

Amyloid Oligomers as Blood Biomarkers in the Diagnosis of Alzheimer's Disease

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Abstract

Oligomeric forms of the peptide, β -amyloid, ($A\beta$) are known to be toxic to human and rodent neurones, and have been identified as possible causative agents in the loss of cognitive function in Alzheimer's Disease (AD). An ELISA assay has been developed capable of detecting oligomeric forms of $A\beta$ in biological fluids, but not detecting monomeric species. The ELISA has been validated with a number of synthetic variant sequences of $A\beta$ and the effects of known inhibitors of $A\beta$ oligomer formation. Significantly raised levels of oligomers were detected in sera samples from AD patients, compared to age-matched control sera.

Introduction

Alzheimer's Disease (AD) is a complex neurodegenerative disease, with a present incidence of about 24 million in the global population, and 800,000 in the UK population. Current medications, which include acetyl cholinesterase inhibitors such as Aricept, and NMDA receptor antagonists [1], such as memantine, treat the symptoms but do not slow neurodegeneration [2]. Brain imaging studies with PET reagents that detect $A\beta$ deposits [3] have shown that pathophysiological changes in patients with AD precede the manifestation of clinical symptoms by decades, suggesting that biomarkers may be useful in prediction of the disease in people with mild cognitive impairment (MCI), or prodromal AD, who are yet to develop measurable cognitive symptoms. Deposition of the peptide β -amyloid has been cited as a prime possible cause of the neurodegeneration seen in the disease. This 40-42 residue peptide is released by proteolysis from its transmembrane precursor protein APP, and aggregates in stages. First β -amyloid forms small soluble oligomers, then larger soluble protofibrils and finally precipitates as insoluble protofibrils or fibrils, which are components of the plaques seen in post-mortem sections from brains of Alzheimer's patients [4]. Dimers of β -amyloid have been

shown to block long-term potentiation and reduce dendritic spine density in the rat hippocampus [5]. The Dutch cerebral haemorrhage mutated form of $A\beta$ is also studied. Hereditary cerebral haemorrhage with amyloidosis, Dutch type (HCHWA-D), is a cerebral amyloidosis characterized by prominent vascular deposits and fatal haemorrhages. The disorder is caused by a point mutation in codon 693 of the gene encoding the amyloid precursor protein (APP), resulting in a Glu-->Gln amino acid substitution at position 22 of the amyloid beta-protein ($A\beta$) region The

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crossed β -structure of β -amyloid in fibrils gives rise to the characteristic birefringence seen in post-mortem AD brain sections stained with Congo red. Therapy involving vaccination against β -amyloid has shown some success in early, moderate Alzheimer's patients, and is in clinical trials, and a number of other strategies that lead to decreased β -amyloid production are under investigation [6]. Reliable biomarkers are essential for detection of signs of AD at the prodromal or MCI stages, when treatment and prevention are likely to be most effective. Biomarkers are generally measured in body fluids, such as CSF and blood, but images collected as a result of MRI or PET scanning of brain, are also useful biomarkers. The possibility is arising of asymptomatic treatment for Alzheimer's at the preclinical stages, when A β in the aggregated form is accumulating, to prevent irreversible neuronal damage that occurs gradually, leading to cognition and memory problems. An economical and reliable blood test for A β aggregates at the preclinical stages, would enable screening of large numbers of people, leading to further analyses of CSF samples, supplemented with confirmatory PET brain scans for deposited brain A β and/or hyperphosphorylated versions of the microtubule-associated protein tau aggregated into neurofibrillary tangles, on selected at-risk individuals. Uptake of the PET tau reagent into the medial temporal lobe has been correlated with loss of memory performance in older individuals [7].

Materials and Methods

Solid-phase ELISA

A non-competitive direct solid-phase ELISA assay was developed which allows the selective measurement of the oligomerisation of A β peptides in solution. The assay involves an antibody raised against the N-terminus of the A β peptide sequence, and is used in the same assay in both the biotinylated and non-biotinylated forms. The assay detects oligomers as N-terminal epitopes of β -amyloid. A β oligomers bind to the non-biotinylated antibody-coated wells and then the biotinylated form of the antibody binds to additional N-terminal epitopes present in oligomers. The levels of bound biotin are measured after the addition of peroxidase-avidin, by the colour change produced on addition of peroxidase substrate. Monomers are not detected as the biotinylated antibody is not able to bind the single epitope already occupied by binding to the non-biotinylated anti-NTA4 antibody immobilising A β to the wells.

Preparation of Antibodies.

Affinity-purified anti-NTA4 antibody prepared by immunisation of rabbits with an N-terminal 10-residue synthetic peptide sequence of A β , affinity purified on immobilised peptide, and preservation in 0.05% thiomersal/PBS at -20°C was prepared as described in [8].

Preparation of Biotinylated Anti-NTA4

Anti-NTA4, an affinity-purified polyclonal rabbit antibody raised to the N-terminal ten residues of the A β -peptide, (NTA4) [8] was biotinylated. The affinity-purified antibodies stored at -20°C in PBS were isolated from thiomersal preservative by gel filtration on a Sephadex G-25 gel column in 0.1M sodium borate buffer, and re-concentrated to 0.3mg/ml using a microcentrifuge filter (Centisart-C4). N-Hydroxysuccinimide biotin-caproate (Sigma-Aldrich; B2643) in a stock solution of 10mg/ml in dimethyl sulfoxide was added to the antibody solution to give a molar excess over antibody of 165. The mixture was incubated on a rotating wheel at 20°C for 4 hours. 20 μ l of 1M ammonium chloride was then added to quench the reaction, and incubation continued at room temperature for 10 minutes. The mixture was then subject to gel filtration on Sephadex G-25 in PBS to remove any excess uncoupled biotin. The biotinylated antibody was collected and stored in 200 μ l aliquots at -20°C.

ELISA Assay

An ELISA plate was coated with 5 μ g /ml of non-biotinylated anti-NTA4 antibody (100 μ l /well), in PBS/0.05% sodium azide at 4°C for 24 hours. Any excess antibody remaining was decanted. The plate was then washed twice with blocking buffer (PBS/1% BSA/0.05% Tween (pH 7.4)), and incubated with blocking buffer for 1 hour at room temperature to block any unreactive sites and to prevent the stacking of several layers of antibody. Excess blocking buffer was decanted, and dilutions of A β solutions to be assayed, prepared in blocking buffer, then added (100 μ l /well). The plate was incubated at 37°C for 2 hours. After washing four times with blocking buffer, 100 μ l biotinylated anti-NTA4 antibody, diluted 1 to 500 in blocking buffer, was added to each well, and incubated at 37°C for 1.5 hours. Wells were washed with blocking buffer three times and incubated with extraavidin-peroxidase (E2886; Sigma) diluted 1 to 2000 in blocking buffer (100 μ l /well) at 37°C for 1.5 hours. The plate was washed twice with blocking buffer and three times with PBS/0.05% Tween before adding substrate for peroxidase,

TMB Chromagen, (Thermo-Fisher)(100µl per well). A colour change was allowed to develop for 7 to 10 minutes. 1M H₂SO₄ (100µl /well) was then added to the plate, and the plate was read on an ELISA reader at 450nm [9].

Peptides

Five synthetic Aβ peptides used in the experiments were synthesised on solid-phase using the Fmoc method on a Milligan 9050 peptide synthesiser [10] and then purified on HPLC.

1. Aβ1-40 The sequence is shown below:

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV

2. Aβ1-42 The sequence is shown below:

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVIA

3. E22Q Aβ The underlined E residue in the Aβ1-42 sequence is mutated to Q in E22Q, the sequence found in patients with Dutch Cerebral Haemorrhage (E22Q).

4. Y10orthoNO₂Y Aβ The nitrosylated form of Aβ has a nitro group on residue Y in position 10 of the Aβ1-40 sequence. Nitro-tyrosine residues are found in the region of senile plaques when Aβ reacts with copious amounts of nitric oxide and superoxide present in these lesions.

5. N-Terminal peptide DAEFRHDSGY (residues 1 to 10 of the N-terminal of the Aβ peptide, NTA4)

Aβ1-40, E22Q Aβ and nitrosylated Aβ was prepared in sterile distilled water followed by addition of a 10% volume of sterile 1M Tris-HCl (pH 7.4) to obtain their final concentrations. Aβ1-42 was dissolved in 10mM NaOH and sterile distilled water added, followed by a 10% volume of sterile 1M Tris-HCl (pH 7.4) to obtain the required concentration of Aβ1-42.

Inhibitors

Synthetic peptide inhibitors were tested to see whether they inhibited the formation of oligomers. The synthetic peptide inhibitors tested include Om1, Om2, acetylated Om1 (AcOm1), acetylated Om2 (AcOm2), and

retro-inverse Om2 (RIOm2), which is the D-form of Om2 (lower case letters represent D-amino acids). They were synthesised on solid-phase using the Fmoc method on a Milligan 9050 peptide synthesiser and then purified on HPLC, as described in [11]. Inhibitors were made up to required concentrations in sterilised distilled water. Their sequences are shown below [11]:

Om1	RGKLVFFGR
AcOm1	AcRGKLVFFGR
Om2	RGKLVFFGR-NH ₂
AcOm2	AcRGKLVFFGR-NH ₂
RIOm2	rGffvlkGr-NH ₂

Sites and Subject Recruitment

44 consecutive patients attending memory clinics in Barnes Hospital, with a diagnosis of AD were recruited for this study, along with 16 age-matched carer controls, were consented for the study according to the protocol in "Proteomic studies of Alzheimer's Disease", passed by the St George's Healthcare Ethics Committee, in Sep, 2004. Blood samples (5.0ml) were collected from 44 Alzheimer's patients, aged 65+ (ICD10 and NINCDS criteria; [12]) and 16 age-matched controls with no recorded memory problems, who had accompanied patients to the clinics. Blood was obtained by venepuncture, transferred to centrifuge tubes, incubated at room temperature for 15 mins to clot, and then centrifuged in a bench centrifuge at 2000xg for 15 mins. The clear supernatant serum was transferred to 1.5ml polypropylene tubes, on ice, stored at -20°C for two weeks, then transferred at -20°C to a -70°C freezer. On the same day as assay, samples were thawed by warming rapidly to room temperature, some samples diluted 1 to 100 with blocking buffer (PBS/1% BSA/0.05% Tween (pH 7.4)), before subjecting to the Elisa measurement for oligomers.

Western Blotting

Sera samples from Alzheimer's patients and control patients were removed from the -70°C freezer and left to thaw on ice. Once thawed 10 µl of sample was added to 20 µl of tricine sample buffer containing dithiothreitol (Invitrogen). Samples were then vortexed and heated for

about 5 minutes at 95°C. Equal volumes (25µl) were electrophoresed on 16% Tricine gels (Invitrogen), together with samples of synthetic A μ 42, or low molecular weight markers (Invitrogen). After electrophoresis, proteins were transferred to PVDF membrane by transverse semi-dry electrophoresis. After transfer, the membrane was placed into a 50 ml falcon tube, completely covered with PBS and placed in a boiling water bath for ten minutes. The membrane was left to cool and then washed twice (five minutes each wash) in PBS Tween (0.05% v/v). A western blocking buffer was prepared by measuring out 2.5g of milk powder and adding this to 50ml of PBS Tween. The membrane was then put into 10ml of this blocking buffer and left for an hour on the rocker. Primary antibody was anti-mouse A β 6E10 (Covance) monoclonal antibody, diluted 1 to 1000 in blocking buffer, and secondary anti-mouse IgG linked to peroxidase (Sigma) diluted 1 to 2000 in blocking buffer.

Results

An ELISA was designed to detect soluble oligomers of β -amyloid. The assay used anti-NTA4, an antibody to the N-terminus of β -amyloid to attach oligomers of A β to wells in an ELISA plate and further epitopes present in oligomers were detected by biotinylated form of the anti-NTA4 antibody. The response of the ELISA, as measured by the absorbance values obtained at 450nm shows the amount of soluble oligomers present in solution. The higher the value of absorbance, the greater the level of oligomerisation.

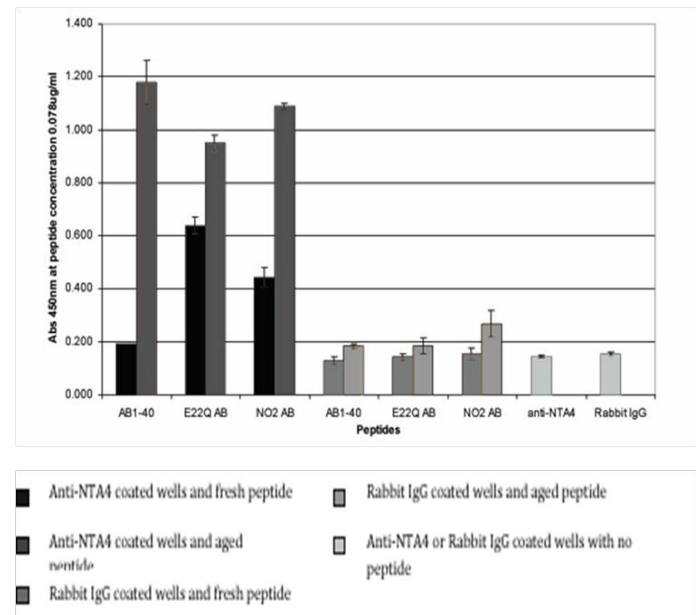
Comparison of A β 1-40, E22Q A β , and Nitrosylated A β

The response of fresh and aged solutions of A β 1-40, E22Q A β , and nitrosylated A β (NO $_2$) were compared (figure 1). Wells of a plate were coated with either unbiotinylated anti-NTA4 or control rabbit IgG antibody.

Wells of a plate were coated with either non-biotinylated anti-NTA4 antibody or control rabbit IgG (0.5µg/ml in PBS/azide) and left overnight at 4°C before removal. Wells were washed in PBS, then fresh peptide solutions of A β 1-40, E22Q A β , and NO $_2$ A β were prepared in sterilised distilled water, to which a 10% volume of sterile 1M Tris-HCl (pH 7.4) had been added to give a final concentrations of 5µg/ml, and tested in the ELISA immediately. Aged solutions were prepared in the same way but were incubated at 37°C for 1 hr before testing. Wells were washed with 1xPBS/1% BSA/0.05% Tween

buffer, and the ELISA developed with biotinylated NTA4 as described in Methods above... The mean of triplicate measurements and the standard deviation for each sample are shown.

Figure 1: ELISA response using antibody to peptide NTA4 (anti-NTA4), compared to control non-specific Rb IgG as coating antibodies, with aged or fresh solutions of A β (1-40) peptides, followed by detection of multiple epitopes by biotinylated NTA4.



The graph shows that fresh samples of A β 1-40, E22Q A β and NO $_2$ A β gave much lower responses than A β solutions that had been aged at pH 7.4. When the peptide solutions were added to wells coated with control rabbit IgG, the response was much lower for both aged and fresh samples – there was no more response than in wells coated with either anti-NTA4 or rabbit IgG to which no A β peptide had been added.

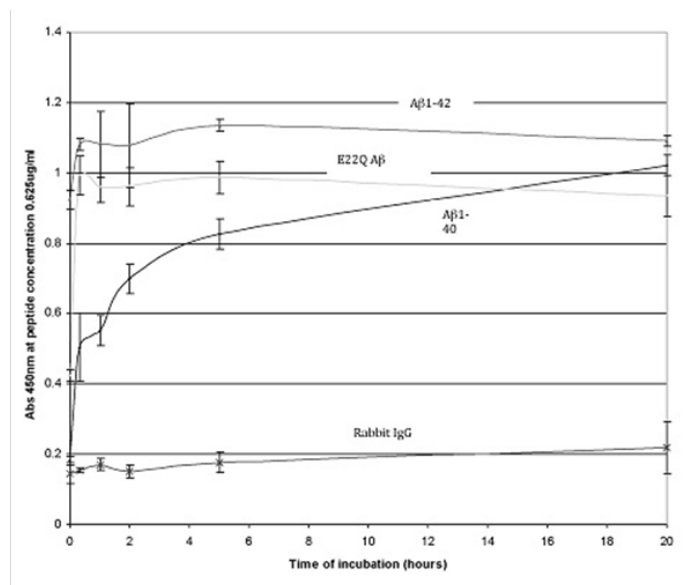
From the ELISA response of wells coated with anti-NTA4, it can be seen that aged A β 1-40 solutions contained higher levels of oligomers than aged E22Q A β or NO $_2$ A β . As the ELISA response of fresh E22Q A β was higher than that of A β , it seems that the Dutch mutated peptide formed oligomers more rapidly and greater extent than native A β E22Q.

Comparison of A β 1-40, A β 1-42 and E22Q A β Oligomer Formation Over Time

An assay was carried out to look at how the levels of soluble A β oligomers in solution vary over a 20-hour period of incubation of the A β peptides. Fresh samples of

A β 1-40, A β 1-42 and E22Q A β were prepared and then incubated in 0.1M Tris at pH7.4, with samples being taken at different times over a 20-hour period. Rabbit IgG coated wells containing just A β 1-42 were used as controls.

Figure 2: Wells of a plate were coated with either non-biotinylated anti-NTA4 or control rabbit IgG (5ug/ml). Samples of A β 1-40, A β 1-42 and E22Q A β (0.5mg/ml), were prepared as described in the methods and then incubated at 37°C. At time points of 0 minutes, 20 minutes, 1, 2, 5, and 20 hours, samples of the peptide solutions were removed and diluted 1 to 800 in cold (+4°C) 1xPBS/1% BSA/0.05% Tween buffer. All three peptides were added to the NTA4 coated wells, but only A β 1-42 was added to the rabbit IgG coated wells to act as a control. The remainder of the ELISA was performed as described in the methods. The mean of triplicate measurements and the standard deviation for each time point is shown.



The amounts of oligomer formed from A β 1-40 was maximum after the first 5 hours of pre-incubation, with a slight further increase at 20 hours. E22Q oligomers were formed more rapidly, reaching their maximum level after just 20 minutes of pre-incubation. Slight decreases were measured up to 20 hours, indicating that further changes such as precipitation may have occluded the epitopes. A β 1-42 oligomers were at high levels after short periods of incubation, rising rapidly in the first 20 minutes, followed by a further slight increases up to 5 hours, than a decline after 20 hours incubation, indicating that some epitopes in the aggregates were also occluded, perhaps by precipitation. Low absorbance values were achieved with A β 1-42 added to control rabbit IgG coated wells.

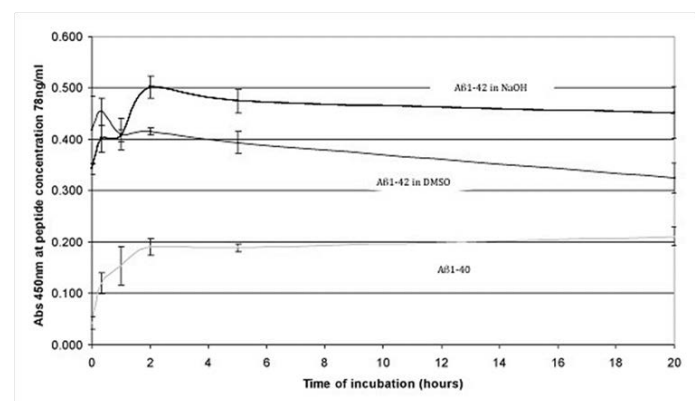
It was interesting to see that the ELISA responses from solutions of A β 1-42 and E22Q A β decrease after 5 hours pre-incubation, possible caused by precipitation

of aggregates. This precipitation of the peptide probably impedes the binding of biotinylated NTA4 antibody as the epitopes are occluded, decreasing the response of the assay.

The data suggests that the rates of oligomerisation between the peptides differ, with A β 1-42 and E22Q A β forming oligomers at the fastest rate, and A β 1-40 the slowest. The high absorbance of A β 1-42 solutions at the beginning of incubation, after 3 mins, suggest that oligomers are formed immediately when A β 1-42 is diluted into blocking buffer.

It was decided to compare A β 1-42 oligomer formation in solutions prepared by dilution into Tris from NaOH with A β 1-42 oligomers prepared in the disaggregating solvent DMSO. It was thought that if any oligomers were present in A β 1-42 before it was made soluble, DMSO would convert them into monomers. Fresh samples of A β 1-40 and A β 1-42 were prepared as described in the methods. A β 1-42 was dissolved in DMSO (100 v/w) and sterilised water was added followed by addition of a 10% volume of sterilised 1M Tris-HCl (pH 7.4) to obtain the required concentration. Solutions were aged, and samples taken over a 20-hour period, and subjected to the ELISA after dilution into blocking buffer. A β 1-40 was used as a control.

Figure 3: Wells of a plate were coated with non-biotinylated anti-NTA4 antibody and left overnight at 4°C. 0.5mg/ml samples of A β 1-40 and A β 1-42 were prepared in 0.1M Tris (pH 7.4) as described in the methods. A β 1-42 was also prepared in 0.1M NaOH or DMSO (50% w/v) and sterilised distilled water (50%w/v) followed by addition of 10% volume of sterilised 1M Tris-HCl (pH 7.4) to give a concentration of A β 1-42 of 0.5mg/ml. The peptides were incubated at 37°C. At time points of 0 minutes, 20 minutes, 1, 2, 5, and 20 hours samples of the peptide solutions were taken and diluted 1 to 800 in 1xPBS/1% BSA/0.05% Tween buffer... The remainder of the ELISA was performed as described in the methods. The mean of triplicate measurements and the standard deviation for each time point is shown.

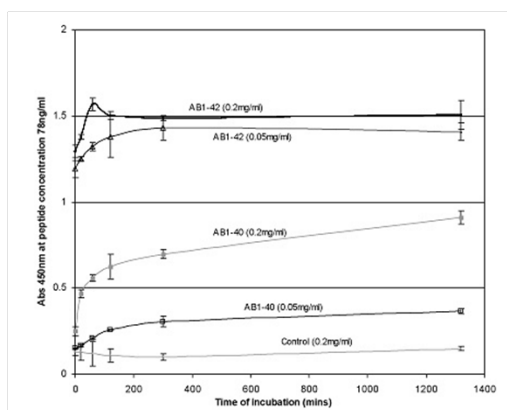


The high absorbance of fresh Aβ1-42 was not affected by predissolving it in DMSO (figure 3). The absorbance value of Aβ1-42 when initially dissolved in DMSO and diluted to 0.1M Tris was as high as when initially dissolved in NaOH. It likely that oligomers are formed from DMSO when it is diluted in the PBS/1%BSA/0.05% Tween, a solution which is compatible with the ELISA test. Aβ1-40 showed a similar response to the previous experiment, reaching a maximum concentration of oligomers after about 4 hours incubation, increasing slightly to 20 hours. The results here suggest that Aβ1-42 forms oligomers at a much faster rate than Aβ1-40, occurring immediately when it is put into solution in 0.1M Tris (pH7.4).

Comparison of Aβ1-40 and Aβ1-42 at Different Concentrations

It was decided to compare the oligomerisation rates of Aβ1-40 and Aβ1-42 at different concentrations over a 20-hour time period. Fresh solutions of the peptides were prepared at concentrations of 0.2 and 0.05mg/ml, and then aged at 37°C. A peptide, the N-terminal 10 residues of Aβ, pre-incubated under the same conditions was used as a control.

Figure 4: Solutions of Aβ1-40 and Aβ1-42 at 0.2mg/ml or 0.05mg/ml, prepared as in methods, were incubated at 37°C. The N-terminal 10 residues of Aβ were incubated under the same conditions and used as a control. At time points of 0 minutes, 20 minutes, 1, 2, 5, and 20 hours a sample of the peptide solutions was taken and diluted 1 to 800 in 1xPBS/1% BSA/0.05% Tween buffer. Samples were incubated on an ELISA plate already coated with immobilised anti-NTA4 antibody. The remainder of the assay was performed as described in the methods. The mean of triplicate measurements and the standard deviation for each time point is shown.



Solutions of Aβ1-40 at 0.05mg/ml showed a gradual increase in binding biotinylated antibody as

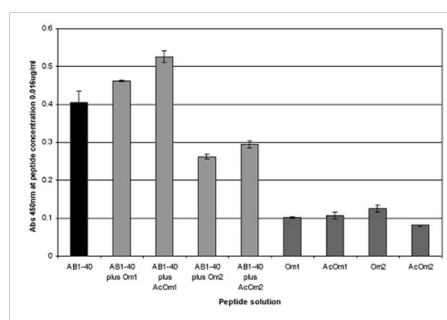
the time of pre-incubation was increased (figure 4). The same is true at a concentration of 0.2mg/ml. In contrast to Aβ1-40, Aβ1-42 gave high absorbance values initially, which increased by only 15% as the pre-incubation time was increased. Solutions incubated at 0.2mg/ml gave only slightly more oligomers than solutions incubated at 0.05mg/ml. A control peptide, NTA4 containing the N-terminal ten residues of Aβ, which binds the anti-NTA4 antibody, but does not oligomerise, did not increase in absorption in the ELISA upon incubation.

It seems that peptides at a higher concentration have an increased rate of oligomerisation and produce high levels of soluble oligomers. Thus the process of oligomerisation is dependent upon concentration.

Inhibitory Activity of Synthetic Peptides on Aβ Oligomerisation

The aim of these assays was to characterise oligomer formation by including peptides previously shown to inhibit cellular toxicity of both Aβ1-40 and Aβ1-42 [11]. Fresh peptide mixtures of Aβ1-40 and peptide inhibitors Om1, AcOm1, Om2, and AcOm2 [11] were prepared. The inhibitor solutions were incubated with Aβ1-40 in an equimolar ratio and then assayed. Aged Aβ1-40 incubated in the absence of inhibitor was used as a control, as were the peptide inhibitors on their own (no Aβ).

Figure 5: Wells of a plate were coated with non-biotinylated anti-NTA4 antibody and left overnight at 4°C. Freshly prepared peptide solutions of Aβ1-40, Om1, AcOm1, Om2, and AcOm2 were prepared in sterile distilled water, to which a 10% volume of sterilised 1M Tris-HCl (pH 7.4) was added to give a peptide concentration of 2mg/ml. Equal volumes of each inhibitor and Aβ1-40 were added to each other so the final concentration of the peptide and inhibitor solution was 1mg/ml. Aged Aβ1-40 and inhibitors on their own (all at 1 mg/ml) were also prepared to act as controls. All solutions were incubated at 37°C for 2 hours. Results are reported after serial dilution to 1µg/ml of Aβ, using both fresh and aged samples, were prepared in 1xPBS/1% BSA/0.05% Tween buffer. The ELISA was performed as described in the methods. The mean of triplicate measurements and the standard deviation for each peptide point are shown.



The results show that compared to the control of A β 1-40 pre-incubated on its own, Om1 and AcOm1 increase the amount of oligomerisation of A β 1-40, rather than inhibit (figure 5). Om2 and AcOm2 in contrast decrease aggregation of A β 1-40. Overall it can be seen that the non-acetylated inhibitors are more effective at reducing the level of oligomerisation than the acetylated forms, and Om2 RGKLVFFGR-NH₂, the amidated form, is more effective than Om1, RGKLVFFGR. Thus the most effective of the synthetic peptides tested here is non-acetylated Om2.

Since Om2 had been found to be the most effective synthetic peptide, it was synthesised in the retro-inverted form, (rGffvlkGr-NH₂), (lower case letters represent D-forms of amino acids), in which the native sequence is inverted, and compared for inhibitory activity on both A β 1-40 and A β 1-42 oligomer formation, and at a greater range of peptide: inhibitor ratios (results are shown in [13]). The retroinverted form, RIOM2, was more resistant to proteolytic degradation than the naturally occurring L-form [13], and may therefore serve as the basis of a preferred drug in vivo rather than the native L-form.

Peptides A β 1-40 and A β 1-42 with inhibitors Om2 and RIOM2 were tested at a range of peptide: inhibitor molar ratios (1:1, 2:1, 4:1, 8:1, and 16:1) with A β peptide concentration constant. A β 1-40 and A β 1-42 solutions were also prepared on their own to act as controls. Each solution was aged for 20 hours after an initial sample had been taken.

It was found that pre-incubated solutions gave a higher absorbance value than fresh for both A peptides (figure 6). Fresh values of A β 1-42 were again much higher than fresh values of A β 1-40. This was consistent with previous experiments. The values of both fresh solutions of peptides were unaffected by the addition of inhibitors, suggesting that the inhibitory effect is not immediate.

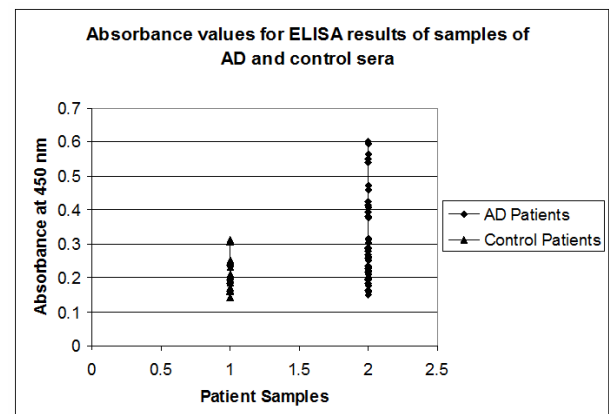
Comparing ELISA response of A β 1-40 pre-incubated with RIOM2 it can be seen that the inhibitor is most effective at peptide: inhibitor molar ratios of 1:1, 2:1 or 4:1 (constant peptide concentration) reducing the ELISA response by 58%, 53%, and 55% respectively. It has little or no effect at ratios of 8:1 and 16:1. When RIOM2 was pre-incubated with A β 1-42 a similar effect is seen at peptide: inhibitor molar ratios of 1:1, 2:1 and 4:1. Om2 inhibits the ELISA response when pre-incubated with both A β 1-40 and A β 1-42 at peptide: inhibitor ratios of either 1:1 (43% with A β 1-40; 50% with A β 1-42) or 2:1 (42% with A β 1-40; 51% with A β 1-42). At peptide: inhibitor ratios greater

than this it does little to inhibit oligomerisation of either A β peptides. Overall RIOM2 inhibits the ELISA response to a greater degree than Om2 especially at low peptide: inhibitor molar ratios.

Elisa Measurement of Oligomers in Sera Samples of Alzheimer's Patients and Controls

Patients sera, or standard preincubated aged A β 42 (1mg/ml) were diluted into blocking buffer and incubated in plates treated with antibody (NTA4), After 24hr incubation at 40C, plates were processed for by the ELISA described in the methods section. A β oligomer concentrations were calculated from measured ELISA ODs using known concentrations of A β 42 as standard. The mean concentrations of oligomers in patients sera was determined as 53.49 + 9.14ng/ml, whereas the mean concentration in age-matched controls was 23.03 + 7.55ng/ml. The significance of the differences between the two groups was calculated as p=0.000652.

Figure 6: Absorbance (450nm) levels from double ELISA performed on sera samples from AD patients and age-matched controls.



The serum levels of β -amyloid oligomers were then plotted as a receiver operating characteristic (ROC curve) (Figure 7) for two groups, patients and controls. The area under the ROC curve was 0.816; this is interpreted that a random patient selected from the AD group would have levels of A β -oligomers higher than a random individual from the control group 81.6% of the time. The 95% confidence level between 0.640 and 0.930 is the interval in which the true area under the ROC curve lies with 95% confidence. The p-value(0.001) is the probability that the sample area 0.816 is found when the actual area is 0.5, providing evidence that the ELISA does have the ability to

distinguish AD patients from controls. The sensitivity of the assay is 79.55%.

Figure 7: ROC curve of true positive rate (sensitivity) as a function of false-positive rate (100-specificity) for different cut-off points for oligomer concentrations in sera.

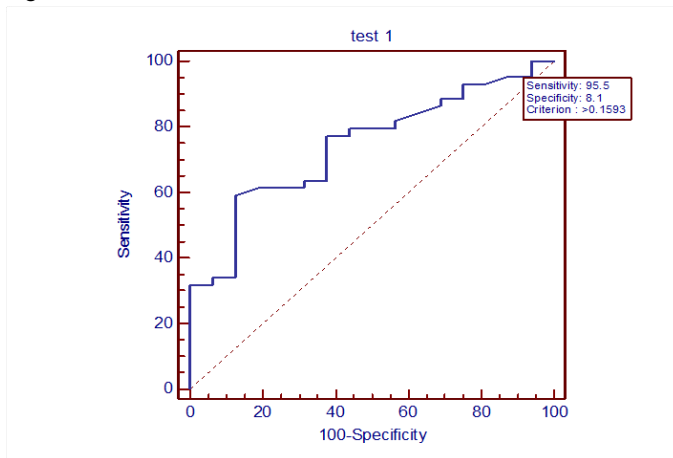
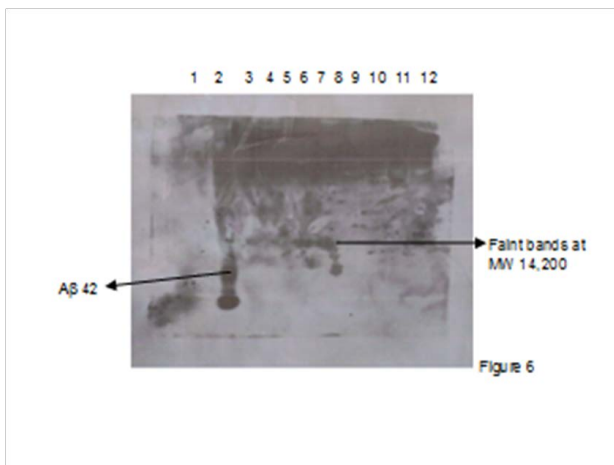


Figure 8: Western blots of sera samples and synthetic peptides using 6E10 anti A β antibodies



Lane 2 A β 42 synthetic peptide

Lanes 3 to 7. Sera of Alzheimer's patients

Lanes 8 to 11 Sera from age-matched controls

Results showed that Alzheimer's sera analysed in lanes 3 to 7 contained a faint band cross reacting with antibody to A β of 14.2kDa, which was not seen in control sera. An A β oligomer of this molecular size would correspond to that of a trimer of A β 42 (MW 13,542). The top of the gel also showed cross reactive material which

could be higher aggregates.

Discussion

It has been established that brain pathological changes occur many years before clinical symptoms of Alzheimer's disease develop. This gives opportunity for intervention to prevent neurodegeneration taking place, using drug therapy or applying behavioural changes, such as increased physical exercise. However, for these to be applied, it will be necessary to simplify and cut the costs of diagnosis so that it may be applied to large populations of at risk individuals. Screening programs such as the ones required have been applied for early detection of other disorders such as faecal testing for bowel cancer. PET imaging agents have been developed that show that build-up of the protein β -amyloid in brain occurs about ten years before cognitive decline shows up in memory tests. However the PET imaging procedure is costly, and makes use of labile, radioactive agents which may be unacceptable to some patients. A β has been measured in CSF, without differentiation of the state or extent of its oligomerisation. Levels appear to be lower in CSF when compared to normal controls or patients with other dementia types [14]. Phosphorylated tau protein I a component of neurofibrillary tangles, seen as intraneuronal deposits in post-mortem sections of AD patients. In CSF, phosphorylated tau is increased in AD patients compared to normal controls. Differences become more pronounced when ratios of phosphorylated tau to A β are calculated. CSF analysis is useful in Alzheimer's disease detection, but is invasive. In contrast, sampling of plasma and serum for biochemical markers is the gold standard for diagnostic screening, and is readily tolerated by most individuals. Most other studies have revealed that levels of total A β , which would include monomeric forms as well as soluble, oligomeric forms in plasma are similar in AD patients than in age-matched controls [5]. Thus increases in A β oligomers are a better indicator of Alzheimer's Disease than monomeric levels, which may indicate that in brain these oligomeric species may have toxic actions against synaptic connections and neuronal integrity which will if left untreated lead to less of cognition. The ELISA assay used does not recognise monomeric A β , or insoluble A β fibrils. Further work is required to see if oligomeric species are elevated at prodromal or MCI stages of the disease, and could therefore be added to a battery of tests aimed at defining currently normal individuals who will go on to develop AD with time.

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