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A Sandwich Enzyme Immunoassay for Measuring AD7C-NTP as an Alzheimer's Disease Marker: AD7C Test

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A simple, fast, reliable, and specific immunoassay has been developed to detect and measure AD7C-NTP, a biochemical marker for Alzheimer's disease, in cerebrospinal fluid (CSF). This assay, called the AD7C Test, is an enzyme-linked sandwich immunoassay (ELSIA) using 96 well microtiter plates. The plate surface is coated with a monoclonal antibody (N_3I_4) which has a high affinity and specificity for AD7C-NTP, capturing it effectively from CSF samples. The detection was achieved using a polyclonal antibody (ADRI). Both N_3I_4 and ADRI were generated using recombinantly produced AD7C-NTP. The assay is highly sensitive (30–50 pg), linear to 2.0 ng ($r^2 > 0.99$), and reproducible (C.V. < 10%). The utility of the assay has been demonstrated using CSF specimens from early Alzheimer's disease patients and age matched controls (sensitivity of 89% and specificity of 89%). J. Clin. Lab. Anal. 12:223–226, 1998. © 1998 Wiley-Liss, Inc.

Key words: NTP; Alzheimer's diagnostic test; Alzheimer's marker

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease characterized by a chronic deteriorating course of impaired intellectual function and memory loss. The definitive diagnosis of AD is made by pathologic examination of postmortem brain tissue (1). Antemortem diagnosis, however, is by exclusion (2), as there are currently no reliable antemortem biochemical tests available. Apolipoprotein E_4 ($ApoE_4$) allele is merely a risk factor consideration (3). Amyloid β-protein level in CSF has been reported to decrease in AD, and Tau protein level (4–11) in CSF has been reported by several groups to increase in AD patients. However, a significant overlap between AD and non-AD levels limits the usefulness of both amyloid β-protein and Tau. An Alzheimer's test based on the combination of amyloid β-protein and Tau has been suggested, but it still suffers from excessive overlap of AD and non-AD and has no diagnostic value when both levels are either high or low (8).

AD7C-NTP is present in the long axonal processes which emerge from the nerve cell body and is found in large amounts associated with neurofibrillary tangles of Alzheimer's disease (12, 13). There is evidence that AD7C-NTP is involved in neuronal repair and regeneration in the brain. AD7C-NTP gene expression is also elevated in Alzheimer's brain tissue (in situ hybridization). The gene encoding AD7C-NTP has been cloned and sequenced, and the protein has been produced by recombinant techniques (14). This protein has been used to generate both monoclonal and polyclonal antibodies to Neuronal Thread Protein (NTP). We have developed a reliable test for AD based on NTP using AD7C protein, a recombinant version of NTP.

MATERIALS AND METHODS

Materials

Sodium chloride, potassium chloride, Tween-20, casein, and Trizma base were purchased from Sigma™ Chemicals. Comassie protein assay dye reagent was purchased from Bio-Rad™. Bovine gamma globulin standard, bovine serum albumin standard, o-phenylenediamine dihydrochloride (OPD) tablets, stable hydrogen peroxide (H_2O_2), goat antirabbit IgG HRP, and purified Tween 20 were purchased from Pierce Chemicals. Sodium hydroxide (2N), hydrochloric acid (6N), high and low binding 96 well ELISA plates, and sulfuric acid (2.5N) were purchased from Fisher™ Scientific.

Buffers and Solutions

coating buffer: TBS pH 7.5

blocking buffer: 0.5% casein in TBS pH 7.5

antibody and standards buffer: 0.1% casein in TBS pH 7.0 with 0.005% purified Tween-20

sample buffer: 0.2% casein in TBS pH 7.0 with 0.01% purified Tween-20

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wash solution: TBS pH 7.0 with 0.05% Tween-20

color substrate: OPD tablets in deionized water with H_2O_2

stop solution: 2.5N H_2SO_4

Equipment

Centrifuge: Marathon™ 13 K/M microcentrifuge

ELISA reader: Bio-Rad™ Model 550 microplate reader

Incubator: Boekel™ Model #133000 dry incubator

Plate washer: Nunc™-Immunowash 12 wash block

Antibodies to AD7C-NTP

Monoclonal antibodies (MAbs) were produced against recombinant AD7C-NTP as described by de la Monte, et al (13). The MAbs were purified on protein G columns (Pharmacia™). Polyclonal antibodies were also raised against recombinant AD7C-NTP. Rabbits were immunized with purified recombinant AD7C-NTP and boosted according to a monthly schedule. Bleeds were screened using direct ELISA by coating plate wells with AD7C-NTP in the coating buffer. After desired antibody titers were achieved, the rabbit antisera was purified by protein A/G affinity chromatography (Pierce™). All antibody solutions are centrifuged for 3 min at 10,000 rpm immediately before use.

Coating the Plates

Microtiter plate wells were coated with MAb using 0.25 µg antibody per well in the coating buffer and incubated for 60 min at 37°C. The wells were then washed with the wash solution and blocked with the blocking buffer for 60 min at 37°C.

Sample Collection and Preparation

The cerebrospinal fluid (CSF) samples were drawn by lumbar puncture. Small aliquots (about one mL), in duplicate, were stored frozen and shipped frozen to our laboratories. The samples were stored at –80° C and thawed just before testing. CSF samples were examined for color, obvious contaminants, and any other abnormal physical characteristics. All samples were assayed for total protein by Comassie protein assay to ensure appropriate total protein content. The samples were then centrifuged (0.25–0.50 ml) in a microcentrifuge tube for 3 min at 10,000 rpm.

Protein Assay

The total protein content of the AD7C-NTP stock solution and CSF samples was determined by using the Comassie protein assay per instructions.

The Assay

The assay was performed according to a detailed CLIA approved Standard Operating Procedure (available from the authors). The plate format is illustrated in Figure 1. The standard and sample well volume was 200 µl, with the exception of 300 µl in all wells for the blocking step. CSF samples were diluted with equal volume of the sample buffer. Blanks were the same as standards except for no AD7C-NTP.

Samples, standards, and blank wells on the coated and blocked plate were filled with 200 µl of the appropriate solutions. The plate was covered and incubated overnight at 4°C. The plate was washed 5 times with the wash solution. The appropriate, diluted ADRI was placed into all wells. The plate was covered and incubated for 60 min at 37°C. The plate was washed 5 times with the wash solution. The goat antirabbit

	1	2	3	4	5	6	7	8	9	10	11	12
A				W	A T	E R	O	N L	Y			
B	W	NTP 2.0ng	NTP 2.0ng	NTP 2.0ng	B	Sample 1	Sample 1	Sample 7	Sample 7	Sample 13	Sample 13	W
C	A T	1.0ng	1.0ng	1.0ng	L	Sample 2	Sample 2	Sample 8	Sample 8	Sample 14	Sample 14	A T
D	E R	0.50ng	0.50ng	0.50ng	A	Sample 3	Sample 3	Sample 9	Sample 9	Sample 15	Sample 15	E R
E	O	0.25ng	0.25ng	0.25ng		Sample 4	Sample 4	Sample 10	Sample 10	Sample 16	Sample 16	O
F	N L	0.125ng	0.125ng	0.125ng	N	Sample 5	Sample 5	Sample 11	Sample 11	Sample 17	Sample 17	N L
G	Y	0.063ng	0.063ng	0.063ng	K	Sample 6	Sample 6	Sample 12	Sample 12	Sample 18	Sample 18	Y
H				W	A T	E R	O	N L	Y			

Fig. 1. Microplate format for AD7C-NTP assay. Samples 1, 2, and 3 are usually pooled CSF controls at low, medium, and high respectively.

IgG enzyme conjugate was diluted per insert and added to all wells. The plate was covered and incubated at 37°C for 60 min. The plate was washed 5 times and 200 µl of the OPD solution, prepared as per insert, was added to each well. The plate was covered and incubated at 37°C for 60 min. Finally, the substrate reaction was stopped by adding 100 µl of 2.5N sulfuric acid. The plates were read at 492 nm in the microplate reader and absorbance values were analyzed.

The microplate reader's data analysis program calculates the mean of the blank values, the background corrected means (± SD) and CVs for standards and samples, plots the standard curve, constructs a linear curve fit equation, and calculates the AD7C-NTP concentration for all samples. The formatted and default plate readings and all other important data are both printed as a hard copy and saved in the computer.

RESULTS

Antibody Selection

The MAbs were screened in the assay for higher affinity and selectivity as well as for their specificity. Over 100 MAbs were screened and two were chosen as most useful: N_3I_4 and N_3C_{11} (13). The polyclonals were chosen to give the highest titer against AD7C-NTP and to produce the highest signal using AD CSF samples. Various permutations of MAb and ADRI were used in the ELSIA format and were optimized to have low total (reagent plus assay) background (about 0.150–0.200 absorbance units), high assay sensitivity, and a working standard curve. A panel of CSF samples was assayed to assure equivalence of recombinant AD7C-NTP and native AD7C-NTP.

Assay Optimization

All parameters in the assay were varied and studied in exhaustive permutations. The optimization included buffers (type, molarity, pH), blocking solutions and agents, sample buffers and volume, wash solutions, enzyme buffers, enzyme concentrations, number of washes, temperature studies, and the duration of individual incubations. The goal of optimization was to have low background, high signal, and minimal noise while maintaining the relative speed, ease, and practicality of the assay as a reproducible routine test.

Linearity

Figure 2 shows a typical standard curve. The assay is linear in the working range (0–2 ng) with $r^2 > 0.99$ and a negligible Y-intercept (0.003). The clinical CSF samples tested so far had levels of up to 2ng/well of AD7C-NTP. The assay is reproducible from plate to plate and operator to operator with CVs usually less than 10% (see Table 1).

Sensitivity

The sensitivity was calculated as being the AD7C-NTP concentration corresponding to 2X standard deviation of absorbance readings for zero NTP blanks (n = 10) and was found to consistently be between 30 and 50 pg. Considering the previously reported cutoff between AD and non-AD samples (200 pg/well), the sensitivity is quite adequate.

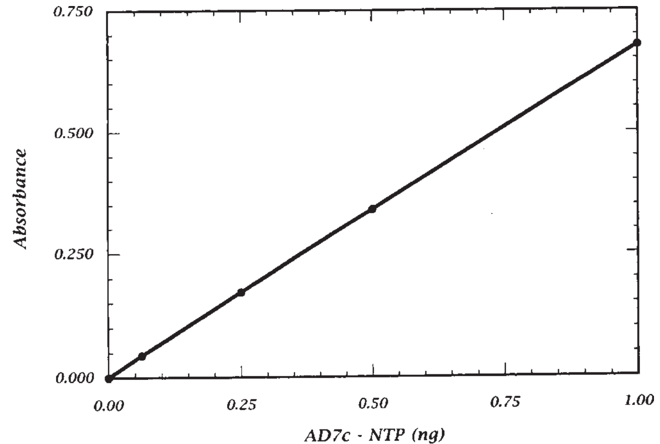

Fig. 2. Standard curve for AD7C-NTP assay. The r^2 is 1.000, the Y-intercept is 0.003 absorbance units, and the slope is 0.674.

Specificity

Specificity of the assay and AD7C-NTP as a biochemical marker for AD have recently been reported to be 89% and 94% in studies involving early AD versus normal controls (14) and possible/probable AD versus demented controls respectively. Earlier published assay data for NTP utilized inferior crossreacting MAbs and did not have adequate specificity for routine use (15).

Clinical Utility

In a clinical study, 89 early AD cases and 18 age matched controls were tested for AD7C-NTP in their CSF samples. The mean for AD group was 4.6 ng/ml (range 0 to 19) and for control group was 1.2 ng/ml (range 0 to 2.2). Levels of AD7C-NTP above 2.0 ng/ml were observed in 89% of AD and only in 11% of aged controls (14).

Reproducibility

Table 1 summarizes the precision study. Intraplate, interplate, and interoperator CVs are less than 10% with the exception of the lowest concentration.

Recovery

Known amounts of recombinant AD7C-NTP stock solution were added to AD, non-AD, and pooled CSF samples and the recovery of added standard was determined to be 90–103%.

DISCUSSION

The method described in this article reliably measures AD7C-NTP in CSF and possibly other body fluids. The as-

TABLE 1. Reproducibility of the AD7C-NTP Assay

		Absorbance reading for:							
Plate	Operator/day	1.000ng	0.500ng	0.250ng	0.125ng	0.063ng	0.000ng	CSF 1	CSF 2
1	1/1	0.814	0.520	0.356	0.284	0.231	0.185	1.144	ND
2	1/1	0.848	0.545	0.361	0.295	0.243	0.205	1.171	ND
3	1/1	0.840	0.512	0.341	0.257	0.206	0.166	1.090	ND
4	1/1	0.838	0.567	0.365	0.265	0.224	0.164	0.983	ND
5	2/1	0.843	0.524	0.353	0.274	0.234	0.189	ND	0.703
6	2/2	0.981	0.587	0.428	0.281	0.228	0.186	ND	0.822
7	2/3	0.881	0.575	0.390	0.299	0.216	0.205	ND	0.768
8	2/4	0.909	0.579	0.433	0.309	0.235	0.218	ND	0.786
Mean	operator 1	0.835	0.536	0.356	0.275	0.226	0.180	1.097	N/A
(%CV)		(1.75)	(4.66)	(2.95)	(6.31)	(6.85)	(10.65)	(7.58)	
Mean	operator 2	0.904	0.566	0.401	0.291	0.228	0.200	N/A	0.770
(%CV)		(6.45)	(5.05)	(9.31)	(5.53)	(3.83)	(7.46)		(6.48)
Mean	operators 1+2	0.869	0.551	0.378	0.283	0.227	0.190	N/A	N/A
(%CV)		(6.18)	(5.38)	(9.27)	(6.21)	(5.15)	(10.01)		

ND = not done
N/A = not applicable
CV = coefficient of variation
ng = nanogram of AD7C-NTP per well
CSF 1 & 2 = 100 µl of pooled CSF samples (high and medium) per well

say can readily and totally be automated using available instrumentation. In its current semiautomated format, a single operator may assay up to 1,000 samples per week (approximately 4,000 data points). The utility of the assay has been demonstrated using CSF samples from early AD cases and age matched controls (sensitivity of 89% and specificity of 89%). The assay provides a unique biochemical laboratory test (AD7C Test) that can help physicians in either the early and more accurate diagnosis of AD or the ruling out of AD as the cause of dementia, with enormous time and cost saving implications.

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