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High-Affinity Rabbit Monoclonal Antibodies Specific for Amyloid Peptides Amyloid-β_{40} and Amyloid-β_{42}

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Abstract. Antibodies that specifically bind to either amyloid-β peptide (Aβ) isoform $A\beta_{40}$ or $A\beta_{42}$ contribute to the study of Alzheimer's disease (AD) pathology and to the development of cerebrospinal fluid-based tests for the probable diagnosis of AD. Polyclonal rabbit anti-Aβ antibodies possess high affinity and specificity, but their generation requires a long immunization period, and the resulting antibodies exhibit variable specificities and affinities. To secure a continuing supply of antibodies with uniform properties, we generated and partially characterized rabbit monoclonal antibodies specific for either $A\beta_{40}$ or $A\beta_{42}$. These antibodies possess nanomolar or sub-nanomolar dissociation constants and are at least 3,000-fold more selective for one isoform over the other. These antibodies are suitable for immunoblotting and, in a sandwich ELISA, RabmAb42 (anti-$A\beta_{42}$) is sensitive enough to measure plasma levels of $A\beta_{42}$. In addition, these antibodies have been applied to the immunohistology of Down syndrome and AD brain tissues, where they reveal fibrillar and diffuse amyloid deposits and are almost free of non-specific staining. The data indicate that diffuse amyloid deposits contain only minute amounts of $A\beta_{40}$. Thus these rabbit monoclonal anti-Aβ antibodies can be widely applied in AD and Down syndrome research and diagnosis.

Keywords: Alzheimer's disease, amyloid-β peptides, anti-Aβ antibodies, ELISA, immunohistology, peptide-antibody dissociation constants, rabbit monoclonal antibodies

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INTRODUCTION

There is a continuing interest in the accurate and precise immunochemical quantification of the isoforms of amyloid-β peptide (Aβ) in brain tissues and body fluids of patients with Alzheimer's disease (AD) [1–6]. In particular, the levels of $A\beta_{42}$ in plasma and cerebrospinal fluid (CSF) have been correlated to the probable diagnosis of AD-type dementia and to the progression of the disease [7–12]. The presence of Aβ plaques in postmortem brains is one of the fundamental findings for the diagnosis of AD [13–15].

The Aβ peptides comprise a family of peptides that are cleaved from a large plasma membrane-associated protein, the amyloid-β peptide precursor (AβPP), by membrane-associated proteases called secretases [15]. The concerted action of these proteases yields peptides possessing heterogeneous amino- and carboxyl-terminal residues. These peptides aggregate to form fibrillar deposits in the cerebral blood vessels and brain parenchyma of individuals afflicted with AD.

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The amyloidogenic region of AβPP is located between residues Asp-597 and Thr-639 of the brain form of AβPP, $A\beta PP_{695}$. The amino acid sequence of this region, expressed in the single-letter amino acid code, is:

$$D_1AEFRHDSGYE_{11}VHHQKLVFFA_{21}$$

$$EDVGSNKGAI_{31}IGLMVGGVVIAT_{43}.$$

The predominant variants associated with AD are those terminating with Gly-38, Val-40, and Ala-42. Mouse monoclonal and rabbit polyclonal antibodies raised to peptide subsequences corresponding to these variants are widely used to aid the diagnosis of probable AD and for disease mechanism studies. In this report, the peptides named $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{43}$ denote the sequences beginning with Asp-1 and ending, respectively, with Gly-38, Val-40, and Ala-42.

The common experience of many investigators is that high-affinity anti-Aβ antibodies are difficult to generate. The root of this difficulty is that the Aβ amino acid sequence is highly conserved among higher organisms, and their immune systems prevent the generation of antibodies to self-antigens. Nevertheless it has been found that when immunized with Aβ peptides for 9-12 months, rabbits or mice will produce anti-Aβ antibodies. Currently, antibodies from each species have certain advantages. Investigators have found that the rabbit antibodies have higher affinities than the mouse antibodies, which allows the more sensitive detection of Aβ variants [16, 17]. However, monoclonal antibodies are readily generated from mice and they provide a limitless source with reproducible properties.

Using the methods of Spieker-Polet et al. [18], we have generated and partially characterized rabbit monoclonal antibodies to $A\beta_{40}$ and $A\beta_{42}$. These antibodies possess high affinity and high specificity for each Aβ variant, and they can be produced with uniform properties in amounts not limited by the constraints of production in animals.

MATERIALS AND METHODS

The peptides $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{43}$ were obtained from Bachem (King of Prussia, PA). KLH and maleimide-activated KLH were obtained from Pierce Endogen (Rockford, IL). Peptides AP-22 (C-aminohexanoyl-IGLMVGGVVoh), AP-23 (C-aminohexanoyl-GLMVGGVVIAoh), and AP-53 (C-aminohexanoyl-MVGGVVIAoh) were synthesized by American Peptide Co. (Sunnyvale, CA). Epoxy-activated agarose came from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibody 6E10 is a product of Signet Laboratories (Dedham, MA). Rabbit mAb BA3-9 was purchased from Covance (Emeryville, CA). Rabbit polyclonal antibodies R359 (against $A\beta_{40}$), and R226 and R422 (both against $A\beta_{42}$), were generated and characterized at IBR [19].

Generation of rabbit monoclonal antibodies

Our previous publication [19] contains the details of the methods for the immunization of rabbits, the purification of antibodies, and the immunoblotting and ELISA procedures. In brief, to generate antibodies against $A\beta_{40}$, 4-5-lb male New Zealand white rabbits were immunized for a period of 9–12 months with a KLH conjugate of AP-22 (Table 1). The KLH–AP-23 conjugate similarly was employed to generate anti-$A\beta_{42}$ antibodies. During this period we measured the titers, specificity, and affinities of the maturing antibodies. When these titers and affinities reached levels similar to those previously reported, the rabbits were euthanized and their spleens were removed, placed in cold Iscove's medium, and shipped on ice to Epitomics (Burlingame, CA), which performed the fusions, cloning, and initial ELISA screening. Clones that appeared to secrete anti-Aβ antibodies were recloned and amplified, and their secreted antibodies were characterized by titer and affinity. Those clones that secreted antibodies of relatively high titer, specificity, and affinity were amplified and subcloned for several weeks to assure that they were stable. The antibodies were adsorbed from culture media on an AP-53-agarose matrix (for anti-$A\beta_{42}$ antibodies) or an AP-22-agarose matrix (for $A\beta_{40}$ antibodies). The purified antibodies were eluted with 4.5 M $MgCl_2$ and were dialyzed against PBS.

Dissociation constant measurements

Antibody-antigen dissociation constants were determined by the ELISA method of Friguet et al. [20]. In brief, 100 μL aliquots of a 0.1 nM solution of the antibody were incubated with increasing concentrations (0.1 nM–100 nM) of $A\beta_{40}$ or $A\beta_{42}$ for 18 h at 4°C.

Table 1
Synthetic peptides for immunization and antibody purification

Peptide	Sequence
AP-22	nh_2C-ahx-IGLMVGGVVoh
AP-23	nh_2C-ahx-GLMVGGVVIAoh
AP-53	nh_2C-ahx-MVGGVVIAoh

The reactions were conducted in triplicate. Aliquots of these solutions were transferred to wells coated with 22 pmol per well $A\beta_{40}$ or $A\beta_{42}$. After a second incubation of 60 min at ambient temperature, the aliquots were removed, the wells were washed, and the amounts of bound antibody were determined with an anti-rabbit-IgG-alkaline phosphatase conjugate (Biosource, http://www.invitrogen.com). This method may be compromised by the dissociation of the initial antibody-Aβ complex during the second incubation. To assess this possibility, the aliquot withdrawn after the 60-min incubation was reapplied to a second well and re-assayed. The first and second assays were similar and were less than 10% of the total antibody in the aliquot; therefore, the measurements were considered to be valid. Corrections for the bivalency of the antibodies could be applied [21]; however, these were deemed not to be important for the characterization reported here.

ELISA inhibition assays

Details of the ELISA inhibition assay appeared in an earlier publication [19]. In brief, wells of a microtiter plate were coated with either $A\beta_{40}$ or $A\beta_{42}$ (200 pmol in 100 μL 50 mM Na_2CO_3, pH 9.6). Hundred pM antibody was mixed with the test inhibitor peptide at 0–10 μM concentration and then 100 μL of the mixture were incubated in the wells at 4°C overnight. The wells were washed and the amounts of bound antibody were measured as described in the preceding paragraph. Absorbance measurements on 3 wells were averaged; deviations from the mean were less than 10%. Concentrations of peptide that inhibited antibody binding by 50% were calculated from plots of percent inhibition versus peptide concentration.

Quantification of plasma $A\beta_{40}$ and $A\beta_{42}$

Anonymous plasma samples from AD and control patients were obtained from the Alzheimer's Disease Research Center, NYU School of Medicine, New York. $A\beta_{40}$ and $A\beta_{42}$ levels were measured in 30 AD and control plasmas in a double-antibody sandwich ELISA using a combination of mouse monoclonal antibody 6E10 (specific to an epitope present on amino acid residues 3 to 11 of Aβ) as the capture antibody and either rabbit monoclonal or polyclonal antibodies specific for $A\beta_{40}$ and $A\beta_{42}$ as the detection antibodies. This method had previously been employed using rabbit polyclonal antibodies [22]. Briefly, wells of microtiter plates were coated overnight at 4°C with 100 μL of 6E10 (2.5 μg/ml) diluted in carbonate-bicarbonate buffer, pH 9.6. The plates were washed with PBS-Tween, blocked for 1 h with 1% BSA, and 100 μL of standard ($A\beta_{40}$ or $A\beta_{42}$) ranging from 625 pg/ml to 5 pg/ml or undiluted plasma were applied and incubated at 4°C overnight. After washing, plates were incubated with 100 μl of biotinylated rabbit antibody specific for $A\beta_{40}$ or $A\beta_{42}$ respectively. The plates were allowed to stand for 2 h at room temperature and, after washing, 100 μl of neutravidin horseradish peroxidase conjugate was added. Plates were washed again and 100 μL of 3,3′, 5,5′-tetramethylbenzidine substrate solution was added. The reaction was stopped by adding 100 μL of 1 M phosphoric acid. The optical density (OD) was measured at 450 nm in a microplate ELISA reader.

Tissues examined by immunohistochemistry

Eight brain hemispheres (four samples from Down syndrome (DS) subjects from 48 to 66 years of age and four samples from AD subjects from 72 to 84 years of age) were examined in two protocols. Two brain hemispheres of subjects with AD and two of subjects with DS were formalin-fixed and embedded in paraffin. Another two brain hemispheres of AD subjects and two of DS subjects were formalin fixed and embedded in polyethylene glycol (PEG). The paraffin protocol was used to evaluate application of these antibodies in routine studies requiring 6–8-μm-thick sections. The PEG protocol was used to evaluate the penetration of antibodies into 50-μm-thick sections used in morphometric studies.

PEG protocol

The hemisphere was fixed in 10% buffered formalin for several months and then dissected into 30-mm-thick frontal slabs. The tissue blocks were dehydrated in a graded series of ethanol: 5 days in 70% ethanol; 2 days in 80% ethanol; 1 week in 96% ethanol. The dehydrated tissue was infiltrated with polyethylene glycol (PEG) 400 (Merck #807 485) for 6 days (two changes of 3 days each at room temperature) and with PEG 1000 for another 6 days (two changes of 3 days each at 42°C). Slabs were embedded in fresh PEG 1000 [23] and stored at 4°C. Tissue blocks were then cut at a temperature of 18°C into 50-μm-thick serial sections. They were stored in 70% ethanol at room temperature and used for immunocytochemical and morphometric studies.

Neuropathological examination

Neuropathological evaluation was based on gross brain description and a standard immunohistochemical examination using mAb 4G8 of 8-μm-thick sections from 16 brain regions. Alzheimer-type pathology was evaluated using criteria developed at a consensus conference organized by the National Institute on Aging and the Reagan Institute of the National Alzheimer Disease Association [13]. Both AD and DS specimens presented neuropathological changes typical for Braak and Braak neocortical stage VI [24].

Immunostaining

Serial sections were washed with PBS and immunostained as free-floating sections. To enhance the immunoreactivity of Aβ, sections were treated with 70% formic acid for 20 min [25]. Aβ in plaques was detected with rabbit monoclonal antibodies $A\beta_{40}$ or $A\beta_{42}$. Results were compared with immunostaining with mouse mAb 4G8 detecting amino acid residues 17–24 of Aβ. The endogenous peroxidase in the sections was blocked with 0.2% hydrogen peroxide in methanol. Non-specific binding was blocked with 10% fetal bovine serum in PBS for 30 min. The antibodies were diluted in 10% fetal bovine serum in PBS and were incubated with the sections overnight at 4°C. The sections were washed and treated for 30 min with biotinylated sheep anti-rabbit IgG serum diluted 1 : 200. The sections were treated with an extravidin peroxidase conjugate (1 : 200) for 1 h, and the product of the reaction was visualized with diaminobenzidine (0.5 mg/mL with 1.5% hydrogen peroxide in PBS). After immunostaining, the sections were lightly counterstained with hematoxylin. Adjacent sections were stained with thioflavin S and examined by fluorescence microscopy to distinguish fibrillar and non-fibrillar plaques.

Adsorption of antibodies with peptides $A\beta_{40}$ and $A\beta_{42}$

To confirm the specificity of the immunostaining, the reactivities with antibodies adsorbed with $A\beta_{40}$ or $A\beta_{42}$ were examined and compared to those of unadsorbed antibodies. For adsorption, antibody at dilutions of 75–126 ng/mL (the dilutions at which consistently positive results are achieved on sections) was combined with excess of blocking peptide (1.68 pmol of antibody and 2.5 nmol of $A\beta_{40}$ or $A\beta_{42}$; US Peptide Inc., Rancho Cucamonga, CA). The mixture was incubated for 1 h at room temperature and used for immunocytochemical tests.

The Institutional Review Board at the New York State Institute for Basic Research in Developmental Disabilities approved the methods applied in this study. All brain tissue samples were identified by case number and examined blind to clinical and demographic information.

RESULTS

We screened a panel of rabbit monoclonal hybridomas for antibodies of high affinity and we further screened the antibodies for those of high specificity for the Aβ isoform. We selected for further characterization two anti-$A\beta_{42}$ antibodies, namely, mAb 1-11-3 and mAb 1-11-4. Only one anti-$A\beta_{40}$ antibody clone was selected – mAb 5-139. The cells secreting these antibodies have been passaged many times and have remained stable for more than 1 year.

The antibodies secreted by these clones were purified from the culture media on peptide–agarose matrices. Using the ELISA method of Friguet et al. [20], we determined the dissociation constants of mAb 1-11-3 and mAb 1-11-4 each to be 0.7 ± 0.2 nM and that of RabmAb40 to be 4 ± 0.5 nM (Fig. 1). In the additional assays described in the following text mAb 1-11-3 and mAb 1-11-4 have indistinguishable properties; whether these are structurally identical has not yet been determined. As a mnemonic aid, in the following text mAb 5-139 is designated RabmAb40 and mAb 1-11-3 and mAb 1-11-4 are designated RabmAb42.

ELISA inhibition assays confirmed that the epitope of RabmAb42 is located within the $A\beta_{42}$ sequence of AP-23 (Table 2). Peptide AP-53, which is 2 residues shorter that AP-23, inhibits antibody binding at a 350 to 500-fold higher concentration. This indicates that Gly-33 or Leu-34 contributes to antibody binding. $A\beta_{40}$ inhibits the binding of each antibody at 3,000-fold higher concentrations than does $A\beta_{42}$. Thus the C-terminal Ile-Ala sequence is also an important part of the epitope. This makes RabmAb42 sufficiently specific that it can be used to quantify $A\beta_{42}$ in the presence of a large excess of $A\beta_{40}$. $A\beta_{43}$ (possessing a C-terminal Thr) competed with $A\beta_{42}$ binding at a 15-fold higher concentration. This finding suggests that the complementarity-determining region possesses a strong but not absolute requirement for the terminal carboxyl group. Since $A\beta_{43}$ rarely has been found in biological samples, this cross-reactivity does not limit the antibodies' utility for detecting and quantifying $A\beta_{42}$.

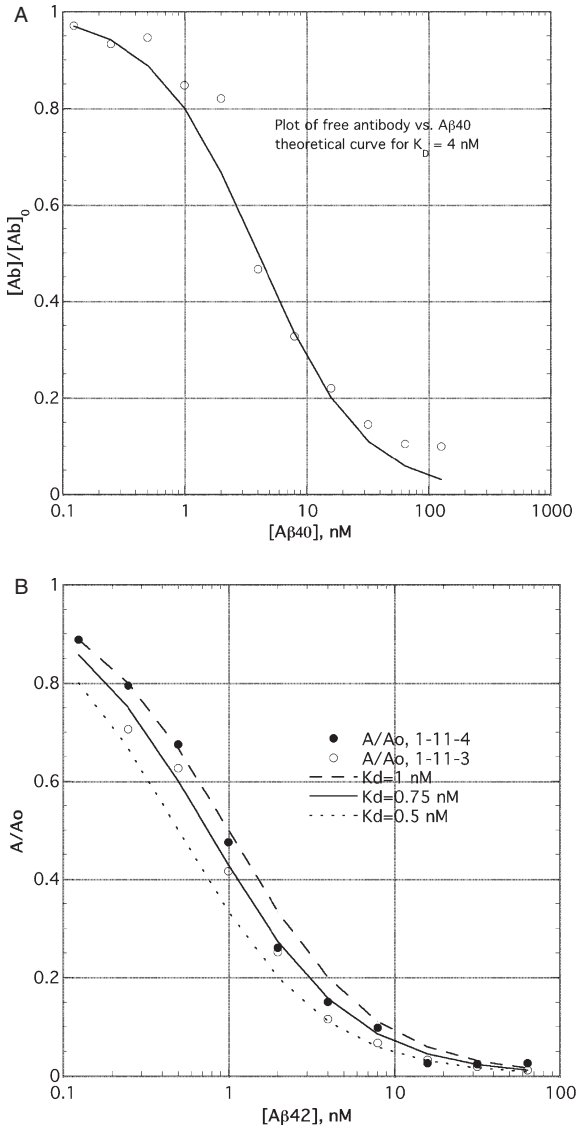

Fig. 1. Dissociation constant determinations for Aβ-antibody complexes. A) Binding of $A\beta_{40}$ to RabmAb40. The fraction of unbound antibody (A/Ao) in equilibrium with the Aβ-antibody complex was plotted versus the concentration of unbound peptide. The data points (open circles) are averages of 3 measurements with a range of less than 5%. The solid curve was calculated for an adsorption isotherm with $K = 4$ nM. B) Binding of $A\beta_{42}$ to mAb 1-11-3 (open circles) and 1-11-4 (filled circles). The curves are calculated for adsorption isotherms with $K = 0.5$ nM (dotted line), 0.75 nM (solid line) or 1.0 nM (dashed line).

Similar inhibition assays confirmed that the epitope of RabmAb40 lies within the $A\beta_{40}$ sequence of the immunogen, AP-22 (Table 2). Surprisingly, RabmAb40 binds more tightly to $A\beta_{40}$ than to the immunogen. This antibody is specific for the C-terminal Val-Val sequence of $A\beta_{40}$. Neither $A\beta_{38}$ nor

Table 2
50% inhibition concentrations for the antibodies

Peptide	mAb 1-11-3 C_I (nM)	mAb 1-11-4 C_I (nM)	RabmAb40 C_I (nM)
$A\beta_{40}$	3000	3000	7
$A\beta_{42}$	1	1	≫2200
$A\beta_{43}$	12	16	nd
AP-23	<1	<1	nd
AP-53	350	500	nd
$A\beta_{38}$	nd	nd	≫2400
AP-22	nd	nd	180

Inhibition ELISA experiments were performed as described in Methods. The wells were coated with $A\beta_{42}$ for determinations with mAb 1-11-3 and mAb 1-11-4, and with $A\beta_{40}$ for experiments with RabmAb40. The left-hand column lists the inhibitor peptide; successive columns list the peptide concentration that inhibited antibody binding to the wells by 50%. nd: not determined.

$A\beta_{42}$ measurably inhibited the binding of RabmAb40 to $A\beta_{40}$, even at concentrations 300-fold greater than the $A\beta_{40}$ concentration that produces 50% inhibition (Table 2). Thus, in ELISA procedures, RabmAb40 is highly specific for $A\beta_{40}$.

Utility of the antibodies in immunoblot analyses

Anti-Aβ antibodies are commonly used in immunoblotting procedures to measure sub-picomolar amounts of the peptides. The sensitivities of these rabbit monoclonal anti-Aβ antibodies initially were determined by slot-blotting various amounts of peptides on a nitrocellulose membrane and developing the blot with various concentrations of the antibodies. The amounts of $A\beta_{42}$-antibody complex were quantified using chromogenic detection followed by photodensitometric scanning (Table 3). When developed with 200 ng/mL of anti-$A\beta_{42}$ antibody, 1 fmol of $A\beta_{42}$ was detectable; whereas, 200 fmol of $A\beta_{40}$ gave no detectable signal. The sensitivities of mAb 1-11-3 and mAb 1-11-4 were similar. Even at an anti-$A\beta_{42}$ IgG concentration of 25 ng/mL, 2 fmol of $A\beta_{42}$ was detectable (result not shown). In a similar assay, a blot developed with RabmAb40 at 200 ng/mL revealed 1 fmol of $A\beta_{40}$ but 200 fmol of $A\beta_{42}$ gave no signal (Table 3).

These antibodies can also be used to detect amyloid deposits in tissue extracts. Normal human fibroblast homogenates containing 10 μg of protein were mixed with 4 fmol of $A\beta_{42}$, and the mixture was resolved by gel electrophoresis followed by western blotting. The blots were developed with anti-$A\beta_{42}$ antibodies (Fig. 2) and the band densities were quantified (Table 4). Monoclonal antibodies 1-11-3 and 1-11-4 can readily reveal 4 fmol of $A\beta_{42}$. Although these anti-

Table 3
Quantification of $A\beta_{42}$ and $A\beta_{40}$ by the rabbit mAbs

Peptide		mAb 1-11-3	mAb 1-11-4	RabmAb40
Name	fmol/slot	200 ng/mL	200 ng/mL	200 ng/mL
$A\beta_{40}$	1	nd	nd	2200
$A\beta_{40}$	5	nd	nd	4600
$A\beta_{40}$	200	0	0	nd
$A\beta_{42}$	1	230	400	nd
$A\beta_{42}$	5	3000	3700	nd
$A\beta_{42}$	200	nd	nd	0

Peptides listed in the left-hand column were adsorbed to a nitrocellulose membrane in the amounts listed in the next column. The membranes were developed as described in Methods, and the alkaline phosphatase reaction product was quantified by photodensitometric scanning. The densities are the uncorrected output from the scanner expressed in arbitrary units. nd: not done.

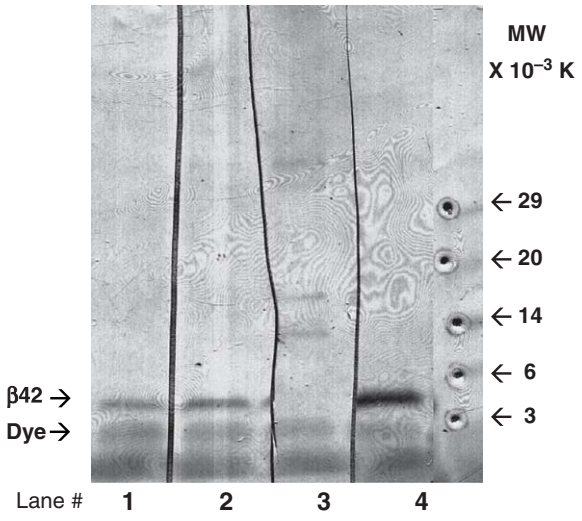

Fig. 2. Immunodetection of $A\beta_{42}$ in the presence of other brain proteins. Four femtomoles of $A\beta_{42}$ were added to an aliquot of a normal human fibroblast homogenate containing 10 micrograms of proteins, and the mixture was resolved by PAGE and electroblotted. The blot was sectioned and developed with the primary antibodies listed in Table 4 followed by goat anti-rabbit IgG – alkaline phosphatase. The primary antibody used to develop lane 3, BA3-9, is a commercially available anti-$A\beta_{42}$ rabbit mAb. This antibody does not detect $A\beta_{42}$ at the 4 fmol level. It serves here as a non-immune control.

Table 4
Western blotting conditions for Fig. 2

Lane no.	$A\beta_{1\text{-}42}$ added (fmol)	Homogenate added (μg protein)	Antibody	[IgG][1] μg/mL	Scanned density[2]
1	4	10	mAb 1-11-3	0.1	2,907
2	4	10	mAb 1-11-4	0.1	3,782
3	4	10	Ab BA3-9	1.0	0
4	4	10	Ab R226	0.05	9,049

[1]Concentraton of primary antibody used to develop the blot. [2]Densitometer output in arbitrary units corrected for a blank scan of 50 units.

bodies are somewhat less sensitive than R226, our best polyclonal antibody [19], they exhibit negligible reactivity with other proteins in a cell homogenate. The hippocampus from AD brain contains as much as 10 fmol of $A\beta_{42}$ per μg of tissue [19]; therefore, RabmAb42 could be used to detect $A\beta_{42}$ isoforms in brain extracts.

Use of RabmAb40 and RabmAb42 in ELISA determinations of Aβ isoforms

For clinical applications the ELISA technique requires that the antibodies be of high affinity and sensitivity because total Aβ concentration in CSF is about 2 nM (10 ng per mL) and the Aβ concentration in plasma is 100-fold lower [26–28]. Moreover, the antibodies must be highly specific because, in these fluids, the molar ratio of $A\beta_{40}/A\beta_{42}$ is 10–20.

To test the sensitivity and specificity of the rabbit monoclonal antibodies in ELISA, the microtiter plate wells were coated with the capture antibody, mouse mAb 6E10, which binds all isoforms containing Aβ amino acid residues Phe-4–Glu-11. Solutions containing either $A\beta_{40}$ or $A\beta_{42}$ were allowed to equilibrate with the antibody coating the wells, and then the bound Aβ was probed with RabmAb42. This antibody detected 4 fmol/mL of $A\beta_{42}$, but 2.4 pmol/mL of $A\beta_{40}$ gave no detectable absorbance. This result is consistent with the competitive inhibition assay, where $A\beta_{42}$ bound more than 3,000 times more tightly than $A\beta_{40}$. Thus in ELISA of body fluids RabmAb42 can measure $A\beta_{42}$ at a level of about 4 pmol/mL without interference by $A\beta_{40}$ in body fluids.

Comparison of rabbit monoclonal and polyclonal antibodies in the measurement of plasma Aβ levels

Many investigations have already used mouse monoclonal or rabbit polyclonal antibodies to measure $A\beta_{40}$ and $A\beta_{42}$ in plasma; however, the measured levels vary considerably among laboratories. To determine whether our monoclonal and polyclonal antibodies gave results similar to each other, we tested 30 AD and control plasma samples using the sandwich ELISA with both polyclonal and monoclonal antibodies against $A\beta_{40}$ and $A\beta_{42}$. When the results were compared (Fig. 3) for each set of antibodies there was a strong correlation between the measured levels of Aβ [($r = 0.86$, $p < 0.001$ for anti-$A\beta_{40}$ antibody R359 vs. RabmAb40, Fig. 3A) and ($r = 0.96$, $p < 0.001$ for anti-$A\beta_{42}$ antibody R422 vs. RabmAb42, Fig. 3B)].

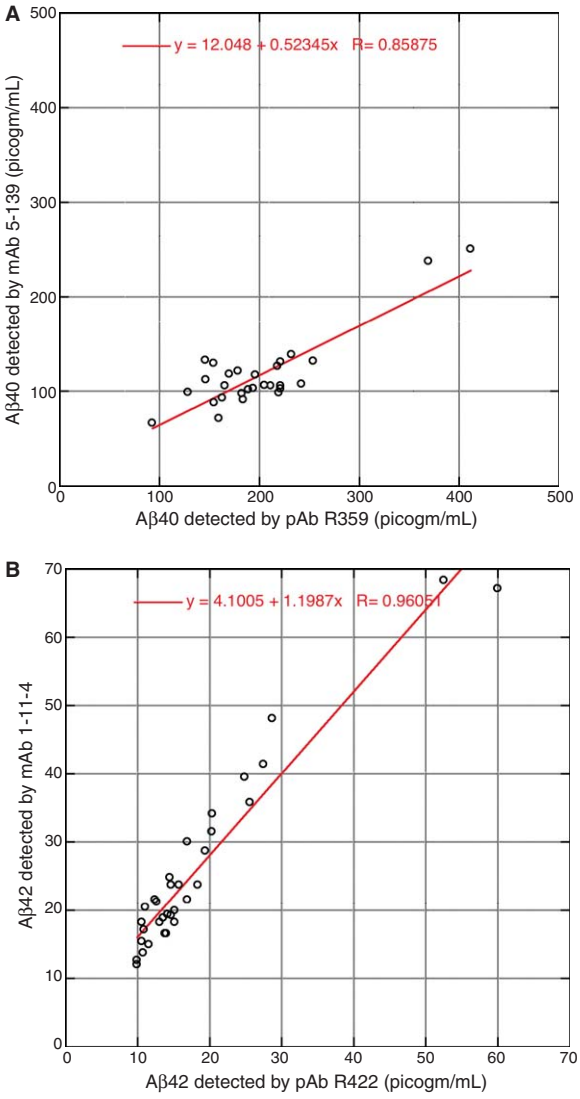

Fig. 3. Correlations of plasma Aβ measurements determined by monoclonal and polyclonal antibodies. AD and control plasma samples were applied to ELISA wells coated with mAb 6E10. The plates were washed and the wells were treated with either polyclonal antibodies (R359 for $A\beta_{40}$ or R422 for $A\beta_{42}$) or with the monoclonal antibodies, and the amounts of bound antibody were determined as described in the text. Aβ concentrations were calculated using a standard curve of absorbance vs. [$A\beta_{40}$] or [$A\beta_{42}$]. The values were displayed on scatter-plots. A) RabmAb40 vs R359. B) RabmAb42 vs. R422.

Use of RabmAb40 and RabmAb42 in immunohistology

Samples of both AD and DS brains were processed by 2 protocols. Paraffin-embedded samples were cut into thin sections, and PEG-embedded samples were cut into thick sections. The results in Figs 4 and 5 were obtained with PEG-embedded samples. These thick sections are liable to be subject to non-specific background staining; however, the results show that RabmAb40 and RabmAb42 produce negligible non-specific staining. The immunostaining of brain sections with mAbs RabmAb40 and RabmAb42 revealed patterns of plaques in the hippocampus (Fig. 4A and B) similar to those seen in sections stained with mouse mAb 4G8, an antibody that binds both amyloid peptides (data not shown); however, somewhat fewer plaques were detected in sections stained with RabmAb40.

The results were similar with paraffin-embedded tissue (see Supplementary Data, Figs S1 and 2; available online: http://www.j-alz.com/issues/23/vol23-2.html#supplementarydata01). The amounts of deposited Aβ detected in the 8-μm paraffin sections were lower than those detected in the 50-μm PEG sections. The relative amounts are roughly proportional to the relative thickness of the sections. Comparing the images from the 2 protocols, one can conclude that the distribution patterns of fibrillar and diffuse amyloid deposits, and their immunoproperties, are similar.

Immunostaining with antibodies pre-adsorbed with synthetic peptides $A\beta_{40}$ (RabmAb40) and $A\beta_{42}$ (RabmAb42) did not reveal any plaques, which indicated that adsorption was complete and there is no non-specific staining (Fig. 4C and D, respectively). Adsorption of RabmAb40 with $A\beta_{42}$ and RabmAb42 with $A\beta_{40}$ revealed patterns of plaque distribution comparable with unadsorbed antibodies (Fig. 4E and F, respectively), which indicated that RabmAb40 does not react with $A\beta_{42}$, and RabmAb42 does not react with $A\beta_{40}$.

Similar tests on sections from cerebellum containing diffuse, nonfibrillar (thioflavin S-negative) plaques revealed that antibodies adsorbed with their cognate peptide do not label diffuse amyloid; whereas, adsorption with the non-cognate peptide did not change the pattern of immunostaining of diffuse Aβ deposits (Fig. 5A–F).

DISCUSSION

The generation of polyclonal antibodies specific for Aβ C-terminal isoforms is time consuming, and the resulting antisera can differ widely in their affinities and specificities. In our experience, among rabbits immunized for periods up to one year, only about one in twenty produced high-affinity antibodies. Moreover, polyclonal antisera to Aβ C-terminal

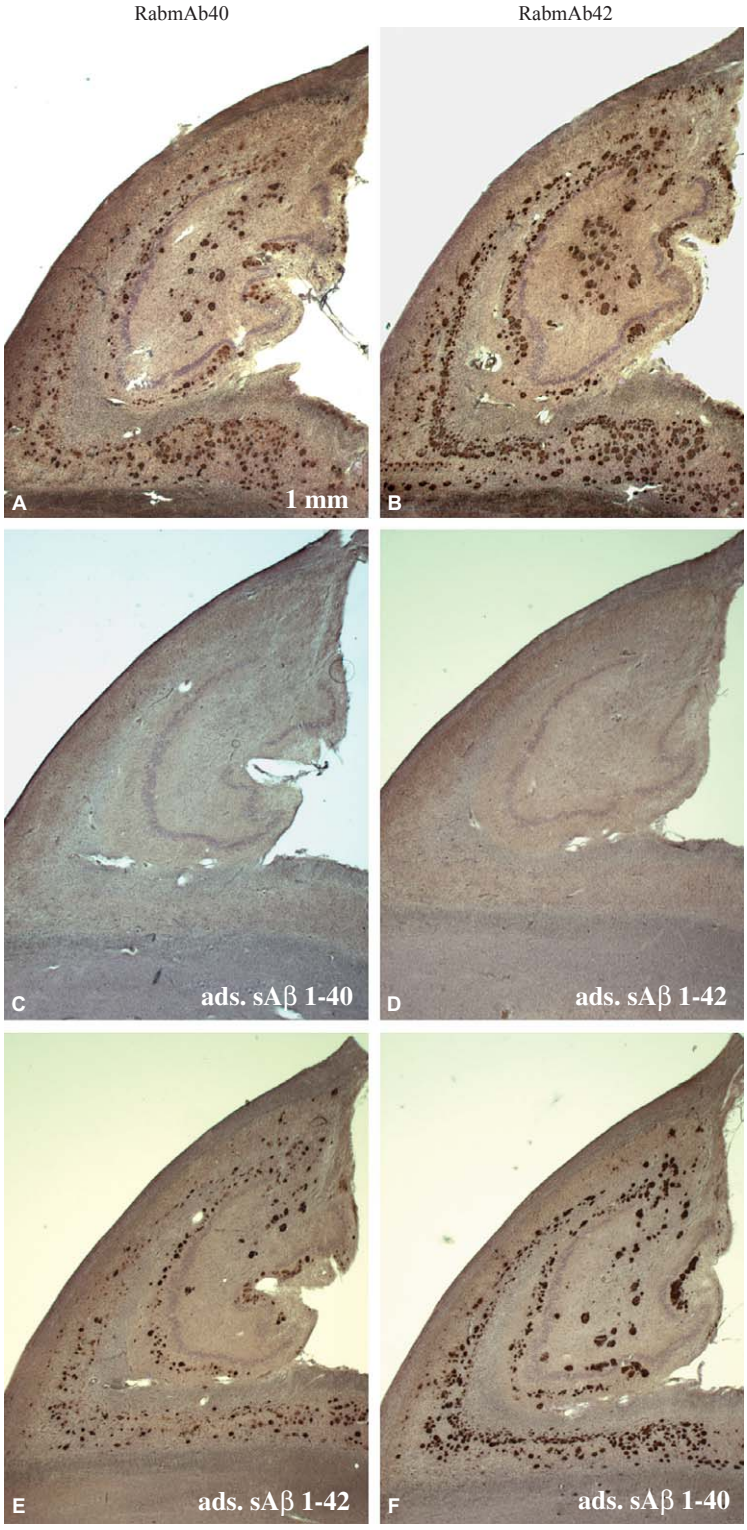

Fig. 4. Sections from the cornu Ammonis of a 75-year-old DS subject diagnosed with AD immunostained with rabbit RabmAb40 (A) or RabmAb42 (B). Numerous Aβ-positive fibrillar plaques show characteristic cornu Ammonis (CA) and dentate gyrus (DG) layer distributions. Figure (C) and (D) respectively illustrate the lack of immunoreactivity in sections incubated with RabmAb40 adsorbed with $A\beta_{40}$ or RabmAb42 adsorbed with $A\beta_{42}$. Adsorption of RabmAb40 with $A\beta_{42}$ (E) or adsorption of RabmAb42with $A\beta_{40}$ (F) does not block immunostaining.

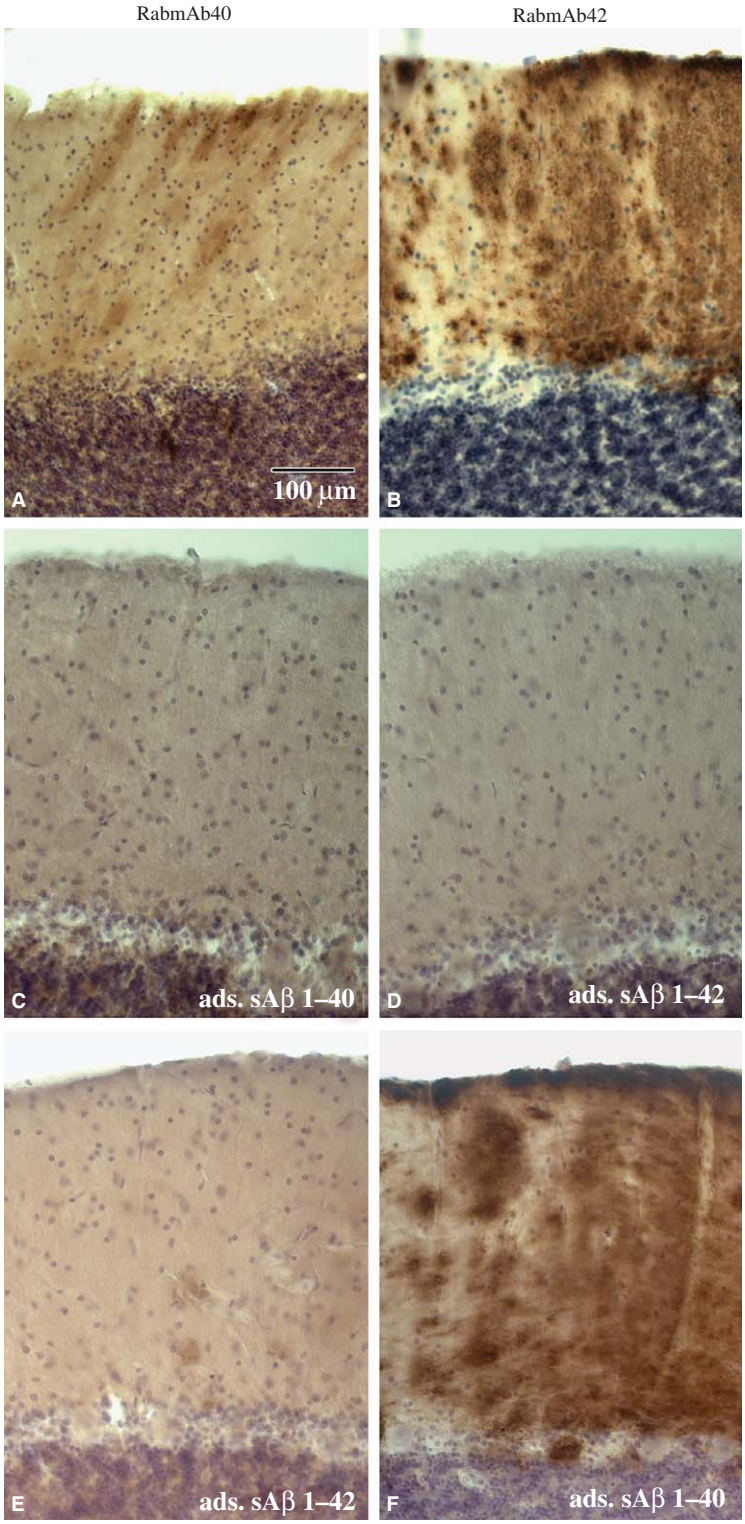

Fig. 5. Sections from the DS subject showing differences in detection of diffuse non-fibrillar Aβ deposits in the molecular layer of cerebellar cortex with RabmAb40 (A) and RabmAb42 (B). Sections incubated with RabmAb40 adsorbed with $A\beta_{40}$ (C) or RabmAb42 adsorbed with $A\beta_{42}$ (D) show greatly reduced immunoreactivity. Adsorption of RabmAb40 with $A\beta_{42}$ (E) or adsorption of RabmAb42 with $A\beta_{40}$ (F) did not change their immunoreactivity, which confirmed the specificity of the antibodies.

isoforms that appear to be specific in immunoblots and ELISA procedures often give discrepant results in immunohistological procedures. To obtain a reliable supply of antibodies possessing consistent affinity and specificity, we isolated hybridomas generated from rabbits that produced high-affinity antisera to $A\beta_{40}$ or $A\beta_{42}$.

Judging by the results in Table 2, RabmAb42 possesses sufficient selectivity to allow the quantification of $A\beta_{42}$ in the presence of at least a 500-fold excess of $A\beta_{40}$. RabmAb40 was similarly specific for $A\beta_{40}$. Using sandwich ELISA we found that RabmAb42 detected 4 fmol/mL of $A\beta_{42}$, but 2.4 pmol/mL $A\beta_{40}$ produced no absorbance (data not shown). These results indicate that RabmAb42 could detect $A\beta_{42}$ with no interference from $A\beta_{40}$ in body fluids, even though the level of $A\beta_{40}$ is 10–20 fold higher than the level of $A\beta_{42}$ [26–28].

The dissociation constants of RabmAb42 clones 1-11-3 and 1-11-4 were determined to be about 0.7 nM. These dissociation constants are nearly as low as those of our best polyclonal antibodies, for example, R226 [19]. These constants are not precise thermodynamic values. Although we used what we consider to be the most reliable commercially available preparations, there are uncertainties in the concentrations of $A\beta_{40}$ and $A\beta_{42}$. Moreover, the degree of aggregation of $A\beta_{42}$ and $A\beta_{40}$ may affect their immunochemical properties. Although we used DMSO-dissociated peptides to make our solutions, self-aggregation or binding to BSA or to reaction vessel walls may have lowered the effective peptide concentrations.

For the complex mAb 6E10-$A\beta_{40}$ we can roughly compare our determination of the K_D (by the ELISA method of Friguet et al. [20]) with that determined by Ramakrishnan et al. [29] by surface plasmon resonance. We measured the K_D to be 4 nM (corrected to 25°C, data not shown); whereas, Ramakrishnan et al. measured the K_D to be 22 nM at 25°C. Considering the variables that affect thermodynamic measurements on Aβ solutions, it is not now possible to compare the absolute values of Aβ-antibody dissociation constants determined by different methods in different laboratories.

One of our primary aims was to obtain a continuing source of antibodies for the measurement of Aβ isoforms in plasma, CSF, and cell culture media. In our ELISA procedure, RabmAb42 and polyclonal antibody R422 gave similar measurements of the level of $A\beta_{42}$ in various plasma samples. Moreover, the results were highly correlated. In the comparative measurements of $A\beta_{40}$ (Fig. 3A), the amounts determined with RabmAb40 were somewhat lower than those determined with R359; however, the results again were highly correlated, so that the relative levels of $A\beta_{40}$ measured were similar.

These antibodies were successfully used in immunohistology. To illustrate regiospecific differences in the immunoreactivity of amyloid deposits in human brain, serial sections from the brains of DS individuals were immunostained with antibodies specific for $A\beta_{40}$ and $A\beta_{42}$. The brain sections revealed comparable immunostaining of fibrillar amyloid with both RabmAb40 and RabmAb42, which indicated the presence of both $A\beta_{40}$ and $A\beta_{42}$ in fibrillar deposits. Weak immunoreactivity of diffuse amyloid deposits with RabmAb40 but strong immunoreactivity with RabmAb42 indicated that $A\beta_{40}$ is only a minute fraction of the total Aβ in diffuse plaques. Lack of immunoreaction in sections stained with each antibody pre-adsorbed with its cognate synthetic Aβ peptide – and strong reaction in sections stained with antibody pre-adsorbed with the non-cognate peptide – indicate that antibodies are highly specific in such immunohistochemical applications.

DS subjects develop Alzheimer's type pathology (with brain amyloidosis and neurofibrillary degeneration) at age about 40 years of age, some 20 years before the onset of such pathology in sporadic AD. Overexpression of AβPP, overproduction of Aβ, and early intracellular accumulation of Aβ [30] have been considered to be the foundations for this early onset of AD pathology and functional deterioration [31, 32].

The loss of intraneuronal Aβ immunoreactivity in areas of plaque formation has led to the conclusion that neurons release intracellular Aβ, which initiates a seeding process leading to plaque formation [33]. In sporadic AD, and in brains of DS subjects, two major forms of amyloid deposit are observed: fibrillar and diffuse. Fibrillar amyloid deposition is the predominant form of brain amyloidosis with approximately 41 plaques per square mm in the neocortex in severe AD [34]. Diffuse, non-fibrillar Aβ-positive plaques develop in the molecular layer of the cerebellum in the absence of fibrillar deposits [35, 36], the parvocellular layer of the presubiculum [37] and the caudate nucleus/putamen [38]. These persist in diffuse, nonfibrillar form until the end-phase of AD, which suggests that these diffuse amyloid deposits represent a separate pathological process from that resulting in the fibrillar amyloid deposits within archi- and neo-cortex and subcortical gray matter. Both species- and region-specific factors appear to determine the nature of amyloid deposition [34].

There is continuing interest in the development of biomarkers to aid the diagnosis of probable AD and mild cognitive impairment (MCI) and to track the conversion of MCI to probable AD [1, 5, 39, 40]. A related objective is to identify individuals at high risk of developing dementia. Most studies find that CSF $A\beta_{42}$ levels are lower in patients with probable AD than in nondemented age-matched control subjects [3, 4, 26, 28, 41]. CSF levels of $A\beta_{42}$ and microtubule-associated protein tau are useful markers to support the diagnosis of probable AD [5, 40, 42, 43].

A recent multicenter study found that patients with incipient AD could be identified by their CSF $A\beta_{42}$ levels; however, the measurements of the $A\beta_{42}$ levels varied among the participating laboratories [1, 3, 40, 42]. The report concluded that it is necessary to standardize the assay to lower the variability [43]. Part of this variability might be eliminated by the use of the same antibody sets. The addition of the highly specific and sensitive RabmAb42 could be useful for this purpose.

Another application of rabbit monoclonal antibodies such as RabmAb40 and RabmAb42 could be to quantify Aβ levels in human plasma, where the levels of $A\beta_{40}$ and $A\beta_{42}$ are 100-fold lower than in CSF [27, 28]. High affinity antibodies are necessary to measure these levels. Lui et al. examined $A\beta_{40}$ and $A\beta_{42}$ levels in a large number of plasma samples using commercial multiplex assay and ELISA [44]. In addition, they compared $A\beta_{40}$ and $A\beta_{42}$ levels with amyloid load derived from positron-emission tomography (PET) with the Pittsburgh compound B (PiB). They observed lower $A\beta_{1\text{-}42}$ levels and $A\beta_{1\text{-}42/1\text{-}40}$ ratios in patients with AD and these were inversely correlated with the Aβ load. Since two different assay methodologies significantly affected the interpretation of data, the authors concluded that the cross sectional analysis of plasma Aβ isoform levels are not sufficient to diagnose individuals with AD. Longitudinal studies might determine whether plasma Aβ levels are useful to aid the diagnosis of AD.

Using ELISA or multiplex technologies, several longitudinal studies measured plasma levels of $A\beta_{40}$ and $A\beta_{42}$ [7–10, 12, 45, 46] however, the results of these studies were inconsistent. Among several possible factors, methodological issues may have contributed to the discrepancies. One of these may have been the use of lower affinity mouse monoclonal antibodies. As shown in Fig. 3B, RabmAb42 is sensitive enough to measure plasma levels of $A\beta_{42}$.

To summarize, these rabbit monoclonal antibodies are widely applicable for immunochemical research on AD and its expression in DS, and for the development of methods to aid the early diagnosis and to track the progression of the disease.

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