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PLASMA ANTIBODIES TO Aß40 AND Aß42 IN PATIENTS WITH ALZHEIMER'S DISEASE AND NORMAL CONTROLS

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Abstract Antibodies to amyloid ß protein (Aß) are present naturally or after Aß vaccine therapy in human plasma. To clarify their clinical role, we examined plasma samples from 113 patients with Alzheimer's disease (AD) and 205 normal controls using the tissue amyloid plaque immunoreactivity (TAPIR) assay. A high positive rate of TAPIR was revealed in AD (45%) and age-matched controls (41%), however, no significance was observed. No significant difference was observed in the MMS score or disease duration between TAPIR-positive and negative samples. TAPIR-positive plasma reacted with the Aß40 monomer and dimer, and the Aß42 monomer weakly, but not with the Aß42 dimer. TAPIR was even detected in samples from young normal subjects and young Tg2576 transgenic mice. Although the Aß40 level and Aß40/42 ratio increased, and Aß42 was significantly decreased in plasma from AD groups when compared to controls, no significant correlations were revealed between plasma Aß levels and TAPIR grading. Thus an immune response to Aß40 and immune tolerance to Aß42 occurred naturally in humans without a close relationship to the Aß burden in the brain. Clarification of the mechanism of the immune response to Aß42 is necessary for realization of an immunotherapy for AD.

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Introduction

Recent studies suggested that Aß immunotherapy is the most promising among the many candidate therapies for AD. Schenk and others showed that an Aß42 peptide vaccine clearly reduced the Aß amyloid burden in transgenic model mice[1)]. Passive immunization using anti-Aß antibodies was also shown to be effective for reduction of the Aß amyloid burden[2)]. These findings suggest peripheral antibodies to Aß may serve a protective role against AD. A detectable increase in antibodies to Aß42 was observed in about 25% of patients who received AN1792 in a Phase I study[3,4)]. Analysis of serum samples by ELISA indicated that 15 of 18 patients experiencing meningoencephalitis in a Phase II study had antibodies against Aß42. CSF antibodies to Aß42 were present in 6 of 8 patients tested after the onset of encephalitis. However, titers of antibodies to Aß42 were not correlated with the occurrence or severity of symptoms or relapses[4)]. An autoantibody to Aß40 was first detected in human B cell lines from AD patients[5)]. Naturally occurring antibodies to synthetic Aß40 were confirmed by ELISA in the CSF and plasma of non-immunized humans and titers were significantly higher in healthy controls than in patients with AD[6)]. Titers of anti-Aß42 peptide antibodies were lower in AD patients compared with healthy individuals[7)], or elevated in AD patients and elder transgenic mice[8)]. Naturally occurring anti-Aß42 antibodies were detected at very low levels by ELISA in over 50% of elderly individuals and at modest levels in 5% of them. Neither the presence nor the amount of naturally occurring anti-Aß42 antibodies correlated with the presence, or age of AD onset, or the plasma levels of Aß40 and Aß42[9)]. Normal levels of

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antibodies to Aß42 and Aß40 were present in both AD and control groups, even in a young population[10]. Thus, the previous reports suggested complex relationships for naturally occurring antibodies to Aß.

Here, we examined 113 AD cases and 155 age-matched normal controls by TAPIR assay in order to clarify the positive rates, antibody characters, correlations with clinical symptoms, and clinical roles of naturally occurring antibodies against ß-amyloid plaques. Modification of plasma Aß40 and Aß42 concentrations by antibodies to Aß was also studied based on age- or AD-dependent alterations of plasma Aß levels.

Materials and Methods

After informed consent was given, blood samples were collected into 0.1% EDTA from a total of 318 subjects including 113 patients with AD (AD group) and 205 normal controls (total normal control group: tNC group). As age- matched controls (aNC group), 115 samples from subjects over 43 years old were selected from the tNC group. The clinical diagnosis of AD was based on NINCDS-ADRDA criteria. The clinical severity of AD was evaluated using the Mini-Mental State Examination (MMS). AD patients were divided into 3 subgroups according to clinical stages: early stage MMS score >20, moderate stage MMS score 10~20, advanced stage MMS score <10.

Five µm serial paraffin sections of brains from Tg2576 mice or Alzheimer's patients were used. Sections were incubated at 4°C overnight with human or mouse plasma diluted with blocking solution (1:100). Sections were then treated with Vectastain Elite ABC kit (Vector, Burlingame, CA). Immunostaining with Ab9204 (1:1,000, antibody to a synthetic Aß peptide) or without the primary antibody were used as positive and negative controls, respectively.

TAPIR findings were classified into 4 levels: negative -, no senile plaque core; weakly positive ±, senile plaque cores were stained weakly and less than 5 cores were stained in each brain section on a slide; positive +, ≥5 senile plaque cores were stained clearly in at least one brain section per slide; strongly positive ++, most senile plaque cores were strongly labeled when compared to Ab9204 immunostaining.

About 2 g of gray matter of the AD brain was homogenized with 4 volumes of TBS with protease inhibitors (1 µg/ml Leupeptin, 1 µg/ml TLCK, 0.1µg/ml Pepstain A, 1mM phenylmethysulfonyl fluoride and 1mM EDTA), and centrifuged at 100,000g for 1 hour. The resulting pellet was extracted with 10 ml of 10% SDS in TBS and then with 1ml of 99% formic acid (FA). The final supernatant was lyophilized, dissolved with 20 µl of 99% DMSO, and stored at -80°C until use (formic acid soluble amyloid Aß fraction: FAß).

20 µl of protein G agarose was washed 3 times with 1 ml RIPA buffer. Prewashed protein G agarose was mixed with 600 ng synthetic Aß40, 600 ng synthetic Aß42 or 300 ng FAß in 1 ml of RIPA buffer and incubated at room temperature for 30 minutes. After centrifugation, the resulting supernatant was mixed again with 20 µl of prewashed protein G agarose and 10 µl of plasma, incubated at room temperature for 3 hours, and then centrifuged. The pellet was boiled with 1 x NuPage LDS sample buffer containing 0.1 M dithiothreitol for 10 minutes at 70°C and separated on a 4 to 12% NuPage Bis-Tris gel (Invitrogen, CA). After electro-transfer, the blot membrane was incubated with monoclonal 6E10 (specific to Aß1-16, 1:1000) at 4°C overnight. After washing and incubation with horseradish- peroxidase-conjugated goat anti-mouse IgG (1:2000) at RT for 2 hours, the signal was developed by SuperSignal west Dura extended duration substrate (Pierce Biotechology, CA).

Sandwich ELISA was used to specifically quantify whole plasma Aß, as previously

described. Microplates were pre-coated with monoclonal BNT77 (IgA, anti-Aß11-28, specific Aß11-16) and sequentially incubated with 100 µl of samples followed by horseradish- peroxidase-conjugated BA27 (anti-Aß1-40, specific Aß40) or BC05 (anti-Aß35-43, specific Aß42 and Aß43).

Results

In the AD group, 42 cases (37.2%) were TAPIR -, 20 (17.8%) were ±, 44 (38.9%) were grading +, and 7 (6.2%) were ++. Fifty one of 113 AD patients were ++ and +, suggesting frequent appearance (45.1%) of naturally occurring antibodies to amyloid plaque cores. In the aNC group, 54 cases (34.8%) were TAPIR -, 37 (23.9%) were ±, 44 (28.4%) were +, and 20 (12.9%) were ++. Sixty-four cases of 155 aNC group (41.3%) were TAPIR ++ or +. No significant differences were detected by Mann-Whitney's U tests in the positive rates of naturally occurring antibodies to amyloid plaque cores among groups (p= 0.77), or comparisons between the positive AD group (++ and +), negative AD group (± and -), positive aNC group (++ and +) and negative aNC (± and -) group (p = 0.54).

There were no significant differences in gender or mean age in both AD and aNC groups. No significant differences were observed in MMS scores and disease duration among the TAPIR -, ±, +, ++ subgroups of AD samples. There were also no significant differences in the progressive decline of MMS scores among these AD subgroups. The presence of naturally occurring antibodies to Aß as detected by TAPIR may therefore not improve prognosis of AD.

As indicated in Fig 1, freshly prepared Aß40 and Aß42 were composed of monomers and dimers. However, formic acid extractable Aß (FAß) exhibited polymerization as shown by the higher molecular mass of its oligomers (Fig 1, left panel). Immunoprecipitation with TAPIR ++/+ plasma obtained from the AD and aNC groups retrieved Aß40 monomers and dimers as well as higher molecular mass polymers. Immunodetection of monomeric Aß42 using 6E10 was very weak, whereas no dimeric form of Aß42 was detected (Fig 1 right panels). These findings suggest that TAPIR- positive plasma reacts with Aß, but its reactivity to Aß42 is very weak.

In order to clarify when these antibodies against Aß appear, we additionally examined the remaining 50 plasma samples from subjects younger than 43 yeas old in the tNC group. Surprisingly, TAPIR revealed that antibodies to Aß appeared in a 2 year-old child and also in some young subjects (TAPIR +; Fig 2A, B and C). TAPIR positive rates were 57% by 10 years old (n=7; 4 TAPIR+), 64% by 20 years old (n=11; 6TAPIR +), 20% by 30 years old (n=10; 2 TAPIR +) and 10% by 40 years old (n=10; 1 TAPIR+). To confirm further this early appearance of antibodies to Aß, immunoprecipitation was performed. Essentially identical finding to those seen in the AD and aNC groups were revealed (Fig 2D~F). Aß40 and FAß monomers and dimers were strongly immunoprecipitated (arrows). However, immunoprecipitation of the Aß42 monomer was also weak and the Aß42 dimer was absent in TAPIR-positive plasma from younger controls.

Plasma from younger and older Tg2576 mice labeled amyloid cores in AD brains (Fig 2G~I). The appearance rate was 1/3 at 4 months old (1 TAPIR+), 3/3 at 8 months old (1 TAPIR ++ and 2 TAPIR +), 1/1 at 16 months old (1 TAPIR++) and 1/1 at 23 months old mice (1 TAPIR+). Finally, we summarized age- dependent TAPIR-positive rates (TAPIR grading + and ++) in 10 year increments in both AD and tNC groups (Fig 2J). TAPIR-positive rates were high in young subjects (1~20 years old), low during adulthood (21~50 years old) and then increased again after 50. No differences were observed between AD and tNC samples from 50 to 91 years old. Thus,

the appearance of antibodies to Aß preceded Aß amyloid deposition in human and model mouse brains.

To examine the effect of antibodies to Aß on plasma Aß concentrations, we measured levels of Aß40 and Aß42 in 318 plasma samples by specific ELISA. In the tNC group, plasma Aß40 levels increased after 40 years of age (Fig 3A; P<0.0001). On the contrary, plasma Aß42 levels increased between the teens and twenties, then gradually declined with age (Fig 3B; P=0.0158). The Aß ratio (Aß40/Aß42) was stable until ~30 years old and then gradually increased (Fig 3C; P<0.0001).

Significantly increased levels of plasma Aß40 were observed in the AD group (112 ± 39.51 pmol/L) compared to the aNC group (95.38 ± 32.30; p<0.0002; Fig 3D). Aß42 levels were significantly decreased in the AD group (10.29 ±13.80 pmol/L) compared to the aNC group (12.13 ± 12.29; p<0.0001; Fig 3E). Based on these changes, the Aß ratio (Aß40/Aß42) was more strongly increased in the AD group (14.42 ± 10.00) than in the aNC group (8.34 ± 3.83; p<0.0001; Fig 3F). ROC analysis of the Aß ratio indicated that the significant cut off value was

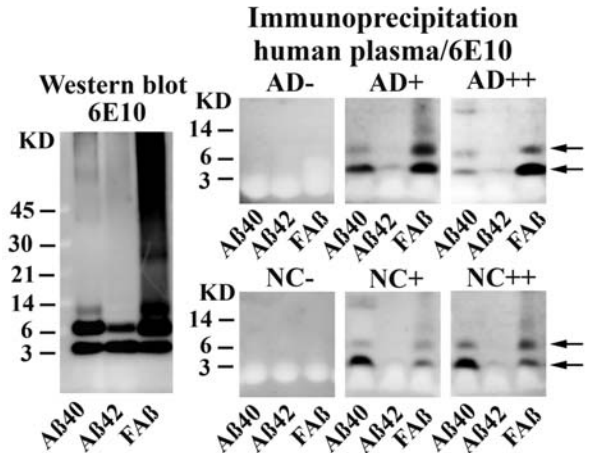

Figure 1 TAPIR-positive plasma immunoprecipitated Aß40 and amyloid Aß, but Aß42 very weakly.

On direct western blotting of synthetic Aß40, Aß42, and FAß from the AD brain, antibody 6E10 detected monomers and dimers of Aß40, Aß42 and brain amyloid Aß with smear aggregates (left panel). Immunoprecipitations of Aß40, Aß42, and FAß using TAPIR–, +, and ++ plasma from the AD group (right upper panel, AD) or the aNC group (right lower panel, NC) were labeled by antibody 6E10, showing that monomers (arrow) and dimers (arrow) of Aß40 were recognized by TAPIR positive plasma (grading + and ++) in addition to Aß42 monomers, and brain Aß amyloid monomers and dimers with smear aggregates, which showed weak signals. "Adopted from reference 17."

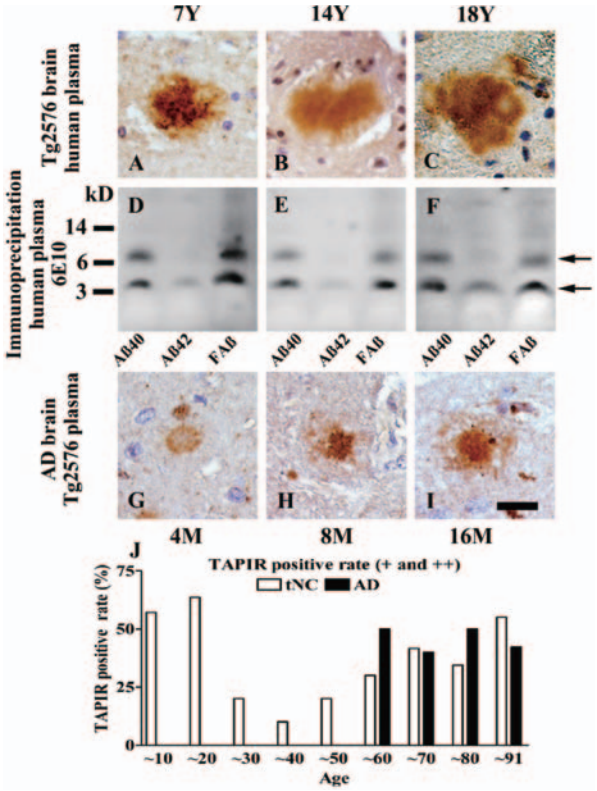

Figure 2 Antibodies to Aß appeared before Aß amyloid deposits in the brain

TAPIR was positive in 7 year old (TAPIR +; A, 7Y), 14 year old (TAPIR +; B, 14Y), and 18 year old young persons (TAPIR +, C, 18Y). TAPIR positive plasma strongly immunoprecipitated monomers and dimers (arrow) of Aß40 and FAß, and weakly immunoprecipitated monomers of Aß42 and Aß amyloid (D, E and F; corresponding plasma of upper panels; D and A 7Y, E and B 14Y and F and C 18Y). Plasma from younger and older Tg2576 mice also labeled amyloid cores in AD brains (G: 4 months old TG; H: 8 months old Tg and I: 16 months old Tg). Bar scale=15µm.
J: TAPIR positive rates in the tNC group according to age. Columns show the TAPIR-positive rate (TAPIR grading + and ++) for 10 year increases in the AD (black columns) and tNC (white columns) groups. TAPIR-positive rates were high in young subjects (1~20 years old), low during adulthood (21~50 years old) and then increased again after age 50. No differences were observed between AD and tNC groups in samples from subjects 50 to 91 years old. "Adopted from reference 17."

9.0, which provided high sensitivity (78.8%) and low specificity (30.3%) for clinical diagnosis of AD. When the mean + 2 SD (15.9) of the aNC group was used as a cutoff value, the sensitivity was 24% and the specificity was 96%. When AD was divided into 3 subgroups according to clinical stage, increasing Aß40 levels and Aß ratio, as well as decreasing Aß42 levels progressed from the early stage to the advanced stage (Fig 3G~I).

Discussion

In our study, a high positive rate of TAPIR was found in both AD and aNC groups, but no significant difference was found between these groups. Essentially the same findings were observed even in strongly positive (++) subgroups of AD and aNC. Non-parametric analysis revealed that neither MMSE score nor disease duration correlated with TAPIR grade, indicating that the physiological impact of naturally occurring anti-Aß antibodies is below clinical significance. Our immunoprecipitation study suggested that TAPIR ++/+ plasma obtained from AD and aNC subjects retrieved Aß40 monomers and dimers as well as higher

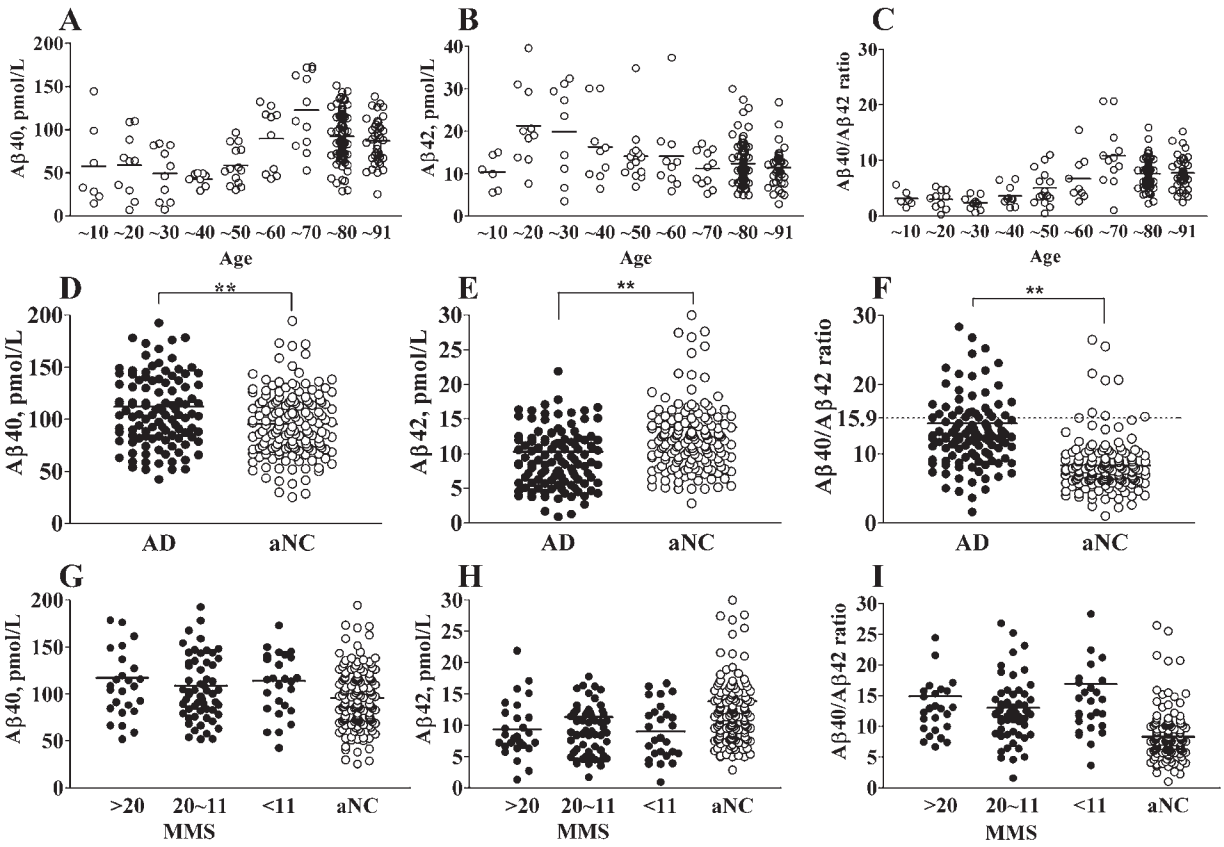

Figure 3 Age-dependent regulation of plasma Aß levels in controls, and their alteration in AD.

Plasma Aß40 and Aß42 levels showed different age-dependent alterations in the tNC group. Aß40 levels increased from age 50 and decreased from age 70 (A). Aß42 levels were high in the teens and twenties, then gradually decreased with age (B). Based on these different changes, the Aß ratio (Aß40/Aß42) progressively increased from age 40 (C). Significantly increased levels of Aß40 (D: $p = 0.0002$) and increased Aß ratio (F: $p < 0.0001$) as well as decreased levels of Aß42 (E: $p < 0.0001$) were shown between the AD and aNC groups. When the mean +2SD of the Aß ratio in the aNC group was used as a diagnostic marker for AD, the cut off value 15.9 (dot line) provided 24% sensitivity and 96% specificity (F). Constant alterations of plasma Aß levels in AD were recognized at the early (MMS score >20), moderate (MMS score 20~11), and advanced stages (MMS score <11) (G~I). A, D, G: Aß40; B, E, H: Aß42; C, F, I: Aß ratio. Bars show mean levels. "Adopted from reference 17."

molecular mass polymers. Immunodetection of monomeric Aß42 using 6E10 was very weak, whereas no dimeric form of Aß42 was detected under our testing conditions. The absence of anti-Aß42 dimer antibodies and the relatively low levels of anti-Aß42 monomers were characteristic of naturally occurring antibodies to Aß. These findings are considered to be another reason why naturally occurring antibodies to Aß are not sufficient for prevention of development of dementia.

Our TAPIR assay also showed that anti-Aß antibodies were naturally present throughout the entire human life span. It is relevant to note that naturally occurring anti-Aß antibodies were unequivocally detected in young human subjects as well as young Tg2576 mice. In relative terms, the positive rates of anti-Aß antibodies were highest in young individuals, lowest in those middle-aged and higher in the elderly. The presence of anti-Aß antibodies in young human subjects was characterized by the subsequent immunoprecipitation study. Anti-Aß antibodies retrieved Aß40 monomers and dimers as well as high molecular mass oligomers in FAß fractions, but they retrieved fewer Aß42 dimers. To our knowledge, this is the first report showing the relatively selective presence of anti-Aß40 antibodies, and reduced amounts of anti-Aß42 antibodies in young individuals.

The exact mechanism underlying spontaneous anti-Aß antibody production remains unknown. Although increased Aß42 levels have been detected in transgenic animal models[11], immune hyporesponsiveness to Aß42 was also shown[12]. Increased T cell reactivity to Aß42 was shown to increase in elderly individuals and patients with AD[13]. However, the previous findings and our results could not show increased titers of anti-Aß42 antibodies in these groups. Thus, hyopoimmunue responses to Aß42, especially to the Aß42 oligomer, actually occurred in AD and healthy populations. Since Aß42 is highly pathogenic and neurotoxic, Aß42 may be sequestered and spontaneous immune responses to Aß may be suppressed in human populations. Significantly increased levels of plasma Aß40, increased Aß ratio and decreased levels of Aß42 were revealed in the AD group when compared to the aNC group. When AD was divided into 3 groups according to clinical stage, the Aß ratio increased progressively from the early stage to the advanced stages of AD. These findings show that plasma Aß ratio can be used as an easy, non-invasive, and useful biomarker for diagnosis and monitoring of clinical symptoms of AD, although the sensitivity and specificity are lower than those in CSF samples[14~16].

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