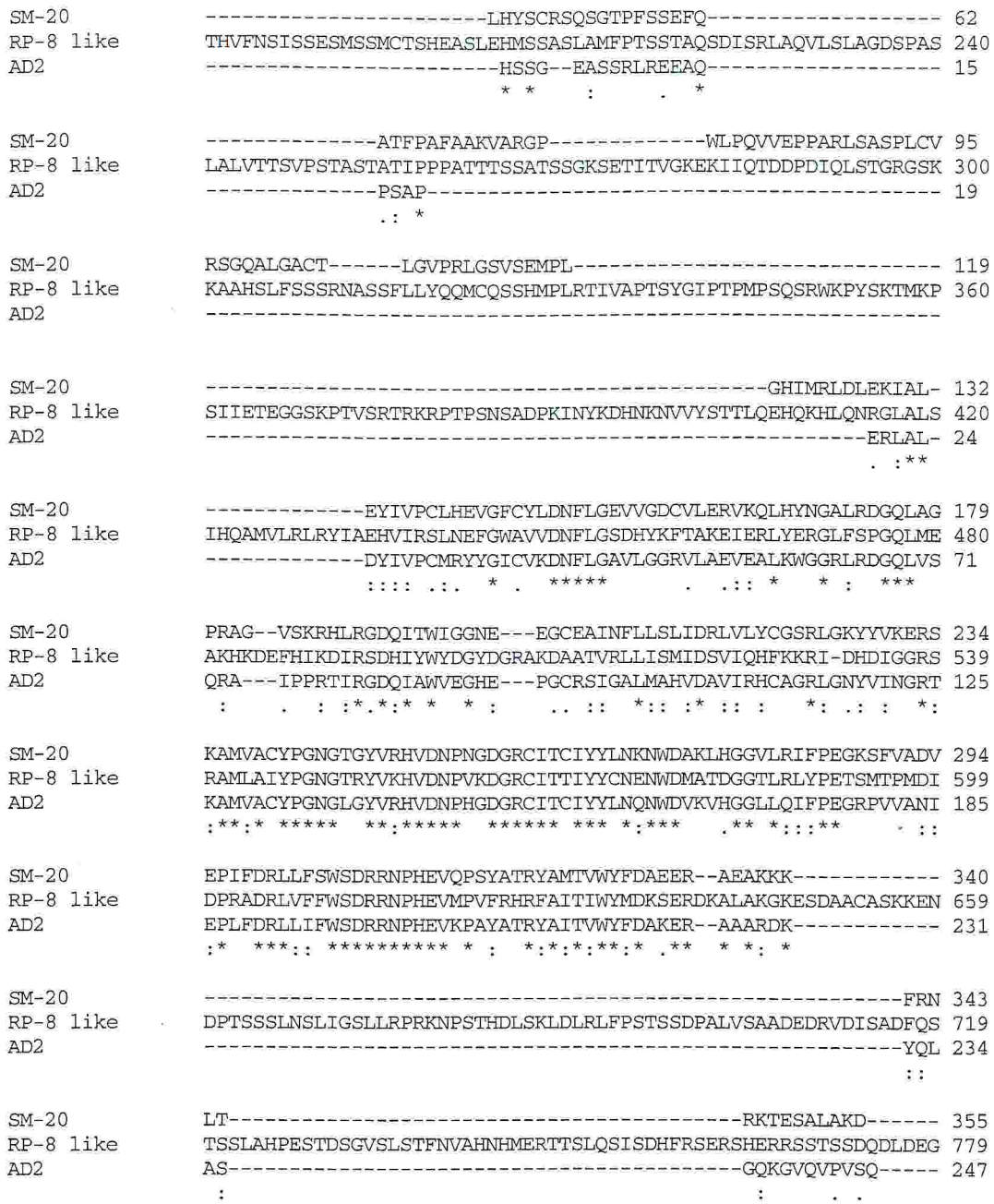


Figure 27. Multiple sequence alignments of AD2, SM-20 and RP-8 like made by CLUSTALW (Higgins, 1994). The multiple sequence alignment was performed between the protein coded by AD2 cDNA, the rat protein SM-20 and the *C.elegans* RP-8 like protein. In the consensus line: "*" indicates identical or conserved residues in all sequences in the alignment; ":" conserved substitutions and "." semi-conserved substitutions.

Analysis of the protein sequence of AD2 for possible post-translational modifications was made by scanning protein against PROSITE (Bucher and Bairoch, 1994; Bairoch *et al.*, 1997). This revealed four putative protein kinase C phosphorylation sites (aa 7-9, aa 71-73, aa 79-81, aa 197-199); two putative casein kinase II phosphorylation sites (aa 2-5, aa 17-20); five putative N-myristoylation sites (aa 44-49, aa 67-72, aa 99-104, aa 134-139) and a cell attachment sequence (aa 81-83). All these sites are indicated in Fig. 24.

Analysis of the amino acid sequence of AD2 made by Expasy (Appel *et al.*, 1994) predicted that this protein is soluble: in fact, it contained no N-terminal signal peptides, no transmembrane domains and results of the k-NN Prediction (Horton and Nakai, 1997) were for a cytoplasmic localization. The instability index calculated according to Guruprasad et al. (Guruprasad *et al.*, 1990) classified the protein as stable, a calculation confirmed by the finding of no potential PEST sequences.

Secondary structure analysis of the deduced amino acid sequence predicted that AD2 protein had primarily an α-helical structure at its N-terminus and multiple β-sheet regions at its C-terminus. These and other calculated features of AD2 are shown in Fig. 28.

It must be noted, however, that it was not known if AD2 clone was a full length clone, so all these predictions were valid at least for the polypeptide coded by AD2 cDNA and subjected to computer analysis.

In conclusion, AD2 is likely to be a long lived cytoplasmic protein, with the potential to be N-glycosylated and phosphorylated, although as mentioned above for AD1 (2.4.1.), it is unlikely that a cytoplasmic protein is glycosylated. The cell attachment sequence found, Arg-Gly-Asp, would predict for AD2 protein a role in cell adhesion since it was found in a number of proteins playing a role in cell adhesion.

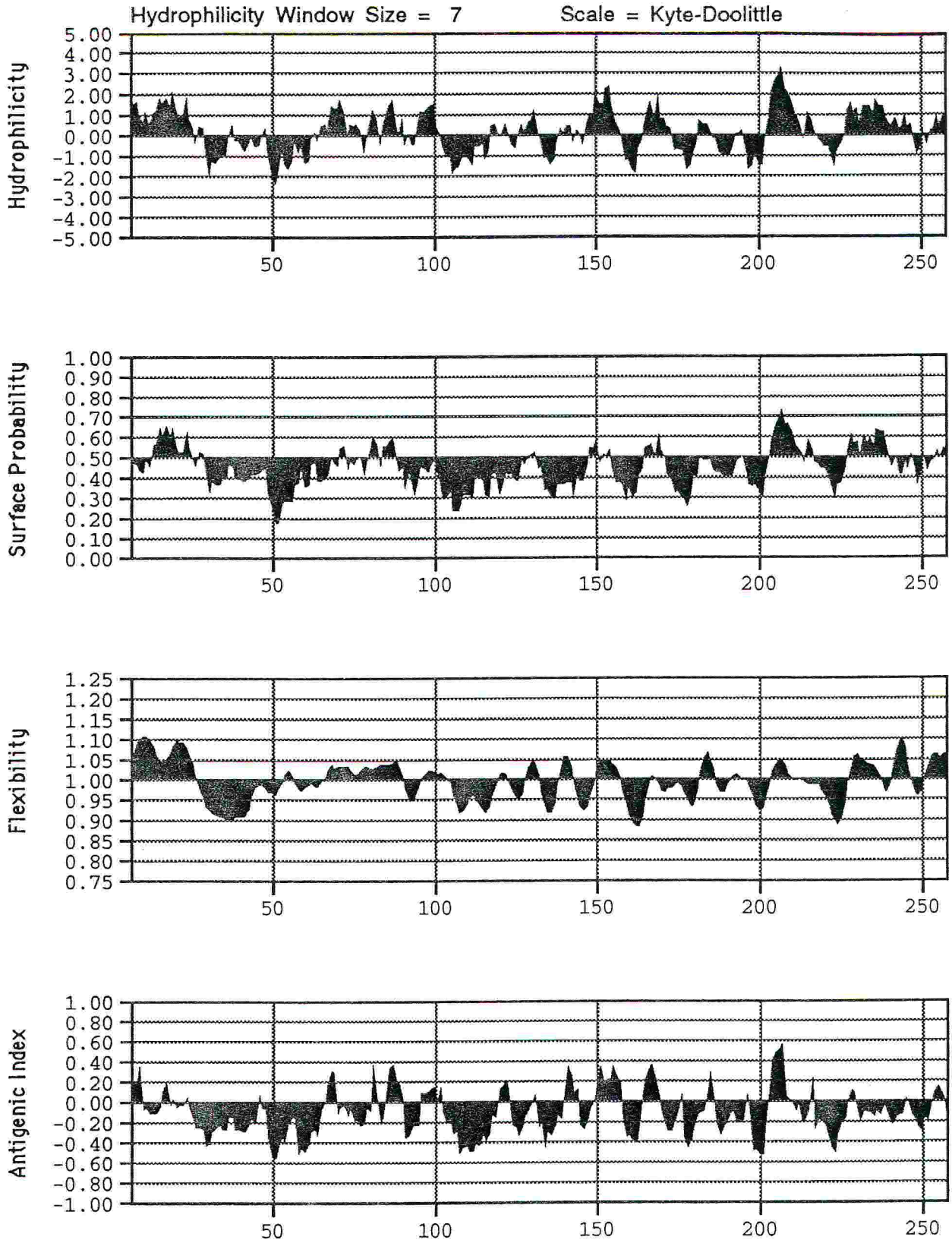


Figure 28. Results of AD2 protein analysis by Mac Vector software.

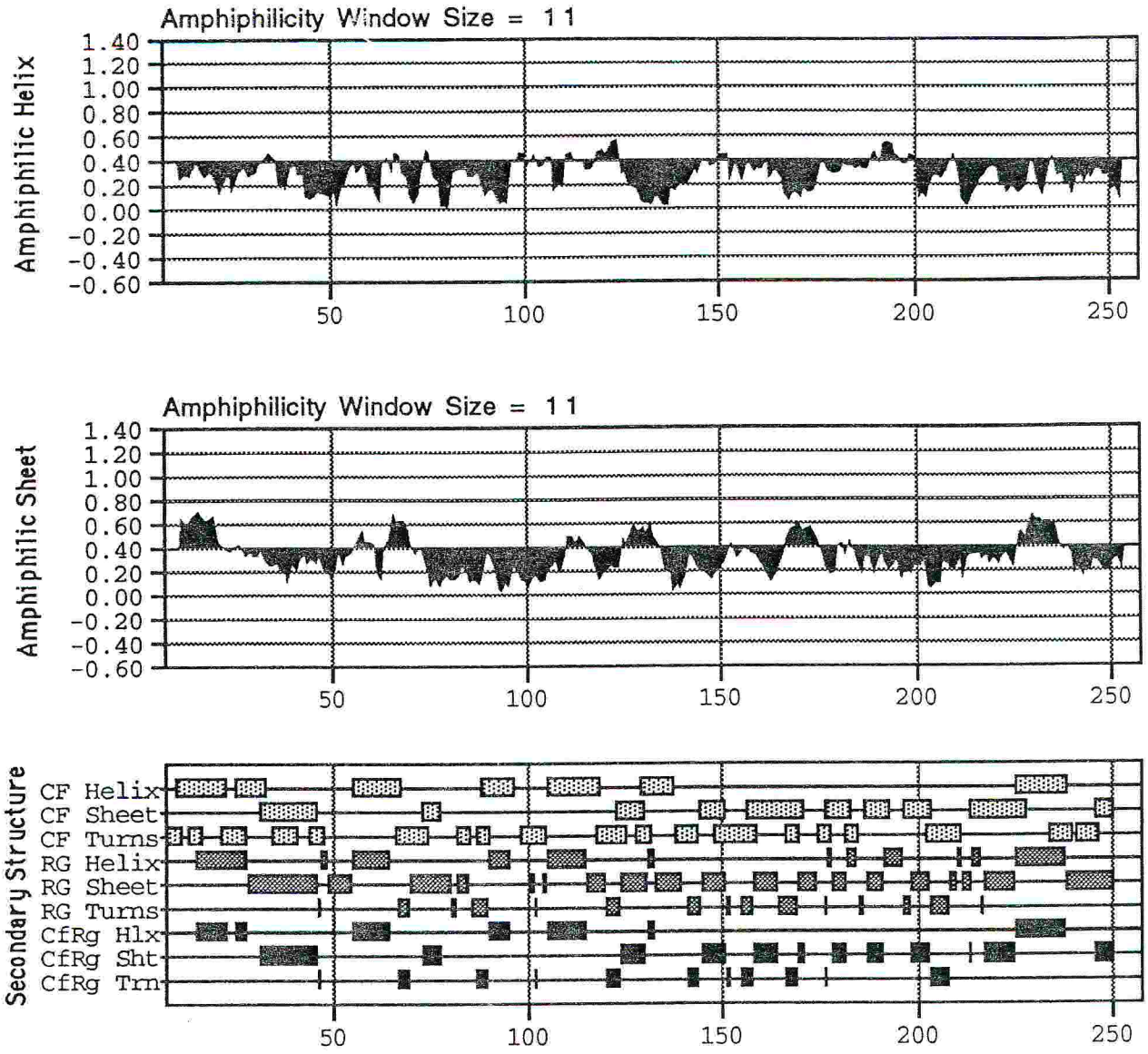


Figure 28. Continued.

2.5. EXPRESSION PATTERNS OF THE NEW AD-SPECIFIC ANTIGENS

The immunological screening of AD hippocampal cDNA library resulted in the isolation of two putative AD-specific antigens (2.3.2.). Identification and computer analysis of the cDNAs isolated (2.4.1. and 2.4.2.) suggested that the two putative AD-specific antigens were not yet identified proteins, at least in humans.

For their further characterization the study of the expression pattern in different tissues was performed by using two different experimental approaches: Northern blot (2.5.1.) and Western blot (2.5.2.) analysis on human tissues.

2.5.1. Northern Blot Analysis

To determine the size of the two transcripts and to define their expression pattern a Northern blot analysis was performed by using a variety of human normal tissues. Attempts were made to compare the expression of the two genes in normal and AD brain but so far they were unsuccessful due to the low quality of mRNA which could be obtained from AD brains (as discussed in 3.1.1.1.).

Before performing conventional Northern blot analysis, a preliminary "electronic" Northern blot was run to get insights into the expression patterns of the two new proteins; the so called "electronic" Northern blot consisted simply in a BLAST search for DNA sequence against the human ESTs database.

In Figs. 29 and 30 the sequence alignments between the full length AD1 (AC002985) and AD2 cDNAs and human ESTs are graphically schematized; only matches having an homology higher than 90% are reported. As shown in Fig. 29, the results of the "electronic" Northern blot predicted that AD1 transcript(s) were expressed in human brain as well as in heart and kidney. Conventional Northern blot analysis in part confirmed the results of "electronic" Northern blot as reported in Fig. 31a: the expression of an approximately 7-kb long AD1 transcript was detected in human kidney, skeletal muscle, heart and brain. AD1 was strongly expressed in heart and skeletal muscle, only weakly in brain and kidney, detectable after longer exposures (data not shown); no expression was detected in peripheral blood leukocytes, lung,

placenta, small intestine, liver, spleen, thymus and colon. From the genomic sequence in GenBank it is not possible to say if the size corresponds to the predicted ORF because the length of the 5' and 3' untranslated regions is not known. The rat homologue transcript, at least for the shortest Vesl isoform, is 6.5 kb long.

For the AD2 transcript(s), on the basis of the results of of the "electronic" Northern blot, the expression was predicted in a much more higher number of human tissues (see Fig. 30); conventional Northern blot results confirmed the expectation (Fig. 31b). An approximately 2-kb long AD2 transcript was detected in all tissues analyzed even if the expression levels varied greatly as shown in Fig. 31b where it can be seen that the highest expression was found in brain, heart, kidney, liver and placenta, followed by skeletal muscle, spleen, small intestine and lung. Finally, AD2 is weakly expressed in colon, thymus and peripheral blood leukocytes.

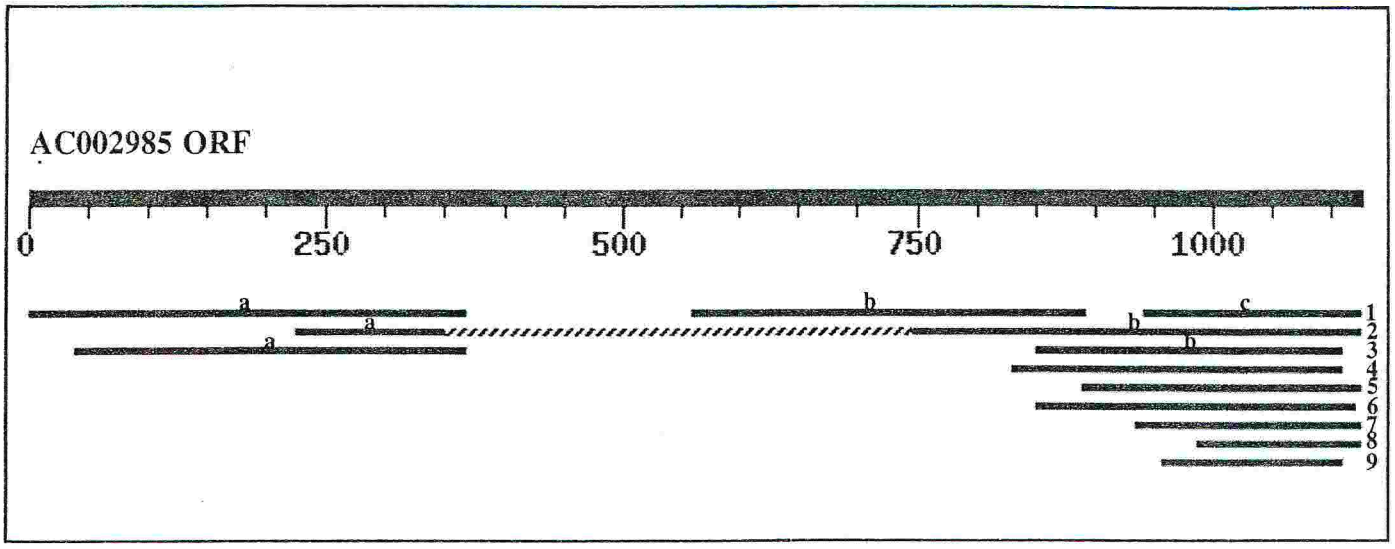


Figure 29. Results of "electronic" Northern blot analysis for the full length AD1 cDNA (AC002985).
AD1 cDNA was submitted to BLAST search against human ESTs database. In the graph are reported the alignments with ESTs having an homology higher than 90% to AD1 cDNA. Each lane is numbered (on the right hand of the panel) and each segment (corresponding to an EST) in a lane is a distinguished by a letter (when more than one segment for lane is present).
1a. brain (99%); 1b. and 1c. senescent fibroblasts (95%); 2a. and 2b. heart (97%); 3a. brain (99%); 3b. pregnant uterus (97%); 4. senescent fibroblasts (95%); 5. kidney (97%); 6. pregnant uterus (91%); 7. parathyroid gland (96%); 8.pharynx (94%); 9. multiple sclerosis lesions

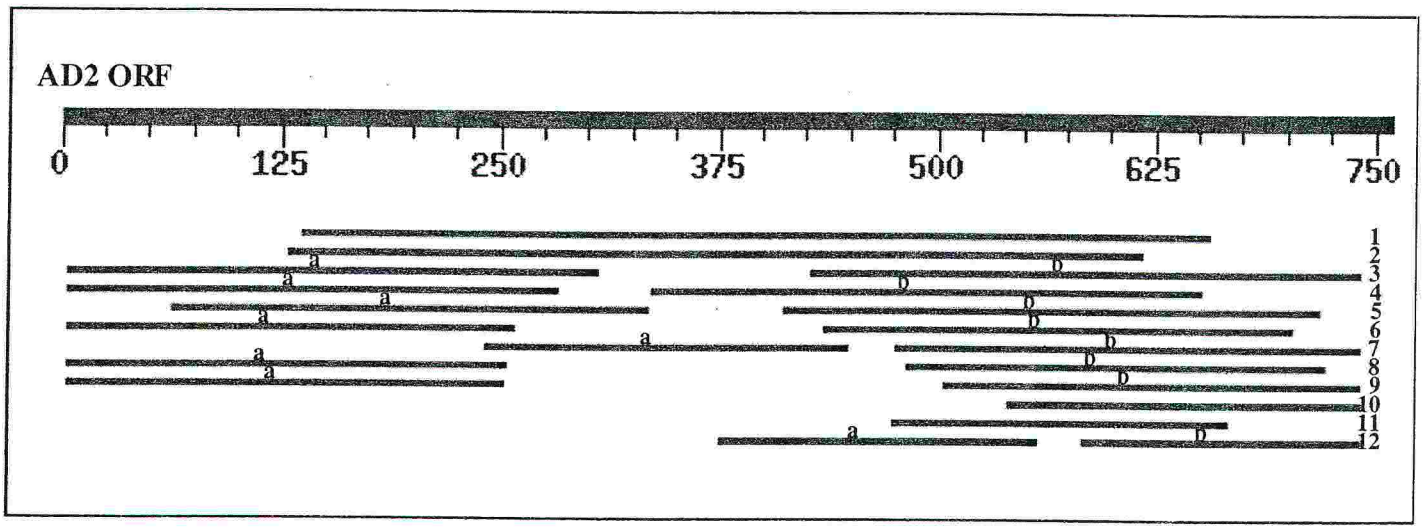


Figure 30. Results of "electronic" Northern blot analysis for AD2 cDNA.
AD2 cDNA was submitted to BLAST search against human ESTs database. In the graph are reported the alignments with ESTs having an homology higher than 90% to AD2 cDNA. Each lane is numbered (on the right hand of the panel) and each segment (corresponding to an EST) in a lane is distinguished by a letter (when more than one segment for lane is present).
1, 2, 4a, 6a, 6b, 12b. human tonsillar cells enriched for germinal center B cells; 3a. B cells; 3b and 5b. T lymphocytes; 4b. ovary; 5a. colon; 7a and 7b, 10. placenta; 8a. heart; 8b. kidney; 9a. senescent fibroblasts; 9b. multiple sclerosis lesions; 11. pancreas; 12a. synovial membrane.

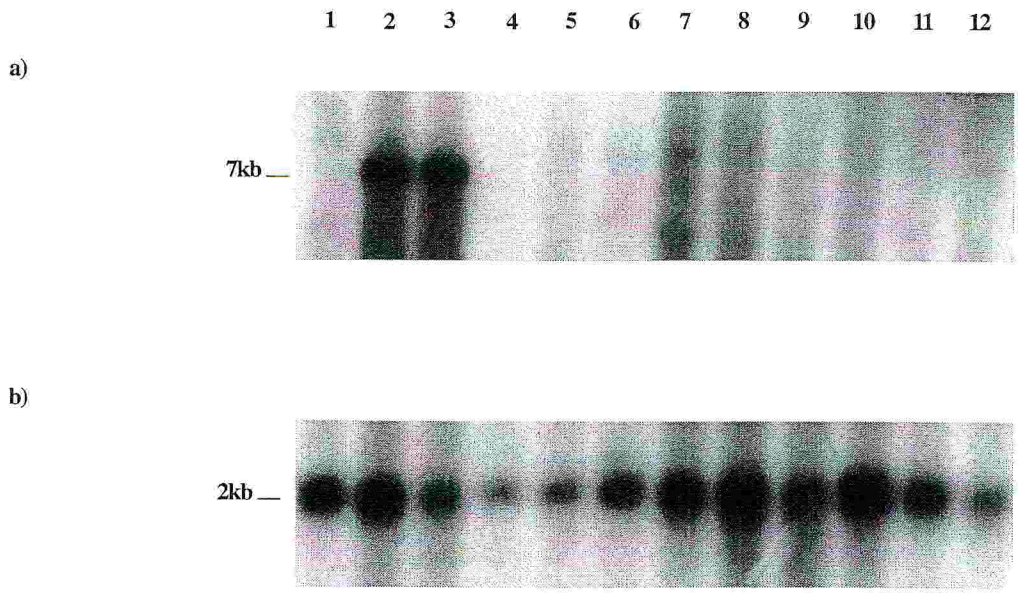


Figure 31. Northern blot analysis of AD1 and AD2 expression.
2 µg of polyA$^+$RNA extracted from different human tissues and blotted onto a nylon membrane (Clontech) were probed with radiolabeled cDNAs. The two probes used were ~600 bp long fragments obtained from AD1 and AD2 cDNA inserts by restriction digestion with *EcoRI* and *PstI*. The filter was probed with the AD1 fragment, exposed to Kodak X-AR film O/N, stripped and riprobed with the AD2 fragment.
The results of hybridization with the AD1 fragment are shown in a), with the AD2 fragment in b). AD1 fragment hybridized with a 7 kb transcript; AD2 with a 2 kb transcript.
Tissues examined were: brain (lane 1), heart (lane 2), skeletal muscle (lane 3), colon (lane 4), thymus (lane 5), spleen (lane6), kidney (lane 7), liver (lane 8), small intestine (lane 9), placenta (lane 10), lung (lane 11) and peripheral blood leukocytes (lane 12).

2.5.2. Western Blot Analysis

By Northern blot analysis the size of the trascripts was determined together with their expression patterns; the aim of the Western blot analysis is the determination of the size of the two proteins isolated as well as the study of their expression patterns in a panel of human tissues in comparison with those found by Northern blot.

Western blot analysis is still underway. Before extending the analysis to a panel of human tissues, preliminary experiments were performed on brain homogenates to state if it was possible to detect the proteins in tissue homogenates using either AD sera which detected the proteins in immunoscreening or rabbit antisera obtained by immunization with the recombinant proteins expressed and purified as His-tagged fusion proteins (2.6.1.1.).

In Fig. 32 the results of these preliminary experiments are shown. As can be seen from the figure, no specific band was detected following incubation with human sera which recognized the antigens in immunoscreening, even after an overnight incubation, neither on AD (lane 2 in panels a and b) nor NDC brain extracts (lane 4 in panels a and b). Of the two polyclonal antisera, rabbit anti-AD1 was not able to detect any band neither on AD (lane 6 in panel a) nor NDC brain (lane 8 in panel a) - this result was not surprising on the basis of the low level of AD1 expression observed in the brain by Northern blot analysis (as shown in Fig. 31a); rabbit anti-AD2 recognized a band both on AD as well as on NDC brain (lanes 6 and 8 in panel b) at the approximately the same heigth of the recombinant protein (lane 1 in panel b); this band was not detected by rabbit preimmune serum neither on AD nor on NDC brain (lanes 7 and 9 in panel b).

From these Western blots it cannot be concluded neither that the expression of the two antigens isolated is AD specific nor was there an appreciable difference in their expression level between normal and AD brain. As already stressed, these experiments were only the preliminary attempts, they will be repeated and confirmed by using both antisera after affinity purification and extended to a larger panel of tissues to gain further insight on the expression of the two proteins.

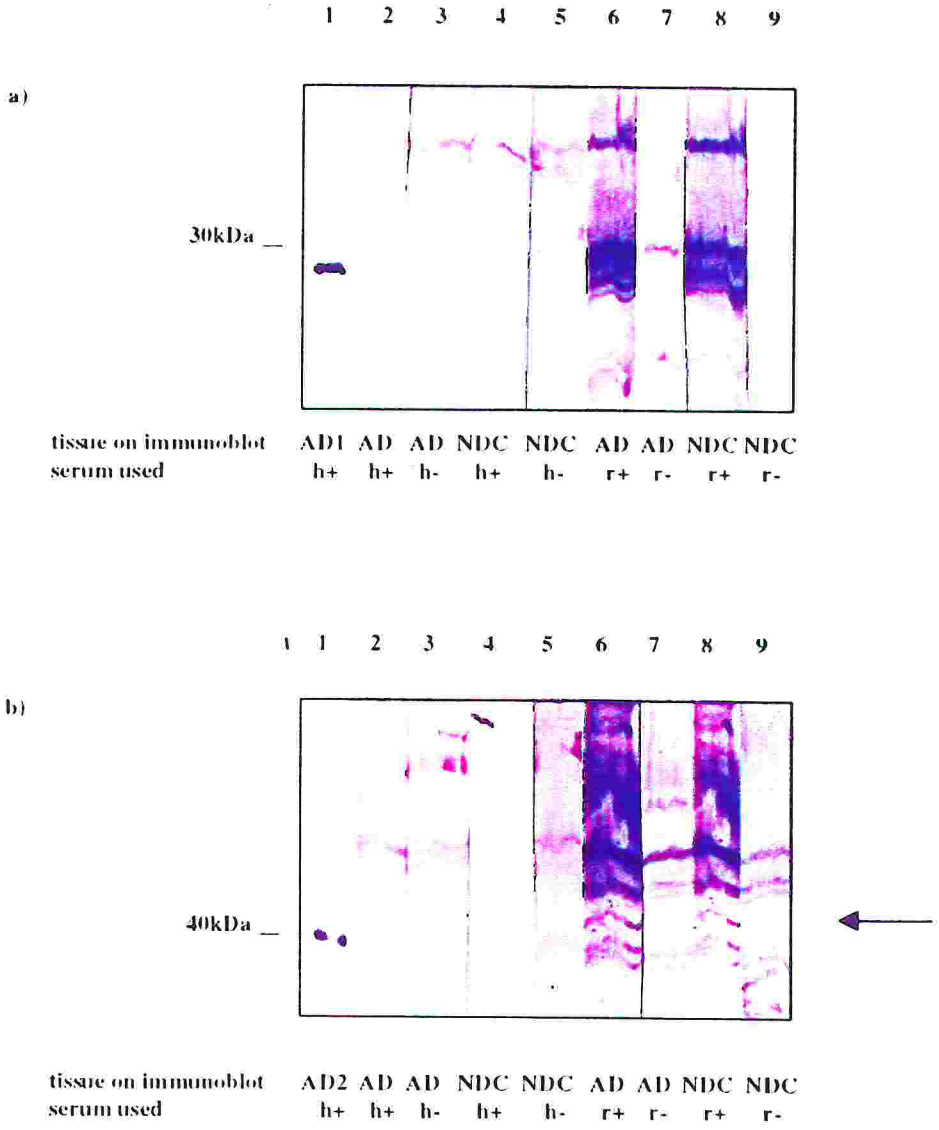


Figure 32. Western blot analysis of brain homogenates.
Samples from AD and normal brains were homogenised and subjected to SDS-PAGE as described in Materials and Methods(4.7.2.)Blots were incubated with human sera or rabbit antisera, an anti-human or an anti-rabbit IgG alkaline-phosphatase conjugated respectively and developed.
In a) the recombinant AD1 protein (lane 1) and homogenates from AD and NDC brains were incubated with human sera (h) and rabbit (r) antisera. Human sera are from the AD patient (8006) who recognized AD1 protein in immunoscreening (h+), and an age-matched negative control (h-). Rabbit antisera were from rabbit before (r-) and after (r+) immunization with the recombinant AD1 protein.
In b) the recombinant AD2 protein (lane 1) and brain homogenates from AD and NDC brains were incubated with human sera from the AD patient (9016) who recognized AD2 protein in immunoscreening (h+), and an age-matched negative control (h-) and antisera from rabbit before (r-) and after (r+) immunization with the recombinant AD2 protein.

2.6. OCCURRENCE OF ANTIBODIES AGAINST AD-SPECIFIC ANTIGENS IN PATIENTS' SERA.

To detemine the occurrence of antibodies against the newly defined AD antigens, sera from AD patients and healthy controls were tested by SEREX approach (2.3.). The results of immunoscreenings were shown in Table 11 (2.3.2.): antibodies which recognize AD1 were detected in 2 out of 10 patients' sera and in none of the healthy age-matched controls; the same result was obtained for AD2 with the only difference that the 2 patients' sera which recognized AD1 were different from the 2 patients' sera which recognized AD2.

To ascertain the specificity of these antibodies in AD sera, the experimental approach needs to be changed because, as it was shown in Fig. 12, the preabsorption procedure necessary to perform the phage assay was ineffective for the most of AD sera.

2.6.1. Testing Serum Responses By ELISA

An ELISA was therefore established to evaluate the presence of anti-AD1 and anti-AD2 antibodies in sera from AD patients and healthy age-matched controls. For this purpose the two antigens are expressed and purified as recombinant His-tagged fusion proteins (as described in 4.8.1. of Materials and Methods). In Fig. 33 the results of expression and purification for the two antigens are shown.

Preliminary ELISA experiments revealed the system needs further modification to reduce the high background probably due to bacterial contaminants present in the purified proteins which are recognized by antibodies in both normal and AD subjects. This was revealed by high ELISA signals against the irrelevant protein purified in the same way and used as a negative control.

2.6.1.1. Production of recombinant His-tagged proteins

The prokaryotic expression of the two clones as His-tagged fusion proteins yielded two products with apparent molecular masses of approximately 30 kDa for AD1 protein

and 40 KDa for AD2 protein respectively, consistent with the expected molecular masses for polypeptides coded by the two cDNA inserts (Fig. 33). Another irrelevant protein was purified in the same conditions as His-tagged fusion protein to serve as internal negative control in ELISA experiments. The two recombinant AD1 and AD2 proteins are recognized both by AD sera which identified them in immunoscreening (as shown in the right panels of Fig. 33) as well as by rabbit polyclonal antisera raised against them (data not shown).

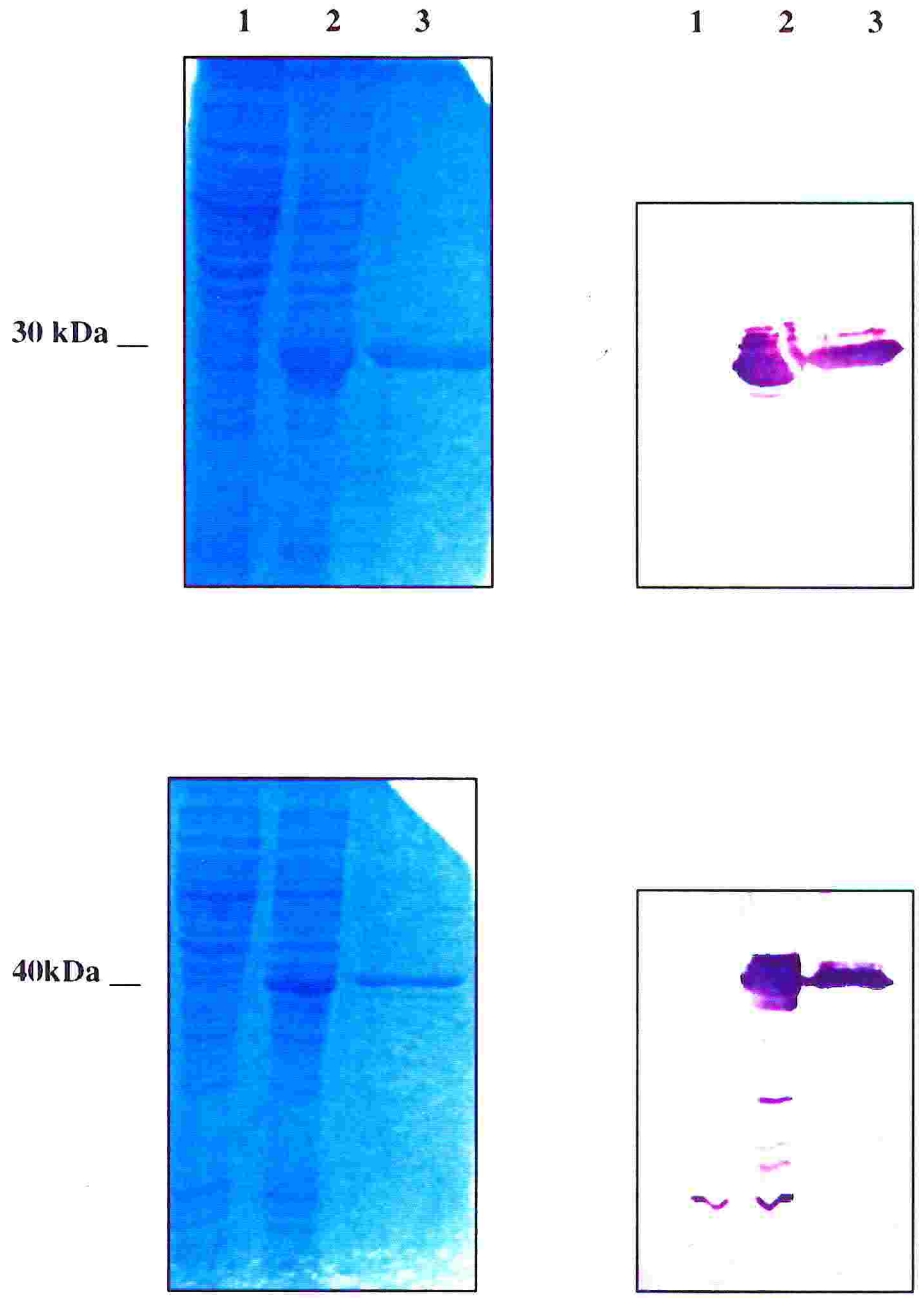


Figure 33. Prokaryotic expression and purification of recombinant His-tagged AD1 and AD2 proteins.
The cDNAs coding for the His-tagged AD1 and AD2 proteins were expressed in *E.coli* (as described in. 4.8.1. of Materials and Methods. Lysates of bacteria induced for expression containing the empty vector alone (lanes 1) and the recombinant proteins (lanes 2) were analyzed by Comassie staining and Western blot after electrophoresis. Recombinant proteins were purified by affinity cromatography and specific eluates were analyzed as lysates (lanes 3). The results of expression and purification of AD1 are shown in the upper panel: those of AD2 in the lower panel. The Western blots shown in the right panels of the figure were performed with the two sera which identified the antigens in the immunoscreening (8006 for AD1 and 9016 for AD2, respectively): the same results were observed by using the rabbit antisera (data not shown).

3. DISCUSSION

3.1. HUNTING FOR AD-SPECIFIC ANTIGENS: WHY THE SEREX APPROACH

As already mentioned in the Introduction, it is now a common view to state that AD should be viewed as a syndrome with different sub-types of the disease, one of which may involve defective immune regulation and/or autoimmunity.

Such a hypothesis was supported by several findings which have shown impairments of cell-mediated and humoral immunity in AD (for a review see Singh, 1997).

Among the immunological factors which could be implicated in the pathogenesis and/or progression of AD, brain autoantibodies may play an important role. Independently of their involvement in the pathogenesis of the disease, autoantibodies specifically present in AD sera and/or CSFs could be employed as biological markers for the disease if any correlation is demonstrated between their presence in serum and/or CSF and the progression of the disease.

Nevertheless, even though several brain-specific autoantibodies in AD sera as well as CSFs have been described, very few AD-specific antigens that elicit such immune responses in AD patients have been defined at the molecular level. This was probably due to the inadequacy of the methodologies used to detect them: attempts to specifically stain brain tissue using sera have not been successful due to the high background staining observed with control sera. Furthermore, immunocytochemical staining does not permit the molecular identification of putative AD-specific antigens, being a semi-quantitative technique unable to reveal different concentrations of anti-neuronal antibodies in AD sera and/or CSFs with respect to healthy age-matched individuals. More sensitive techniques such as ELISA would detect such differences more precisely but require knowledge of the identity of the specific antigen(s).

All reported attempts to identify antigens recognized by AD specific sera (Table 3, 1.6.3.2. of the Introduction) suggest that newer methodologies are needed to demonstrate the presence of specific autoantibodies in AD patients.

The SEREX approach (described in 1.9.1. of the Introduction) could be one such methodology, since it was set up and used successfully for the systematic identification of disease-specific antigens recognized by sera from patients with tumours.

The immunological screening of cDNA libraries has already been used as a tool for the identification of autoantigens in several different autoimmune diseases (Pietropaolo *et al.*, 1993; Sohda *et al.*, 1994; Despres *et al.*, 1995), but the SEREX approach, involving a stringent serological detection system which is limited to antigens that elicit high-titer IgG responses in the patient *in vivo*, should reduce the detection of aspecific autoantibodies not strictly linked to the disease, overcoming the controversial question of a generalized increase of organ (and non-organ)-specific autoantibodies with aging (Juby *et al.*, 1994; Candore *et al.*, 1997).

3.1.1. Construction Of The AD Hippocampal cDNA Library

The SEREX approach implies, as the first step, the construction of a cDNA library from fresh tissue specimens, which in our case was from AD brain.

As already mentioned in the Results, the few AD cDNA libraries reported in the literature (Goedert, 1987; Octave *et al.*, 1988; Salim *et al.*, 1988; Vitek *et al.*, 1988) were constructed from patients' cerebral cortices (except the commercial one from Clontech, which is no longer available, constructed from AD hippocampus), so the construction of a cDNA library from AD hippocampus was undertaken.

AD hippocampus was chosen as a starting material because, besides being together with temporal cortex one of the first areas affected by the specific pathological lesions of the disease, including NFT, SP, and loss of neurons (1.3 and 1.3.1. of the Introduction), it also contains the greatest number of different cell types. As a result it would be expected to represent the greatest antigenic diversity, and so the greatest number of antigens which could be recognized by sera.

To find proteins specifically recognized by antibodies present in AD sera, in theory a cDNA library from normal brain could also be used, but an AD hippocampal cDNA library would give additional information about proteins specifically present in

AD brain and not in normal brain such as proteins from infectious agents (if any) or induced by disease. As already mentioned in the Introduction (1.6.5.), the involvement of viral agents, such as HSV, in AD is still a controversial question.

3.1.1.1. Problems With RNA From Post Mortem Tissues In General And In AD In Particular.

Comparing the characteristics of the AD hippocampal cDNA library constructed with those of the only other AD hippocampal cDNA library so far reported, the commercial one from Clontech (Table 12), the only parameter which differs significantly is the size of the library, which is approximately five times smaller. This difference is likely to be due to the limiting amount of starting material used (AD hippocampus) coupled with the problems associated with RNA isolated from post mortem tissues in general, and in AD in particular.

TABLE 12. Comparison of the characteristics of the two AD hippocampal cDNA libraries so far reported.

LIBRARY CHARACTERISTICS	CLONTECH	MINE
Library mRNA Source	hippocampus from: 70-yr-old female with AD	hippocampus from: 61-yr-old female with AD 97-yr-old female with AD 84-yr-old female with AD
Priming Method	oligo(dT)-primed	oligo(dT)-primed
Vector	λgt11	λZAPII
Independent Clones	$8.1x10^5$	$1.7x10^5$
Insert Size Range (Avg.)	0.3 - 3.1 kb (1.1 kb)	0.3 - 2.8 kb (0.8 kb)
% of Recombinants	87%	90%

It is well known from the literature that the yield in total RNA and in particular in poly(A)$^+$RNA extracted from postmortem brain tissues is reduced due to RNA degradation. In the case of AD brain this reduction is likely to be higher due to the neuronal loss occuring in the disease (Guillemette *et al.*, 1986; Johnson *et al.*, 1986;

Goedert, 1987; McLachlan *et al.*, 1988; Clark and Parhad, 1989; Iacopino and Christakos, 1990).

Various hypotheses have been proposed to explain this degradation: the time between death and freezing of the tissue; mechanical damage during dissection (Johnson *et al.*, 1986); the storage time, the freezing/thawing of the tissue; the age of the subjects (Alberghina *et al.*, 1988); and the premortem suffering (e.g. prolonged anoxia or ischemia) (Roses, 1987; Morrison, 1988), hippocampus being especially vulnerable to ischaemic damage (Pulsinelli, 1985).

The hippocampal tissue used here was frozen after a relatively short post-mortem delay (see Table 13, 4.4.1. of Materials and Methods). It is likely that better quality mRNA could be obtained if it could be prepared immediately without freezing. However, given the unpredictable nature of death, and the fact that samples were obtained from The Netherlands Brain Bank, this is not presently possible.

Whatever the cause of the RNA degradation observed in human brain, it occurs and its extent is variable: differing results have also been reported on a supposed increased degradation in diseased brain compared with normal tissue (Sajdel-Sulkowska and Marotta, 1984; Doebler *et al.*, 1989; Ross *et al.*, 1992), suggesting that loss of mRNA may reflect reduced neuronal metabolic activity occurring in response to a variety of etiological events.

In addition, several reports have documented reduced poly(A)$^+$RNA content specifically in AD brains (Guillemette *et al.*, 1986; Marotta *et al.*, 1986; Taylor *et al.*, 1986), with a distribution largely coincident with those regions, hippocampus and temporal cortex, which are most severely affected by AD (Harrison *et al.*, 1991). Furthermore, of this reduced poly(A)$^+$RNA only a small proportion, approximately 5-10%, compared to the 30% obtained in the case of fresh rat tissues, could be reverse transcribed into first strand cDNA (Salim *et al.*, 1988; Kobayashi *et al.*, 1990), indicating that mRNA from AD brains is in some unexplained way different from that found in normal brain. The results obtained in this study confirm that the conversion of

mRNA into cDNA is far less efficient with AD mRNA than freshly prepared rat brain mRNA.

Despite these problems, the characteristics of the library obtained (as shown in Table 12) suggested that it was suitable for the immunological screening with AD patients' sera, a conclusion confirmed by the screening results obtained.

3.1.2. Immunological screening Of AD Hippocampal cDNA Library

Since a definitive diagnosis of AD is possible only at autopsy, and it is difficult to obtain sera from such confirmed AD cases, a preselection of the patients used to provide the sera for this study was made. Only those patients with at least one APOEε4 allele were used (with one exception, as shown in Tables 8 and 9 in 2.3. and 2.3.1. of the Results), as it has been previously demonstrated that suspected AD patients with at least one APOEε4 allele have a diagnosis of AD at autopsy with a greater than 90% probability (Welsh-Bohmer *et al.*, 1997).

Despite the problems encountered in sera preabsorption (described in 2.3.1. of the Results and discussed in 3.1.2.1.), the immunological screening of the library eventually resulted in the isolation of two putative AD-specific antigens, that is, antibodies recognizing these antigens are specifically present in 20% of AD patients examined and not in healthy age-matched controls (as shown in Table 11, 2.3.2. of the Results).

3.1.2.1. Preabsorption Problems

When carrying out the immunological screening of the AD hippocampal cDNA library using sera from AD patients, it was observed that such sera were much more difficult to preabsorb than normal age-matched controls or tumour patients (as shown in Fig. 12, 2.3.1. of the Results). These findings raised the question on the possible causes of this difficulty, which may in itself reflect some aspect of the AD process. It may be interesting to investigate this phenomenon more closely to see if a correlation, if any,

exists between preabsorption problems and clinical diagnoses or the progressive stage of the disease.

One can speculate that preabsorption problems were due to two possible reasons:

1) a higher number of antibodies against bacterial antigens in AD sera or,

2) the presence of an as yet unidentified serum component, which would confer greater stickiness to AD immunoglobulins.

The first hypothesis would imply an increased susceptibility to bacterial infections in AD patients, reasonable if you consider that AD patients eventually become so mentally debilitated that they are unable to take care of themselves, and usually end life in an institution.

An alternative view is suggested by the findings of high serum cholesterol level as a significant risk factor for AD (Notkola *et al.*, 1998) together with altered phospholipid (Miller *et al.*, 1991) and lipoprotein (Montine *et al.*, 1997) metabolism in patients with AD, which may be correlated to the second hypothesis .

Both hypotheses, however, are highly speculative, and will require much more work to test and eventually confirm or refute.

3.2. IDENTIFICATION OF THE TWO AD-SPECIFIC ANTIGENS

In order to identify the two putative AD-specific antigens isolated by immunological screening of the AD hippocampal cDNA library, positive clones identified in the library were sequenced. This revealed that the two cDNAs coded for two unknown proteins, at least in humans, so the second step in their identification was the study of their expression in a large panel of human tissues (3.2.1.).

3.2.1. Results Of Sequencing Analysis And Expression Studies

As illustrated in the Results (2.4.1.), the first clone isolated, named AD1, was identical to a putative gene identified in a human genomic sequence deposited in GenBank. So far, the existence of the putative protein was assumed on the basis of the

gene structure and the presence of a number of highly homologous human ESTs, found in different human tissues (as shown in Fig. 29, 2.5.1. of the Results).

The isolation of the AD1 cDNA confirms that the gene is effectively transcribed and processed. Interestingly, the genomic sequence identified in GenBank, is found on chromosome 19; which is the same chromosome on which the ApoE gene is located (Olaisen *et al.*, 1982).

Surprisingly, AD1 mRNA is highly expressed in heart and skeletal muscle and very weakly in brain (as shown in Fig.31a, 2.5.1. of the Results). Unfortunately it proved impossible to perform satisfactory Northern blots on AD brain mRNA, due to the low quality of mRNA which could be obtained from AD brains.

From further searches in the DNA/protein databases, it became clear that AD1 is the human homologue of the Vesl family of proteins described in the rat, which are in turn similar to the Mena/VASP family of proteins (3.2.1.1.).

The second clone isolated, named AD2, was not identical to any deposited DNA sequence although a number of highly homologous human ESTs expressed in a wide range of human tissues were identified (as shown in Fig. 30, 2.5.1. of the Results). The expression patterns described by the ESTs were confirmed by Northern blot analysis, indicating that AD2 has a similar distribution to these ESTs. By Northern blot an approximately 2-kb long AD2 transcript was detected in all tissues analyzed: the highest expression was found in brain, heart, kidney, liver and placenta, followed by skeletal muscle, spleen, small intestine and lung and, finally, an weak expression was detected in colon, thymus and peripheral blood leukocytes (as shown in Fig. 31b, 2.5.1. of the Results).

The AD2 protein is homologous to SM-20, a rat protein expressed in VSMCs (3.2.1.2.) and to a lesser degree to a putative *C.elegans* protein named "Weak similarity with apoptosis protein RP-8", not yet isolated but just presumed on the basis of a predicted gene in a genomic sequence.

3.2.1.1. TheVesl Family Of Rat Proteins

Vesl (VASP/Ena-related gene up-regulated during seizure and LTP)/Homer was isolated as a synaptic plasticity-regulated gene from rat hippocampus (Brakeman *et al.*, 1997; Kato *et al.*, 1997). Very recently, Kato *et al.* (1998) reported additional members of the Vesl/Homer family; thereafter, the Vesl/Homer cDNA that codes for the 186 aa long protein, the first isoform to be isolated, was denoted as Vesl-1S.

The Vesl-1S/Homer protein interacts with mGluR1 and mGluR5, G protein-coupled glutamate receptors that mediate phosphatidylinositol turnover, by means of a PDZ-like domain (Brakeman *et al.*, 1997). Vesl-1S/Homer is the first PDZ protein that is found to be up-regulated by extracellular stimulation such as LTP (Long Term Potentiation). Thus, Vesl-1S/Homer may function as a modulator for mGluR signaling.

PDZ domains are so called because they occur in PSD-95, Dlg and ZO-1, all members of MAGUKs (membrane-associated guanylate kinases); they were originally termed GLGF domains (Gly-Leu-Gly-Phe being a relatively conserved element of their sequences) or DHR domains (Discs-large homologous region), being initially found within the *Drosophila lethal(1) discs large-1* (*Dlg*) tumour suppressor gene product. They constitute an interface for certain kinds of protein-protein interactions, specifically interactions with integral membrane proteins such as receptors (for reviews, see Gomperts, 1996; Sheng, 1996; Ziff, 1997). One of the well known functions of PDZ proteins is their ability to cluster associated integral membrane proteins at synapses (Kim *et al.*, 1995; Doyle *et al.*, 1996).

Subsequent to the report by Brakeman *et al.* (1997), others noted the stronger homology of Vesl-1S/Homer to the recently described EVH1 domain (Enabled/VASP homology) (Gertler *et al.*, 1996; Kato *et al.*, 1997; Ponting and Phillips, 1997). Proteins that encode the EVH1 domain include *Drosophila enabled* (termed Mena in mouse) (Gertler *et al.*, 1996), yeast Bee1p (Li, 1997), VASP (Haffner *et al.*, 1995), and the Wiscott-Aldrich Syndrome Protein (WASP) (Symons *et al.*, 1996). These proteins appear to fuction in the transduction of cell surface signals to the actin-based cytoskeleton. It is important to point out that the PDZ domain constitutes a part of the

longer EVH1 domain (as shown in Fig. 17, 2.4.1. of the Results), but that not all PDZ domains are EVH1 domains, i.e. many PDZ domains lack the extra elements required to be described as EVH1 domains.

Vesl-1S/Homer is a splicing variant of Vesl-1L, which lacks the C-terminal portion present in Vesl-1L (191 amino acids). The N-terminal portions of Vesl-1S/Homer and Vesl-1L (175 amino acids) are derived from the same exon. Vesl-2 is closely related to Vesl-1L: specifically, the N-terminal portion, which contains the R*XXXXX*GLGF motif of the PDZ domain and the EVH1-homologous region, is highly conserved between Vesl-1L and Vesl-2 (86% identity in 120 amino acid residues). As expected, given their structural similarity, all these proteins were able to bind to mGluR1 and mGluR5, probably through the R*XXXXX*GLGF motif. The homology in the amino acid sequences between Vesl-1L and Vesl-2 decreases at the C-terminal half but is, nevertheless, still significant (34% identical in 246 amino acids). Specifically, both Vesl-1L and Vesl-2 have at the C-terminal end an highly conserved region homologous to the MCC protein, a putative colorectal tumour suppressor (Kinzler *et al.*, 1991), and a leucine-zipper domain, suggesting that Vesl-1L and Vesl-2 can dimerise with other leucine zipper domains, in fact, Vesl-1L and Vesl-2, but not Vesl-1S, which lacks the leucine zipper domain, are able to interact with one another both homotypically and heterotypically.

In addition to mGluRs, the N-terminal portions of the Vesl family, which contain the EVH1 domains, were shown to interact with several other unidentified hippocampal proteins detected in an overlay experiment described by Kato *et al.* (1998). This suggests that they serve as an interface that may mediate more than one protein-protein interaction. Given their interaction with mGluRs, it is striking that the Vesl mRNAs are expressed at such low levels in neuronal tissue. In fact, the data described here show that the Vesl proteins are far more abundant in heart and skeletal muscle than brain.

The Vesl family of proteins is unique in that it contains an inducible form, the Vesl-1S protein, that is upregulated during seizure and LTP in the adult hippocampus,

whereas Vesl-1L and Vesl-2 transcripts were constitutively expressed in the adult hippocampus. Inversely, throughout the development of the hippocampus, the level of Vesl-1S mRNA was low, whereas Vesl-1L mRNA was expressed at P8. The expression profile of Vesl-2 mRNA was similar to that of Vesl-1L, relatively high at P8 and low at P15 and P22. Taken together, the expression of Vesl-1L and Vesl-2 mRNAs was regulated during hippocampal development but not during LTP of adult hippocampus. In marked contrast, Vesl-1S expression was up-regulated following LTP induction, but not during development.

3.2.1.2. Rat SM-20 Gene And Protein

SM-20 is a member of the immediate early gene (IEG) family that is induced by growth factors and vasoactive agonists in VSMCs.

VSMCs are a major constituent of the blood vessel wall and are responsible for the maintenance of vascular tone. These cells also play an important role in several disease processes. Analyses of arteries from hypertensive models have demonstrated both increased number (hyperplasia) and increased size (hypertrophy) of VSMCs (Owens and Schwartz, 1982; Lee *et al.*, 1983; Owens and Reidy, 1985).

SM-20 is an unusual immediate early gene induced by growth factors and vasoactive agonists because it is expressed in VSMCs and not in fibroblasts. High levels of SM-20 mRNA are seen in skeletal, cardiac and smooth muscle as well as in the brain and PC12 cells, although it was not determined which specific cell types express SM-20 and to what extent the vascular component of the various tissues contributed to the signals observed (Wax *et al.*, 1994).

An SM-20 antigen of approximately 40 kDa was localized to filaments in the cytoplasm of cultured rat aortic SMCs and detected in several rat tissues. In human atherosclerotic coronary arteries, SM-20 antigen predominated in the SMC of the intimal plaques and was not detected in macrophages, endothelium, or adventitial cells (Wax *et al.*, 1996).

3.2.2. Hypotheses On The Function Of The Two AD-Specific Antigens

On the basis of the homologies reported above, it is interesting to speculate on the possible function of the two proteins isolated; these hypotheses will need to be confirmed by further studies addressed to identify their location and function (3.4.).

On the basis of the homology with the Vesl family of rat proteins (3.2.1.1.), AD1 may modulate mGluR signaling and this function, as occurs in other PDZ proteins (Bannert *et al.*, 1996), may be regulated by protein kinase-mediated phosphorylation. An examination of AD1 protein sequence using PROSITE (Bucher and Bairoch, 1994; Bairoch *et al.*, 1997) identified a number of potential phosphorylation sites (see 2.4.1. of the Results). Interestingly, metabotropic receptor signaling, together with having implications in several forms of activity-dependent synaptic plasticity (Bortolotto *et al.*, 1994; O'Connor *et al.*, 1994), is also implicated in neurodegenerative diseases (Nicoletti *et al.*, 1996; Conn and Pin, 1997) and, more specifically, in AD, since severe dysfunctions in the phosphoinositide signalling pathway have been reported (Pacheco and Jope, 1996; Fowler, 1997). As a result, a role for these proteins in the pathogenesis of SAD has been hypothesized. It is very interesting in this regard, that in the preliminary results described here, 20% of AD patients appear to have antibodies in their serum which recognize AD1, a Vesl protein.

AD1 seems to be the human homologue of the longer rat isoforms, Vesl 1L and Vesl 2, as it contains the C-terminal end which is homologous to the MCC protein and the leucine-zipper domain, which could imply, as is the case for Vesl-1L and Vesl-2, homo- or heterodimerization with other putative members of the family (3.2.1.1.). Consequently, its expression would not be expected to be upregulated during seizures and LTP in the adult hippocampus, as occurs with Vesl-1S, but only during hippocampal development. Unfortunately, we have not been able to examine the level of AD1 expression in AD brains, due to the low quality and quantity of the RNA we could obtain.

On the other hand, the high conservation at the N-terminus between all members of the Vesl family would predict also for the human homologue the possibility to

interact with several other proteins in addition to the group 1 mGluRs, prediction confirmed by the fact that the AD1 transcript is expressed at high levels in tissues that do not express mGluRs, such as heart and skeletal muscle.

More speculative are the hypotheses on the putative function(s) of AD2 protein since a lower homology was found with already known proteins, none being human. The rat homologous transcript is widely expressed like the human one; in both cases it remains to be determined which specific cell types express SM-20 and to what extent the signals observed are due to the vascular component of the various tissues examined. Besides this, the rat SM-20 antigen has a molecular mass of approximately 40 kDa, the same observed both for the AD2 antigen detected in brain extracts as well as for the recombinant AD2 protein (shown in Figs. 32 and 33 in 2.5.2 and 2.6.1.1. respectively of the Results).

The location of SM-20 antigen on blood vessel wall is very intriguing because cerebrovascular Aβ deposition is a key pathological feature of AD and also because there is a growing body of evidence to suggest a vascular involvement in the pathogenesis of AD (Choi *et al.*, 1998; Perry *et al.*, 1998; Stewart, 1998; Suo *et al.*, 1998).

Significantly, between the autoantibodies found in sera of AD patients so far reported (shown in Table 3, 1.6.3.2 of the Introduction), antibodies which immunoreact to vascular structures in rat and bovine brain have been described (Fillit *et al.*, 1987; Foley *et al.*, 1988). Nevertheless, others have found antibodies which recognize smooth muscle both in AD as well as in non demented controls and in vascular forms of dementia (VaD and CVD) (Ounanian *et al.*, 1990; Lopez *et al.*, 1991; Lopez *et al.*, 1992), these may represent the normal increase in the presence of autoantibodies known to occur with age.

For all these reasons, special care must be taken to specifically correlate the presence of the relative autoantibodies to AD and other forms of dementia, such as VaD

and CVD, and more in general to vascular diseases, including atherosclerosis and thrombosis.

3.3. POSSIBLE ROLES FOR AUTOANTIBODIES IN ALZHEIMER DISEASE

As already mentioned in the Introduction (1.9.), autoantibodies in autoimmune diseases can be directly involved in the pathogenesis of the disease, mark the presence of the real etiologic agent or be a consequence of the disease process. To state their role in the disease, one of the most crucial factors is the location of the presumptive target antigen: if they are directed against extracellular or cell surface molecules, it is more likely that they would be pathogenic. If, on the other hand, their targets are intracellular, they are usually not pathogenic unless it can be unequivocally demonstrated that (a) the antigen is released from within the cell so that it can bind onto a cell surface receptor or other extracellular location; (b) the antigen moves to an aberrant site on the cell surface; or (c) a cross-reactive molecule, the actual target, is at an accessible location. Antibodies directed against intracellular antigens are more likely to be due to a result of cellular damage by other means which results in the release and exposure of intracellular antigens to the immune system.

3.3.1. AD-Specific Antibodies As Diagnostic Tools

Independently of the involvement in the pathogenesis of the disease, if the presence and titre of AD-specific antibodies in patients' sera and/or CFSs could be associated with clinically relevant features or with the progression of the disease, they may be used for the serodiagnosis of AD, as prognostic markers, or as an endpoint in the evaluation of therapeutic interventions. As stressed in the Introduction (1.7.), sensitive biological markers useful in the diagnosis and therapy of AD are badly needed.

Such a possible function for the autoantibodies detected in this study needs to be further investigated: on the basis of the results of this thesis, 20% of AD patients and no healthy age-matched individuals analyzed were positive for each of the two

autoantibodies found and the patients whose serum contain one of the two autoantibodies does not contain the other. The analysis of a higher number of individuals, not only between those affected by AD but also by other forms of dementia, neurodegenerative diseases and healthy age-matched controls will provide further insight into the relevance and specificity of these antibodies in AD and their possible function as diagnostic markers. Preliminary experiments in this regard have been started in an ELISA format, but appear to be plagued by the presence of antibodies in both normal and AD subjects which recognize bacterial contaminants present in the purified proteins.

3.3.2. AD-Specific Antibodies As Effectors Of The Disease

An important factor which must be considered in evaluating the plausibility of autoantibodies or other immune factors as effectors of neuropathology, is the existence of the blood-brain and blood-CSF barriers (BBB and BCB), which are assumed to restrict passive entry of immunoglobulins and other large molecules into the CNS. Given these conditions, it could be predicted that autoimmune-mediated neuropathology could occur only under circumstances involving physiologic challenge or injury to the BBB.

There is a diversity of opinions concerning the functionality of the BBB and the BCB in aging, AD and other neuropsychiatric disorders: in particular, there is disagreement as to whether an age-related increase in permeability actually occurs in healthy humans and/or in various pathological conditions (for a review see Shah and Mooradian, 1997). Injury to the BBB and BCB has been suggested to occur in subsets of patients with AD (Fillit *et al.*, 1987; Hampel *et al.*, 1997; Skoog *et al.*, 1998). These findings are confirmed by several others which reported the presence of T-helper and T-cytotoxic suppressor (CD8) lymphocytes (Itagaki *et al.*, 1988; Luber-Narod and Rogers, 1988) as well as complement activation (McGeer *et al.*, 1989) in AD brain.

T lymphocytes together with activated microglia, which have been shown to vigorously express the MHC class II surface glycoprotein HLA-DR (McGeer *et al.*, 1987; McGeer

et al., 1988; Rogers *et al.*, 1988), in AD brain represent the appropriate tissue elements for a cell-mediated immune response.

The antibodies detected by our screening procedure were of the IgG subclass, implying that cognate helper T-cell immunity might be present and operative in patients with a respective B-cell response; so it remains to be found which cells are responsible for the initiation of such a B-cell response since both in normal as well as in AD brain few B lymphocytes and IgGs are found, indicating that significant local immunoglobulin synthesis is unlikely to occur. These findings are confirmed by PCR experiments performed in our laboratory in which it proved impossible to amplify immunoglobulin V genes from AD brain tissue.

It could be hypothesized that an increased permeability of the BBB will permit infiltration of B lymphocytes too, which could synthesize autoantibodies against neuronal antigens, although this is not supported by the data. Alternatively, the leakage of antigens from the CNS to the systemic circulation may promote the access of antigen to immunocompetent cells, allowing production of antibodies against CNS antigens in the periphery. Both the detection of infiltrating T lymphocytes and the hypothesis of infiltrating B lymphocytes would be in agreement with the findings of this thesis about the high homology of AD2 cDNA with several human ESTs expressed by B and T lymphocytes. AD2 mRNA could be expressed by B (or T) lymphocytes occurring in the brain for a dysfunction of the BBB or, alternatively, constitutively present; this last hypothesis would remain to demonstrate.

The observation that the two AD-specific antigens isolated are both intracellular raises a further question. The initiation of a B-cell response needs the exposure of the antigen to the immune system: in normal conditions, intracellular antigens are inaccessible by the immune system but, since an extensive neuronal death occurs in AD, it can be hypothesized that these antigens encounter immune cells following release by dying cells - in the brain in the hypothesis of infiltrating B lymphocytes; in

the systemic circulation if CNS antigen leakage occurs. In either case, this hypothesis excludes a pathogenic role for the corresponding autoantibodies in the disease.

Another hypothesis which can be formulated is that the two AD-specific antigens isolated contact the immune system following release from sites different than human brain, sites more easily contacted by the immune cells. This hypothesis is supported by the results of the expression studies showing a very wide spectrum of expression for both the antigens isolated, particularly for AD1 which is expressed at such low levels in human brain. It is also in agreement with the model of neuroautoimmunity (NAI) proposed by Singh (1997) and diagrammatically sketched out in Fig. 34. The NAI model has three novel features: 1) a blood-borne acute phase which leads to a chronic disease phase in the brain; 2) cytotoxic T lymphocytes (CD8+) directly through cell-mediated immunity (CMI) or indirectly through glia-activation (microglial and astroglial cells) induce target cell-cytotoxicity, and 3) glia-activation leads to inflammatory nonspecific tissue damage, but brain-specific autoantibodies direct CMI towards neuron-specific neurodegeneration as in AD. This model is supported by a number of findings which suggest several immune dysfunctions in AD (well reviewed by Singh, 1997); it remains to be established which is(are) the antigen(s) responsible for the observed immune activation. AD1 and AD2 widely expressed in several tissues other than brain could be the possible candidates.

The confirmation of such an hypothesis would strengthen the conception of AD as a systemic disorder based on numerous abnormalities found in tissues other than brain such as alterations of Ca^{2+} metabolism in fibroblasts (Ito *et al.*, 1994), deposition of Aβ in non-neural tissues, including skin, subcutaneous tissue and intestine (Joachim *et al.*, 1989) and several changes in systemic immunity (Pirttila *et al.*, 1992). In this regard, it is very interesting to observe that both antigens are highly expressed in the heart since several reports suggested a correlation between heart disease, cardiovascular diseases and AD: the premature presence of senile plaques in coronary artery disease (CAD), and neurofibrillary tangles as well as senile plaques in hypertension, suggest a

neuropathologic link between CAD, hypertension, and AD (Sparks, 1997); furthermore, in patients with probable AD, APOEε4 is associated with coronary atherosclerosis (Kosunen *et al.*, 1995), and cardiac disease (van der Cammen *et al.*, 1998), ApoE being a major component of the genetic basis of cardiovascular disease, as well as AD (Contois *et al.*, 1996). Although the pathogenic role of apoEε4 in both AD and cardiovascular disease has been well described, it is possible that the link between cardiovascular disease and AD extends beyond this known correlation.

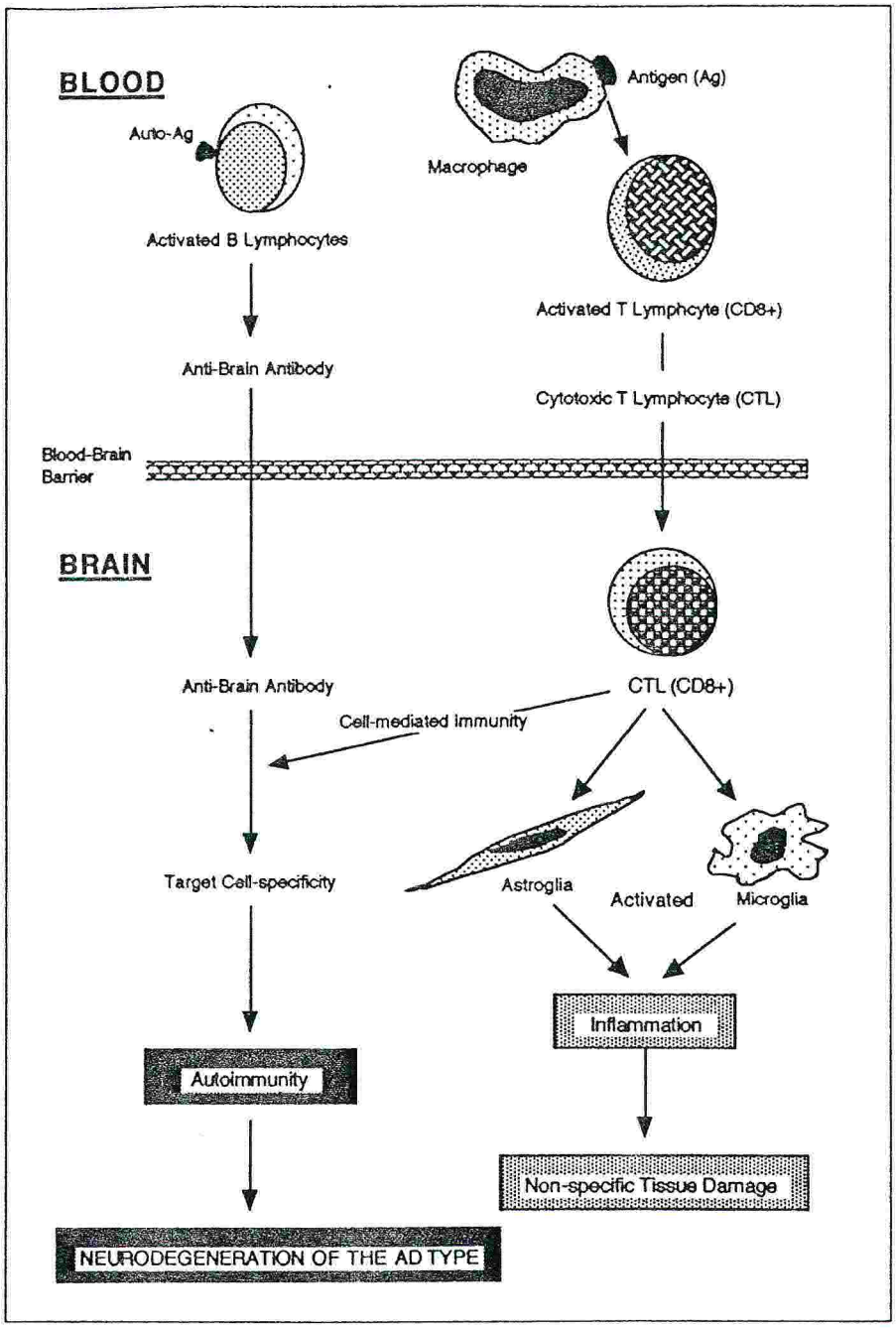


Figure 34. Diagrammatic sketch of the neuroautoimmunity (NAI) model proposed by Singh. (from Singh, 1997).

The question remains as to the significance of these antibodies, when present. For CSF anti-microglial antibodies McRae *et al.* (1996) suggested the possibility that, in some patients with AD, these antibodies could bind to neurons and activate the classical complement cascade; in addition to complement activation, antibody binding to neuronal antigens could potentially damage neurons by other mechanisms, including disruption of neuronal physiology, or opsonization of neuronal antigens to facilitate phagocytosis by microglia. Although it is clear that for this hypothesis to be feasible, a prior damage of the BBB to permit entry of complement components is required. In this case, the primary cause of BBB damage may provide initial damage which is then further exacerbated by activation of the immune system.

In this regard, both of the antibodies found in this study are directed against intracellular targets, so at the functional level they would not be expected to affect normal cell function. In the presence of a leaky BBB, they could interact with their respective antigens after neuronal death, either in or out of the CNS. It is likely, therefore, that they may contribute to exacerbate, rather than start, neurodegenerative processes as described above.

3.4. FUTURE PERSPECTIVES

Most of the work described in this thesis is just the beginning of the studies that should be continued to give final answers to questions such as: what are the functions of these two new proteins? Do they have a role in the brain? Are the antibodies directed against these proteins specifically present in AD patients? And do they influence the progression of the disease? What happens to the levels and activities of these proteins in AD and other neurological disorders?

A first step to answer these questions will come from the continuation of the expression studies by *in situ* hybridization to detect the localization and distribution of the relative transcripts and immunohistochemical analysis to detect the location of the two proteins. Both experiments will be performed in parallel on human brain samples from healthy age-matched individuals, disease controls and AD patients. The finding of an overexpression in AD would help to explain the presence of autoantibodies directed against these antigens because it is known that overexpressed genes elicit an immune response by overriding thresholds critical for the maintenance of tolerance (Viola and Lanzavecchia, 1996). In addition to looking for differences in levels/forms of AD1 and AD2 in AD brain, it may also be important to look at levels/forms of these proteins in other tissues, in which they are known to be expressed, as well as in fibroblast models derived from AD patients (Gasparini *et al.*, 1998), as the immunological defect which has allowed the creation of these antibodies may exist in the periphery rather than in the brain, as discussed above.

The second step will address the possibility to use these autoantibodies as diagnostic markers by testing a much more higher number of patients' sera in comparison with other diseased patients and healthy age-matched controls: this will be perfomed by the ELISA experiments, already started, on the single antigens.

4. MATERIALS AND METHODS

4.1. CHEMICALS AND MOLECULAR BIOLOGY REAGENTS

Enzymes for modification of RNA or DNA were obtained from Boehringer Manheim, Gibco BRL, New England Biolabs, Pharmacia and Promega.

Other chemicals, immunochemicals and enzymes were purchased from the companies indicated in brackets.

4.2. METHODS OF MOLECULAR BIOLOGY

Standard methods of molecular biology were used as described in Sambrook *et al.* (1989).

4.3. BACTERIAL STRAINS, PLASMIDS AND BACTERIOPHAGE

XL1-Blue MRF' Strain Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q}Z*ΔM15* Tn*10* (Tet^{r})] (Stratagene)

XLOLR Strain Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q}Z*ΔM15* Tn*10* (Tet^{r})] Su^{-} (nonsuppressing) λ^{r} (lambda resistant) (Stratagene)

ExAssist^{TM} interference-resistant helper phage (Stratagene)

TOP10 Strain F- *mcr*A*Δ*(*mrr-hsd*RMS-*mcr*BC)*Φ*80*lacZ*ΔM15 *DlacX*74 *deo*R *rec*A1 *ara*D139 *Δ*(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G (Invitrogen)

pTrcHisB (Invitrogen).

4.4. CONSTRUCTION OF THE cDNA LIBRARY FROM AD HIPPOCAMPUS

4.4.1. Brain Samples

Human brain samples from AD patients were obtained from the Netherland Institute for Brain Research. All AD patients met clinical and neuropathological criteria for the diagnosis of AD. In all cases requisite sections were removed, snap frozen in liquid nitrogen, and stored at -70 °C until used.

In particular, for the construction of the cDNA library I have used the pieces described in Table 13.

Table 13. Brain samples used for the construction of the cDNA library.

Brain area	Brain weight (g)	pH CSF	PMD	Sex	Age	Neuropathological diagnosis
hippocampus	846	7.22	4:15	F	61	AD
hippocampus	1149	7.70	3:30	F	97	AD
hippocampus	1094	6.81	4:00	F	84	AD

4.4.2. Isolation Of Total RNA From AD Hippocampus

Total RNA was isolated following the guanidine-isothiocyanate procedure (Chomczynski and Sacchi, 1987). Extracted RNA was quantitated spectrophotometrically by measuring the optical density at 260 nm. Its quality was checked spectrophotometrically by measuring the OD_{260}/OD_{280} ratio and confirmed electrophoretically on a 1% denaturing formaldehyde agarose gel and by Northern blot (4.7.1.) using as probes β-actin and GAPDH.

4.4.3. Purification Of Poly(A)$^+$RNA From Total RNA

Poly(A)$^+$RNA was affinity-purified using Fast-Track^R 2.0 mRNA extraction kit (Invitrogen), according to the manifacturers instructions. Quantity and quality of poly(A)$^+$RNA was tested as for total RNA (4.4.2).

4.4.4. Construction Of A cDNA Library From AD Hippocampus

The unidirectional cDNA library was constructed using the ZAP Express™ cDNA Synthesis and Gigapack® II Gold Cloning Kits (Stratagene).

First-strand cDNA synthesis was performed using the 50-base oligonucleotide linker-primer (Fig. 35b). The oligonucleotide was designed with a "GAGA" sequence to protect the *XhoI* restriction enzyme recognition site and an 18-base poly(dT) sequence. The restriction site allowed the finished cDNA to be cloned into the ZAP Express vector (Fig. 35c) in a sense orientation (using *EcoRI* and *XhoI*) with respect to the lacZ promoter. The poly(dT) region hybridized to the 3' poly(A)$^+$ region of the mRNA template, and first-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and 5-methyl dCTP. The use of 5-methyl dCTP during first-strand synthesis hemimethylated the cDNA, protecting it from the subsequent digestion with *XhoI* restriction endonuclease.

During second-strand synthesis, RNase H nicked the RNA bound to the first-strand cDNA to produce a multitude of fragments, which served as primers for DNA polymerase I. DNA polymerase I "nick-translated" these RNA fragments into second-strand cDNA. The second-strand nucleotide mixture was supplemented with dCTP to reduce the probability of 5-methyl dCTP becoming incorporated in the second-strand and ensuring that the restriction sites in the linker-primer were susceptible to restriction with *XhoI*. The uneven termini of the double-stranded cDNA were nibbled back or filled with *Pfu* DNA polymerase and *EcoRI* adapters (Fig. 35d) were ligated to the blunt ends. These adapters were composed of 9- and 13-mer oligonucleotides, which were complementary to each other and which formed *EcoRI* cohesive ends following annealing. The 9-mer oligonucleotide was kinased (to allow it to ligate to other blunt termini available in the form of cDNA and other adapters) and 13-mer was kept dephosphorylated to prevent it from ligating to other cohesive ends. After adapter ligation, the ligase was heat inactivated, and the 13-mer oligonucleotide kinased to enable its ligation into the dephosphorylated vector arms.

The subsequent *XhoI* digestion released the *EcoRI* adapter and residual linker-primers from the 3' end of the cDNA. These two fragments as well as cDNAs smaller than 500 bp were separated on a Sephacryl S-500 spin column. The size-fractionated cDNA was then precipitated and ligated to the ZAP Express vector arms.

The ZAP Express vector library was packaged using a high-efficiency *in vitro* packaging system (Gigapack II^R Gold Packaging Extract kit, Stratagene) and plated in the *E.coli* cell line XL1-Blue MRF' (which is McrA$^-$McrB$^-$ strain and does not digest hemimethylated DNA).

The final library from the AD hippocampus contained approximately $1.7x10^5$ pfu/ml and was amplified to a titer of approximately $1x10^8$ pfu/ml with an average insert size of 0.8 kb.

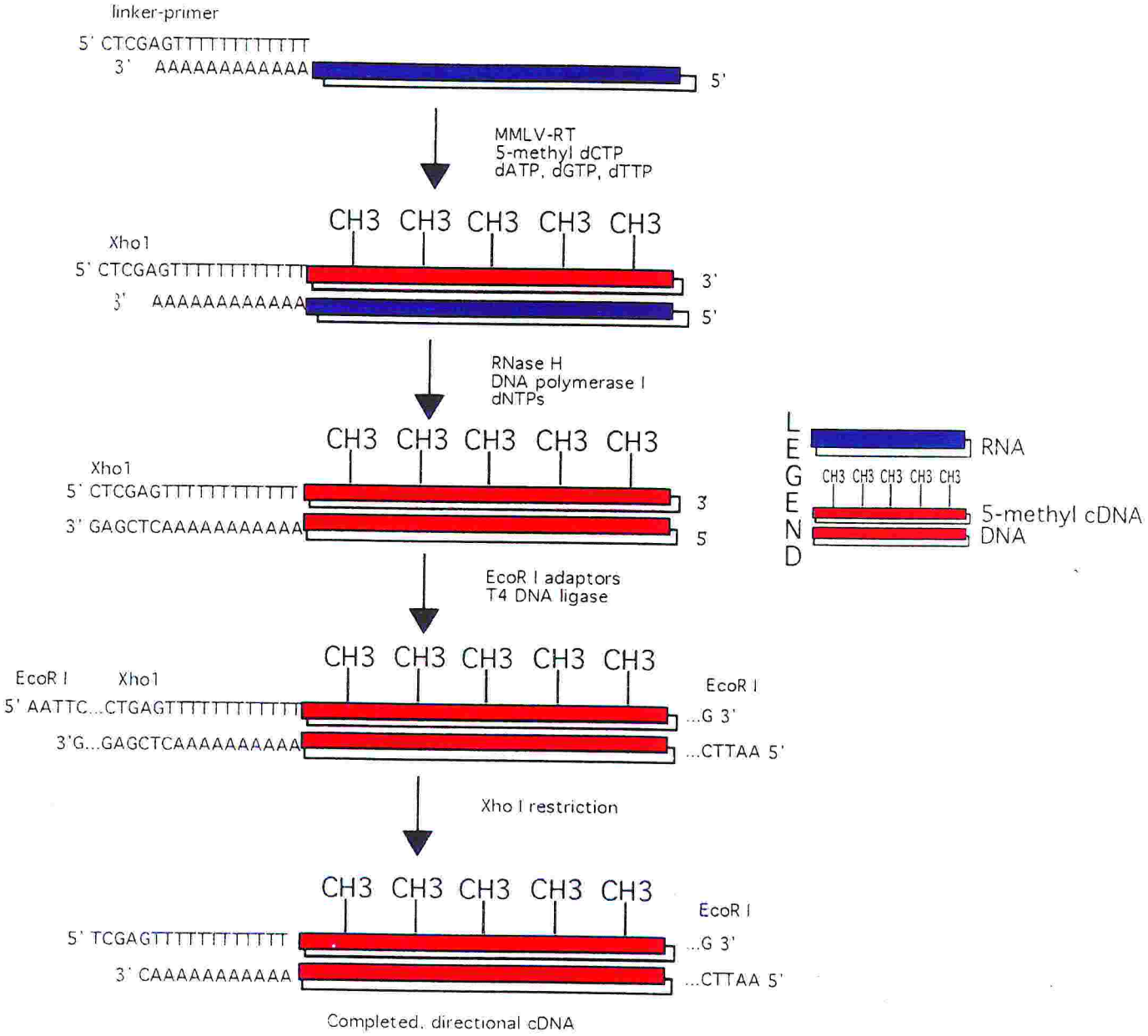
4.4.5. Visualization Of The Insert Size Of Clones From The cDNA Library

Single plaques were picked up and dissolved in 1 ml of SM buffer with chloroform (2% v/v), the PCR was carried out on 1 ml of these primary phage stocks using T3 and T7 commercial primers and AmpliTaq polymerase (Qiagen) with the following reaction parameters: 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min; 55 °C for 1 min; 72 °C for 2 min, on a programmable thermocycler (MJ Research, Inc). PCR fragments' sizes were determined electrophoretically on 1% TBE agarose gels.

The same protocol was used to amplify *in vivo* excised clones, picking sigle colonies directly into the reaction mix.

In the next two pages, Figure 35. Construction of the cDNA library in ZAP Express vector.
In a) the cDNA synthesis flow chart is shown. The sequences of the oligonucletide linker-primer and adapters are shown in b) and c). The expression cassette in ZAP Express and pBK-CMV vectors is shown in d). In e) the ZAP Express vector excision is shown. Individual λ phage or an amplified library are allowed to infect *E. coli* cells which are co-infected with filamentous helper phage. Inside the cell, trans-acting proteins from helper phage recognize initiator (I) and terminator (T) domains within the ZAP Express vector arms. Both of these signals are recognized by the helper phage gene II protein and a new DNA strand is synthesized, displacing the existing strand. The displaced strand is circularized by the helper phage proteins, and secreted from the cell as a phagemid. pBK-CMV plasmids are recovered by infecting an F' strain and growing it in the presence of kanamycin.

A. cDNA synthesis flow chart



B. Oligonucleotide linker-primer

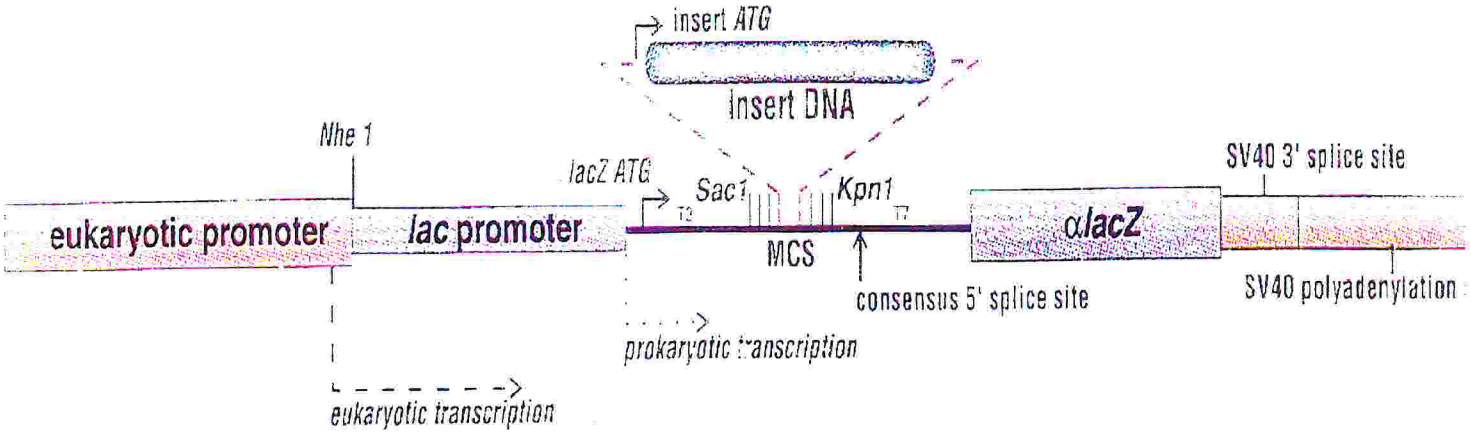
5' GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTT 3'

"GAGA" Sequence Xho I Poly(dT)

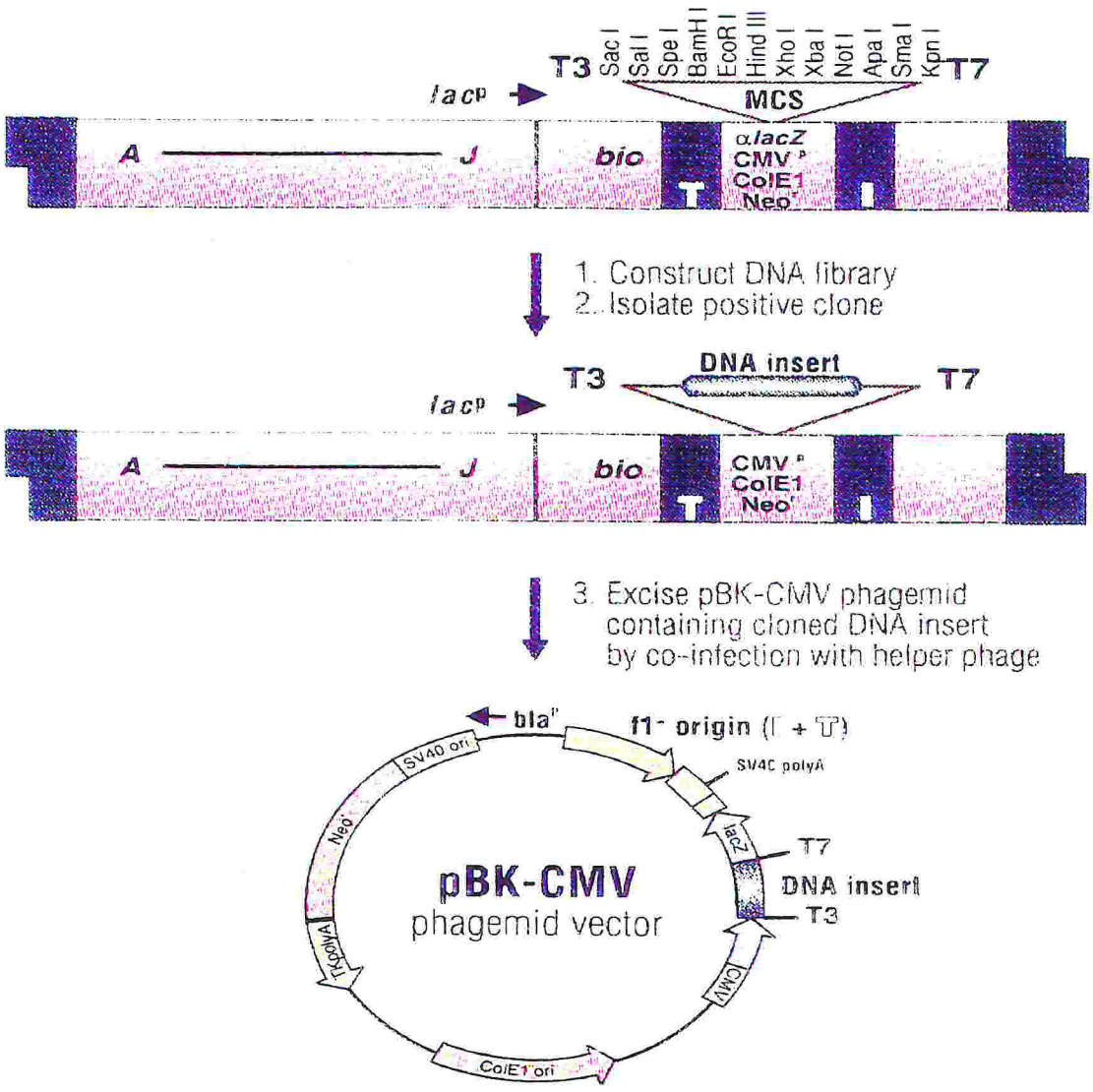
C. Adapters

5' AATTCGGCACGAG 3'
3' GCCGTGCTC 5'

D.


insert ATG
Insert DNA
Nhe 1
lacZ ATG
Sac1
Kpn1
SV40 3' splice site
eukaryotic promoter
lac promoter
MCS
αlacZ
consensus 5' splice site
SV40 polyadenylation
prokaryotic transcription
eukaryotic transcription

E.


T3
Sac I
Sal I
Spe I
BamH I
EcoR I
Hind III
Xho I
Xba I
Not I
Apa I
Sma I
Kpn I
T7
MCS
lacp
A
J
bio
αlacZ
CMV
ColE1
Neo
1. Construct DNA library
2. Isolate positive clone
DNA insert
3. Excise pBK-CMV phagemid containing cloned DNA insert by co-infection with helper phage
bla
f1 origin
SV40 ori
SV40 polyA
lacZ
DNA insert
pBK-CMV
phagemid vector
Neo
TK poly A
CMV
ColE1 ori

4.5. IMMUNOLOGICAL SCREENING OF THE cDNA LIBRARY

4.5.1. Human Sera

Human sera were obtained from the Netherland Institute for Brain Research and from the I.R.C.C.S. San Giovanni di Dio, Sacred Heart Hospital-FBF, Brescia, Italy. Sera were obtained from peripheral blood after centrifugation and stored at -70 °C until use.

In Table 14 are listed all sera samples available for this study.

TABLE 14. Sera available for this study.

CODE NUMBER	CLINICAL DIAGNOSIS	AGE	SEX	APOE GENOTYPE	ORIGIN
1017	MILD AD	53	F	ε3ε4	ITALY (BS)
1028	MILD AD	78	F	ε3ε4	ITALY (BS)
1030	MILD AD	65	F	ε4ε4	ITALY (BS)
1035	MILD AD	84	F	ε3ε4	ITALY (BS)
1037	MILD AD	71	F	ε3ε4	ITALY (BS)
1039	MILD AD	76	F	ε3ε3	ITALY (BS)
1046	MILD AD	73	F	ε3ε3	ITALY (BS)
1047	CVD	86	F	ε3ε3	ITALY (BS)
8001	AD	80	F	ε3ε3	ITALY (BS)
8003	AD+CVD	91	F	ε3ε4	ITALY (BS)
8004	AD	82	F	ε3ε4	ITALY (BS)
8005	DVI	89	F	ε3ε4	ITALY (BS)
8006	AD	72	F	ε3ε4	ITALY (BS)
8008	AD	90	F	ε3ε3	ITALY (BS)
8009	PD	72	M	ε3ε3	ITALY (BS)
8010	AD	80	F	ε3ε4	ITALY (BS)
8011	AD	78	F	ε3ε4	ITALY (BS)

TABLE 14. Continued.

CODE NUMBER	CLINICAL DIAGNOSIS	AGE	SEX	APOE GENOTYPE	ORIGIN
8012	AD	72	F	ε3ε4	ITALY (BS)
8013	AD	71	F	ε3ε4	ITALY (BS)
8014	AD			ε3ε4	ITALY (BS)
9013	SDAT		F	ε3ε3	ITALY (PV)
9014	SDAT		F	ε4ε4	ITALY (PV)
9015	SDAT		F	ε3ε4	ITALY (PV)
9016	SDAT		M	ε4ε4	ITALY (PV)
	SEVERE AD	58	F	ε3ε3	HOLLAND
	MODERATE AD	74	F		HOLLAND
	AMNESIA	75	M	ε2ε3	HOLLAND
	SEVERE AD	65	F		HOLLAND
	SEVERE AD	75	F		HOLLAND
K	SEVERE AD	73	F	ε4ε4	HOLLAND
	MILD AD	60	M		HOLLAND
	MODERATE AD	81	F		HOLLAND
J	SEVERE AD	66	F	ε4ε4	HOLLAND
	MIXED DEMENTIA	72	M	ε3ε3	HOLLAND
W	MILD AD	76	M	ε3ε4	HOLLAND
	MILD AD	80	F		HOLLAND
V	MODERATE AD	79	M	ε4ε4	HOLLAND
	MILD AD	70	M	ε2ε4	HOLLAND
	MODERATE AD	69	M	ε3ε3	HOLLAND

4.5.2. Sera Preadsorption

Before use in immunological screening human sera were extensively preabsorbed against bacterial and phage proteins to remove the background due to antibodies directed against bacterial and phage antigens. Preabsorption procedure consisted of at least three steps:

1) preabsorption against *E.coli* proteins

2a) and b) preabsorption against phage and phage induced *E.coli* proteins.

The whole (or single steps of the) procedure were repeated until sera were sufficienly preabsorbed. To test the quality of sera a pilot immunoscreening on a few plates was performed as described in 4.5.3.: sera are considered suitable for immunoscreening when they give a visible but low background on bacterial lawn and no background on lytic plaques.

4.5.2.1. Preabsorption Against E.coli Proteins

A bacterial lysate from a 50 ml overnight culture of XL1 Blue MRF' was obtained by sonication and allowed to bind to glutardialdehyde activated silica matrix (Boehringer Mannheim).

Serum 1:10 diluted in TBS containing 0.1% low-fat milk, 0.05% NaN_3 and 0.05% Thimerosal was incubated with the lysate bound to the matrix at 4 °C overnight end over end rotating.

4.5.2.2. Preabsorption Against Phage And Phage-Induced E.coli Proteins

a) 50 ml of a culture of XL1 Blue MRF' are grown to OD_{600} of 0.5 and infected with unrecombinant phage (empty vector used for the construction of the library). A bacterial lysate from this 50 ml overnight culture was obtained and incubated with serum as described above (4.5.2.1).

b) Unrecombinant phage (empty vector used for the construction of the library) were plated at a density of about 10.000 pfu/140 mm plate on LB plates with XL1-Blue

MRF' lawn bacteria and processed as in the primary screening (described in 4.5.3.) with the only differences that 1:10 diluted serum from the above preabsorption steps was used as a primary antibody and no incubation with the secondary antibody was performed. Each serum was incubated with at least five different filters.

4.5.3. Primary Screening

Approximately 10^5 pfu from the AD hippocampus cDNA library were plated at a density of about 5000 pfu/140 mm plate on LB plates with XL1-Blue MRF' lawn bacteria (600 µl of cells at an OD_{600} of 0.5) induced with 50 mM IPTG (Talent). Plates were incubated at 37 °C overnight, then blotted onto nitrocellulose filters (Schleicher & Schuell) at 37 °C for 3-4 hours and chilled at 4 °C for 1-2 hours to prevent the LB top agar from sticking to the filters. Then filters were extensively washed in TBST (TBS added with 0.05% Tween 20), blocked for 1 hr at room temperature with 5% low-fat milk in TBS and incubated overnight at room temperature with preabsorbed sera 1:100 diluted in TBS containing 0.1% low-fat milk, 0.05% NaN_3 and 0.05% Thimerosal.
After washings in TBS, filters were incubated with an alkaline phosphatase-conjugated goat anti-human IgG Fc_γ fragment specific (Dianova) and positive plaques were visualized by staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Boehringer Mannheim).

4.5.4. Secondary And Tertiary Screening

A square centimeter "window" around clones identified as primary "putative" positives was taken from the primary plates and dissolved in SM buffer with chloroform (2% v/v) (SM buffer, 1 l: 5.8 g of NaCl; 2 g of $MgSO_4x7H_2O$; 50 ml of 1M Tris-HCl, pH 7.5, 5 ml of 2% gelatin in H_2O). Phage from this primary phage stocks were titered and plated on secondary 90 mm LB plates (with the same host cells as in the primary screening) to obtain about 500-1000 well isolated plaques. Secondary screening was then performed as described above (4.5.3.).

Clones which were positive after secondary screening were picked up from secondary plates if well isolated or the whole procedure was repeated once again to obtain well isolated plaques and the individual positive clones were picked up from tertiary plates. Purity and insert size of the positive clones was checked by PCR (as described above, 4.4.5.).

4.5.5. Screening With Anti-Human IgG

Clones which were positive after secondary screening were screened with the secondary antibody as a primary antibody to exclude they are human IgGs by using the same procedure described above (4.5.3.).

4.5.6. Screening With Other AD And NDC (Non Demented Controls) Sera

Clones which were positive after primary screening with one AD serum were confirmed for that serum by secondary (and if it was necessary by tertiary) screening (4.5.4.) and screened with sera from other AD patients and healthy age-matched controls with the same procedure used for secondary screening (4.5.4.). These screenings were performed on primary putative positive clones to ensure the presence on the plates of internal negative controls.

4.6. SEQUENCE ANALYSIS OF POSITIVE CLONES

Positive clones subcloned to monoclonality were submitted to *in vivo* excision (4.6.1.). The nucleotide sequence of cDNA inserts was determined (4.6.2.) and sequence data analyzed (4.6.3.).

4.6.1. *In vivo* Excision Of The pBK-CMV Vector From The ZAP Express Vector.

The ZAP Express vector has been designed to allow simple, efficient *in vivo* excision and recircularization of any insert cloned within the vector. Following *in vivo* excision, the insert is found within a phagemid pBK-CMV, which permits both

prokaryotic and eukaryotic expression (Short *et al.*, 1988). The protocol included the use of the ExAssist helper phage with XLOLR *E.coli* strain, to increase the efficiency of excision. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a nonsuppressing *E.coli* strain such as XLOLR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of productive co-infection from the ExAssist helper phage. Since the ExAssist helper phage cannot replicate in the XLOLR strain, single-stranded rescue cannot be performed in this strain using this helper phage. XLOLR cells are also resistant to λ infection, preventing λDNA contamination after excision. The excision was performed following the manifacturers instructions. Briefly, plaque of interest was picked up from the agar plate and incubated in 500 µl of SM buffer and 20 µl of chloroform overnight at 4 °C. 250 µl of the phage stock was mixed with 200 µl of XL1-Blue MRF' cells at OD_{600} of 1.0 and 1 µl of the ExAssist helper phage (> $1x10^6$ pfu/µl) and incubated at 37°C for 15 min. 3 ml of NZY broth were added and incubated for further 2-3 hours at 37 °C with shaking. The mixture was heated at 65-70 °C for 20 min and spin at 1000xg for 15 min. The supernatant was stored at 4 °C. To plate the excised phagemids, 200 µl of XLOLR cells (OD_{600} of 1.0) was added to 100 µl of the phage supernatant and incubated for further 45 min. XLOLR cells containing pBK-CMV double-stranded phagemid vector with cloned DNA insert were plated on LB-kanamycin agar plates.

4.6.2. Sequencing

Plasmids were purified by using QIAQuick Minipreps Kit (Qiagen) and sequencing was performed using the SequiTherm EXCEL™ II Long-Read™ DNA Sequencing Kit-LC (Epicentre Technologies) with an automatic DNA sequencer (model 4000L by MWG-BIOTECH) according to the manufacter's instructions.

4.6.3. Sequence Alignments

Sequence data were analyzed with BLAST (Altschul *et al.*, 1997) software on EMBL and PROSITE software (Bucher and Bairoch, 1994; Bairoch *et al.*, 1997) on SIB.

4.7. EXPRESSION STUDIES OF POSITIVE CLONES

To study the expression of the positive clones isolated as mRNAs and proteins a Northern (4.7.1.) and Western blot (4.7.2.) analysis were performed respectively.

4.7.1. Northern Blot Analysis

20 µg of the total RNA extracted from several tissues as described above (4.4.2.) were electrophoresed on 1% denaturing formaldehyde agarose gel and transferred to the nylon membranes (Hybond^{TM}-N membranes - Amersham, Life Science) by classical capillary transfer. The dried membranes were fixed by exposure to UV light (120,000 mJ of UV energy for 30 seconds). After prehybridization the membranes were incubated overnight at 42 °C in a small volume (3-5 ml) of hybridization solution (50% formamyde, 6X SSC, 5X Denhardt's solution, 0.5% SDS, 100 mg/ml of acid-alkali cleaved salmon sperm DNA) with a ^{32}P-labeled specific probe added to a final concentration of >10^6 cpm/ml (10 pmol/ml) of hybridization solution. The membranes were washed at progressively higher stringency, with the final wash in 1X SSC and 0.1% SDS at 65 °C for 20 min and exposed to Kodak X-AR films at least for 16 hours or subjected to analysis by Phosphorimager (Packard). After exposure the filters were stripped and rehybridized with GAPDH to prove RNA integrity.

Alternatively, human 12-Lane Multiple Tissue Northern (MTN^{TM}) from Clontech on which 2 µg of poly(A)$^+$RNA from 12 different human tissues were used according to the manufacters instructions. Briefly, membranes were prehybridized in 5 ml of ExpressHyb Hybridation Solution (Clontech) with continuous shaking at 68 °C for 30 min, hybridized for 1 h with the specific radiolabeled probes and then washed at progressively higher stringency with the final wash in 0.1X SSC and 0.1% SDS at 50°C

for 40 min with one change of fresh solution. Filters are exposed to Kodak X-AR films at least for 16 hours and/or subjected to analysis by Phosphorimager (Packard).

4.7.2. Western Blot Analysis On Brain Homogenates

4.7.2.1. Preparation Of Brain Homogenates

Brain tissues were homogenized in SDS-PAGE loading buffer (5v/wt) on ice, centrifuged and supernatants were passed several times through an insulin needle, centrifuged again, added with β-mercaptoethanol (10% v/v) and stored at -70 °C until used.

4.7.2.2. Production Of Polyclonal Rabbit Antisera

Approximately 1 mg (200 µg per immunization) of each purified His-tagged fusion protein (4.8.1.) was used to immunize a rabbit. Sera before and after immunization were tested by Western blot for reactivity with the His-tagged fusion protein used for immunization.

4.7.2.3. Western Blot Analysis

SDS-PAGE was perfomed as described by Laemmli (Laemmli, 1970). After electrophoresis, proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell) in a semi-dry system (Tris-glycine-methanol). After blocking with 5% low-fat milk in TBS for 1 hr at room temperature, membranes were incubated with the primary antibody (human sera or rabbit antisera) appropriately diluted in 5% low-fat milk in TBS overnight at room temperature (human sera) or 4 °C (rabbit antisera). After washes in TBS, the blots were incubated for 1 hr at room temperature with the secondary antibody (an alkaline-phosphatase conjugated anti-human IgG, Dianova, for human sera; an alkaline-phosphatase conjugated anti-rabbit IgG, Sigma, for rabbit antisera) and detected by staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

4.8. DETECTION OF AD1- AND AD2-SPECIFIC ANTIBODIES IN HUMAN SERA

By immunoscreening of the AD hippocampal cDNA library two clones (named AD1 and AD2) were isolated, each of them was recognized by 2 out of 10 AD sera and 0 out of 4 control sera. To test an higher number of human sera an ELISA experiment was set up (4.8.3.) by using the single antigens purified (4.8.1.).

4.8.1. Production Of His-Tagged Recombinant Proteins

The expression and purification of His-tagged recombinant proteins were performed using the Xpress™ System Protein Expression TrcHis and Purification kits (Invitrogen).

Positive cDNAs were recovered from pBK-CMV phagemid vector by restriction digestion with the enzymes *BamHI* and *KpnI*, gel-purified using the QiaQuick Gel Extraction kit (Qiagen) and ligated in frame to *BamHI -KpnI* digested, dephosphorylated, and gel-purified pTrcHisB (Invitrogen), allowing for the translation of a fusion protein bearing a 6-histidine tail at the N-terminus. To express the His-tagged recombinant proteins, the constructs were transformed into *E.coli* TOP10 strain and selected on ampicillin-containing plates. The presence of cDNA inserts was checked by *BamHI -KpnI* restriction analysis and their identity was verified by Western blot analysis of bacterial pellets expressing recombinant proteins (4.8.2.).

An unrelated His-tagged protein whom cDNA was available in the lab was also purified for using as negative control in successive ELISA experiments (4.8.3.).

To determine the optimal time for expression of the two recombinant proteins as well as their solubility properties a pilot expression experiment was performed. Single recombinant colonies from the two cDNA clones and the vector alone (as a negative control) were grown in liquid medium supplemented with ampicillin (50 µg/ml) and induced with 1 mM IPTG (Talent). Aliquots were removed at different times and bacterial pellets and supernatants were analyzed by SDS-PAGE. From this pilot experiment it was assessed that both proteins are in bacterial pellets so they must be

purified under denaturing conditions and that the optimal time to harvest the cells after induction with IPTG is about five hours. After that, a large-scale induction of recombinant proteins from the respective clones was performed. Cells were harvested 5 hr after induction with 1 mM IPTG. Lysis was performed in Guanidinium Lysis Buffer (6 M Guanidine Hydrochloride; 20 mM Sodium Phosphate; 500 mM Sodium Chloride), pH 7.8, for 10 minutes at room temperature. Cell lysates were sonicated on ice and insoluble debris removed by centrifugation. Supernatants were loaded onto pre-equilibrated ProBond™ Columns (Invitrogen) and allowed the polyHis-containing proteins to fully bind. Washes and elutions were performed using a Denaturing Buffer (8 M Urea; 20 mM Sodium Phosphate; 500 mM Sodium Chloride) with progressively lower pHs, starting from pH 7.8 for binding, to pH 6.0 and 5.3 for washes, to pH 4.0 for elution. Pooled fractions which containing the peak absorbance at 280 nm were dialyzed against 10 mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4 °C to remove the urea. Protein quantification was done by Bio-Rad D_C Protein Assay (BIO-RAD) kit based on the protein quantification method of Lowry (Lowry, 1951).

4.8.2. Western Blot Analysis

SDS-PAGE and Western blot analysis were perfomed as described in 4.7.2.3. Bacterial pellets from 1 ml of culture were directly resuspended in 100 µl of SDS loading buffer added with 10% β-mercaptoethanol and loaded on polyacrylamide gels.

4.8.3. ELISA Experiments

Following production and purification of the three His-tagged proteins, detection of AD1- and AD2-specific antibodies in sera was carried out by ELISA as follows. His-tagged proteins were dissolved in 0.05 M bicarbonate buffer (pH 9.6) at a concentration of 10 µg/ml. The solution (100 µl) was dispensed into Nunc 96-well plates and incubated overnight at 4 °C. After washes with PBST (PBS added with 0.1% Tween 20) plates were incubated with a 5% low-fat milk in PBS solution (120 µl in each well) for 1 h at room temperature, washed with PBST, incubated with 100 µl of human sera

diluted appropriately in 5% low-fat milk in PBS for 1 h at room temperature, washed again with PBST and incubated with 100 µl of the secondary antibody, an anti-human IgG horseradish-peroxidase conjugated (Pierce) 1:20.000 diluted in 5% low-fat milk PBS for 1 h at room temperature. After washes detection was accomplished using 70 µl of TMB (Techna). The reaction was stopped after 1 h incubation with 70 µl of 1 M H_2SO_4 and the absorbance at 450 nm was measured by an ELISA reader (SLT-SPECTRA).

5. REFERENCES

Abraham, C. R., Selkoe, D. J., and Potter, H., 1988, Immunochemical identification of the serine protease inhibitor alpha 1- antichymotrypsin in the brain amyloid deposits of Alzheimer's disease, *Cell* **52**(4):487-501.

Adler, M. J., Coronel, C., Shelton, E., Seegmiller, J. E., and Dewji, N. N., 1991, Increased gene expression of Alzheimer disease beta-amyloid precursor protein in senescent cultured fibroblasts, *Proc Natl Acad Sci U S A* **88**(1):16-20.

Aisen, P. S., and Davis, K. L., 1997, The search for disease-modifying treatment for Alzheimer's disease, *Neurology* **48**(5 Suppl 6):S35-41.

Alafuzoff, I., Iqbal, K., Friden, H., Adolfsson, R., and Winblad, B., 1987, Histopathological criteria for progressive dementia disorders: clinical- pathological correlation and classification by multivariate data analysis, *Acta Neuropathol (Berl)* **74**(3):209-25.

Alberghina, M., Viola, M., Insirello, L., and Giuffrida Stella, A. M., 1988, Age-related changes of RNA and lipid synthesis in vitro by retina and optic nerve of the rat, *Neurochem Pathol* **8**(2):131-48.

Allinquant, B., Moya, K. L., Bouillot, C., and Prochiantz, A., 1994, Amyloid precursor protein in cortical neurons: coexistence of two pools differentially distributed in axons and dendrites and association with cytoskeleton, *J Neurosci* **14**(11 Pt 2):6842-54.

Alonso, A. C., Grundke-Iqbal, I., Barra, H. S., and Iqbal, K., 1997, Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration:

sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau, *Proc Natl Acad Sci U S A* **94**(1):298-303.

Alonso, A. C., Grundke-Iqbal, I., and Iqbal, K., 1996, Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules, *Nat Med* **2**(7):783-7.

Alonso, A. C., Zaidi, T., Grundke-Iqbal, I., and Iqbal, K., 1994, Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease, *Proc Natl Acad Sci U S A* **91**(12):5562-6.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J., 1997, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res* **25**(17):3389-402.

Altstiel, L. D., Lawlor, B., Mohs, R., Schmeidler, J., Dalton, A., Mehta, P., and Davis, K., 1995, Elevated alpha 1-antichymotrypsin serum levels in a subset of nondemented first-degree relatives of Alzheimer's disease patients, *Dementia* **6**(1):17-20.

Amaducci, L. A., Fratiglioni, L., Rocca, W. A., Fieschi, C., Livrea, P., Pedone, D., Bracco, L., Lippi, A., Gandolfo, C., Bino, G., and et al., 1986, Risk factors for clinically diagnosed Alzheimer's disease: a case- control study of an Italian population, *Neurology* **36**(7):922-31.

Andersen, K., Launer, L. J., Ott, A., Hoes, A. W., Breteler, M. M., and Hofman, A., 1995, Do nonsteroidal anti-inflammatory drugs decrease the risk for Alzheimer's disease? The Rotterdam Study, *Neurology* **45**(8):1441-5.

Andreadis, A., Brown, W. M., and Kosik, K. S., 1992, Structure and novel exons of the human tau gene, *Biochemistry* **31**(43):10626-33.

Aoki, T., Gibbs, C. J., Jr., Sotelo, J., and Gajdusek, D. C., 1982, Heterogeneic autoantibody against neurofilament protein in the sera of animals with experimental kuru and Creutzfeldt-Jakob disease and natural scrapie infection, *Infect Immun* **38**(1):316-24.

Appel, R. D., Bairoch, A., and Hochstrasser, D. F., 1994, A new generation of information retrieval tools for biologists: the example of the ExPASy WWW server, *Trends Biochem Sci* **19**(6):258-60.

Arai, H., Lee, V. M., Hill, W. D., Greenberg, B. D., and Trojanowski, J. Q., 1992, Lewy bodies contain beta-amyloid precursor proteins of Alzheimer's disease, *Brain Res* **585**(1-2):386-90.

Arai, H., Lee, V. M., Otvos, L., Jr., Greenberg, B. D., Lowery, D. E., Sharma, S. K., Schmidt, M. L., and Trojanowski, J. Q., 1990, Defined neurofilament, tau, and beta-amyloid precursor protein epitopes distinguish Alzheimer from non-Alzheimer senile plaques, *Proc Natl Acad Sci U S A* **87**(6):2249-53.

Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., and Hyman, B. T., 1992, Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease, *Neurology* **42**(3 Pt 1):631-9.

Bairoch, A., Bucher, P., and Hofmann, K., 1997, The PROSITE database, its status in 1997, *Nucleic Acids Res* **25**(1):217-21.

Bannert, N., Baier, M., Werner, A., and Kurth, R., 1996, Interleukin-16 or not? [letter], *Nature* **381**(6577):30.

Bartus, R. T., Dean, R. L. d., Beer, B., and Lippa, A. S., 1982, The cholinergic hypothesis of geriatric memory dysfunction, *Science* **217**(4558):408-14.

Beffert, U., Bertrand, P., Champagne, D., Gauthier, S., and Poirier, J., 1998, HSV-1 in brain and risk of Alzheimer's disease [letter; comment] [see comments], *Lancet* **351**(9112):1330-1.

Behl, C., Davis, J., Cole, G. M., and Schubert, D., 1992, Vitamin E protects nerve cells from amyloid beta protein toxicity, *Biochem Biophys Res Commun* **186**(2):944-50.

Behl, C., Davis, J. B., Lesley, R., and Schubert, D., 1994, Hydrogen peroxide mediates amyloid beta protein toxicity, *Cell* **77**(6):817-27.

Benditt, E. P., 1989, What role(s) may extracellular matrix, particularly heparan sulfate, play in amyloid of Alzheimer's disease, *Neurobiol Aging* **10**(5):506; discussion 510-2.

Beyer, C. F., Tepper, D. J., and Hill, J. M., 1989, Cryogenic induced ocular HSV-1 reactivation is enhanced by an inhibitor of the lipoxygenase pathway, *Curr Eye Res* **8**(12):1287-92.

Bird, T. D., 1995, Apolipoprotein E genotyping in the diagnosis of Alzheimer's disease: a cautionary view [editorial; comment] [see comments], *Ann Neurol* **38**(1):2-4.

Blennow, K., 1998, Biochemical markers aid the diagnosis of Alzheimer's disease, in: *6th International Conference on Alzheimer's Disease and Related Disorders*, Neurobiology of Aging, **19** (4S), Amsterdam, pp. S233.

Blennow, K., Wallin, A., Agren, H., Spenger, C., Siegfried, J., and Vanmechelen, E., 1995, Tau protein in cerebrospinal fluid: a biochemical marker for axonal degeneration in Alzheimer disease?, *Mol Chem Neuropathol* **26**(3):231-45.

Bongioanni, P., Boccardi, B., Borgna, M., Castagna, M., and Mondino, C., 1997, T-cell interferon gamma binding in patients with dementia of the Alzheimer type, *Arch Neurol* **54**(4):457-62.

Bongioanni, P., Boccardi, B., Borgna, M., and Rossi, B., 1998, T-lymphocyte interleukin 6 receptor binding in patients with dementia of Alzheimer type, *Arch Neurol* **55**(10):1305-8.

Boon, T., Cerottini, J. C., Van den Eynde, B., van der Bruggen, P., and Van Pel, A., 1994, Tumor antigens recognized by T lymphocytes, *Annu Rev Immunol* **12**:337-65.

Bortolotto, Z. A., Bashir, Z. I., Davies, C. H., and Collingridge, G. L., 1994, A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation, *Nature* **368**(6473):740-3.

Boyles, J. K., Zoellner, C. D., Anderson, L. J., Kosik, L. M., Pitas, R. E., Weisgraber, K. H., Hui, D. Y., Mahley, R. W., Gebicke-Haerter, P. J., Ignatius, M. J., and et al., 1989, A role for apolipoprotein E, apolipoprotein A-I, and low density lipoprotein receptors in cholesterol transport during regeneration and remyelination of the rat sciatic nerve, *J Clin Invest* **83**(3):1015-31.

Bowen, D. M., White, P., Spillane, J. A., Goodhardt, M. J., Curzon, G., Iwangoff, P., Meier-Ruge, W., and Davison, A. N., 1979, Accelerated ageing or selective neuronal loss as an important cause of dementia?, *Lancet* **1**(8106):11-4.

Braak, H., and Braak, E., 1991, Neuropathological stageing of Alzheimer-related changes, *Acta Neuropathol (Berl)* **82**(4):239-59.

Braak, H., and Braak, E., 1994, Morphological criteria for the recognition of Alzheimer's disease and the distribution pattern of cortical changes related to this disorder, *Neurobiol Aging* **15**(3):355-6; discussion 379-80.

Braak, H., and Braak, E., 1996, Development of Alzheimer-related neurofibrillary changes in the neocortex inversely recapitulates cortical myelogenesis, *Acta Neuropathol (Berl)* **92**(2):197-201.

Braak, H., and and Braak, E., 1997, Pattern of Cortical Lesions in Alzheimer's Disease, in: *Alzheimer's Disease: Biology, Diagnosis and Therapeutics* (W. B. Iqbal K, Nishimura T, Takeda M and Wisniewski /HM, ed.), John Wiley & Sons Ltd, Chicester, pp. 227-237.

Braak, H., Braak, E., Grundke-Iqbal, I., and Iqbal, K., 1986, Occurrence of neuropil threads in the senile human brain and in Alzheimer's disease: a third location of paired helical filaments outside of neurofibrillary tangles and neuritic plaques, *Neurosci Lett* **65**(3):351-5.

Brakeman, P. R., Lanahan, A. A., O'Brien, R., Roche, K., Barnes, C. A., Huganir, R. L., and Worley, P. F., 1997, Homer: a protein that selectively binds metabotropic glutamate receptors [see comments], *Nature* **386**(6622):284-8.

Brass, N., Heckel, D., Sahin, U., Pfreundschuh, M., Sybrecht, G. W., and Meese, E., 1997, Translation initiation factor eIF-4gamma is encoded by an amplified gene and induces an immune response in squamous cell lung carcinoma, *Hum Mol Genet* **6**(1):33-9.

Breen, K. C., Bruce, M., and Anderton, B. H., 1991, Beta amyloid precursor protein mediates neuronal cell-cell and cell- surface adhesion, *J Neurosci Res* **28**(1):90-100.

Breitner, J. C., Gau, B. A., Welsh, K. A., Plassman, B. L., McDonald, W. M., Helms, M. J., and Anthony, J. C., 1994, Inverse association of anti-inflammatory treatments and Alzheimer's disease: initial results of a co-twin control study, *Neurology* **44**(2):227-32.

Breitner, J. C., Welsh, K. A., Helms, M. J., Gaskell, P. C., Gau, B. A., Roses, A. D., Pericak-Vance, M. A., and Saunders, A. M., 1995, Delayed onset of Alzheimer's disease with nonsteroidal anti- inflammatory and histamine H2 blocking drugs, *Neurobiol Aging* **16**(4):523-30.

Brenner, D. E., Kukull, W. A., van Belle, G., Bowen, J. D., McCormick, W. C., Teri, L., and Larson, E. B., 1993, Relationship between cigarette smoking and Alzheimer's disease in a population-based case-control study, *Neurology* **43**(2):293-300.

Brugge, K., Katzman, R., Hill, L. R., Hansen, L. A., and Saitoh, T., 1992, Serological alpha 1-antichymotrypsin in Down's syndrome and Alzheimer's disease, *Ann Neurol* **32**(2):193-7.

Bucher, P., and Bairoch, A., 1994, A generalized profile syntax for biomolecular sequence motifs and its function in automatic sequence interpretation, *Ismb* **2**:53-61.

Bush, A. I., Pettingell, W. H., Multhaup, G., d Paradis, M., Vonsattel, J. P., Gusella, J. F., Beyreuther, K., Masters, C. L., and Tanzi, R. E., 1994a, Rapid induction of Alzheimer A beta amyloid formation by zinc [see comments], *Science* **265**(5177):1464-7.

Bush, A. I., Pettingell, W. J., de Paradis, M., Tanzi, R. E., and Wasco, W., 1994b, The amyloid beta-protein precursor and its mammalian homologues. Evidence for a zinc-modulated heparin-binding superfamily, *J Biol Chem* **269**(43):26618-21.

Butterfield, D. A., Hensley, K., Harris, M., Mattson, M., and Carney, J., 1994, beta-Amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: implications to Alzheimer's disease, *Biochem Biophys Res Commun* **200**(2):710-5.

Cai, X. D., Golde, T. E., and Younkin, S. G., 1993, Release of excess amyloid beta protein from a mutant amyloid beta protein precursor [see comments], *Science* **259**(5094):514-6.

Candore, G., Di Lorenzo, G., Mansueto, P., Melluso, M., Frada, G., Li Vecchi, M., Esposito Pellitteri, M., Drago, A., Di Salvo, A., and Caruso, C., 1997, Prevalence of organ-specific and non organ-specific autoantibodies in healthy centenarians, *Mech Ageing Dev* **94**(1-3):183-90.

Capell, A., Grunberg, J., Pesold, B., Diehlmann, A., Citron, M., Nixon, R., Beyreuther, K., Selkoe, D. J., and Haass, C., 1998, The proteolytic fragments of the Alzheimer's disease-associated presenilin-1 form heterodimers and occur as a 100-150-kDa molecular mass complex, *J Biol Chem* **273**(6):3205-11.

Chao, C. C., Ala, T. A., Hu, S., Crossley, K. B., Sherman, R. E., Peterson, P. K., and Frey, W. H. n., 1994, Serum cytokine levels in patients with Alzheimer's disease, *Clin Diagn Lab Immunol* **1**(4):433-6.

Chapman, J., Bachar, O., Korczyn, A. D., Wertman, E., and Michaelson, D. M., 1989, Alzheimer's disease antibodies bind specifically to a neurofilament protein in Torpedo cholinergic neurons, *J Neurosci* **9**(8):2710-7.

Chapman, J., Korczyn, AD, Hareuveni, M and Michaelson, DM, 1986, Antibodies to cholinergic cell bodies in Alzheimer's disease, in: *Alzheimer's disease and Parkinson's disease* (H. I. a. L. C. Fisher A, ed.), Plenum Press, New York, pp. 329-336.

Choi, J. Y., Morris, J. C., and Hsu, C. Y., 1998, Aging and cerebrovascular disease, *Neurol Clin* **16**(3):687-711.

Chomczynski, P., and Sacchi, N., 1987, Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction, *Anal Biochem* **162**(1):156-9.

Christie, R. H., Chung, H., Rebeck, G. W., Strickland, D., and Hyman, B. T., 1996, Expression of the very low-density lipoprotein receptor (VLDL-r), an apolipoprotein-E receptor, in the central nervous system and in Alzheimer's disease, *J Neuropathol Exp Neurol* **55**(4):491-8.

Clark, A. W., and Parhad, I. M., 1989, Expression of neuronal mRNAs in Alzheimer type degeneration of the nervous system, *Can J Neurol Sci* **16**(4 Suppl):477-82.

Clay, M. A., Anantharamaiah, G. M., Mistry, M. J., Balasubramaniam, A., and Harmony, J. A., 1995, Localization of a domain in apolipoprotein E with both cytostatic and cytotoxic activity, *Biochemistry* **34**(35):11142-51.

Cleveland, D. W., Hwo, S. Y., and Kirschner, M. W., 1977a, Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly, *J Mol Biol* **116**(2):227-47.

Cleveland, D. W., Hwo, S. Y., and Kirschner, M. W., 1977b, Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin, *J Mol Biol* **116**(2):207-25.

Conn, P. J., and Pin, J. P., 1997, Pharmacology and functions of metabotropic glutamate receptors, *Annu Rev Pharmacol Toxicol* **37**:205-37.

Contois, J. H., Anamani, D. E., and Tsongalis, G. J., 1996, The underlying molecular mechanism of apolipoprotein E polymorphism: relationships to lipid disorders, cardiovascular disease, and Alzheimer's disease, *Clin Lab Med* **16**(1):105-23.

Cook, D. G., Sung, J. C., Golde, T. E., Felsenstein, K. M., Wojczyk, B. S., Tanzi, R. E., Trojanowski, J. Q., Lee, V. M., and Doms, R. W., 1996, Expression and analysis of presenilin 1 in a human neuronal system: localization in cell bodies and dendrites, *Proc Natl Acad Sci U S A* **93**(17):9223-8.

Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L., and Pericak-Vance, M. A., 1993, Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families [see comments], *Science* **261**(5123):921-3.

Crews, D. E., Kamboh, M. I., Mancilha-Carvalho, J. J., and Kottke, B., 1993, Population genetics of apolipoprotein A-4, E, and H polymorphisms in Yanomami Indians of northwestern Brazil: associations with lipids, lipoproteins, and carbohydrate metabolism, *Hum Biol* **65**(2):211-24.

Crutcher, K., Tolar, M, Harmony, JAK, and Marques, MA, 1997, A New Hypothesis for the Role of Apolipoprotein E in Alzheimer's disease Pathology, in: *Alzheimer's*

Disease: Biology, Diagnosis and Therapeutics (K. Iqbal, Winblad, B, Nishimura, T, Takeda, M, Wisniewski, HM, ed.), John Wiley & Sons, Chichester, pp. 543-552.

Crutcher, K. A., Clay, M. A., Scott, S. A., Tian, X., Tolar, M., and Harmony, J. A., 1994, Neurite degeneration elicited by apolipoprotein E peptides, *Exp Neurol* **130**(1):120-6.

Curtiss, L. K., Forte, T. M., and Davis, P. A., 1984, Cord blood plasma lipoproteins inhibit mitogen-stimulated lymphocyte proliferation, *J Immunol* **133**(3):1379-84.

D'Angelo, C. D. A. a. G., 1986, Anticorpi verso tangles e placche senili, simili ad anticorpi antineurofilamento, in sieri di dementi presenili, senili e sieri di pazienti affetti da sindrome di Down, *EOS-ROV-Imm.* **6**:8.

Dahlstrom, A., Wigander, A., Lundmark, K., Gottfries, C. G., Carvey, P. M., and McRae, A., 1990, Investigations on auto-antibodies in Alzheimer's and Parkinson's diseases, using defined neuronal cultures, *J Neural Transm Suppl* **29**:195-206.

Davidsson, P., Jahn, R., Bergquist, J., Ekman, R., and Blennow, K., 1996, Synaptotagmin, a synaptic vesicle protein, is present in human cerebrospinal fluid: a new biochemical marker for synaptic pathology in Alzheimer disease?, *Mol Chem Neuropathol* **27**(2):195-210.

Davies, P., and Maloney, A. J., 1976, Selective loss of central cholinergic neurons in Alzheimer's disease [letter], *Lancet* **2**(8000):1403.

De Sauvage, F., and Octave, J. N., 1989, A novel mRNA of the A4 amyloid precursor gene coding for a possibly secreted protein, *Science* **245**(4918):651-3.

De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K., and Van Leuven, F., 1998, Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein [see comments], *Nature* **391**(6665):387-90.

Delacourte, A., and Buee, L., 1997, Normal and pathological Tau proteins as factors for microtubule assembly, *Int Rev Cytol* **171**:167-224.

Despres, N., Talbot, G., Plouffe, B., Boire, G., and Menard, H. A., 1995, Detection and expression of a cDNA clone that encodes a polypeptide containing two inhibitory domains of human calpastatin and its recognition by rheumatoid arthritis sera, *J Clin Invest* **95**(4):1891-6.

Doebler, J. A., Rhoads, R. E., Anthony, A., and Markesbery, W. R., 1989, Neuronal RNA in Pick's and Alzheimer's diseases. Comparison of disease- susceptible and disease-resistant cortical areas, *Arch Neurol* **46**(2):134-7.

Doll, R., 1993, Review: Alzheimer's disease and environmental aluminium [see comments], *Age Ageing* **22**(2):138-53.

Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R., 1996, Crystal structures of a complexed and peptide-free membrane protein- binding domain: molecular basis of peptide recognition by PDZ, *Cell* **85**(7):1067-76.

Drubin, D. G., and Kirschner, M. W., 1986, Tau protein function in living cells, *J Cell Biol* **103**(6 Pt 2):2739-46.

Eikelenboom, P., and Stam, F. C., 1982, Immunoglobulins and complement factors in senile plaques. An immunoperoxidase study, *Acta Neuropathol (Berl)* **57**(2-3):239-42.

Ershler, W. B., 1993, Interleukin-6: a cytokine for gerontologists, *J Am Geriatr Soc* **41**(2):176-81.

Ewins, D. L., Rossor, M. N., Butler, J., Roques, P. K., Mullan, M. J., and McGregor, A. M., 1991, Association between autoimmune thyroid disease and familial Alzheimer's disease, *Clin Endocrinol (Oxf)* **35**(1):93-6.

Fagan, A. M., Bu, G., Sun, Y., Daugherty, A., and Holtzman, D. M., 1996, Apolipoprotein E-containing high density lipoprotein promotes neurite outgrowth and is a ligand for the low density lipoprotein receptor- related protein, *J Biol Chem* **271**(47):30121-5.

Fang, Q., Kannapell, C. C., Fu, S. M., Xu, S., and Gaskin, F., 1995, VH and VL gene usage by anti-beta-amyloid autoantibodies in Alzheimer's disease: detection of highly mutated V regions in both heavy and light chains, *Clin Immunol Immunopathol* **75**(2):159-67.

Farrer, L. A., Cupples, L. A., Haines, J. L., Hyman, B., Kukull, W. A., Mayeux, R., Myers, R. H., Pericak-Vance, M. A., Risch, N., and van Duijn, C. M., 1997, Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium [see comments], *Jama* **278**(16):1349-56.

Farrer, L. A., Cupples, L. A., van Duijn, C. M., Kurz, A., Zimmer, R., Muller, U., Green, R. C., Clarke, V., Shoffner, J., Wallace, D. C., and et al., 1995, Apolipoprotein E genotype in patients with Alzheimer's disease: implications for the risk of dementia among relatives, *Ann Neurol* **38**(5):797-808.

Feldman, H., Kennard, M., Yamada, T., Adams, S., and Jefferies, W., 1997, Serum Levels of the Iron Binding Protein p97: A Novel Biological Marker of Alzheimer's Disease, in: *Alzheimer's Disease: Biology, Diagnosis and Therapeutics* (K. Iqbal, Winblad, B.,Nishimura, T.,Takeda, M., and Wisniewski, H.M., ed.), John Wiley & Sons, Chichester, pp. 189-196.

Fernandez-Shaw, C., Marina, A., Cazorla, P., Valdivieso, F., and Vazquez, J., 1997, Anti-brain spectrin immunoreactivity in Alzheimer's disease: degradation of spectrin in an animal model of cholinergic degeneration, *J Neuroimmunol* **77**(1):91-8.

Fillit, H., 1994, Estrogens in the pathogenesis and treatment of Alzheimer's disease in postmenopausal women, *Ann N Y Acad Sci* **743**:233-8; discussion 238-9.

Fillit, H., Luine, VN, Reisberg, B, Amador, R, McEwen, B and Zabriskie, JB, 1985, Studies of the specificity of antibrain antibodies in Alzheimer's disease, in: *Senile Dementia of the Alzheimer's Type* (H. J. a. K. AD, ed.), Liss, New York, pp. 307-318.

Fillit, H. M., Kemeny, E., Luine, V., Weksler, M. E., and Zabriskie, J. B., 1987, Antivascular antibodies in the sera of patients with senile dementia of the Alzheimer's type, *J Gerontol* **42**(2):180-4.

Foley, P., Bradford, H. F., Docherty, M., Fillit, H., Luine, V. N., McEwen, B., Bucht, G., Winblad, B., and Hardy, J., 1988, Evidence for the presence of antibodies to cholinergic neurons in the serum of patients with Alzheimer's disease, *J Neurol* **235**(8):466-71.

Foundas, A. L., Eure, K. F., and Seltzer, B., 1996, Conventional MRI volumetric measures of parietal and insular cortex in Alzheimer's disease, *Prog Neuropsychopharmacol Biol Psychiatry* **20**(7):1131-44.

Fowler, C. J., 1997, The role of the phosphoinositide signalling system in the pathogenesis of sporadic Alzheimer's disease: a hypothesis, *Brain Res Brain Res Rev* **25**(3):373-80.

Franceschi, M., Comola, M., Nemni, R., Pinto, P., Iannaccone, S., Smirne, S., and Canal, N., 1989, Neuron-binding antibodies in Alzheimer's disease and Down's syndrome, *J Gerontol* **44**(4):M128-30.

Frederickson, R. C., 1992, Astroglia in Alzheimer's disease, *Neurobiol Aging* **13**(2):239-53.

French, L. R., Schuman, L. M., Mortimer, J. A., Hutton, J. T., Boatman, R. A., and Christians, B., 1985, A case-control study of dementia of the Alzheimer type, *Am J Epidemiol* **121**(3):414-21.

Friedland, R. P., Jagust, W. J., Huesman, R. H., Koss, E., Knittel, B., Mathis, C. A., Ober, B. A., Mazoyer, B. M., and Budinger, T. F., 1989, Regional cerebral glucose transport and utilization in Alzheimer's disease, *Neurology* **39**(11):1427-34.

Furuta, A., Price, D. L., Pardo, C. A., Troncoso, J. C., Xu, Z. S., Taniguchi, N., and Martin, L. J., 1995, Localization of superoxide dismutases in Alzheimer's disease and Down's syndrome neocortex and hippocampus, *Am J Pathol* **146**(2):357-67.

Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., and et al., 1995, Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein [see comments], *Nature* **373**(6514):523-7.

Garver, T. D., Lehman, R. A., and Billingsley, M. L., 1996, Microtubule assembly competence analysis of freshly-biopsied human tau, dephosphorylated tau, and Alzheimer tau, *J Neurosci Res* **44**(1):12-20.

Gaskin, F., Finley, J., Fang, Q., Xu, S., and Fu, S. M., 1993, Human antibodies reactive with beta-amyloid protein in Alzheimer's disease, *J Exp Med* **177**(4):1181-6.

Gaskin, F., and Fu, S. M., 1992, Antineurofibrillary tangle, antineural and anti-beta-amyloid-protein in Alzheimer's disease and related disorders, *Res Immunol* **143**(6):668-70.

Gaskin, F., Kingsley, B. S., and Fu, S. M., 1987, Autoantibodies to neurofibrillary tangles and brain tissue in Alzheimer's disease. Establishment of Epstein-Barr virus-transformed antibody-producing cell lines [published erratum appears in J Exp Med 1987 Mar 1;165(3):937], *J Exp Med* **165**(1):245-50.

Gasparini, L., Racchi, M., Binetti, G., Trabucchi, M., Solerte, S. B., Alkon, D., Etcheberrigaray, R., Gibson, G., Blass, J., Paoletti, R., and Govoni, S., 1998, Peripheral markers in testing pathophysiological hypotheses and diagnosing Alzheimer's disease [see comments], *Faseb J* **12**(1):17-34.

Geldmacher, D. S., and Whitehouse, P. J., 1996, Evaluation of dementia [see comments], *N Engl J Med* **335**(5):330-6.

Gertler, F. B., Niebuhr, K., Reinhard, M., Wehland, J., and Soriano, P., 1996, Mena, a relative of VASP and Drosophila Enabled, is implicated in the control of microfilament dynamics, *Cell* **87**(2):227-39.

Geula, C., Mesulam, M-M, 1994, , in: *Alzheimer Disease* (R. Terry, Katzman, R, Bick, KL, ed.), Raven Press, New York, pp. 263-291.

Geula, C., Wu, C. K., Saroff, D., Lorenzo, A., Yuan, M., and Yankner, B. A., 1998, Aging renders the brain vulnerable to amyloid beta-protein neurotoxicity [In Process Citation], *Nat Med* **4**(7):827-31.

Ghiso, J., Matsubara, E., Koudinov, A., Choi-Miura, N. H., Tomita, M., Wisniewski, T., and Frangione, B., 1993, The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex, *Biochem J* **293**(Pt 1):27-30.

Gibson, G. E., Sheu, K. F., Blass, J. P., Baker, A., Carlson, K. C., Harding, B., and Perrino, P., 1988, Reduced activities of thiamine-dependent enzymes in the brains and peripheral tissues of patients with Alzheimer's disease, *Arch Neurol* **45**(8):836-40.

Goedert, M., 1987, Neuronal localization of amyloid beta protein precursor mRNA in normal human brain and in Alzheimer's disease, *Embo J* **6**(12):3627-32.

Goedert, M., and Jakes, R., 1990, Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization, *Embo J* **9**(13):4225-30.

Goedert, M., Spillantini, M. G., Cairns, N. J., and Crowther, R. A., 1992, Tau proteins of Alzheimer paired helical filaments: abnormal phosphorylation of all six brain isoforms, *Neuron* **8**(1):159-68.

Goedert, M., Spillantini, M. G., Potier, M. C., Ulrich, J., and Crowther, R. A., 1989, Cloning and sequencing of the cDNA encoding an isoform of microtubule- associated

protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain, *Embo J* **8**(2):393-9.

Goedert, M., Wischik, C. M., Crowther, R. A., Walker, J. E., and Klug, A., 1988, Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau, *Proc Natl Acad Sci U S A* **85**(11):4051-5.

Golde, T. E., Estus, S., Usiak, M., Younkin, L. H., and Younkin, S. G., 1990, Expression of beta amyloid protein precursor mRNAs: recognition of a novel alternatively spliced form and quantitation in Alzheimer's disease using PCR, *Neuron* **4**(2):253-67.

Goldgaber, D., Lerman, M. I., McBride, O. W., Saffiotti, U., and Gajdusek, D. C., 1987, Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease, *Science* **235**(4791):877-80.

Gomperts, S. N., 1996, Clustering membrane proteins: It's all coming together with the PSD- 95/SAP90 protein family, *Cell* **84**(5):659-62.

Gong, C. X., Shaikh, S., Wang, J. Z., Zaidi, T., Grundke-Iqbal, I., and Iqbal, K., 1995, Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain, *J Neurochem* **65**(2):732-8.

Gong, C. X., Singh, T. J., Grundke-Iqbal, I., and Iqbal, K., 1993, Phosphoprotein phosphatase activities in Alzheimer disease brain, *J Neurochem* **61**(3):921-7.

Goodman, Y., and Mattson, M. P., 1994, Secreted forms of beta-amyloid precursor protein protect hippocampal neurons against amyloid beta-peptide-induced oxidative injury, *Exp Neurol* **128**(1)**:**1-12.

Graves, A. B., White, E., Koepsell, T. D., Reifler, B. V., van Belle, G., and Larson, E. B., 1990, The association between aluminum-containing products and Alzheimer's disease, *J Clin Epidemiol* **43**(1)**:**35-44.

Gravina, S. A., Ho, L., Eckman, C. B., Long, K. E., Otvos, L., Jr., Younkin, L. H., Suzuki, N., and Younkin, S. G., 1995, Amyloid beta protein (A beta) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at A beta 40 or A beta 42(43), *J Biol Chem* **270**(13)**:**7013-6.

Gregg, R. E., and Brewer, H. B., Jr., 1986, In vivo metabolism of apolipoprotein E in humans, *Methods Enzymol* **129:**482-97.

Griffin, W. S., Stanley, L. C., Ling, C., White, L., MacLeod, V., Perrot, L. J., White, C. L. d., and Araoz, C., 1989, Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease, *Proc Natl Acad Sci U S A* **86**(19)**:**7611-5.

Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., and Binder, L. I., 1986, Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology, *Proc Natl Acad Sci U S A* **83**(13)**:**4913-7.

Grundke-Iqbal, I., Vorbrodt, A. W., Iqbal, K., Tung, Y. C., Wang, G. P., and Wisniewski, H. M., 1988, Microtubule-associated polypeptides tau are altered in Alzheimer paired helical filaments, *Brain Res* **464**(1)**:**43-52.

Guillemette, J. G., Wong, L., Crapper McLachlan, D. R., and Lewis, P. N., 1986, Characterization of messenger RNA from the cerebral cortex of control and Alzheimer-afflicted brain, *J Neurochem* **47**(3):987-97.

Guruprasad, K., Reddy, B. V., and Pandit, M. W., 1990, Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence, *Protein Eng* **4**(2):155-61.

Haass, C., 1997, Presenilins: genes for life and death, *Neuron* **18**(5):687-90.

Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B., and et al., 1992, Amyloid beta-peptide is produced by cultured cells during normal metabolism [see comments], *Nature* **359**(6393):322-5.

Haass, C., and Selkoe, D. J., 1993, Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide, *Cell* **75**(6):1039-42.

Haass, C., and Selkoe, D. J., 1998, Alzheimer's disease. A technical KO of amyloid-beta peptide [news; comment], *Nature* **391**(6665):339-40.

Haffner, C., Jarchau, T., Reinhard, M., Hoppe, J., Lohmann, S. M., and Walter, U., 1995, Molecular cloning, structural analysis and functional expression of the proline-rich focal adhesion and microfilament-associated protein VASP, *Embo J* **14**(1):19-27.

Hampel, H., Kotter, H. U., and Moller, H. J., 1997, Blood-cerebrospinal fluid barrier dysfunction for high molecular weight proteins in Alzheimer disease and major depression: indication for disease subsets, *Alzheimer Dis Assoc Disord* **11**(2):78-87.

Hardy, J. A., and Higgins, G. A., 1992, Alzheimer's disease: the amyloid cascade hypothesis, *Science* **256**(5054):184-5.

Harris, M. E., Carney, J. M., Cole, P. S., Hensley, K., Howard, B. J., Martin, L., Bummer, P., Wang, Y., Pedigo, N. W., Jr., and Butterfield, D. A., 1995, beta-Amyloid peptide-derived, oxygen-dependent free radicals inhibit glutamate uptake in cultured astrocytes: implications for Alzheimer's disease, *Neuroreport* **6**(14):1875-9.

Harrison, P. J., Barton, A. J., Najlerahim, A., McDonald, B., and Pearson, R. C., 1991, Regional and neuronal reductions of polyadenylated messenger RNA in Alzheimer's disease, *Psychol Med* **21**(4):855-66.

Heinonen, O., Syrjanen, S., Soininen, H., Talasniemi, S., Kaski, M., Mantyjarvi, R., Syrjanen, K., and Riekkinen, P., Sr., 1993, Circulating immune complexes in sera from patients with Alzheimer's disease, multi-infarct dementia and Down's syndrome, *Neurosci Lett* **149**(1):67-70.

Henderson, V. W., Paganini-Hill, A., Emanuel, C. K., Dunn, M. E., and Buckwalter, J. G., 1994, Estrogen replacement therapy in older women. Comparisons between Alzheimer's disease cases and nondemented control subjects, *Arch Neurol* **51**(9):896-900.

Hensley, K., Carney, J. M., Mattson, M. P., Aksenova, M., Harris, M., Wu, J. F., Floyd, R. A., and Butterfield, D. A., 1994, A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease, *Proc Natl Acad Sci U S A* **91**(8):3270-4.

Heyman, A., Wilkinson, W. E., Stafford, J. A., Helms, M. J., Sigmon, A. H., and Weinberg, T., 1984, Alzheimer's disease: a study of epidemiological aspects, *Ann Neurol* **15**(4):335-41.

Higgins, D. G., 1994, CLUSTAL V: multiple alignment of DNA and protein sequences, *Methods Mol Biol* **25**:307-18.

Hilbich, C., Kisters-Woike, B., Reed, J., Masters, C. L., and Beyreuther, K., 1991, Aggregation and secondary structure of synthetic amyloid beta A4 peptides of Alzheimer's disease, *J Mol Biol* **218**(1):149-63.

Hinds, T. R., Kukull, W. A., Van Belle, G., Schellenberg, G. D., Villacres, E. C., and Larson, E. B., 1994, Relationship between serum alpha 1-antichymotrypsin and Alzheimer's disease, *Neurobiol Aging* **15**(1):21-7.

Hofman, A., Schulte, W., Tanja, T. A., van Duijn, C. M., Haaxma, R., Lameris, A. J., Otten, V. M., and Saan, R. J., 1989, History of dementia and Parkinson's disease in 1st-degree relatives of patients with Alzheimer's disease, *Neurology* **39**(12):1589-92.

Holtzman, D. M., Pitas, R. E., Kilbridge, J., Nathan, B., Mahley, R. W., Bu, G., and Schwartz, A. L., 1995, Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line, *Proc Natl Acad Sci U S A* **92**(21):9480-4.

Honjo, H., Ogino, Y., Naitoh, K., Urabe, M., Kitawaki, J., Yasuda, J., Yamamoto, T., Ishihara, S., Okada, H., Yonezawa, T., and et al., 1989, In vivo effects by estrone sulfate on the central nervous system-senile dementia (Alzheimer's type), *J Steroid Biochem* **34**(1-6):521-5.

Horton, P., and Nakai, K., 1997, Better prediction of protein cellular localization sites with the k nearest neighbors classifier, *Ismb* **5:**147-52.

Hoyer, S., 1998, Glucose/energy metabolism and its significance in sporadic Alzheimer's disease, in: *6th International Conference on Alzheimer's Disease and Related Disorders*, Neurobiology of Aging, **19** (4S), Amsterdam, pp. S4.

Hui, D. Y., Harmony, J. A., Innerarity, T. L., and Mahley, R. W., 1980, Immunoregulatory plasma lipoproteins. Role of apoprotein E and apoprotein B, *J Biol Chem* **255**(24)**:**11775-81.

Hung, A. Y., and Selkoe, D. J., 1994, Selective ectodomain phosphorylation and regulated cleavage of beta- amyloid precursor protein, *Embo J* **13**(3)**:**534-42.

Huynh, D. P., Vinters, H. V., Ho, D. H., Ho, V. V., and Pulst, S. M., 1997, Neuronal expression and intracellular localization of presenilins in normal and Alzheimer disease brains, *J Neuropathol Exp Neurol* **56**(9)**:**1009-17.

Hyman, B. T., and Gomez-Isla, T., 1994, Alzheimer's disease is a laminar, regional, and neural system specific disease, not a global brain disease, *Neurobiol Aging* **15**(3)**:**353-4; discussion 379-80.

Hyman, B. T., Van Hoesen, G. W., and Damasio, A. R., 1990, Memory-related neural systems in Alzheimer's disease: an anatomic study, *Neurology* **40**(11)**:**1721-30.

Iacopino, A. M., and Christakos, S., 1990, Specific reduction of calcium-binding protein (28-kilodalton calbindin- D) gene expression in aging and neurodegenerative diseases, *Proc Natl Acad Sci U S A* **87**(11)**:**4078-82.

Ignatius, M. J., Gebicke-Harter, P. J., Skene, J. H., Schilling, J. W., Weisgraber, K. H., Mahley, R. W., and Shooter, E. M., 1986, Expression of apolipoprotein E during nerve degeneration and regeneration, *Proc Natl Acad Sci U S A* **83**(4):1125-9.

Ignatius, M. J., Shooter, E. M., Pitas, R. E., and Mahley, R. W., 1987, Lipoprotein uptake by neuronal growth cones in vitro, *Science* **236**(4804):959-62.

Iqbal, K., Grundke-Iqbal, I., Smith, A. J., George, L., Tung, Y. C., and Zaidi, T., 1989, Identification and localization of a tau peptide to paired helical filaments of Alzheimer disease, *Proc Natl Acad Sci U S A* **86**(14):5646-50.

Iqbal, K., Grundke-Iqbal, I., Zaidi, T., Merz, P. A., Wen, G. Y., Shaikh, S. S., Wisniewski, H. M., Alafuzoff, I., and Winblad, B., 1986, Defective brain microtubule assembly in Alzheimer's disease [published erratum appears in Lancet 1986 Nov 15;2(8516):1174], *Lancet* **2**(8504):421-6.

Iqbal, K., Zaidi, T., Bancher, C., and Grundke-Iqbal, I., 1994, Alzheimer paired helical filaments. Restoration of the biological activity by dephosphorylation, *FEBS Lett* **349**(1):104-8.

Irizarry, M. C., Soriano, F., McNamara, M., Page, K. J., Schenk, D., Games, D., and Hyman, B. T., 1997, Abeta deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse, *J Neurosci* **17**(18):7053-9.

Ishii, T., and Haga, S., 1975, Identification of components of immunoglobulins in senile plaques by means of fluorescent antibody technique, *Acta Neuropathol (Berl)* **32**(2):157-62.

Ishii, T., and Haga, S., 1976, Immuno-electron microscopic localization of immunoglobulins in amyloid fibrils of senile plaques, *Acta Neuropathol (Berl)* **36**(3):243-9.

Ishii, T., and Haga, S., 1984, Immuno-electron-microscopic localization of complements in amyloid fibrils of senile plaques, *Acta Neuropathol (Berl)* **63**(4):296-300.

Itagaki, S., McGeer, P. L., and Akiyama, H., 1988, Presence of T-cytotoxic suppressor and leucocyte common antigen positive cells in Alzheimer's disease brain tissue, *Neurosci Lett* **91**(3):259-64.

Ito, E., Oka, K., Etcheberrigaray, R., Nelson, T. J., McPhie, D. L., Tofel-Grehl, B., Gibson, G. E., and Alkon, D. L., 1994, Internal Ca2+ mobilization is altered in fibroblasts from patients with Alzheimer disease, *Proc Natl Acad Sci U S A* **91**(2):534-8.

Itzhaki, R. F., Lin, W. R., Shang, D., Wilcock, G. K., Faragher, B., and Jamieson, G. A., 1997, Herpes simplex virus type 1 in brain and risk of Alzheimer's disease [see comments], *Lancet* **349**(9047):241-4.

Iwangoff, P., Armbruster, R., Enz, A., and Meier-Ruge, W., 1980, Glycolytic enzymes from human autoptic brain cortex: normal aged and demented cases, *Mech Ageing Dev* **14**(1-2):203-9.

Iwatsubo, T., Mann, D. M., Odaka, A., Suzuki, N., and Ihara, Y., 1995, Amyloid beta protein (A beta) deposition: A beta 42(43) precedes A beta 40 in Down syndrome [see comments], *Ann Neurol* **37**(3):294-9.

Iyo, M., Namba, H., Fukushi, K., Shinotoh, H., Nagatsuka, S., Suhara, T., Sudo, Y., Suzuki, K., and Irie, T., 1997, Measurement of acetylcholinesterase by positron emission tomography in the brains of healthy controls and patients with Alzheimer's disease, *Lancet* **349**(9068):1805-9.

Jacobsen, J. S., Blume, A. J., and Vitek, M. P., 1991, Quantitative measurement of alternatively spliced amyloid precursor protein mRNA expression in Alzheimer's disease and normal brain by S1 nuclease protection analysis, *Neurobiol Aging* **12**(5):585-92.

Jankovic, B. D., and Djordjijevic, D., 1991, Differential appearance of autoantibodies to human brain S100 protein, neuron specific enolase and myelin basic protein in psychiatric patients, *Int J Neurosci* **60**(1-2):119-27.

Jefferies, W. A., Food, M. R., Gabathuler, R., Rothenberger, S., Yamada, T., Yasuhara, O., and McGeer, P. L., 1996, Reactive microglia specifically associated with amyloid plaques in Alzheimer's disease brain tissue express melanotransferrin, *Brain Res* **712**(1):122-6.

Jensen, M., Basun, H., and Lannfelt, L., 1995, Increased cerebrospinal fluid tau in patients with Alzheimer's disease, *Neurosci Lett* **186**(2-3):189-91.

Joachim, C. L., Mori, H., and Selkoe, D. J., 1989, Amyloid beta-protein deposition in tissues other than brain in Alzheimer's disease, *Nature* **341**(6239):226-30.

Johnson, S. A., Morgan, D. G., and Finch, C. E., 1986, Extensive postmortem stability of RNA from rat and human brain, *J Neurosci Res* **16**(1):267-80.

Josefsson, E., Tarkowski, A., and Carlsten, H., 1992, Anti-inflammatory properties of estrogen. I. In vivo suppression of leukocyte production in bone marrow and redistribution of peripheral blood neutrophils, *Cell Immunol* **142**(1):67-78.

Juby, A. G., Davis, P., McElhaney, J. E., and Gravenstein, S., 1994, Prevalence of selected autoantibodies in different elderly subpopulations, *Br J Rheumatol* **33**(12):1121-4.

Kalaria, R. N., 1992, Serum amyloid P and related molecules associated with the acute-phase response in Alzheimer's disease, *Res Immunol* **143**(6):637-41.

Kalaria, R. N., 1993, The immunopathology of Alzheimer's disease and some related disorders, *Brain Pathol* **3**(4):333-47.

Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K., and Muller-Hill, B., 1987, The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor, *Nature* **325**(6106):733-6.

Kang, J., and Muller-Hill, B., 1990, Differential splicing of Alzheimer's disease amyloid A4 precursor RNA in rat tissues: PreA4(695) mRNA is predominantly produced in rat and human brain, *Biochem Biophys Res Commun* **166**(3):1192-200.

Karcher, D., Federsppiel, B. S., Lowenthal, F. D., Frank, F., and Lowenthal, A., 1986, Anti-neurofilament antibodies in blood of patients with neurological diseases, *Acta Neuropathol (Berl)* **72**(1):82-5.

Kato, A., Ozawa, F., Saitoh, Y., Fukazawa, Y., Sugiyama, H., and Inokuchi, K., 1998, Novel members of the Vesl/Homer family of PDZ proteins that bind metabotropic glutamate receptors, *J Biol Chem* **273**(37):23969-75.

Kato, A., Ozawa, F., Saitoh, Y., Hirai, K., and Inokuchi, K., 1997, vesl, a gene encoding VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis, *FEBS Lett* **412**(1):183-9.

Khachaturian, Z. S., 1985, Diagnosis of Alzheimer's disease, *Arch Neurol* **42**(11):1097-105.

Kim, E., Niethammer, M., Rothschild, A., Jan, Y. N., and Sheng, M., 1995, Clustering of Shaker-type K+ channels by interaction with a family of membrane-associated guanylate kinases, *Nature* **378**(6552):85-8.

Kim, T. W., Pettingell, W. H., Jung, Y. K., Kovacs, D. M., and Tanzi, R. E., 1997, Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease, *Science* **277**(5324):373-6.

Kingsley, B. S., Gaskin, F., and Fu, S. M., 1988, Human antibodies to neurofibrillary tangles and astrocytes in Alzheimer's disease, *J Neuroimmunol* **19**(1-2):89-99.

Kinzler, K. W., Nilbert, M. C., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hamilton, S. R., Hedge, P., Markham, A., and et al., 1991, Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers [see comments], *Science* **251**(4999):1366-70.

Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., and Ito, H., 1988, Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity, *Nature* **331**(6156):530-2.

Kobayashi, H., Sakimura, K., Kuwano, R., Sato, S., Ikuta, F., Takahashi, Y., Miyatake, T., and Tsuji, S., 1990, Stability of messenger RNA in postmortem human brains and construction of human brain cDNA libraries, *J Mol Neurosci* **2**(1):29-34.

Kondo, K., Yamashita, I, 1990, , in: *Psychogeriatrics: medical and social advances* (k. Hasegawa, Homma,A, ed.), Excerpta medica, Amsterdam, pp. 49-53.

Kondoh, Y., Nagata, K., Sasaki, H., and Hatazawa, J., 1997, Dynamic FDG-PET study in probable Alzheimer's disease, *Ann N Y Acad Sci* **826**:406-9.

Kopke, E., Tung, Y. C., Shaikh, S., Alonso, A. C., Iqbal, K., and Grundke-Iqbal, I., 1993, Microtubule-associated protein tau. Abnormal phosphorylation of a non- paired helical filament pool in Alzheimer disease, *J Biol Chem* **268**(32):24374-84.

Kosunen, O., Talasniemi, S., Lehtovirta, M., Heinonen, O., Helisalmi, S., Mannermaa, A., Paljarvi, L., Ryynanen, M., Riekkinen, P. J., Sr., and Soininen, H., 1995, Relation of coronary atherosclerosis and apolipoprotein E genotypes in Alzheimer patients, *Stroke* **26**(5):743-8.

Kounnas, M. Z., Moir, R. D., Rebeck, G. W., Bush, A. I., Argraves, W. S., Tanzi, R. E., Hyman, B. T., and Strickland, D. K., 1995, LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation, *Cell* **82**(2):331-40.

Kovacs, D. M., Fausett, H. J., Page, K. J., Kim, T. W., Moir, R. D., Merriam, D. E., Hollister, R. D., Hallmark, O. G., Mancini, R., Felsenstein, K. M., Hyman, B. T., Tanzi, R. E., and Wasco, W., 1996, Alzheimer-associated presenilins 1 and 2: neuronal expression in brain and localization to intracellular membranes in mammalian cells, *Nat Med* **2**(2):224-9.

Kumar, A., Schapiro, M. B., Grady, C. L., Matocha, M. F., Haxby, J. V., Moore, A. M., Luxenberg, J. S., St George-Hyslop, P. H., Robinette, C. D., Ball, M. J., and et al., 1991, Anatomic, metabolic, neuropsychological, and molecular genetic studies of three pairs of identical twins discordant for dementia of the Alzheimer's type, *Arch Neurol* **48**(2):160-8.

Kumar, M., Cohen, D., and Eisdorfer, C., 1988, Serum IgG brain reactive antibodies in Alzheimer disease and Down syndrome, *Alzheimer Dis Assoc Disord* **2**(1):50-5.

L'Hernault, S. W., and Arduengo, P. M., 1992, Mutation of a putative sperm membrane protein in Caenorhabditis elegans prevents sperm differentiation but not its associated meiotic divisions, *J Cell Biol* **119**(1):55-68.

Laemmli, U. K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**(259):680-5.

Laping, N. J., Morgan, T. E., Nichols, N. R., Rozovsky, I., Young-Chan, C. S., Zarow, C., and Finch, C. E., 1994, Transforming growth factor-beta 1 induces neuronal and astrocyte genes: tubulin alpha 1, glial fibrillary acidic protein and clusterin, *Neuroscience* **58**(3):563-72.

Ledesma, M. D., Bonay, P., Colaco, C., and Avila, J., 1994, Analysis of microtubule-associated protein tau glycation in paired helical filaments, *J Biol Chem* **269**(34):21614-9.

Lee, P. N., 1994, Smoking and Alzheimer's disease: a review of the epidemiological evidence, *Neuroepidemiology* **13**(4):131-44.

Lee, R. M., Garfield, R. E., Forrest, J. B., and Daniel, E. E., 1983, Morphometric study of structural changes in the mesenteric blood vessels of spontaneously hypertensive rats, *Blood Vessels* **20**(2):57-71.

Lee, V. M., Balin, B. J., Otvos, L., Jr., and Trojanowski, J. Q., 1991, A68: a major subunit of paired helical filaments and derivatized forms of normal Tau, *Science* **251**(4994):675-8.

Lehericy, S., Hirsch, E. C., Cervera-Pierot, P., Hersh, L. B., Bakchine, S., Piette, F., Duyckaerts, C., Hauw, J. J., Javoy-Agid, F., and Agid, Y., 1993, Heterogeneity and selectivity of the degeneration of cholinergic neurons in the basal forebrain of patients with Alzheimer's disease, *J Comp Neurol* **330**(1):15-31.

Lehtovirta, M., Laakso, M. P., Soininen, H., Helisalmi, S., Mannermaa, A., Helkala, E. L., Partanen, K., Ryynanen, M., Vainio, P., Hartikainen, P., and et al., 1995, Volumes of hippocampus, amygdala and frontal lobe in Alzheimer patients with different apolipoprotein E genotypes, *Neuroscience* **67**(1):65-72.

Lemaire, H. G., Salbaum, J. M., Multhaup, G., Kang, J., Bayney, R. M., Unterbeck, A., Beyreuther, K., and Muller-Hill, B., 1989, The PreA4(695) precursor protein of Alzheimer's disease A4 amyloid is encoded by 16 exons, *Nucleic Acids Res* **17**(2):517-22.

Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D. M., Oshima, J., Pettingell, W. H., Yu, C. E., Jondro, P. D., Schmidt, S. D., Wang, K., and et al., 1995, Candidate gene for the chromosome 1 familial Alzheimer's disease locus [see comments], *Science* **269**(5226):973-7.

Li, J., Xu, M., Zhou, H., Ma, J., and Potter, H., 1997, Alzheimer presenilins in the nuclear membrane, interphase kinetochores, and centrosomes suggest a role in chromosome segregation, *Cell* **90**(5):917-27.

Li, Q. X., Evin, G., Small, D. H., Multhaup, G., Beyreuther, K., and Masters, C. L., 1995, Proteolytic processing of Alzheimer's disease beta A4 amyloid precursor protein in human platelets, *J Biol Chem* **270**(23):14140-7.

Li, R., 1997, Bee1, a yeast protein with homology to Wiscott-Aldrich syndrome protein, is critical for the assembly of cortical actin cytoskeleton, *J Cell Biol* **136**(3):649-58.

Loeffler, D. A., Brickman, C. M., Juneau, P. L., Perry, M. F., Pomara, N., and Lewitt, P. A., 1997, Cerebrospinal fluid C3a increases with age, but does not increase further in Alzheimer's disease, *Neurobiol Aging* **18**(5):555-7.

Loeffler, D. A., Juneau, P. L., Nguyen, H. U., Najman, D., Pomara, N., and LeWitt, P. A., 1997, Immunocytochemical detection of anti-hippocampal antibodies in Alzheimer's disease and normal cerebrospinal fluid, *Neurochem Res* **22**(2):209-14.

Loffler, J., and Huber, G., 1992, Beta-amyloid precursor protein isoforms in various rat brain regions and during brain development, *J Neurochem* **59**(4):1316-24.

Lopez, O. L., Rabin, B. S., and Huff, F. J., 1991, Serum auto-antibodies in Alzheimer's disease, *Acta Neurol Scand* **84**(5):441-4.

Lopez, O. L., Rabin, B. S., Huff, F. J., Rezek, D., and Reinmuth, O. M., 1992, Serum autoantibodies in patients with Alzheimer's disease and vascular dementia and in nondemented control subjects, *Stroke* **23**(8):1078-83.

Lowry, O., Rosebrough, NJ, Farr, AL, and Randall, RJ, 1951, *J.Biol.Chem.* **193:**265-275.

Luber-Narod, J., and Rogers, J., 1988, Immune system associated antigens expressed by cells of the human central nervous system, *Neurosci Lett* **94**(1-2):17-22.

Lucca, U., Tettamanti, M., Forloni, G., and Spagnoli, A., 1994, Nonsteroidal antiinflammatory drug use in Alzheimer's disease, *Biol Psychiatry* **36**(12):854-6.

Ma, J., Yee, A., Brewer, H. B., Jr., Das, S., and Potter, H., 1994, Amyloid-associated proteins alpha 1-antichymotrypsin and apolipoprotein E promote assembly of Alzheimer beta-protein into filaments [see comments], *Nature* **372**(6501):92-4.

Mahley, R. W., 1988, Apolipoprotein E: cholesterol transport protein with expanding role in cell biology, *Science* **240**(4852):622-30.

Mann, D. M., 1989, Cerebral amyloidosis, ageing and Alzheimer's disease; a contribution from studies on Down's syndrome, *Neurobiol Aging* **10**(5):397-9; discussion 412-4.

Marotta, C. A., Majocha, R. E., Coughlin, J. F., Manz, H. J., Davies, P., Ventosa-Michelman, M., Chou, W. G., Zain, S. B., and Sajdel-Sulkowska, E. M., 1986, Transcriptional and translational regulatory mechanisms during normal aging of the mammalian brain and in Alzheimer's disease, *Prog Brain Res* **70:**303-20.

Marques, M. A., Tolar, M., Harmony, J. A., and Crutcher, K. A., 1996, A thrombin cleavage fragment of apolipoprotein E exhibits isoform- specific neurotoxicity, *Neuroreport* **7**(15-17):2529-32.

Mash, D. C., Flynn, D. D., and Potter, L. T., 1985, Loss of M2 muscarine receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation, *Science* **228**(4703):1115-7.

Mathew, S., Scanlan, M. J., Mohan Raj, B. K., Murty, V. V., Garin-Chesa, P., Old, L. J., Rettig, W. J., and Chaganti, R. S., 1995, The gene for fibroblast activation protein alpha (FAP), a putative cell surface-bound serine protease expressed in cancer stroma and wound healing, maps to chromosome band 2q23, *Genomics* **25**(1):335-7.

Matsubara, E., Hirai, S., Amari, M., Shoji, M., Yamaguchi, H., Okamoto, K., Ishiguro, K., Harigaya, Y., and Wakabayashi, K., 1990, Alpha 1-antichymotrypsin as a possible biochemical marker for Alzheimer- type dementia, *Ann Neurol* **28**(4):561-7.

Matsuo, E. S., Shin, R. W., Billingsley, M. L., Van deVoorde, A., O'Connor, M., Trojanowski, J. Q., and Lee, V. M., 1994, Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau, *Neuron* **13**(4):989-1002.

Mattila, K. M., and Frey, H., 1995, Two-dimensional analysis of qualitative and quantitative changes in blood cell proteins in Alzheimer's disease: search for extraneuronal markers, *Appl Theor Electrophor* **4**(4):189-96.

Mayeux, R., Stern, Y., Ottman, R., Tatemichi, T. K., Tang, M. X., Maestre, G., Ngai, C., Tycko, B., and Ginsberg, H., 1993, The apolipoprotein epsilon 4 allele in patients with Alzheimer's disease [see comments], *Ann Neurol* **34**(5):752-4.

McCaddon, A., Davies, G., Hudson, P., Tandy, S., and Cattell, H., 1998, Total serum homocysteine in senile dementia of Alzheimer type, *Int J Geriatr Psychiatry* **13**(4):235-9.

McGeer, P. L., Akiyama, H., Itagaki, S., and McGeer, E. G., 1989, Activation of the classical complement pathway in brain tissue of Alzheimer patients, *Neurosci Lett* **107**(1-3):341-6.

McGeer, P. L., Itagaki, S., Tago, H., and McGeer, E. G., 1987, Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR, *Neurosci Lett* **79**(1-2):195-200.

McGeer, P. L., Itagaki, S., Tago, H., and McGeer, E. G., 1988, Occurrence of HLA-DR reactive microglia in Alzheimer's disease, *Ann N Y Acad Sci* **540**:319-23.

McGeer, P. L., McGeer, E., Rogers, J., and Sibley, J., 1990, Anti-inflammatory drugs and Alzheimer disease [letter] [see comments], *Lancet* **335**(8696):1037.

McGeer, P. L., Rogers, J., and McGeer, E. G., 1994, Neuroimmune mechanisms in Alzheimer disease pathogenesis [see comments], *Alzheimer Dis Assoc Disord* **8**(3):149-58.

McKee, A. C., Kosik, K. S., and Kowall, N. W., 1991, Neuritic pathology and dementia in Alzheimer's disease, *Ann Neurol* **30**(2):156-65.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., and Stadlan, E. M., 1984, Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease, *Neurology* **34**(7):939-44.

McLachlan, D. R., Lukiw, W. J., Wong, L., Bergeron, C., and Bech-Hansen, N. T., 1988, Selective messenger RNA reduction in Alzheimer's disease, *Brain Res* **427**(3):255-61.

McRae, A., Blennow, K, Gottfies, CG, Wallin, A and Dahlstrom, A, 1990, Brain-specific antibodies in the CSF of patients with Alzheimer's disease and other types of dementias, in: *Biological markers in dementia of Alzheimer's type* (F. C. e. al., ed.), Smith-Gordon, pp. 135-148.

McRae, A., Dahlstrom, A., Polinsky, R., and Ling, E. A., 1993, Cerebrospinal fluid microglial antibodies: potential diagnostic markers for immune mechanisms in Alzheimer's disease, *Behav Brain Res* **57**(2):225-34.

McRae, A., Ling, E. A., Polinsky, R., Gottfries, C. G., and Dahlstrom, A., 1991, Antibodies in the cerebrospinal fluid of some Alzheimer's disease patients recognize amoeboid microglial cells in the developing rat central nervous system, *Neuroscience* **41**(2-3):739-52.

McRae, A., Ling, E. A., Wigander, A., and Dahlstrom, A., 1996, Microglial cerebrospinal fluid antibodies. Significance for Alzheimer disease, *Mol Chem Neuropathol* **28**(1-3):89-95.

McRae-Degueurce, A., Booj, S., Haglid, K., Rosengren, L., Karlsson, J. E., Karlsson, I., Wallin, A., Svennerholm, L., Gottfries, C. G., and Dahlstrom, A., 1987, Antibodies in cerebrospinal fluid of some Alzheimer disease patients recognize cholinergic neurons in the rat central nervous system, *Proc Natl Acad Sci U S A* **84**(24):9214-8.

McRae-Degueurce, A., and Geffard, M., 1986, One perfusion mixture for immunocytochemical detection of noradrenaline, dopamine, serotonin and acetylcholine in the same rat brain, *Brain Res* **376**(1):217-9.

Mecocci, P., Ekman, R., Parnetti, L., and Senin, U., 1993, Antihistone and anti-dsDNA autoantibodies in Alzheimer's disease and vascular dementia, *Biol Psychiatry* **34**(6):380-5.

Mecocci, P., Parnetti, L., Donato, R., Santucci, C., Santucci, A., Cadini, D., Foa, E., Cecchetti, R., and Senin, U., 1992, Serum autoantibodies against glial fibrillary acidic protein in brain aging and senile dementias, *Brain Behav Immun* **6**(3):286-92.

Mecocci, P., Parnetti, L., Romano, G., Scarelli, A., Chionne, F., Cecchetti, R., Polidori, M. C., Palumbo, B., Cherubini, A., and Senin, U., 1995, Serum anti-GFAP and anti-S100 autoantibodies in brain aging, Alzheimer's disease and vascular dementia, *J Neuroimmunol* **57**(1-2):165-70.

Miller, A. E., Neighbour, P. A., Katzman, R., Aronson, M., and Lipkowitz, R., 1981, Immunological studies in senile dementia of the Alzheimer type: evidence for enhanced suppressor cell activity, *Ann Neurol* **10**(6):506-10.

Miller, B. L., Read, S., Tang, C., and Jenden, D., 1991, Differences in red blood cell choline and lipid-bound choline between patients with Alzheimer disease and control subjects, *Neurobiol Aging* **12**(1):61-4.

Milward, E. A., Papadopoulos, R., Fuller, S. J., Moir, R. D., Small, D., Beyreuther, K., and Masters, C. L., 1992, The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth, *Neuron* **9**(1):129-37.

Mochizuki, Y., Oishi, M., Hara, M., and Takasu, T., 1996, Amino acid concentration in dementia of the Alzheimer type and multi- infarct dementia, *Ann Clin Lab Sci* **26**(3):275-8.

Monning, U., Sandbrink, R., Weidemann, A., Banati, R. B., Masters, C. L., and Beyreuther, K., 1995, Extracellular matrix influences the biogenesis of amyloid precursor protein in microglial cells, *J Biol Chem* **270**(13):7104-10.

Montine, T. J., Montine, K. S., and Swift, L. L., 1997, Central nervous system lipoproteins in Alzheimer's disease, *Am J Pathol* **151**(6):1571-5.

Moran, M. A., Mufson, E. J., and Gomez-Ramos, P., 1993, Colocalization of cholinesterases with beta amyloid protein in aged and Alzheimer's brains, *Acta Neuropathol (Berl)* **85**(4):362-9.

Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Titani, K., and Ihara, Y., 1993, Ubiquitin is conjugated with amino-terminally processed tau in paired helical filaments, *Neuron* **10**(6):1151-60.

Morrison, M., 1988, Messenger RNA levels in neurological diseases, in: *The Molecular Biology of Neurological Diseases* (R. R. a. H. AE, ed.), Butterworth, Stoneham, pp. 135-152.

Mortimer, J. A., French, L. R., Hutton, J. T., and Schuman, L. M., 1985, Head injury as a risk factor for Alzheimer's disease, *Neurology* **35**(2):264-7.

Mountjoy, C. Q., Rossor, M. N., Iversen, L. L., and Roth, M., 1984, Correlation of cortical cholinergic and GABA deficits with quantitative neuropathological findings in senile dementia, *Brain* **107**(Pt 2):507-18.

Mountjoy, C. Q., Roth, M., Evans, N. J., and Evans, H. M., 1983, Cortical neuronal counts in normal elderly controls and demented patients, *Neurobiol Aging* **4**(1):1-11.

Muller, T. J., Thome, J., Chiaramonti, R., Dierks, T., Maurer, K., Fallgatter, A. J., Frolich, L., Scheubeck, M., and Strik, W. K., 1997, A comparison of qEEG and HMPAO-SPECT in relation to the clinical severity of Alzheimer's disease, *Eur Arch Psychiatry Clin Neurosci* **247**(5):259-63.

Multhaup, G., Bush, A. I., Pollwein, P., and Masters, C. L., 1994, Interaction between the zinc (II) and the heparin binding site of the Alzheimer's disease beta A4 amyloid precursor protein (APP), *FEBS Lett* **355**(2):151-4.

Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E., and Ikeda, K., 1991, Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease, *Brain Res* **541**(1):163-6.

Nandy, K., 1978, Brain-reactive antibodies in aging and senile dementia, in: *Alzheimer's disease: Senile Dementia and Related Disorders* (T. R. a. B. Katzman R, ed.), Raven Press, New York, pp. 503-514.

Narindrasorasak, S., Lowery, D. E., Altman, R. A., Gonzalez-DeWhitt, P. A., Greenberg, B. D., and Kisilevsky, R., 1992, Characterization of high affinity binding between laminin and Alzheimer's disease amyloid precursor proteins, *Lab Invest* **67**(5):643-52.

Nathan, B. P., Bellosta, S., Sanan, D. A., Weisgraber, K. H., Mahley, R. W., and Pitas, R. E., 1994, Differential effects of apolipoproteins E3 and E4 on neuronal growth in vitro, *Science* **264**(5160):850-2.

Nathan, B. P., Chang, K. C., Bellosta, S., Brisch, E., Ge, N., Mahley, R. W., and Pitas, R. E., 1995, The inhibitory effect of apolipoprotein E4 on neurite outgrowth is associated with microtubule depolymerization, *J Biol Chem* **270**(34):19791-9.

Neve, R. L., Dawes, L. R., Yankner, B. A., Benowitz, L. I., Rodriguez, W., and Higgins, G. A., 1990, Genetics and biology of the Alzheimer amyloid precursor, *Prog Brain Res* **86**:257-67.

Nicoletti, F., Bruno, V., Copani, A., Casabona, G., and Knopfel, T., 1996, Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders?, *Trends Neurosci* **19**(7):267-71.

Notkola, I. L., Sulkava, R., Pekkanen, J., Erkinjuntti, T., Ehnholm, C., Kivinen, P., Tuomilehto, J., and Nissinen, A., 1998, Serum total cholesterol, apolipoprotein E epsilon 4 allele, and Alzheimer's disease, *Neuroepidemiology* **17**(1):14-20.

Novak, M., Kabat, J., and Wischik, C. M., 1993, Molecular characterization of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament, *Embo J* **12**(1):365-70.

O'Connor, J. J., Rowan, M. J., and Anwyl, R., 1994, Long-lasting enhancement of NMDA receptor-mediated synaptic transmission by metabotropic glutamate receptor activation, *Nature* **367**(6463):557-9.

Octave, J. N., de Sauvage, F., Macq, A. F., and Maloteaux, J. M., 1988, Cloning of the cDNA from normal brain and brain of patients with Alzheimer's disease in the expression vector lambda GT 11, *Prog Neuropsychopharmacol Biol Psychiatry* **12**(5):813-20.

Ohm, T. G., Muller, H., Braak, H., and Bohl, J., 1995, Close-meshed prevalence rates of different stages as a tool to uncover the rate of Alzheimer's disease-related neurofibrillary changes, *Neuroscience* **64**(1):209-17.

Oishi, M., Mochizuki, Y., Yoshihashi, H., Takasu, T., and Nakano, E., 1996, Laboratory examinations correlated with severity of dementia, *Ann Clin Lab Sci* **26**(4):340-5.

Olaisen, B., Teisberg, P., and Gedde-Dahl, T., Jr., 1982, The locus for apolipoprotein E (apoE) is linked to the complement component C3 (C3) locus on chromosome 19 in man, *Hum Genet* **62**(3):233-6.

Oltersdorf, T., Ward, P. J., Henriksson, T., Beattie, E. C., Neve, R., Lieberburg, I., and Fritz, L. C., 1990, The Alzheimer amyloid precursor protein. Identification of a stable intermediate in the biosynthetic/degradative pathway, *J Biol Chem* **265**(8):4492-7.

Ott, A., Slooter, A. J., Hofman, A., van Harskamp, F., Witteman, J. C., Van Broeckhoven, C., van Duijn, C. M., and Breteler, M. M., 1998, Smoking and risk of dementia and Alzheimer's disease in a population- based cohort study: the Rotterdam Study, *Lancet* **351**(9119):1840-3.

Ounanian, A., Guilbert, B., Renversez, J. C., Seigneurin, J. M., and Avrameas, S., 1990, Antibodies to viral antigens, xenoantigens, and autoantigens in Alzheimer's disease, *J Clin Lab Anal* **4**(5):367-75.

Owens, G. K., and Reidy, M. A., 1985, Hyperplastic growth response of vascular smooth muscle cells following induction of acute hypertension in rats by aortic coarctation, *Circ Res* **57**(5):695-705.

Owens, G. K., and Schwartz, S. M., 1982, Alterations in vascular smooth muscle mass in the spontaneously hypertensive rat. Role of cellular hypertrophy, hyperploidy, and hyperplasia, *Circ Res* **51**(3):280-9.

Pacheco, M. A., and Jope, R. S., 1996, Phosphoinositide signaling in human brain, *Prog Neurobiol* **50**(2-3):255-73.

Paganini-Hill, A., and Henderson, V. W., 1994, Estrogen deficiency and risk of Alzheimer's disease in women, *Am J Epidemiol* **140**(3):256-61.

Pappolla, M. A., Omar, R. A., Kim, K. S., and Robakis, N. K., 1992, Immunohistochemical evidence of oxidative [corrected] stress in Alzheimer's disease [published erratum appears in Am J Pathol 1996 Nov;149(5):1770], *Am J Pathol* **140**(3):621-8.

Pappolla, M. A., Sos, M., Omar, R. A., and Sambamurti, K., 1996, The heat shock/oxidative stress connection. Relevance to Alzheimer disease, *Mol Chem Neuropathol* **28**(1-3):21-34.

Pericak-Vance, M. A., Bass, M. P., Yamaoka, L. H., Gaskell, P. C., Scott, W. K., Terwedow, H. A., Menold, M. M., Conneally, P. M., Small, G. W., Vance, J. M., Saunders, A. M., Roses, A. D., and Haines, J. L., 1997, Complete genomic screen in late-onset familial Alzheimer disease. Evidence for a new locus on chromosome 12 [see comments], *Jama* **278**(15):1237-41.

Perry, G., Smith, M. A., McCann, C. E., Siedlak, S. L., Jones, P. K., and Friedland, R. P., 1998, Cerebrovascular muscle atrophy is a feature of Alzheimer's disease, *Brain Res* **791**(1-2):63-6.

Pietropaolo, M., Castano, L., Babu, S., Buelow, R., Kuo, Y. L., Martin, S., Martin, A., Powers, A. C., Prochazka, M., Naggert, J., and et al., 1993, Islet cell autoantigen 69 kD (ICA69). Molecular cloning and characterization of a novel diabetes-associated autoantigen, *J Clin Invest* **92**(1):359-71.

Pirttila, T., Mattinen, S., and Frey, H., 1992, The decrease of CD8-positive lymphocytes in Alzheimer's disease, *J Neurol Sci* **107**(2):160-5.

Poirier, J., 1994, Apolipoprotein E in animal models of CNS injury and in Alzheimer's disease, *Trends Neurosci* **17**(12):525-30.

Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., and Fuller, F., 1988, A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors, *Nature* **331**(6156):525-7.

Ponting, C. P., and Phillips, C., 1997, Identification of homer as a homologue of the Wiskott-Aldrich syndrome protein suggests a receptor-binding function for WH1 domains, *J Mol Med* **75**(11-12):769-71.

Pouplard, A. a. E., J, 1985, New immunological findings in senile dementia, *Interdiscipl.Topics Geront.* **19**:62.

Pulsinelli, W., 1985, Selective neuronal vulnerability: morphological and molecular characteristics, in: *Progress in Brain Research* (S. B. a. W. F. Kogure K Hossmann K-A, ed.), Elsevier Science Publishers, Amsterdam, pp. 29-37.

Quon, D., Wang, Y., Catalano, R., Scardina, J. M., Murakami, K., and Cordell, B., 1991, Formation of beta-amyloid protein deposits in brains of transgenic mice, *Nature* **352**(6332):239-41.

Rall, S. C., Jr., Weisgraber, K. H., and Mahley, R. W., 1982, Human apolipoprotein E. The complete amino acid sequence, *J Biol Chem* **257**(8):4171-8.

Rebeck, G. W., Harr, S. D., Strickland, D. K., and Hyman, B. T., 1995, Multiple, diverse senile plaque-associated proteins are ligands of an apolipoprotein E receptor, the alpha 2-macroglobulin receptor/low- density-lipoprotein receptor-related protein, *Ann Neurol* **37**(2):211-7.

Rechsteiner, M., and Rogers, S. W., 1996, PEST sequences and regulation by proteolysis, *Trends Biochem Sci* **21**(7):267-71.

Rich, J. B., Rasmusson, D. X., Folstein, M. F., Carson, K. A., Kawas, C., and Brandt, J., 1995, Nonsteroidal anti-inflammatory drugs in Alzheimer's disease [see comments], *Neurology* **45**(1):51-5.

Roch, J. M., Shapiro, I. P., Sundsmo, M. P., Otero, D. A., Refolo, L. M., Robakis, N. K., and Saitoh, T., 1992, Bacterial expression, purification, and functional mapping of the amyloid beta/A4 protein precursor, *J Biol Chem* **267**(4):2214-21.

Rogaev, E. I., Sherrington, R., Rogaeva, E. A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., and et al., 1995, Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene, *Nature* **376**(6543):775-8.

Rogers, J., Cooper, N. R., Webster, S., Schultz, J., McGeer, P. L., Styren, S. D., Civin, W. H., Brachova, L., Bradt, B., Ward, P., and et al., 1992, Complement activation by beta-amyloid in Alzheimer disease, *Proc Natl Acad Sci U S A* **89**(21):10016-20.

Rogers, J., Luber-Narod, J., Styren, S. D., and Civin, W. H., 1988, Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease, *Neurobiol Aging* **9**(4)**:**339-49.

Rogers, J. D., Brogan, D., and Mirra, S. S., 1985, The nucleus basalis of Meynert in neurological disease: a quantitative morphological study, *Ann Neurol* **17**(2)**:**163-70.

Rogers, S., Wells, R., and Rechsteiner, M., 1986, Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis, *Science* **234**(4774)**:**364-8.

Rosenberg, S. A., 1997, Cancer vaccines based on the identification of genes encoding cancer regression antigens, *Immunol Today* **18**(4)**:**175-82.

Roses, A., Gilbert, JR, Bartlett, RJ, Pericak-Vance, MA, Dawson, D, and Morrison, MR, 1987, Sib-pair linkage and subtraction hybridazation strategies for Alzheimer's disease, in: *Banbury Report 27, Molecular Neuropathology of Aging*, New York, pp. 443-458.

Roses, A. D., 1995, Apolipoprotein E genotyping in the differential diagnosis, not prediction, of Alzheimer's disease [see comments], *Ann Neurol* **38**(1)**:**6-14.

Roses, A. D., 1998, Genetic associations [letter], *Lancet* **351**(9106)**:**916.

Roses, A. D., Einstein, G., Gilbert, J., Goedert, M., Han, S. H., Huang, D., Hulette, C., Masliah, E., Pericak-Vance, M. A., Saunders, A. M., Schmechel, D. E., Strittmatter, W. J., Weisgraber, K. H., and Xi, P. T., 1996, Morphological, biochemical, and genetic support for an apolipoprotein E effect on microtubular metabolism, *Ann N Y Acad Sci* **777:**146-57.

Ross, B. M., Knowler, J. T., and McCulloch, J., 1992, On the stability of messenger RNA and ribosomal RNA in the brains of control human subjects and patients with Alzheimer's disease, *J Neurochem* **58**(5):1810-9.

Sahin, U., Tureci, O., and Pfreundschuh, M., 1997, Serological identification of human tumor antigens, *Curr Opin Immunol* **9**(5):709-16.

Sahin, U., Tureci, O., Schmitt, H., Cochlovius, B., Johannes, T., Schmits, R., Stenner, F., Luo, G., Schobert, I., and Pfreundschuh, M., 1995, Human neoplasms elicit multiple specific immune responses in the autologous host, *Proc Natl Acad Sci U S A* **92**(25):11810-3.

Saitoh, T., Sundsmo, M., Roch, J. M., Kimura, N., Cole, G., Schubert, D., Oltersdorf, T., and Schenk, D. B., 1989, Secreted form of amyloid beta protein precursor is involved in the growth regulation of fibroblasts, *Cell* **58**(4):615-22.

Sajdel-Sulkowska, E. M., and Marotta, C. A., 1984, Alzheimer's disease brain: alterations in RNA levels and in a ribonuclease-inhibitor complex, *Science* **225**(4665):947-9.

Salim, M., Rehman, S., Sajdel-Sulkowska, E. M., Chou, W. G., Majocha, R. E., Marotta, C. A., and Zain, S. B., 1988, Preparation of a recombinant cDNA library from poly(A+) RNA of the Alzheimer brain. Identification and characterization of a cDNA copy encoding a glial-specific protein, *Neurobiol Aging* **9**(2):163-71.

Sambrook, J., Fritsch, EF, and Maniatis, T, 1989, Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratory Press.

Sanan, D. A., Weisgraber, K. H., Russell, S. J., Mahley, R. W., Huang, D., Saunders, A., Schmechel, D., Wisniewski, T., Frangione, B., Roses, A. D., and et al., 1994, Apolipoprotein E associates with beta amyloid peptide of Alzheimer's disease to form novel monofibrils. Isoform apoE4 associates more efficiently than apoE3, *J Clin Invest* **94**(2):860-9.

Saunders, A. M., Schmader, K., Breitner, J. C., Benson, M. D., Brown, W. T., Goldfarb, L., Goldgaber, D., Manwaring, M. G., Szymanski, M. H., McCown, N., and et al., 1993, Apolipoprotein E epsilon 4 allele distributions in late-onset Alzheimer's disease and in other amyloid-forming diseases [see comments], *Lancet* **342**(8873):710-1.

Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S., 1996, Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease [see comments], *Nat Med* **2**(8):864-70.

Schmechel, D. E., Saunders, A. M., Strittmatter, W. J., Crain, B. J., Hulette, C. M., Joo, S. H., Pericak-Vance, M. A., Goldgaber, D., and Roses, A. D., 1993, Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease, *Proc Natl Acad Sci U S A* **90**(20):9649-53.

Schneider, L. S., Farlow, M. R., Henderson, V. W., and Pogoda, J. M., 1996, Effects of estrogen replacement therapy on response to tacrine in patients with Alzheimer's disease, *Neurology* **46**(6):1580-4.

Scott, W. K., Saunders, A. M., Gaskell, P. C., Locke, P. A., Growdon, J. H., Farrer, L. A., Auerbach, S. A., Roses, A. D., Haines, J. L., and Pericak-Vance, M. A., 1997,

Apolipoprotein E epsilon2 does not increase risk of early-onset sporadic Alzheimer's disease, *Ann Neurol* **42**(3):376-8.

Selkoe, D. J., 1991, The molecular pathology of Alzheimer's disease, *Neuron* **6**(4):487-98.

Selkoe, D. J., 1993, Physiological production of the beta-amyloid protein and the mechanism of Alzheimer's disease, *Trends Neurosci* **16**(10):403-9.

Selkoe, D. J., 1994a, Cell biology of the amyloid beta-protein precursor and the mechanism of Alzheimer's disease, *Annu Rev Cell Biol* **10**:373-403.

Selkoe, D. J., 1994b, Normal and abnormal biology of the beta-amyloid precursor protein, *Annu Rev Neurosci* **17**:489-517.

Selkoe, D. J., 1997, Alzheimer's disease: genotypes, phenotypes, and treatments, *Science* **275**(5300):630-1.

Serot, J. M., Bene, M. C., and Faure, G. C., 1994, Comparative immunohistochemical characteristics of human choroid plexus in vascular and Alzheimer's dementia [see comments], *Hum Pathol* **25**(11):1185-90.

Serot, J. M., Bene, M. C., Gobert, B., Christmann, D., Leheup, B., and Faure, G. C., 1992, Antibodies to choroid plexus in senile dementia of Alzheimer's type, *J Clin Pathol* **45**(9):781-3.

Seshadri, S., Drachman, D. A., and Lippa, C. F., 1995, Apolipoprotein E epsilon 4 allele and the lifetime risk of Alzheimer's disease. What physicians know, and what they should know, *Arch Neurol* **52**(11):1074-9.

Shah, G. N., and Mooradian, A. D., 1997, Age-related changes in the blood-brain barrier, *Exp Gerontol* **32**(4-5):501-19.

Shen, J., Bronson, R. T., Chen, D. F., Xia, W., Selkoe, D. J., and Tonegawa, S., 1997, Skeletal and CNS defects in Presenilin-1-deficient mice, *Cell* **89**(4):629-39.

Sheng, M., 1996, PDZs and receptor/channel clustering: rounding up the latest suspects [comment], *Neuron* **17**(4):575-8.

Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., and et al., 1995, Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease [see comments], *Nature* **375**(6534):754-60.

Sherwin, B. B., and Gelfand, M. M., 1989, A prospective one-year study of estrogen and progestin in postmenopausal women: effects on clinical symptoms and lipoprotein lipids, *Obstet Gynecol* **73**(5 Pt 1):759-66.

Shigematsu, K., McGeer, P. L., and McGeer, E. G., 1992, Localization of amyloid precursor protein in selective postsynaptic densities of rat cortical neurons, *Brain Res* **592**(1-2):353-7.

Shivers, B. D., Hilbich, C., Multhaup, G., Salbaum, M., Beyreuther, K., and Seeburg, P. H., 1988, Alzheimer's disease amyloidogenic glycoprotein: expression pattern in rat brain suggests a role in cell contact, *Embo J* **7**(5):1365-70.

Short, J. M., Fernandez, J. M., Sorge, J. A., and Huse, W. D., 1988, Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties, *Nucleic Acids Res* **16**(15):7583-600.

Singh, V., Yang, YY and Singh, EA, 1992, Immunoblot detection of antibodies to myelin basic protein in Alzheimer's disease patients, *Neurosci.Lett.* **147:**25.

Singh, V. K., 1997, Neuroautoimmunity: pathogenic implications for Alzheimer's disease, *Gerontology* **43**(1-2)**:**79-94.

Singh, V. K., and Fudenberg, H. H., 1986, Detection of brain autoantibodies in the serum of patients with Alzheimer's disease but not Down's syndrome, *Immunol Lett* **12**(5-6)**:**277-80.

Skoog, I., Vanmechelen, E., Andreasson, L. A., Palmertz, B., Davidsson, P., Hesse, C., and Blennow, K., 1995, A population-based study of tau protein and ubiquitin in cerebrospinal fluid in 85-year-olds: relation to severity of dementia and cerebral atrophy, but not to the apolipoprotein E4 allele, *Neurodegeneration* **4**(4)**:**433-42.

Skoog, I., Wallin, A., Fredman, P., Hesse, C., Aevarsson, O., Karlsson, I., Gottfries, C. G., and Blennow, K., 1998, A population study on blood-brain barrier function in 85-year-olds: relation to Alzheimer's disease and vascular dementia, *Neurology* **50**(4)**:**966-71.

Small, D. H., Nurcombe, V., Reed, G., Clarris, H., Moir, R., Beyreuther, K., and Masters, C. L., 1994, A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth, *J Neurosci* **14**(4)**:**2117-27.

Smith, M. A., Kutty, R. K., Richey, P. L., Yan, S. D., Stern, D., Chader, G. J., Wiggert, B., Petersen, R. B., and Perry, G., 1994, Heme oxygenase-1 is associated with the neurofibrillary pathology of Alzheimer's disease, *Am J Pathol* **145**(1)**:**42-7.

Sohda, M., Misumi, Y., Fujiwara, T., Nishioka, M., and Ikehara, Y., 1994, Molecular cloning and sequence analysis of a human 372-kDA protein localized in the Golgi complex, *Biochem Biophys Res Commun* **205**(2):1399-408.

Soininen, H., Syrjanen, S., Heinonen, O., Neittaanmaki, H., Miettinen, R., Paljarvi, L., Syrjanen, K., Beyreuther, K., and Riekkinen, P., 1992, Amyloid beta-protein deposition in skin of patients with dementia [letter], *Lancet* **339**(8787):245.

Sparks, D. L., 1997, Coronary artery disease, hypertension, ApoE, and cholesterol: a link to Alzheimer's disease?, *Ann N Y Acad Sci* **826:**128-46.

Srivastava, P. K., 1996, Do human cancers express shared protective antigens? or the necessity of remembrance of things past, *Semin Immunol* **8**(5):295-302.

Stewart, R., 1998, Cardiovascular factors in Alzheimer's disease [editorial], *J Neurol Neurosurg Psychiatry* **65**(2):143-7.

Stewart, W. F., Kawas, C., Corrada, M., and Metter, E. J., 1997, Risk of Alzheimer's disease and duration of NSAID use [see comments], *Neurology* **48**(3):626-32.

Strittmatter, W. J., Saunders, A. M., Goedert, M., Weisgraber, K. H., Dong, L. M., Jakes, R., Huang, D. Y., Pericak-Vance, M., Schmechel, D., and Roses, A. D., 1994a, Isoform-specific interactions of apolipoprotein E with microtubule- associated protein tau: implications for Alzheimer disease, *Proc Natl Acad Sci U S A* **91**(23):11183-6.

Strittmatter, W. J., Saunders, A. M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G. S., and Roses, A. D., 1993a, Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease, *Proc Natl Acad Sci U S A* **90**(5):1977-81.

Strittmatter, W. J., Weisgraber, K. H., Goedert, M., Saunders, A. M., Huang, D., Corder, E. H., Dong, L. M., Jakes, R., Alberts, M. J., Gilbert, J. R., and et al., 1994b, Hypothesis: microtubule instability and paired helical filament formation in the Alzheimer disease brain are related to apolipoprotein E genotype, *Exp Neurol* **125**(2):163-71; discussion 172-4.

Strittmatter, W. J., Weisgraber, K. H., Huang, D. Y., Dong, L. M., Salvesen, G. S., Pericak-Vance, M., Schmechel, D., Saunders, A. M., Goldgaber, D., and Roses, A. D., 1993b, Binding of human apolipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease, *Proc Natl Acad Sci U S A* **90**(17):8098-102.

Sturchler-Pierrat, C., Abramowski, D., Duke, M., Wiederhold, K. H., Mistl, C., Rothacher, S., Ledermann, B., Burki, K., Frey, P., Paganetti, P. A., Waridel, C., Calhoun, M. E., Jucker, M., Probst, A., Staufenbiel, M., and Sommer, B., 1997, Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology, *Proc Natl Acad Sci U S A* **94**(24):13287-92.

Suo, Z., Tan, J., Placzek, A., Crawford, F., Fang, C., and Mullan, M., 1998, Alzheimer's beta-amyloid peptides induce inflammatory cascade in human vascular cells: the roles of cytokines and CD40 [In Process Citation], *Brain Res* **807**(1-2):110-7.

Symons, M., Derry, J. M., Karlak, B., Jiang, S., Lemahieu, V., McCormick, F., Francke, U., and Abo, A., 1996, Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization, *Cell* **84**(5):723-34.

Tagliavini, F., Pilleri, G., Gemignani, F., and Lechi, A., 1983, Neuronal loss in the basal nucleus of Meynert in progressive supranuclear palsy, *Acta Neuropathol (Berl)* **61**(2):157-60.

Takahashi, H., Kurashima, C., Utsuyama, M., and Hirokawa, K., 1990, Immunohistological study of senile brains by using a monoclonal antibody recognizing beta amyloid precursor protein: significance of granular deposits in relation with senile plaques, *Acta Neuropathol (Berl)* **80**(3):260-5.

Takami, K., Terai, K., Matsuo, A., Walker, D. G., and McGeer, P. L., 1997, Expression of presenilin-1 and -2 mRNAs in rat and Alzheimer's disease brains, *Brain Res* **748**(1-2):122-30.

Takeda, M., Kanayama, G, Niigawa, H, Tanii, H, Taniguchi, N, Ikura, Y, Kashiwagi, Y, Miyamae, Y, and Nishimura, T, 1997, Neurochemical basis of risk factors for Alzheimer's disease: acute-phase protein and neurofilament phosphorylation after repetitive subthreshold brain injury, in: *Alzheimer's disease: biology, diagnosis and therapeutics* (K. Iqbal, Winblad, B, Nishimura, T, Takeda, M, Wisniewski, HM, ed.), John Wiley & Sons, Chichester, pp. 561-566.

Tamaoka, A., Sawamura, N., Odaka, A., Suzuki, N., Mizusawa, H., Shoji, S., and Mori, H., 1995, Amyloid beta protein 1-42/43 (A beta 1-42/43) in cerebellar diffuse plaques: enzyme-linked immunosorbent assay and immunocytochemical study, *Brain Res* **679**(1):151-6.

Tanzi, R. E., Gusella, J. F., Watkins, P. C., Bruns, G. A., St George-Hyslop, P., Van Keuren, M. L., Patterson, D., Pagan, S., Kurnit, D. M., and Neve, R. L., 1987, Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus, *Science* **235**(4791):880-4.

Tanzi, R. E., McClatchey, A. I., Lamperti, E. D., Villa-Komaroff, L., Gusella, J. F., and Neve, R. L., 1988, Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease, *Nature* **331**(6156):528-30.

Tanzi, R. E., Vaula, G., Romano, D. M., Mortilla, M., Huang, T. L., Tupler, R. G., Wasco, W., Hyman, B. T., Haines, J. L., Jenkins, B. J., and et al., 1992, Assessment of amyloid beta-protein precursor gene mutations in a large set of familial and sporadic Alzheimer disease cases, *Am J Hum Genet* **51**(2):273-82.

Tavolato, B., and Argentiero, V., 1980, Immunological indices in presenile Alzheimer's disease, *J Neurol Sci* **46**(3):325-31.

Taylor, G. R., Carter, G. I., Crow, T. J., Johnson, J. A., Fairbairn, A. F., Perry, E. K., and Perry, R. H., 1986, Recovery and measurement of specific RNA species from postmortem brain tissue: a general reduction in Alzheimer's disease detected by molecular hybridization, *Exp Mol Pathol* **44**(1):111-6.

Teller, J. K., Russo, C., DeBusk, L. M., Angelini, G., Zaccheo, D., Dagna-Bricarelli, F., Scartezzini, P., Bertolini, S., Mann, D. M., Tabaton, M., and Gambetti, P., 1996, Presence of soluble amyloid beta-peptide precedes amyloid plaque formation in Down's syndrome [see comments], *Nat Med* **2**(1):93-5.

Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., Hansen, L. A., and Katzman, R., 1991, Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment, *Ann Neurol* **30**(4):572-80.

Terryberry, J. W., Thor, G., and Peter, J. B., 1998, Autoantibodies in neurodegenerative diseases: antigen-specific frequencies and intrathecal analysis, *Neurobiol Aging* **19**(3):205-16.

Theuring, F., Thunecke, M., Kosciessa, U., and Turner, J. D., 1997, Transgenic animals as models of neurodegenerative diseases in humans, *Trends Biotechnol* **15**(8):320-5.

Tilvis, R. S., Erkinjuntti, T., Sulkava, R., and Miettinen, T. A., 1987, Fatty acids of plasma lipids, red cells and platelets in Alzheimer's disease and vascular dementia, *Atherosclerosis* **65**(3):237-45.

Toh, B. H., Gibbs, C. J., Jr., Gajdusek, D. C., Goudsmit, J., and Dahl, D., 1985, The 200- and 150-kDa neurofilament proteins react with IgG autoantibodies from patients with kuru, Creutzfeldt-Jakob disease, and other neurologic diseases, *Proc Natl Acad Sci U S A* **82**(10):3485-9.

Tomlinson, B. E., Blessed, G., and Roth, M., 1970, Observations on the brains of demented old people, *J Neurol Sci* **11**(3):205-42.

Tsuda, T., Lopez, R., Rogaeva, E. A., Freedman, M., Rogaev, E., Drachman, D., Pollen, D., Haines, J., Liang, Y., McLachlan, D. R., and et al., 1994, Are the associations between Alzheimer's disease and polymorphisms in the apolipoprotein E and the apolipoprotein CII genes due to linkage disequilibrium?, *Ann Neurol* **36**(1):97-100.

Tureci, O., Sahin, U., Schobert, I., Koslowski, M., Scmitt, H., Schild, H. J., Stenner, F., Seitz, G., Rammensee, H. G., and Pfreundschuh, M., 1996, The SSX-2 gene, which is involved in the t(X;18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40, *Cancer Res* **56**(20):4766-72.

Tureci, O., Schmitt, H., Fadle, N., Pfreundschuh, M., and Sahin, U., 1997, Molecular definition of a novel human galectin which is immunogenic in patients with Hodgkin's disease, *J Biol Chem* **272**(10):6416-22.

Turjanski, N., and Brooks, D. J., 1997, PET and the investigation of dementia in the parkinsonian patient, *J Neural Transm Suppl* **51**:37-48.

Ueki, A., Kawano, M., Namba, Y., Kawakami, M., and Ikeda, K., 1993, A high frequency of apolipoprotein E4 isoprotein in Japanese patients with late-onset nonfamilial Alzheimer's disease, *Neurosci Lett* **163**(2):166-8.

Uhl, G. R., Hilt, D. C., Hedreen, J. C., Whitehouse, P. J., and Price, D. L., 1983, Pick's disease (lobar sclerosis): depletion of neurons in the nucleus basalis of Meynert, *Neurology* **33**(11):1470-3.

Utermann, G., Vogelberg, K. H., Steinmetz, A., Schoenborn, W., Pruin, N., Jaeschke, M., Hees, M., and Canzler, H., 1979, Polymorphism of apolipoprotein E. II. Genetics of hyperlipoproteinemia type III, *Clin Genet* **15**(1):37-62.

van der Cammen, T. J., Verschoor, C. J., van Loon, C. P., van Harskamp, F., de Koning, I., Schudel, W. J., Slooter, A. J., Van Broeckhoven, C., and van Duijn, C. M., 1998, Risk of left ventricular dysfunction in patients with probable Alzheimer's disease with APOE*4 allele, *J Am Geriatr Soc* **46**(8):962-7.

van Duijn, C. M., Havekes, L. M., Van Broeckhoven, C., de Knijff, P., and Hofman, A., 1995, Apolipoprotein E genotype and association between smoking and early onset Alzheimer's disease, *Bmj* **310**(6980):627-31.

Van Hoesen, G. W., Hyman, B. T., and Damasio, A. R., 1991, Entorhinal cortex pathology in Alzheimer's disease, *Hippocampus* **1**(1):1-8.

Vandermeeren, M., Mercken, M., Vanmechelen, E., Six, J., van de Voorde, A., Martin, J. J., and Cras, P., 1993, Detection of tau proteins in normal and Alzheimer's disease cerebrospinal fluid with a sensitive sandwich enzyme-linked immunosorbent assay, *J Neurochem* **61**(5):1828-34.

Vazquez, J., Fernandez-Shaw, C., Marina, A., Haas, C., Cacabelos, R., and Valdivieso, F., 1996, Antibodies to human brain spectrin in Alzheimer's disease, *J Neuroimmunol* **68**(1-2):39-44.

Vigo-Pelfrey, C., Seubert, P., Barbour, R., Blomquist, C., Lee, M., Lee, D., Coria, F., Chang, L., Miller, B., Lieberburg, I., and et al., 1995, Elevation of microtubule-associated protein tau in the cerebrospinal fluid of patients with Alzheimer's disease, *Neurology* **45**(4):788-93.

Viola, A., and Lanzavecchia, A., 1996, T cell activation determined by T cell receptor number and tunable thresholds [see comments], *Science* **273**(5271):104-6.

Vitek, M. P., Bhattacharya, K., Glendening, J. M., Stopa, E., Vlassara, H., Bucala, R., Manogue, K., and Cerami, A., 1994, Advanced glycation end products contribute to amyloidosis in Alzheimer disease, *Proc Natl Acad Sci U S A* **91**(11):4766-70.

Vitek, M. P., Rasool, C. G., de Sauvage, F., Vitek, S. M., Bartus, R. T., Beer, B., Ashton, R. A., Macq, A. F., Maloteaux, J. M., Blume, A. J., and et al., 1988, Absence of mutation in the beta-amyloid cDNAs cloned from the brains of three patients with sporadic Alzheimer's disease, *Brain Res* **464**(2):121-31.

Vito, P., Lacana, E., and D'Adamio, L., 1996, Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer's disease gene ALG-3, *Science* **271**(5248):521-5.

Wang, J. Z., Gong, C. X., Zaidi, T., Grundke-Iqbal, I., and Iqbal, K., 1995, Dephosphorylation of Alzheimer paired helical filaments by protein phosphatase-2A and -2B, *J Biol Chem* **270**(9):4854-60.

Wang, J. Z., Grundke-Iqbal, I., and Iqbal, K., 1996, Glycosylation of microtubule-associated protein tau: an abnormal posttranslational modification in Alzheimer's disease [see comments], *Nat Med* **2**(8):871-5.

Watts, H., Kennedy, P. G., and Thomas, M., 1981, The significance of anti-neuronal antibodies in Alzheimer's disease, *J Neuroimmunol* **1**(1):107-16.

Wax, S. D., Rosenfield, C. L., and Taubman, M. B., 1994, Identification of a novel growth factor-responsive gene in vascular smooth muscle cells, *J Biol Chem* **269**(17):13041-7.

Wax, S. D., Tsao, L., Lieb, M. E., Fallon, J. T., and Taubman, M. B., 1996, SM-20 is a novel 40-kd protein whose expression in the arterial wall is restricted to smooth muscle, *Lab Invest* **74**(4):797-808.

Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L., and Beyreuther, K., 1989, Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein, *Cell* **57**(1):115-26.

Weinstock, M., 1995, The pharmacotherapy of Alzheimer's disease based on the cholinergic hypothesis: an update, *Neurodegeneration* **4**(4):349-56.

Weisgraber, K. H., Innerarity, T. L., and Mahley, R. W., 1982, Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site, *J Biol Chem* **257**(5):2518-21.

Welsh-Bohmer, K. A., Gearing, M., Saunders, A. M., Roses, A. D., and Mirra, S., 1997, Apolipoprotein E genotypes in a neuropathological series from the Consortium to Establish a Registry for Alzheimer's Disease, *Ann Neurol* **42**(3):319-25.

Wen, G. Y., Wisniewski, H. M., Blondal, H., Benedikz, E., Frey, H., Pirttila, T., Rudelli, R., and Kim, K. S., 1994, Presence of non-fibrillar amyloid beta protein in skin biopsies of Alzheimer's disease (AD), Down's syndrome and non-AD normal persons, *Acta Neuropathol (Berl)* **88**(3):201-6.

Whitehouse, P. J., Hedreen, J. C., White, C. L. d., and Price, D. L., 1983, Basal forebrain neurons in the dementia of Parkinson disease, *Ann Neurol* **13**(3):243-8.

Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., and Delon, M. R., 1982, Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain, *Science* **215**(4537):1237-9.

Wisniewski, H. M., Narang, H. K., and Terry, R. D., 1976, Neurofibrillary tangles of paired helical filaments, *J Neurol Sci* **27**(2):173-81.

Wisniewski, T., Castano, E. M., Golabek, A., Vogel, T., and Frangione, B., 1994, Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro, *Am J Pathol* **145**(5):1030-5.

Wolozin, B., Iwasaki, K., Vito, P., Ganjei, J. K., Lacana, E., Sunderland, T., Zhao, B., Kusiak, J. W., Wasco, W., and D'Adamio, L., 1996, Participation of presenilin 2 in apoptosis: enhanced basal activity conferred by an Alzheimer mutation, *Science* **274**(5293):1710-3.

Wong, P. C., Zheng, H., Chen, H., Becher, M. W., Sirinathsinghji, D. J., Trumbauer, M. E., Chen, H. Y., Price, D. L., Van der Ploeg, L. H., and Sisodia, S. S., 1997, Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm, *Nature* **387**(6630):288-92.

Xu, H., Gouras, G. K., Greenfield, J. P., Vincent, B., Naslund, J., Mazzarelli, L., Fried, G., Jovanovic, J. N., Seeger, M., Relkin, N. R., Liao, F., Checler, F., Buxbaum, J. D., Chait, B. T., Thinakaran, G., Sisodia, S. S., Wang, R., Greengard, P., and Gandy, S., 1998, Estrogen reduces neuronal generation of Alzheimer beta-amyloid peptides, *Nat Med* **4**(4):447-51.

Xu, S., and Gaskin, F., 1997, Increased incidence of anti-beta-amyloid autoantibodies secreted by Epstein-Barr virus transformed B cell lines from patients with Alzheimer's disease, *Mech Ageing Dev* **94**(1-3):213-22.

Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M., and Harigaya, Y., 1988, Diffuse type of senile plaques in the brains of Alzheimer-type dementia, *Acta Neuropathol (Berl)* **77**(2):113-9.

Yamamoto, Y., Nakano, S., Kawashima, S., Nakamura, S., Urakami, K., Kato, T., and Kameyama, M., 1990, Plasma and serum G4 isoenzyme of acetylcholinesterase in patients with Alzheimer-type dementia and vascular dementia, *Ann Clin Biochem* **27**(Pt 4):321-6.

Yan, S. D., Chen, X., Schmidt, A. M., Brett, J., Godman, G., Zou, Y. S., Scott, C. W., Caputo, C., Frappier, T., Smith, M. A., and et al., 1994, Glycated tau protein in Alzheimer disease: a mechanism for induction of oxidant stress, *Proc Natl Acad Sci U S A* **91**(16):7787-91.

Yan, S. D., Yan, S. F., Chen, X., Fu, J., Chen, M., Kuppusamy, P., Smith, M. A., Perry, G., Godman, G. C., Nawroth, P., and et al., 1995, Non-enzymatically glycated tau in Alzheimer's disease induces neuronal oxidant stress resulting in cytokine gene expression and release of amyloid beta-peptide, *Nat Med* **1**(7):693-9.

Yankner, B. A., 1996, Mechanisms of neuronal degeneration in Alzheimer's disease, *Neuron* **16**(5):921-32.

Yoshikai, S., Sasaki, H., Doh-ura, K., Furuya, H., and Sakaki, Y., 1990, Genomic organization of the human amyloid beta-protein precursor gene [published erratum appears in Gene 1991 Jun 30;102(2):291-2], *Gene* **87**(2):257-63.

Zannis, V. I., and Breslow, J. L., 1981, Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modification, *Biochemistry* **20**(4):1033-41.

Ziff, E. B., 1997, Enlightening the postsynaptic density, *Neuron* **19**(6):1163-74.