# Quantification of Alzheimer Amyloid β Peptides Ending at Residues 40 and 42 by Novel ELISA Systems

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## Abstract

**Background:** The amyloid β (Aβ) peptide is a key molecule in the pathogenesis of Alzheimer's disease. Reliable methods to detect and quantify soluble forms of this peptide in human biological fluids and in model systems, such as cell cultures and transgenic animals, are of great importance for further understanding the disease mechanisms. In this study, the application of new and highly specific ELISA systems for quantification of Aβ40 and Aβ42 (Aβ peptides ending at residues 40 or 42, respectively) in human cerebrospinal fluid (CSF) are presented.

**Materials and Methods:** Monoclonal antibodies W0-2, G2-10 and G2-11 were thoroughly characterized by (SPOT) epitope mapping and immunoprecipitation/mass spectrometry. We determined whether aggregation affected the binding capacities of the antibodies to synthetic peptides and whether components of the CSF affected the ability of the antibodies to bind synthetic Aβ1-40 and Aβ1-42 peptides. The stability of Aβ40 and Aβ42 in CSF during different temperature conditions was also studied to optimize sample handling from lumbar puncture to Aβ assay.

**Results:** The detection range for the ELISAs were 20-250 pM. The intra-assay variations were 2% and 3%, and the inter-assay variations were 2% and 10% for Aβ40 and Aβ42, respectively. The antibodies specifically detected the expected peptides with equal affinity for soluble and fibrillar forms of the peptide. The presence of CSF obstructed the recognition of synthetic peptides by the antibodies and the immunoreactivity of endogenous CSF Aβ decreased with increasing storage time and temperature.

**Conclusions:** This study describes highly sensitive ELISAs with thoroughly characterized antibodies for quantification of Aβ40 and Aβ42, an important tool for the understanding of the pathogenesis of Alzheimer's disease. Our results pinpoint some of the difficulties associated with Aβ quantification and emphasize the importance of using a well-documented assay.

## Introduction

The main constituent of senile plaques found in the brains of Alzheimer's disease (AD) patients is the amyloid β peptide (Aβ), a proteolytic cleavage product of the larger, membrane-bound, amyloid precursor protein (APP; 1). Aβ is produced by various cell types and is secreted into blood and cerebrospinal fluid (CSF; 2,3). Soluble Aβ shows variations in the N- as well as the C-termini, but the majority of the peptides end at residue 40 (Aβ40). The amount of peptides ending at residue 42 (Aβ42) is roughly 1/10 of the Aβ40 peptides (4–6). Quantification of soluble Aβ in blood and CSF has been performed with enzyme-

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linked immunosorbent assays (ELISAs) (3,5,7–15), immunoradiometric sandwich assay (16), immunoprecipitation/blotting (17-19) or direct western blots (19). Specific quantification of A$\beta$42 in CSF by ELISA has been performed using the 277-2 antibody (10,12), 21F12 (14,15) and BC-05 (5,11,13). Only one antibody specific for A$\beta$40, BA-27, has been described in ELISA assays quantifying soluble A$\beta$ in CSF (11) and in blood (5,20).

In the present study, we developed new, highly specific ELISA systems for quantification of A$\beta$40 and A$\beta$42 in CSF. The antibodies used were W0-2 for capture and G2-10 and G2-11 for A$\beta$40 and A$\beta$42 detection, respectively, all previously described (19). In addition to previous studies on the antibody specificity (19), we thoroughly investigated the specificity of the three antibodies by several methods. We performed epitope mapping, where binding of antibody to overlapping peptides synthesized on a membrane was tested [the (SPOT) synthesis method (21)]. This method gives detailed information on linear epitopes. Peptides immunoprecipitated by the antibodies were investigated with matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF-MS) to confirm binding of the antibodies to the cognate peptides. We examined the binding capacity of G2-11 to synthetic A$\beta$1-42 in soluble or fibrillar conformation states by direct ELISA. We also investigated the abilities of the antibodies to bind synthetic A$\beta$1-40 and 1-42, which were preincubated with CSF for different lengths of time to test if components in the CSF affected the immunoreactivity of the peptides. Finally, the stability of endogenous A$\beta$40 and A$\beta$42 in CSF was studied during different conditions to optimize the sample handling process from lumbar puncture to A$\beta$ assay.

## Materials and Methods

### Antibody Specificity Experiments

PEPTIDE SYNTHESIS AND EPITOPE MAPPING. SPOT peptide synthesis was performed according to Frank (21). Nitrocellulose membranes were chemically derivatized with Fmoc-$\beta$-alanine to provide anchor function and spacer arms. Decapeptides overlapping each other by one amino acid, covering residues 1-42 of A$\beta$, were synthesized using Fmoc chemistry. Chromatography paper Chr 1 was purchased from Whatman

(Kebo, Stockholm, Sweden). N-N-dimethylformamide (DMF), N-N-methylpyrrolidinone (NMP), piperidine, dichloromethane (DCM), trifluoroacetic acid (TFA), N-N-diisopropylethylamine, acetic anhydride, triisobutylsilane, N-Fmoc-$\beta$-alanine, molecular sieves 4 Å, methanol, carbodiimide (DIC), NaN$_3$, acetic acid, $N$-methyl-imidazole (NMI) and 1-hydroxybenzotriazole (Hobt) were all purchased from Fluka (Stockholm, Sweden). Fmoc amino acid derivatives were purchased from Bachem (Stockholm, Sweden) and bromophenol blue from Amersham (Uppsala, Sweden).

The antibody-binding assay was principally perfomed according to Frank (21), but was modified in several ways. Membranes were transferred from the freezer to a glass dish on a rocking plate and rinsed twice with methanol. The following series of incubations were carried out: Tris buffered saline (TBS) containing 0.05% Tween-20 (T-TBS; 5 min), blocking buffer (5% skim milk powder in T-TBS; 10 min), first antibody (1:10000) in blocking buffer + 0.05% NaN$_3$ (overnight), T-TBS (three times for 5 min each), horseradish peroxidase (HRP)-coupled anti-mouse antibody (1:500) (Amersham) in blocking buffer (30 min), T-TBS (five times for 20 min each), enhanced chemiluminescent protein (ECL) detection (Amersham). The epitope mappings were repeated twice for all three antibodies.

IMMUNOPRECIPITATION/MASS SPECTROMETRY. Antigen specificity of G2-10 and G2-11 was tested by immunoprecipitation-mass spectrometry (IP-MS). The antibodies were incubated with synthetic A$\beta$1-40 and 1-42 and conditioned media from cells expressing human APP. Synthetic A$\beta$ was prepared to a final concentration of 10 nM in TBS. Immunoprecipitation was carried out by incubating 2 $\mu$l of G2-10 (1 mg/ml), 1 $\mu$l of G2-11 (ascites fluid, 2-3 mg/ml) or 1 $\mu$l of 6E10 (3.3 mg/ml, Senetek, Maryland Heights, MO) with 1 ml of the synthetic A$\beta$ solution at 4°C for 3 hr. Mouse neuroblastoma (N2a) cells stably transfected with wild-type human APP and presenilin1 cDNA were incubated with serum-reduced medium for 24 hr. Protease inhibitors (2 mM EDTA-Na$_2$, 10 $\mu$M leupeptin, 1 $\mu$M pepstatin, 1 $\mu$M (PMSF), 0.1 mM (TLCK) and 0.2 mM (TPCK)) were added to the conditioned media. The same amounts of antibodies, as described above, were added to 1 ml of conditioned media and incubated at 4°C for 3 hr. The immunoprecipitated A$\beta$ pep-

tides were analyzed using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) (Voyager-DE STR BioSpectrometry Workstation, PerSeptive Biosystem, Farmingham, MA) as described previously (22).

PRE-INCUBATION OF SYNTHETIC Aβ1-40 AND 1-42 WITH CSF OR H$_2$O AND ANALYSIS WITH DIRECT ELISA. Synthetic Aβ1-40 and 1-42 were solubilized to 1 mg/ml in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), aliquoted in 10 μl aliquots in eppendorf tubes, incubated at room temperature (r.t.) for 30 min and stored at −70°C until assayed. Immediately before analyses, HFIP was removed using a spin-vacuum system. 100 μl of CSF was added to each tube to resolubilize the peptide to 0.1 mg/ml, whereupon, the tubes were incubated in 25°C, 4°C, −20°C or −70°C. In each temperature, the tubes were incubated for 0, 3, 24, 168 or 336 hr before being assayed. Immediately prior to being assayed, the 0.1 mg/ml solutions of peptide in CSF or H$_2$O were further diluted in coating buffer (0.1 M sodium carbonate buffer, pH 9.6) to 5 μg/ml for the G2-10 assay and to 50 μg/ml for the W0-2 and G2-11 assays. 100 μl of the peptide solutions were added to each well on a 96-well plate and each sample was analyzed in duplicate (plates were obtained from Nunc, Denmark). The plates were incubated at 4°C overnight and then washed four times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. Biotinylated (Sulfo-NHS-biotin, Pierce, Rockford, IL) W0-2, G2-10 and G2-11 were diluted 1:40000 in ELISA buffer [4% ELISA blocking reagent (Boehringer Mannheim, Stockholm, Sweden), 0.2% bovine serum albumin (BSA), 2% Tween-20 and 2% goat serum in PBS, pH 7.4] for the assays of Aβ stored in H$_2$O and 1:4000 for the assays of Aβ stored in CSF. 100 μl (0.5 ≤g/ml) antibody were added per well and incubated for 90 min at r.t. on a plate shaker. The plates were washed as before and HRP-conjugated streptavidin was added and incubated for 1 hr. 100 μl solution [1 mg/ml ortho-phenylenediamine (Sigma, Stockholm, Sweden) in a substrate buffer containing 52 mM Na$_2$HPO$_4$, 24 mM citric acid and 0.1% H$_2$O$_2$] was added to each well. After a 10-30 min incubation in the dark, reactions were stopped with 100 μl of 2M H$_2$SO$_4$ and plates were read in a microplate spectrophotometer (Emax, Molecular Devices, Wokingham, UK) at 490 nm. The H$_2$O and CSF

experiments were performed separately and all experiments were done twice with identical plates.

ANALYSIS OF SYNTHETIC Aβ1-40 AND 1-42 DISSOLVED IN CSF WITH SANDWICH ELISA. For these experiments, three different CSF samples to which synthetic Aβ40 or Aβ42 was added, were analyzed by the sandwich ELISA described below. Experiments were performed twice. Each of the three samples were analyzed in six duplicates, three for Aβ40 and three for Aβ42. Synthetic Aβ40 or Aβ42 was dissolved in H$_2$O, diluted and added to the final concentrations of 100 pM, 250 pM or 500 pM in the three duplicates, respectively. CSF samples, in which an equal volume of H$_2$O without synthetic peptide was added, were used as controls. Each plate contained a standard curve with synthetic Aβ 1-40 or 1-42 diluted in ELISA buffer as described below.

Artificial CSF was prepared by mixing solution A (8.66 g NaCl, 0.224 g KCl, 0.206 g CaCl$_2$ • 2 H$_2$O and 0.163 g MgCl$_2$ • 2 H$_2$O in 500 ml water) with solution B (0.214 g Na$_2$HPO$_4$ • 7 H$_2$O and 0.027 g NaH$_2$PO$_4$ • H$_2$O in 500 ml water). Artificial CSF containing albumin was prepared by adding 0.4 mg/ml of human albumin (Sigma, Stockholm, Sweden) to the above solution.

PREPARATION OF FIBRILLAR Aβ42 AND ELECTRON MICROSCOPY OF PEPTIDES. Synthetic Aβ1-42 was dissolved in deionized water at a concentration of 1 mg/ml, vortexed and immediately frozen in liquid nitrogen. To obtain fibrillar Aβ, vials were rapidly thawed and incubated at 37°C for 7 days. 5 μl of Aβ solution were placed on a carbon-coated Formvar-grid (200 mesh) and diluted with 5 μl of 0.5% (v/v) glutaraldehyde solution after 2 min. The total adsorption time was 3 min. Thereafter, the grid was rinsed with deionized water, dried with a piece of filter paper and negatively stained with 5 μl of 2% (w/v) uranylacetate solution for 2 min. Finally, the grid was wicked off, air-dried and examined using a Zeiss EM 10 electron microscope (Carl Zeiss, Jena, Germany).

*Sandwich ELISA*

Plates were coated with 100 μl of W0-2 antibody in 0.1 M sodium carbonate buffer, pH 9.6. The plates were coated at 4°C overnight and then blocked with 100 μl per well of ELISA

blocking reagent (Boehringer Mannheim, Stockholm, Sweden) for 1 hr. Synthetic Aβ1-40 and Aβ1-42 peptides for the standard curves were dissolved at 1 mg/ml in 70% formic acid, aliquoted and frozen at −70°C. Immediately prior to addition of samples to the ELISA plates, the peptides were thawed, diluted 1:1000 in dH$_2$O and further diluted in ELISA buffer, thus, obtaining neutral pH. Aβ peptide solutions were added, in duplicate, at the following concentrations: 250, 125, 62.5, 31.25, 15.63 and 3.91 pM. If more than one plate were analyzed at the same time, the same batch of standard curve dilutions was used for all plates.

The ELISA buffer described above was used as dilution buffer for sample, standard curve and detection antibody. CSF samples were diluted 1:10 and 1:3 with dilution buffer for Aβ40 and Aβ42 assays, respectively. Samples (100 μl per well) were analyzed, in duplicate. Two sample incubation conditions were evaluated, at 37°C for 3 hr and at 4°C overnight. All ELISA plates were normalized to each other by inclusion of three standard CSF samples on all plates.

Biotinylated detection antibodies G2-10 and G2-11 (100 μl per well) were added at a concentration of 4.5–5 nM. Incubation times of 3 hr at 37°C and overnight at 4°C were evaluated. After binding of the biotinylated secondary antibody, streptavidin conjugated HRP (0.5 μg/ml, 100 μl per well) was allowed to bind for 1 hr at r.t. on a plate shaker. HRP activity was determined using orthophenylene-diamine as described above.

*Stability Analysis of Endogenous Aβ in CSF*

Stability of Aβ in CSF was investigated by incubation of samples at 20°C and 4°C for 24 hr, 6 hr and 0 hr to mimic the clinical sample-handling procedure from lumbar puncture to storage of CSF before assay. The effect of repeated freezing/thawing cycles was investigated by thawing samples 5, 10 and 15 times. All samples were thawed once, aliquoted and refrozen before being subjected to the different temperature treatments, which meant that all samples were thawed at least twice. Eight groups with eight CSF samples per group were treated as described in Table 1 and analyzed with ELISA. The values for group 1 (analyzed directly after the second thawing) were used as control values.

**Table 1. Scheme over the stability analysis of endogenous Aβ in CSF**

| Group | No. of Times Thawed | Incubation In 20°C | Incubation In 4°C |
|-------|---------------------|--------------------|--------------------|
| 1 | 2 | | |
| 2 | 5 | | |
| 3 | 10 | | |
| 4 | 15 | | |
| 5 | 2 | 6 hr | |
| 6 | 2 | 24 hr | |
| 7 | 2 | | 6 hr |
| 8 | 2 | | 24 hr |

## Results

*G2-10 and G2-11 Specifically Detect Aβ40 and Aβ42 Peptides In CSF and Cell Culture Media*

In the epitope mapping experiments, G2-10 was found to react strongly with the synthetic peptides covering amino acids 30-39 and 31-40, suggesting the requirement of residues
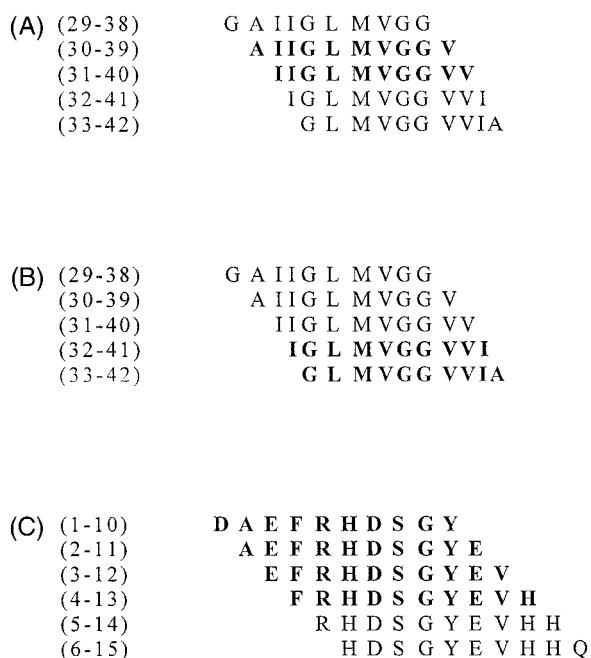


**Fig. 1.   Results of the epitope mapping.** Mapping of (A) G2-10, (B) G2-11 and (C) W0-2. Overlapping 10-mer peptides were synthesized as spots on a nitrocellulose membrane, whereupon, the antibodies were allowed to bind. The peptide sequences are shown to the right and the residue numbers of Aβ spanned by each peptide are shown in parenthesis to the left. The peptides for which the antibodies showed affinity are shown in bold.

39 or 40 for antibody recognition (Fig. 1A). We also found a weak reaction with the synthetic 32-41 and 33-42 peptides, suggesting a weak cross-reaction with the G2-11 epitope. However, in sandwich ELISAs employing G2-10 as a detection antibody, we did not find any cross-reaction with the synthetic 1-42 peptide below 500 pM, well above the concentration range for analysis of diluted CSF (Fig. 2A).

G2-11 reacted strongly with the 32-41 and 33-42 peptides, suggesting residues 41 or 42 in C-terminal position as the epitope for G2-11

(Fig. 1B). In addition, sandwich ELISA with synthetic peptides confirmed the lack of cross-reactivity with peptides lacking these residues (Fig. 2B). Peptides ending at residue 43 were not tested in this epitope mapping.

The W0-2 antibody reacted with the peptides covering residues 1-10, 2-11, 3-12, and 4-13. The common motif for these peptides consisted of residues 4-10, thereby suggesting this region as the W0-2 epitope (Fig. 1C). As peptides ending before residue 10 were not synthesized, we did not know if W0-2 would react with peptides ending at residues 5, 6, 7, 8
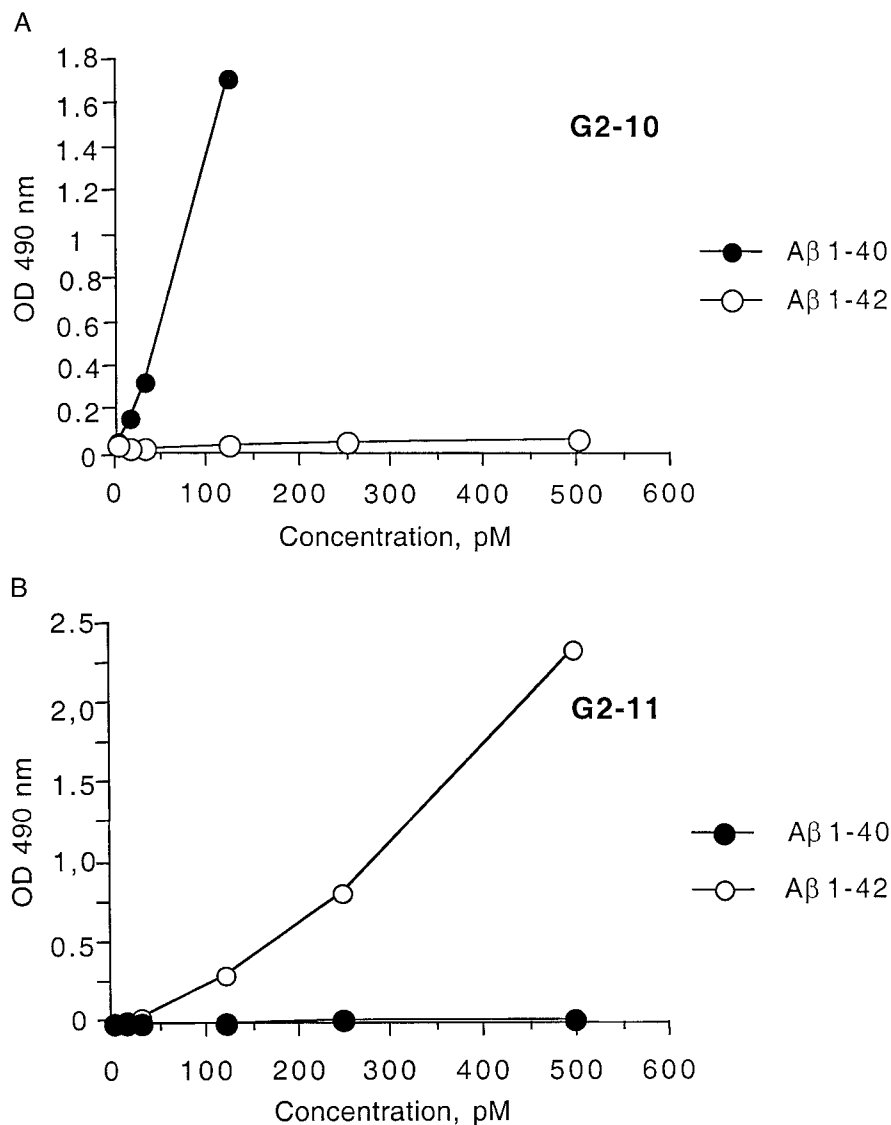


Fig. 2.   Recognition of the Aβ1-40 and 1-42 synthetic peptides. Recognition in the (A) W0-2/G2-10 and (B) W0-2/G2-11 sandwich ELISAs. The range shown for these standard curves correspond to the Aβ levels measured in diluted human CSF. At these concentrations, the W0-2/G2-10 ELISA does not cross-react with Aβ1-42 (A) and the W0-2/G2-11 ELISA does not cross-react with Aβ1-40.

or 9 and we, therefore, could not exclude that the epitope ended before residue 10.

To further establish the specificities of G2-10 and G2-11 by an additional method, IP-MS was performed. At first, synthetic A$\beta$1-40 and 1-42 were immunoprecipitated with the antibodies. G2-10 showed, as indicated with the SPOTs mapping method, strong affinity for A$\beta$1-40 and a slight cross-reactivity with A$\beta$1-

42 (Fig. 3A), similar to the pattern found with the commercial 6E10 antibody (specific for the N-terminus of A$\beta$; Fig. 3B). G2-11 only showed immunoreactivity with the A$\beta$1-42 peptide (Fig. 3C). In conditioned media from N2a cells expressing human wild-type APP and presenilin 1, no cross-reaction of G2-10 with secreted A$\beta$1-42 was found (Fig. 3D); whereas, 6E10 confirmed the presence of A$\beta$1-42 in the
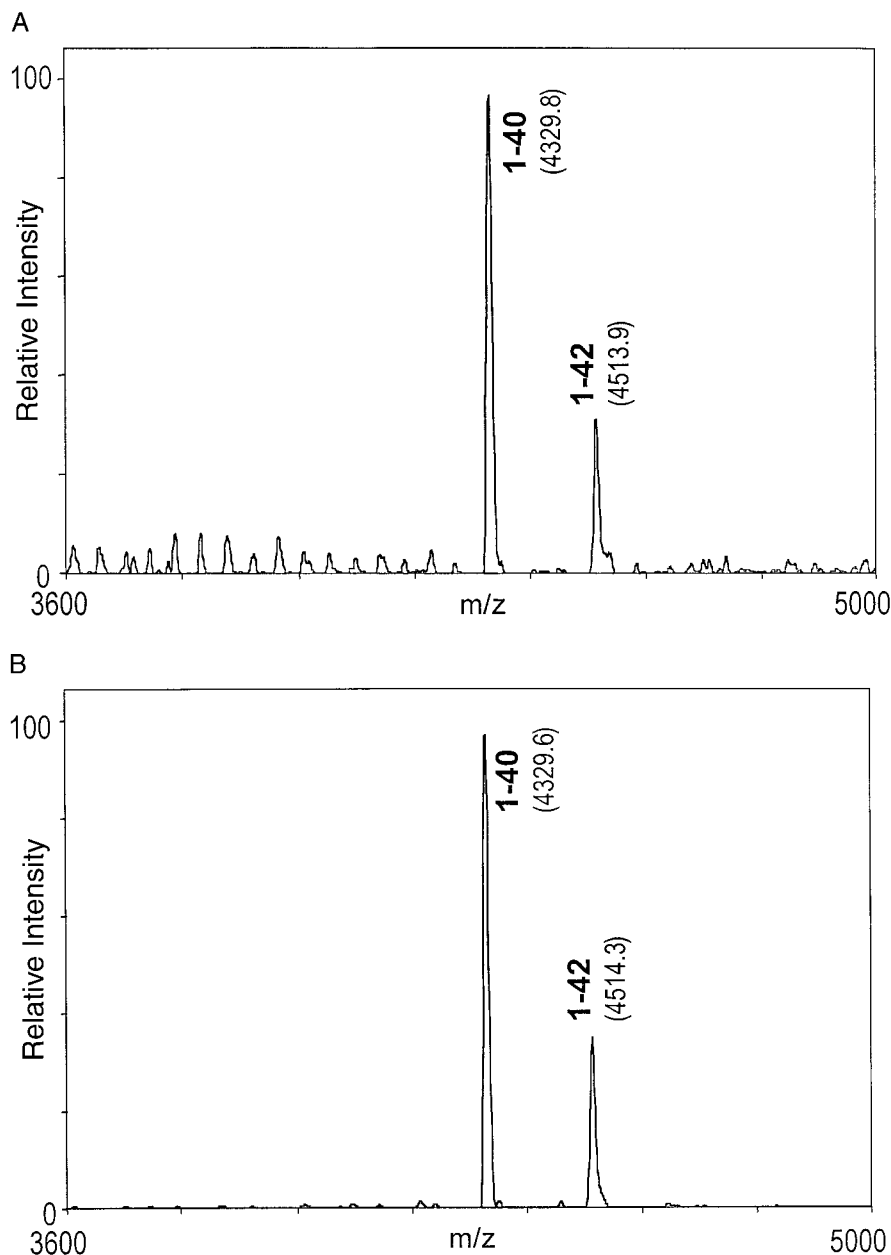


Fig. 3.   Mass spectra results.  For G2-10 (A, D), 6E10 (B, E) and G2-11 (C, F). Figures A–C show spectra of immunoprecipitated synthetic A$\beta$1-40 and A$\beta$1-42 peptides by the respective antibodies. Figures D–F show spectra of A$\beta$ peptides immuno-precipitated from conditioned media from amyloid precursor protein (APP)-expressing cells. The cross-reactivity of G2-10 with synthetic A$\beta$1-42 seen in (A) is not present in media (D).
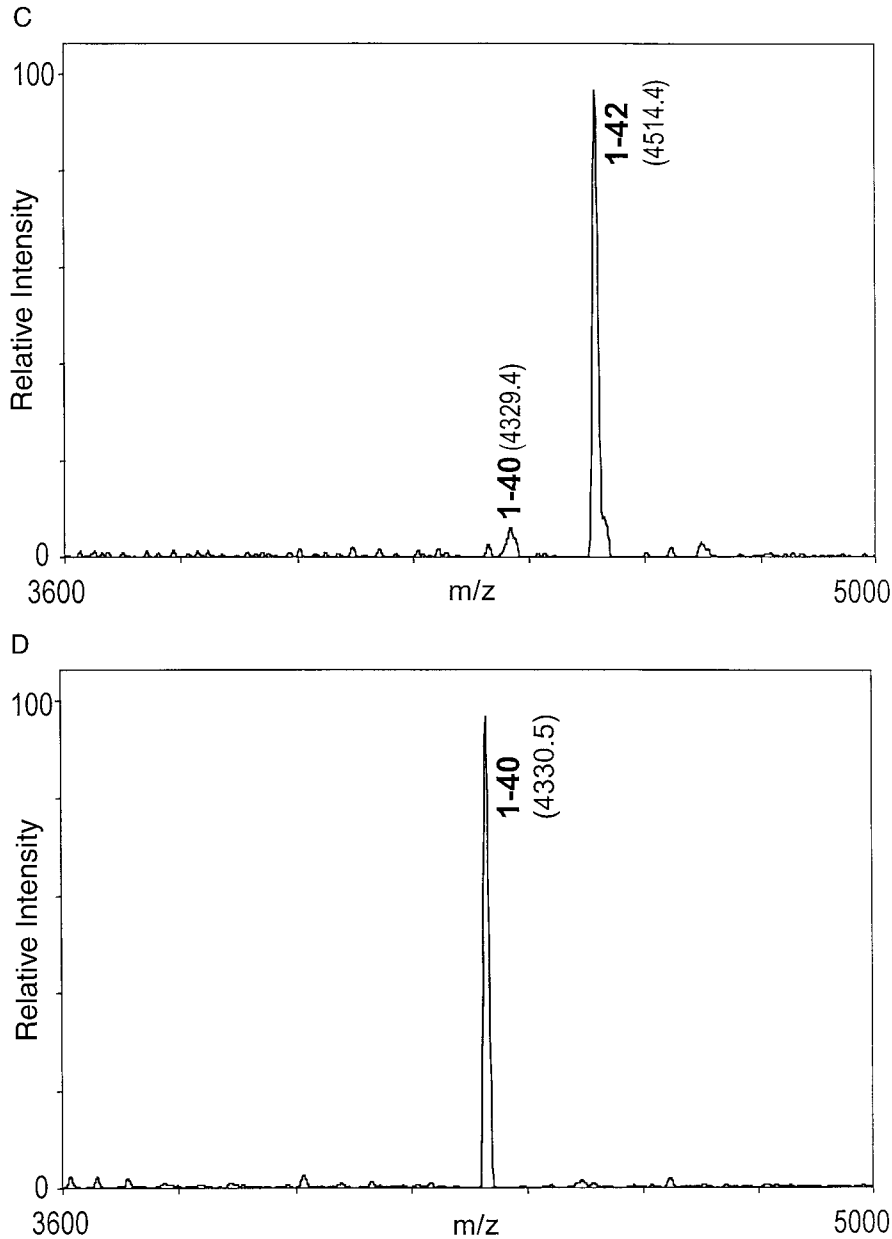
Fig. 3.   Continued

## G2-11 Recognized Soluble and Fibrillar A$\beta$1-42 Equally Well

media (Fig. 3E). G2-11 specifically recognized the secreted A$\beta$1-42 peptide (Fig.3F).

### G2-11 Recognized Soluble and Fibrillar A$\beta$1-42 Equally Well

To analyze the immunoreactivity of G2-11 against A$\beta$1-42 peptides in different states of aggregation, soluble and fibrillar A$\beta$1-42 were prepared. The aggregation states of the two preparations were confirmed by electron microscopy (Fig. 4A and B). Using direct ELISA, we found a similar binding affinity of G2-11 for soluble and fibrillar A$\beta$1-42 (Fig. 5).

### The Parameters of the ELISA Systems

Of the sample incubation times tested, the overnight incubation resulted in higher sensitivity for A$\beta$40, as well as for A$\beta$42. Incubation of the secondary antibodies overnight at 4°C gave a better result, compared with incubation for 3 hr at 37°C. When the assays were optimized, the ELISA systems were found to be able to quantify A$\beta$40 and A$\beta$42 at concentrations ranging from 20 pM up to 250 pM, which was sensitive enough to readily detect A$\beta$42 and A$\beta$40 in diluted human CSF. When assaying A$\beta$40 and A$\beta$42 in CSF, three standard CSF
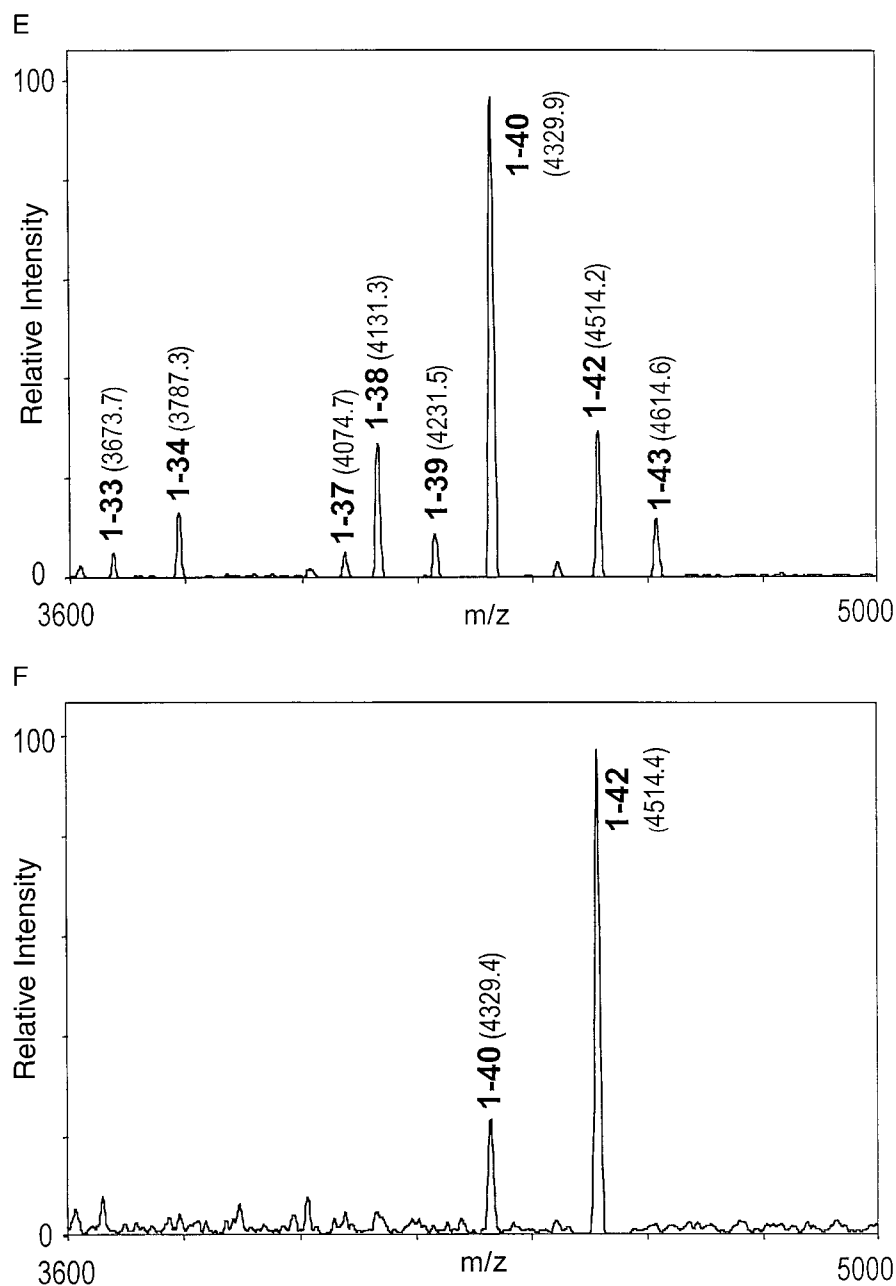
E



F



**Fig. 3.** *Continued*

samples were added to each plate. The plates were normalized against each other by calculating the mean values of the standard CSF samples on all plates, followed by correction of all samples on each plate according to this mean value. The intra-assay variation was determined to 3% and 2% (coefficient of variation) and the inter-assay variation to 10% and 2% for the Aβ42 and Aβ40 assays, respectively.

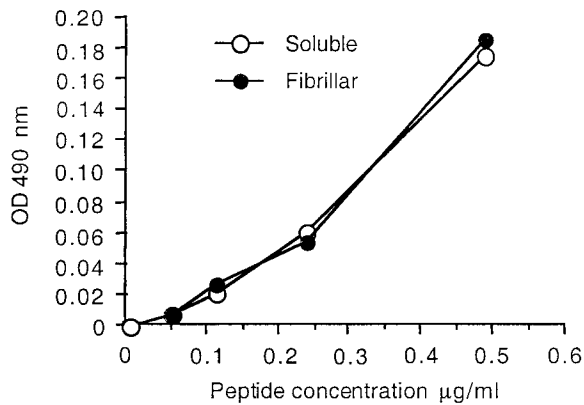## CSF Reduces the Immunoreactivity of Synthetic Aβ1-42 Peptide

Synthetic Aβ1-42 peptide was incubated in CSF or $H_2O$ for different lengths of time and under different temperature settings, to determine if the antigenicity of the peptides would be affected differently by the storage conditions. We found that the antibody-binding capacity of peptides dissolved in CSF immediately prior to the overnight incubation in the ELISA was reduced
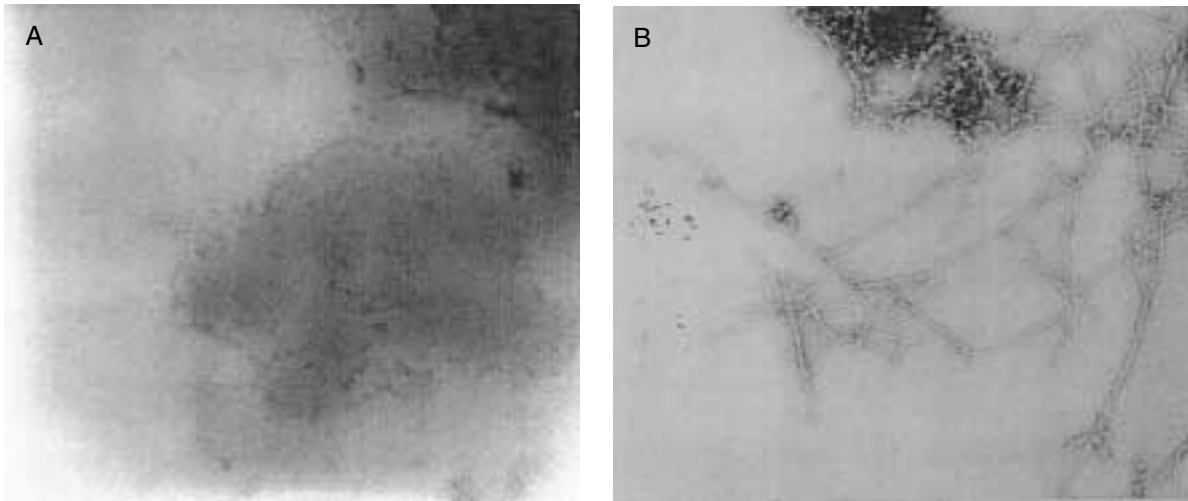
**Fig. 4.    Electron microscopy of synthetic A$\beta$1-42 in H$_2$O.** (A) Immediately analyzed or (B) incubated for 7 days at 37°C.
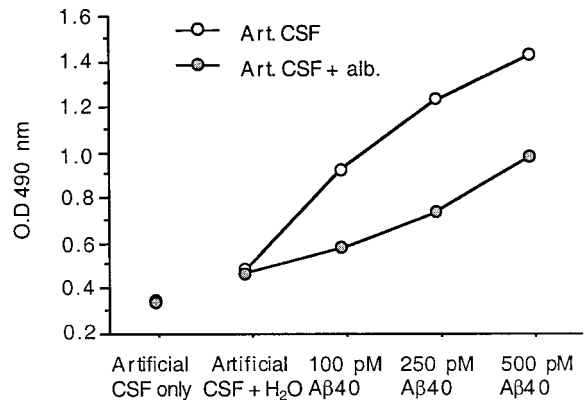
to levels below the detection limit of the assay, even if the concentration of G2-11 was increased 10-fold for the analysis of peptide stored in CSF, compared with peptide stored in H$_2$O (not shown). Next, synthetic A$\beta$1-40 and 1-42 peptides were added to CSF to generate final concentrations of 100, 250 and 500 pM. In accordance with the former experiment, the A$\beta$40 and 42 values measured in the samples were 25–65% of the expected levels. When synthetic peptide was diluted in artificial CSF, with and without physiological concentrations of human albumin, the solution containing albumin showed significantly lower A$\beta$ levels ($p = 0.01$, paired $t$-test), compared with non-albumin containing artificial CSF (Fig. 6).

*Endogenous A$\beta$ Immunoreactivity In CSF Decreases with Time*

Before quantifying endogenous A$\beta$40 and A$\beta$42 in CSF, we wanted to examine the stability of A$\beta$ immunoreactivity in CSF to evaluate the importance of sample handling after lumbar puncture. We investigated the stability over time at 20°C and 4°C, and we also evaluated the effect of repeated freezing/thawing cycles. We found significantly reduced A$\beta$40 and A$\beta$42 immunoreactivity in samples that were stored at 20°C for 24 hr (mean decrease 8%, $p = 0.04$ for A$\beta$40 and mean decrease 10%, $p = 0.02$ for A$\beta$42) or thawed more than 15 times (mean decrease 12%, $p = 0.04$ for A$\beta$40 and mean decrease 19%, $p = 0.02$ for A$\beta$42),



**Fig. 5.    Direct ELISA analysis of soluble and fibrillar A$\beta$.** 96-well plates were coated with peptide of the concentrations indicated and G2-11 was allowed to bind. The antibody showed equal affinity for both peptides.



**Fig. 6.    Synthetic A$\beta$1-40 diluted in artificial CSF with and without human albumin, analyzed with W0-2/G2-10 sandwich ELISA.** The peptide was diluted in H$_2$O and artificial cerebrospinal fluid (Art. CSF) where only H$_2$O was added was used as control.

compared with the control values. For A$\beta$42, a significant decrease was detected after 6 hr at 20°C (mean decrease 10%, $p = 0.04$).

## Discussion

In recent years, the A$\beta$ peptide has been shown to play an important role in AD pathogenesis. With this knowledge, an increased need for a reliable method to quantify A$\beta$40 and A$\beta$42 has arisen. Studies on the cell biological processes underlying AD pathogenesis, as well as investigations of potential biochemical diagnostic markers of the disease are dependent on reliable and specific analytical tools for A$\beta$ quantification. In this study, we present two sensitive ELISAs specifically measuring A$\beta$40 and A$\beta$42 in human CSF. We confirm the specificity of the antibodies previously reported (19) by several methods, experiments that have not been reported for other A$\beta$40 and A$\beta$42 ELISA assays. Results show a weak cross-reaction of the G2-10 antibody with A$\beta$1-42 at high concentrations of synthetic peptide in epitope mapping and IP-MS experiments, but this cross-reaction is not present in conditioned media from cells over-expressing human APP. In addition, it is not seen at the concentrations of synthetic peptide used in ELISA standard curves for quantification of A$\beta$40 in CSF and we, therefore, conclude that G2-10 specifically detects A$\beta$40 in CSF. The difference in cross-reactivity between synthetic and cell-derived A$\beta$ might indicate a difference in the conformation of the peptides. It is also possible that components of the cell culture media might affect the ability of the antibody to recognize its epitope by masking it, as we discuss below. In the epitope mapping experiment, it is possible that the shorter length of the peptides, compared with full-length A$\beta$ might influence the protein folding and, thereby, the exposure of the epitope region. It was recently reported by Pitschke and colleagues (23) that a larger fraction of A$\beta$ in CSF is aggregated in AD, compared with controls. We, therefore, investigated whether our antibodies recognized soluble and fibrillar A$\beta$ differently. We are able to show that G2-11 detects synthetic A$\beta$1-42 peptides of both aggregation states equally well, which suggests that our A$\beta$42 ELISA quantifies all A$\beta$42 peptides with equal affinity, regardless of the aggregation state.

Before sandwich ELISA analysis of endogenous A$\beta$ peptides in human CSF, we determined the stability of synthetic A$\beta$1-42 peptides dissolved in human CSF and H$_2$O, over time and at different temperatures. This analysis was done to test the sensitivity of A$\beta$ to different treatments and was performed with ELISA where the peptide was coated directly on the plates. To our surprise, the immunoreactivity of A$\beta$1-42 stored in CSF almost disappears; whereas, the peptide stored in H$_2$O shows a high immunoreactivity that does not appear to be affected by incubation time or temperature. This suggests that the antibody binding capacity of synthetic A$\beta$1-42 is stable when the peptide is stored in H$_2$O for 336 hr or more, in temperatures ranging from 25°C down to $-70$°C. Peptide stored in CSF might either be immediately degraded or bound by a chaperone protein, thus, hiding the epitope for G2-11. Several CSF proteins have been shown to bind A$\beta$, such as albumin (24,25), lipoproteins (24,26–28), gangliosides (29,30), transthyretin (31), glycosaminoglycans (32) and immunoglobulins (25). Furthermore, binding of synthetic A$\beta$42 peptide to several plasma proteins, which are also present in CSF, was recently found to mask the epitope for an A$\beta$42-specific antibody (33). CSF has also been shown to inhibit A$\beta$ fibril formation, suggesting that a CSF component binds A$\beta$ and, thereby, inhibits aggregation (25,34).

In the sandwich ELISA experiment, the above findings were further investigated by another approach. CSF was "spiked" with synthetic peptide of different concentrations to test whether an increase in immunoreactivity, corresponding to the amount of synthetic peptide added, would be seen. In concordance with the previous experiment, the amount of A$\beta$ in the samples, as estimated by the synthetic peptide standard curve, does not correspond to the amount of synthetic A$\beta$ added in the samples. To analyze whether A$\beta$ is degraded within CSF or if A$\beta$-binding proteins might affect the immunoreactivity of A$\beta$, we mimicked the salt concentrations and pH of CSF in the preparation of artificial CSF with and without physiological concentrations of human albumin. The immunoreactivities of synthetic A$\beta$1-40 and 1-42 are lower in the albumin-containing solution, which supports the study by Kuo and colleagues (35), who found that binding of A$\beta$42 to several proteins, including albumin, inhibited binding of an A$\beta$42-specific antibody to the peptide.

Taken together, the above results suggest that it is the binding of synthetic Aβ to CSF proteins that masks the epitopes of antibodies directed at the C terminus of Aβ, rather than degradation of Aβ in CSF that causes Aβ immunoreactivity in CSF to decrease in our experiments. This protein interaction may render the determination of absolute amounts of Aβ in CSF more difficult and should be kept in mind when this quantification is done. As our IP-MS experiments indicate, a direct comparison between synthetic and cell-derived Aβ should be interpreted with caution.

We also investigated the stability of endogenous Aβ40 and Aβ42 in CSF over time to optimize the sample-handling procedures before assaying for Aβ40 and Aβ42. Our conclusions are that measurements of Aβ40 and Aβ42 in CSF give reproducible results when the CSF is stored at 20°C for up to 6 hr at 4° for at least 24 hr or thawed 2-12 times. The setup of the experiment does not allow us to analyze samples that had never been, or were only once, thawed. Therefore, we can not exclude the possibility that the first thawing affects Aβ immunoreactivity more than the following thawings. The time limit of 6 hr is normally not exceeded during routine procedures for lumbar puncture, but our results indicate that the immunoreactivity of Aβ in CSF does decrease over time. For the most reproducible results, samples should be kept cold and be frozen as fast as possible.

In conclusion, we present highly sensitive ELISA systems for quantification of Aβ42 and Aβ40 in CSF, employing very well-characterized antibodies, and identified some of the difficulties connected with the construction of such an assay. Our data on Aβ stability in CSF might be useful as a guideline for correct treatment of samples from lumbar puncture to ELISA assay.

## Acknowledgments

## References

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