Biochemical Assay of Alzheimer's Disease–Associated Protein(s) in Human Brain Tissue

A Clinical Study

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The concentration of Alzheimer's disease–associated protein (ADAP) was measured in postmortem brain tissue samples of temporal or frontal cortex from 111 human brains using a sandwich immunoassay. Alzheimer's disease–associated protein has three major ALZ-50–reactive subunits, including A-68. This assay utilizes ALZ-50 and a rabbit antibody raised against a highly ADAP-enriched brain protein fraction. The frequently observed cross-reactivity of ALZ-50 with normal brain components in direct immunoassays is minimized by this configuration. There were 27 normal controls, 28 neurologic disease controls, and 56 Alzheimer's disease cases. The normal control and neurologic disease control cases had essentially no detectable level of ADAP, while ADAP was clearly detected in 85.7% of the Alzheimer's disease cases. Clinical dementia, neuritic plaques, and old age per se are not correlated with increased ADAP levels. This biochemical assay of ADAP may prove to be helpful as an adjunct in the clinicopathologic diagnosis of Alzheimer's disease.

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ALZHEIMER'S disease (AD) is a neurodegenerative disease characterized by a chronically deteriorating course of impaired intellectual function and memory loss. The definitive diagnosis of AD is made by pathologic examination of postmortem brain tissue in conjunction with a clinical history of dementia.¹ This diagnosis is based on the presence in brain tissue of neuritic (senile) plaques (NPs) and neurofibrillary tangles (NFTs),

which have been correlated with clinical dementia.² Neuritic plaques are thought to be composed of degenerating axons and nerve terminals as well as possible astrocytic elements,³ and they often exhibit a central amyloid protein core.⁴ The NFTs are intraneuronal aggregates composed of normal and paired helical filaments and presumably consist of several different proteins.⁴

Current diagnostic methods for AD are based on the neurohistopathologic identification and counting of NPs and NFTs, a process that requires staining and microscopic examination of several brain sections.¹ However, there are difficulties with using the NPs and NFTs as the basis for a diagnosis. For instance, the clinical and pathologic progression of AD is marked by a continuing loss of neurons from the cerebral cortex, even though densities of NFTs and NPs within this region do not appear to correlate with the stage of the illness.⁵ Furthermore, NPs and NFTs also occur in nondemented elderly patients.¹ Histochemical staining is not very reproducible, NPs and NFTs are

not uniformly distributed, and histopathologic studies are time-consuming and labor-intensive.⁶ Additionally, some studies have shown that NFTs and NPs are not necessarily correlated with the development of AD.^{5,7,8} Therefore, it would be useful if there were a more rapid assay method for the diagnosis of AD, with comparable reliability.

ALZ-50 is a monoclonal antibody that reacts against brain tissue homogenates from patients with AD; it binds to a protein marker (A-68) highly selective for this disorder.⁹⁻¹¹ Immunocytochemical staining has established that ALZ-50 recognizes an early cytologic change that precedes the formation of NFTs and NPs. This early cytologic change is apparently specific for AD.^{7,12,13} Thus, this antibody may be useful in demonstrating a change in AD that precedes the development of NFTs and NPs. However, ALZ-50 cross-reacts with normal components of the brain in immunoassays and Western blot assays and, less frequently, in immunocytochemical staining.^{11,14} In the present study we used a sandwich enzyme immunoassay (developed at Abbott Laboratories, Abbott Park, Ill) that minimizes ALZ-50 cross-reactivity and detects Alzheimer's disease–associated proteins (ADAP) with high specificity.¹⁵ Alzheimer's disease–associated protein has three major ALZ-50 reactive subunits, including A-68, and is primarily associated with AD.¹⁶

MATERIALS AND METHODS

Analysis

The ALZ-50 antibody used in conjunction with a rabbit polyclonal antibody (PR1) is the basis of a rapid immunoassay (ALZ-EIA) for brain tissue.¹⁵ The rabbit antibody was developed using a highly purified ADAP fraction.

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The assay employs 6.35-mm polystyrene beads coated with a purified (IgG) fraction of PR1. In the assay, the antigen in brain homogenate is first captured on the beads, and, in the second step, ALZ-50 is allowed to bind specifically to the antigen immobilized on the bead. The ALZ-EIA activity is a measure of bound ALZ-50 using an enzyme-conjugated anti-mouse IgM. This configuration appears to minimize complications caused by the cross-reactants from both AD and normal brain homogenates.¹⁵ Temporal or frontocortical areas were used as the source of tissue because these areas were found to have consistently higher ADAP immunoreactivity than other brain areas.¹⁷ The tissue (20 to 200 mg wet weight) was mixed with four volumes of homogenization buffer (tromethamine hydrochloride, 0.05 mol/L, at pH 6.8; ethyleneglycol-bis-[β -aminoethyl-ether]-*N,N,N',N'*-tetraacetic acid, 1 mmol/L; and sodium chloride, 150 mmol/L) and then homogenized gently with a motorized pestle inside a 1.5-mL conical capped tube. The homogenate was centrifuged at 9500g for 5 minutes. A duplicate 50- μ L aliquot of the supernatant (equivalent to 10 mg of wet tissue) was used in the assay. During the analysis the samples were identified only by a number, and they were subsequently decoded.

Diagnosis of AD

The general guidelines used for the diagnosis of AD were those described by Khachaturian.¹ Generally, the clinical diagnosis of AD was made using the following criteria: dementia, insidious onset with a progressive deteriorating course, and subsequent exclusion of other specific causes of dementia by history, physical examination, and laboratory tests. Dementia was considered to be present if there was evidence of impairment of memory and impairment of either abstract thinking or judgment. In all cases, however, the clinical diagnosis was confirmed by histopathologic examination of postmortem brain tissue.¹ In summary, histologic sections of various regions of the brains were stained with hematoxylin-eosin, Masson trichrome, or the silver impregnation technique. The sections were evaluated for the histologic changes characteristic of AD¹ as well as other neuropathologic changes.

In this multicenter study, arriving at a consensus on diagnostic standards for AD was not feasible. Due to widespread variability in the field,⁶ a uniform standard among the centers would not have been desirable. This study is based on diagnostic methods routinely practiced

in some reputable centers for the care of patients with AD.

Specimens

Brain specimens used in the biochemical assay were fresh frozen. All the cases used in this study were clinically evaluated for dementia, and the specimens were evaluated histopathologically by neuropathologists. The frozen brain specimens were obtained from various centers and stored frozen at -70°C . This was a multicenter study, with only biochemical assays carried out at Abbott Laboratories. The cases in the neurologic disease control (NDC) category include Parkinson's disease ($n=16$), multi-infarct dementia ($n=5$), Huntington's disease ($n=2$), amyotrophic lateral sclerosis ($n=2$), Wernicke's encephalopathy ($n=1$), and Korsakoff's syndrome ($n=2$).

The specimens were grouped and categorized according to their corresponding clinicopathologic reports. Hence, the cases designated (by one of the investigators) as senile dementia of Alzheimer type (SDAT) were kept as such in spite of the fact that this notation is no longer acceptable by current AD diagnostic standards and was not used at any of the other sites. The AD group includes the following categories: AD, SDAT, and Down's syndrome with AD neuropathologic characteristics (D/AD). The non-AD group includes the following categories: normal control (NC) and NDC.

Statistical Analysis

Because the measured values of ADAP concentrations are truncated at 0 and 2 and because no assumption of normality can be made, nonparametric statistics were chosen to analyze the data. The Kruskal-Wallis test was used to compare the two categories within the non-AD group (NC and NDC) and to compare the non-AD group with the AD group. The Spearman rank correlation coefficient was used to evaluate the relationship between ADAP concentration and age in the AD group.

RESULTS

Case Description

The clinical data, patient information, pathologic reports, and ADAP assay results for the 111 cases studied are summarized in the Table. Generally the AD cases were older than NC cases (75.8 vs 60.7 years), but the ages of the NDC cases were comparable to those of the AD group (69.8 vs 75.8 years). Considering only cases who were 65 years or older, the average ages become comparable: 74.4, 76.7, and 78.8 years for the NC, NDC, and AD categories, respec-

tively. The D/AD cases were all older than 50 years (average age, 53 years). Postmortem time varied from as short as 1 hour to as long as 96 hours; this information was not available for some cases. Pathologic reports indicated that all specimens in the AD group (AD, SDAT, and D/AD categories) had NP counts ranging from mild to severe, but most were severe. With respect to the severity of NP counts in the non-AD group, NC cases had the lowest density of NPs in a small number of cases, while NDC specimens had a reported density of NPs that was between those of the NC and AD cases. All the cases in the AD group were clinically demented, whereas dementia was not observed in any of the cases in the NC category. The majority of NDC cases were classified as demented.

ADAP Concentration

The ADAP concentration for each of the 111 specimens was determined using ALZ-EIA, and results were reported as absorbance units per 10 mg of wet tissue. All absorbance readings for samples were corrected to net absorbance by subtracting the absorbance reading of the assay when homogenization buffer was used instead of brain homogenate. Any negative net absorbance was reported as zero. The upper limit of the spectrophotometer (Quantum II Dual Wavelength Analyser, Abbott Laboratories) used was 2.0 absorbance. An absorbance over this limit is reported as 2.0; serial dilution indicated absorbance equivalents up to 16. The ADAP concentrations in both the NC and NDC categories are within the experimental error of the method. Therefore, NC and NDC cases as a group had essentially no detectable levels of ADAP. In contrast, the median ADAP concentration in the AD category was 1.39 absorbance units per 10 mg of wet tissue, with a range from 0.03 to 2.0 absorbance units per 10 mg of wet tissue. The D/AD category mimicked the AD category, but the SDAT category demonstrated a wide range of ADAP concentrations (from 0 to 0.64 absorbance units per 10 mg of wet tissue). Within the non-AD group, the ADAP concentrations in the NC ($n=27$) and NDC ($n=28$) categories were not significantly different (Kruskal-Wallis test, $P>.05$). In contrast, ADAP concentrations in the AD group ($n=56$) were higher than in the non-AD group ($n=55$), using the Kruskal-Wallis test ($P<.001$). When the ADAP concentrations in various categories were recalculated using cases 65 years and older, the maximum values for ADAP concentrations in the control categories were ac-

	Non-AD Group		AD Group		
	NC	NDC	AD	SDAT	D/AD
No. of samples	27	28	46	7	3
ADAP immunoreactivity, absorbance units per 10 mg of wet tissue					
Median	0.01	0.01	1.39	0.01	2.0
Range	0.0-0.08	0.0-0.06	0.03-2.0	0.0-0.64	0.70-2.0
Age, y					
Mean	60.7	69.8	75.8	77	53
Range	35-84	40-90	46-92	71-85	52-55
Postmortem time (range), h†	2-64	1-80	1.5-96	1.5-12	18.5-46
Cases 65 y and older					
No.	11	18	40	7	0
Mean age	74.4	76.7	78.8	77	...
ADAP immunoreactivity, absorbance units per 10 mg of wet tissue					
Median	0.01	0.01	0.85	0.01	...
Range	0.0-0.02	0.0-0.04	0.03-2.0	0.0-0.64	...
NP counts, No. of cases					
No NPs	22	12	0	0	0
Mild	4	8	1	2	0
Moderate	1	6	5	3	0
Severe	0	2	40	2	3
Clinical dementia, No. of cases	0/27	16/28	46/46	7/7	1/3
No. of cases with ADAP	0/27	0/28	43/46	2/7	3/3

*ADAP indicates Alzheimer's disease-associated proteins; AD, Alzheimer's disease; NC, normal control; NDC, neurologic disease control; SDAT, senile dementia of Alzheimer type; D/AD, Down's syndrome with AD neuropathologic characteristics; and NPs, neuritic plaques.

†These data were not available for all cases.

tually reduced, and the median ADAP concentration in the AD category was smaller than the overall median: 0.85 vs 1.39 absorbance units per 10 mg of wet tissue (Table).

Individual ADAP concentrations for each diagnostic category are plotted as a scattergram in the Figure. An absorbance value of 0.1 was used as an empirically derived cutoff point between normal and AD cases. The ADAP concentrations for all non-AD cases fell under this value, whereas the ADAP concentrations for 48 of 56 cases in the AD group fell above this value. Only 3 cases in the AD category overlapped with the NC and NDC cases. It is noteworthy that there was no increase in ADAP concentration in the NDC category even though dementia was observed in 16 of 28 cases. The D/AD category also had ADAP levels comparable to those in the AD group, but the SDAT cases had ADAP concentrations in both the normal and AD ranges.

COMMENT

ALZ-EIA Specificity and Sensitivity

The results indicate that the biochemical assay of ADAP (ALZ-EIA) presented in this study produced no false positives in either the NC or NDC cases. There were 8 false negatives among 56 cases in the AD group (sensitivity, 85.7%): 3 of 46 cases in the AD category, 5 of 7 cases in the SDAT category, and no cases in the D/AD category. After this study, six of the authors (G.B., P.D., C.B.N., R.R., D.F.S., and W.O.W.) carried out similar studies in

their own laboratories and obtained similar results (unpublished data).

On further examination of the pathology reports, it was observed that the case with 0.03 absorbance units per 10 mg of wet tissue in the AD category (the lowest value in the AD category) was 83 years old, had a moderate NP value, and was initially thought to have SDAT, but, more important, this patient was found to have a brain tumor. One of the other two false-negative cases had a reported postmortem time of 96 hours (the highest in the study), and the postmortem time for the third false-negative case was not reported (not available), but this patient also was initially thought to have SDAT. The SDAT cases, by convention, would have been diagnosed as AD if the patients had been under 65 years old at the onset of the disease. The histopathologic reports of the 5 false-negative cases in the SDAT group were as follows: patient 1, mild NP value; patient 2, moderate NP value, cerebellar tumor; patient 3, mild NP value, Lewy bodies; patient 4, moderate NP value, no NFTs, Lewy bodies; and patient 5, severe NP value.

Relationship of ADAP to Dementia, NPs, and Age

Alzheimer's disease is characterized by and associated with dementia, NPs, and aging. Dementia alone was not associated with an increase in the ADAP concentration because there were 24 demented cases (16 in the NDC group, 3 in the AD group, and 5 in the SDAT group) with ADAP values in the normal range

(Table and Figure). Furthermore, NPs were not always associated with the presence of ADAP; 29 cases (5 in the NC group, 16 in the NDC group, 3 in the AD group, and 5 in the SDAT group) with NPs had practically no detectable ADAP levels (Table and Figure).

To examine the concentration of ADAP as a function of age, the data for cases 65 years and older were analyzed separately. Based on this analysis, the maximum ADAP value in cases 65 years and older was actually lower in the NC and NDC categories, and the median ADAP value in the AD category decreased from 1.39 to only 0.85 absorbance units per 10 mg of wet tissue for cases 65 years and older (Table). Furthermore, no age-related pattern was apparent (Figure). In fact, the highest values for ADAP immunoreactivity in both the NC and NDC categories corresponded to cases under 65 years. The Spearman correlation test showed that the ADAP concentrations in the AD group ($n=56$) were not significantly correlated with age ($p=0.238$, $P>.05$). The oldest NDC case in this study was a 90-year-old woman with multi-infarct dementia who had a moderate NP value. She had an ADAP concentration of only 0.01 absorbance units per 10 mg of wet tissue, while the youngest AD case in the study was 46 years old and had an ADAP concentration of more than 2.0 absorbance units per 10 mg of wet tissue. Based on the data presented in this study, clinical dementia, NPs, and old age per se do not appear to be associated with the increased ADAP concentra-

Scattergram of Alzheimer's disease-associated protein (ADAP) concentrations for each diagnostic category. Top, A full-scale graph. Bottom, A y-axis scale 10-fold expansion. Closed circles indicate cases 65 years and older; open circles, cases under 65 years. NC indicates normal control; NDC, neurologic disease control; AD, Alzheimer's disease; SDAT, senile dementia of Alzheimer's type; and D/AD, Down's syndrome with AD neurohistologic characteristics. The non-AD group includes NCs and NDCs, and the AD group includes AD, SDAT, and D/AD cases. The circles on the 2.0 line (top) indicate values of 2.0 or higher (up to 16).

tions in the AD group. In conclusion, this biochemical assay of ADAP should be a useful adjunct to the clinicopathologic diagnosis of AD and may contribute to a better understanding of the pathologic characteristics of AD.

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References

1. Khachaturian ZS. Diagnosis of Alzheimer's disease. *Arch Neurol*. 1985;42:1097-1105.
2. Blessed G, Tomlinson BE, Roth M. The association between quantitative measures of dementia and of senile changes in the cerebral grey matter of elderly subjects. *Br J Psychiatry*. 1968;114:797-811.
3. Birecree E, Whetsell W, Stoscheck C, King L, Nanney L. Immunoreactive epidermal growth factor receptors in neuritic plaques from patients with Alzheimer's disease. *J Neuropathol Exp Neurol*. 1988;47:549-560.
4. Davies P. Neurochemical studies: an update on Alzheimer's disease. *J Clin Psychiatry*. 1988;49(suppl):23-28.
5. Mann DMA, Marcyniuk B, Yates PO, Neary D, Snowden JS. The progression of the pathological changes of Alzheimer's disease in frontal and temporal neocortex examined both in biopsy and at autopsy. *Neuropathol Appl Neurobiol*. 1988;14:177-195.
6. Wisniewski M, Rabe A, Zigman W, Silverman W. Neuropathological diagnosis of Alzheimer's disease. *J Neuropathol Exp Neurol*. 1989;48:606-608.
7. Katzman R, Terry R, DeTeresa R, et al. Clinical, pathological and neurochemical changes in dementia: a subgroup with preserved mental status and numerous neocortical plaques. *Ann Neurol*. 1988;23:138-144.
8. Terry RD, Hansen LA, DeTeresa R, Davies P, Tobias H, Katzman R. Senile dementia of the Alzheimer type without neocortical neurofibrillary tangles. *J Neuropathol Exp Neurol*. 1987;46:262-268.
9. Van Hoesen GW, Hyman BT, Wolozin B, Davies P, Damasio AR. A direct demonstration of the involvement of corticocortical pathways in Alzheimer's disease. *Neurology*. 1987;37(suppl 1):332.
10. Wolozin B, Pruchnicki A, Dickson DW, Davies P. A neuronal antigen in the brains of Alzheimer patients. *Science*. 1986;232:648-650.
11. Wolozin B, Davies P. Alzheimer-related neuronal protein A-68: specificity and distribution. *Ann Neurol*. 1987;22:521-526.
12. Hyman BT, Van Hoesen GW, Wolozin B, Davies P, Damasio AR. ALZ-50: recognition of Alzheimer-related neuronal changes before neurofibrillary tangle formation. *Neurology*. 1987;37(suppl 1):225.
13. Hyman BT, Van Hoesen GW, Wolozin B, Davies P, Kromer L, Damasio A. ALZ-50 antibody recognizes Alzheimer-related neuronal changes. *Ann Neurol*. 1988;23:371-379.
14. Ksiezak-Reding H, Davies P, Yen S. ALZ-50: a monoclonal antibody to Alzheimer's disease antigen, cross-reacts with Tau proteins from bovine and normal human brain. *J Biol Chem*. 1988;263:7943-7947.
15. Ghanbari H, Kozuk T, Miller BE, Reising S. A sandwich enzyme immunoassay for detecting and measuring Alzheimer's disease associated proteins (ADAP) in human brain tissue. *J Clin Lab Anal*. 1990;4:189-192.
16. Miller B, Simon A, Ghanbari H. Chromatographic analysis by HPLC of Alzheimer's disease associated proteins. Presented at the 19th Annual Meeting of the Society for Neuroscience; November 2, 1989; Phoenix, Ariz.
17. Ghanbari H, Miller B, Chong J, Haigler H, Whetsell W. Distribution of Alzheimer's disease associated proteins in 10 human brains. Presented at the Second International Conference on Alzheimer's Disease and Related Disorders; July 7, 1990; Toronto, Canada.