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IHC charaterization of mAbs against the N-terminus of Aβ

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Submitted

Abstract

Background

The β amyloid (Aβ) peptide is a key molecule in the pathogenesis of Alzheimer's disease (AD). A reliable method to detect Aβ deposits and associated changes in brain tissue is of great importance for further understanding the disease mechanisms and for diagnostic purposes.

Material and Methods

Two new monoclonal antibodies (mAbs) directed against the amyloid peptide (Aβ), VU-α-Aβ1-17 and IC16, were raised against the N-terminus of Aβ. Specific antigen binding was determined by ELISA. The immunohistochemical properties of VU-α-Aβ1-17, IC16 and two other antibodies (6E10 and 3D6) were evaluated in brain specimens of AD patients and compared with frequently used diagnostic immunohistochemical procedures, with mAbs 4G8 and 6F/3D.

Results

Epitope mapping by competitive ELISA experiments revealed that VU-α-Aβ1-17 and IC16 recognize the N-terminal amino acid sequence 1 to 6 of Aβ. We found that these two mAbs and also 3D6 can be used for immunohistochemical (IHC) detection of Aβ on paraffin embedded tissue without formic acid (FA) pre-treatment. VU-α-Aβ1-17, IC16 and 3D6 are equal (using Tris/EDTA or citrate pre-treatment) and sometimes superior in the detection of Aβ deposits compared to mAbs used for standard diagnostics (=4G8 and 6F/3D).

Conclusion

The N-terminus specific antibodies, VU-α-Aβ1-17 and IC16, are superior to other antibodies in IHC detection of Aβ on paraffin embedded tissue without FA pre-treatment. The omission of FA pre-treatment allows double-/triple-immunohistochemistry for Aβ and antigenic determinants that otherwise would have been are destroyed upon FA pre-treatment.

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease causing progressive impairment of memory and other cognitive functions. Neuropathologically, AD is characterized by neurofibrillary tangles, and extracellular aggregates of β amyloid (Aβ) protein. Assessment of Aβ in brain tissue is essential for neuropathological diagnosis and is crucial for investigating the molecular mechanisms underlying the pathological changes observed in Alzheimer's disease brain. The neuropathological diagnosis of AD is partly based on the detection of Aβ deposits (as diffuse or cored plaques, or in the blood vessels in the cortical areas of the brain). For this reason, the assessment of changes associated with different types of Aβ deposits, in the brain tissue, has become an important facet of diagnostic practice and studies investigating the role of Aβ in AD pathogenesis. Detailed instructions of the Consortium to Establish a Registry for AD (CERAD) [54] and the Braak and Braak staging of AD-related [27, 28, 55] changes are recommended by the National Institute of Aging and Reagan (NIA-Reagan) Institute Working Group [56, 57] to estimate the AD pathologic changes. These recommendations include also specific immunohistochemical (IHC) stainings which make the diagnosis of AD more accurate and reliable [57]. However, besides that no consensus exists on which antibodies to use for the detection of Aβ, IHC stainings for Aβ on paraffin embedded tissue mostly rely on pre-treatment with formic acid (FA) [104-106]. This pre-treatment is a relatively harsh chemical method which results in formylation of proteins and structural modifications of the Aβ peptide and Aβ associated factors. The FA-induced conformational changes of the peptide, of the Aβ plaques and also of the Aβ associated factors [107-114], make the interpretation of double immunostainings of the Aβ plaque difficult [115-117].

To investigate the co-localization of different Aβ forms and Aβ associated factors in various stages of plaque development, it would, therefore, be advantageous to omit FA pre-treatment during IHC stanings [115-117]. In the present study, we describe the biochemical properties of two newly developed monoclonal antibodies (mAbs) VU-α-Aβ1-17 and IC16, raised against the N-terminus of the Aβ peptide. Next we investigated if VU-α-Aβ1-17 and IC16 could be used to detect Aβ by IHC and determined the optimal conditions (pre-treatment of sample, buffers, pH, concentrations of the antibodies). Pre-treatments compared, included FA pre-treatment and citrate or Tris/EDTA combined with autoclave heating. To characterize and to validate these mAbs for IHC, we stained post mortem brain slices of selected AD cases representing the different types of Aβ deposits- diffuse and/or compacted or classical plaques and/or vascular Aβ. For comparison two other mAbs, that are commonly used for AD neuropathological research, 6E10 and 3D6 [88], were used in the same procedures. Since stainings with 4G8 [88] and 6F/3D [118] (with FA pre-treatment) are internationally most frequently used for AD diagnosis [104, 105, 119], we compared these stainings (performed with 4G8 and 6F/3D with FA pre-treatment), with stainings performed with the four different antibodies (mentioned here above: VU-α-Aβ1-17, IC16, 6E10 and 3D6) on serial brain slices (performed with four different pre-treatments) from five different AD cases. We show that VU-α-Aβ1-17, IC16 and 3D6 mAbs directed against the N-terminus detect all described forms of Aβ deposits without FA pre-treatment and can be applied for double-immunohistochemical staining.

Material and Methods

Post mortem brain tissue

Human brain specimens of 5 Alzheimer's Disease cases (see legend supplemental data) were obtained at autopsy with a short post-mortem interval (The Netherlands Brain Bank, Amsterdam, The Netherlands; all patients were males; 73, 86, 83, 82 and 81 years old). Clinical diagnosis was defined according to DSM-III-R criteria and the severity of dementia was evaluated according to the Global Deterioration Scale of Reisberg (GDS) [94]. Neuropathological evaluation was performed on formalin fixed, paraffin embedded tissue from different sites, including the frontal cortex (F2), temporal pole cortex, parietal cortex (superior and inferior lobule), occipital pole cortex and the hippocampus (essentially CA1 and entorhinal area of the parahippocampal gyrus). The distribution and the density of neurofibrillary tangles was determined in Bodian stained sections, while the distribution of senile plaques was determined in sections stained with the methenamine silver method [95]. Staging of AD was evaluated according to Braak and Braak [27, 28, 55]. For the present study we used tissue from the temporal and occipital cortex of cases with Braak stage 6C, 5C, 3C, 5C, and 4C, to study IHC staining of Aβ deposits using several different antibodies combined with alternate methods of tissue pre-treatment.

Peptides

Cys-Aβ1-17, Cys-Aβ33-40, Aβ1-10, Aβ6-15, Aβ11-20 were synthesized (dr Hilkmann; NKI-AVL, Amsterdam, The Netherlands) by Fmoc-chemistry and found to be >95% pure (Aβ1-17 85%) as judged from HPLC reversed phase analysis. Aβ1-42, Aβ1-40, and Aβ1-16 peptides were obtained from Bachem, Bubendorf (Switzerland) and Biosource, Nivelles (Belgium), and Eurogentech (Belgium), respectively.

Monoclonal antibodies

MAbs 6F/3D [118] (directed against epitope 8-17; DAKO, Cytomation, Glostrup, Denmark), 4G8 (directed against epitope 10-24; Signet, Signet, Dedham, MA, USA) and 6E10 (directed against epitope 3-8; Signet, Signet, Dedham, MA, USA) [88] were obtained as indicated. 3D6 was gently provided by Dr Schenk and Dr Seubert (directed against epitope 1-5; Elan/Wyeth Pharmaceuticals, South San Francisco, California) [88]. See supplemental data for concentrations of the mAbs and dilutions for IHC.

Production and screening of monoclonal antibody VU-α-Aβ1-17

VU-α-Aβ1-17 was generated at the clinical chemistry department of the VU University medical centre as follows: The synthetic peptide corresponding to Aβ1-17 was conjugated with bovine serum albumin (BSA) through a cysteine residue added at the C-terminus of the peptide. Mice were subcutaneously (s.c.) immunized with 100µg of the conjugate in complete Freund's adjuvant (first immunization) and another three times with 25 µg antigen without adjuvant with 3 weeks intervals. Three days after the final boost and after testing the sera of the mice for immunity against Aβ1-17 in ELISA, spleen cells and lymph nodes were removed and used for fusion with mouse myeloma SP2/0 cells using polyethylene glycol 4200, 42% (Merck). Fused cells were cultured in Iscove's Modified Dulbecco's Medium (IMEM; Biowhittaker Europe, Verviers, Belgium) selection medium supplemented with

azaserine 5%, heat-inactivated fetal bovine serum (Invitrogen), hypoxanthine, 0.5ng/ml of human interleukin-6 and maintained in an atmosphere of 5% CO2/95% air in a humified incubation at 37°C. Hybridoma supernatants containing antibodies were first screened by solid phase ELISA, and positive cells that secreted antibodies of interest were subcloned by limiting dilution. One clone designated VU-α-Aβ1-17 was selected as being Aβ1-17 N-terminal specific mAb. For large-scale antibody production, hybridomas were cultured in tissue culture roller bottles (Falcon, Becton Dickinson) containing 1L growth medium (IMEM; Biowhittaker Europe, Verviers, Belgium) containing 10% heat-inactivated fetal bovine serum and 0.5ng/ml human interleukin-6. After 4 weeks, supernatants were harvested and concentrated in capillary flow dialysers (Fresensius). Thereafter, monoclonal antibodies were purified from conditioned medium by protein A-sepharose affinity chromatography (Amersham Pharmacia Biotech) according to the instructions of the manufacturer followed by dialysis against PBS, pH 7.4. Wells of a flat-bottomed microtiter plate (Costar 9018, Corning Incorporated NY USA) were coated overnight at room temperature (RT) with 100µl 80 ng/well of Aβ1-17, Aβ1-40, Aβ1-42, and Aβ33-40 in coating buffer (125mM $Na_2CO_3.10H_2O$, 125mM $NaHCO_3$, pH 9.6). The microtiter plates were washed twice with PBT (10 mM phosphate buffered saline, pH 7.2 and 0.02% tween20) and blocked with 200µl 2% low fat milk in PBS (10mM phosphate buffered saline, pH 7.2) for 1 hour at RT. After five washes with PBT, the supernatants (90 µL/well) of the hybridoma supernatants were incubated in different dilutions (1:10, 1:100, 1:1000, 1:10.000, 1:100.000 in 10 mM phosphate buffered saline, pH 7.2 and 0.1% tween20) for 1 h at RT and 500 rpm. Following five washes with PBT the plate was incubated with polyclonal goat anti mouse immunoglobulins conjugated with horseradish peroxidase (DakoCytomation, Glostrup, Denmark) for 1 hour at RT and 500 rpm, and after washing the plate was developed with TMB and the reaction stopped with 1M sulphuric acid. Absorption was determined in a Microplate Spectra Rainbow reader (SLT labinstruments, Groding/Salzburg, Austria) and corrected for background (OD 540)

Production and screening of monoclonal antibody IC16

IC16 was generated at the neuropathology department of the Heinrich University Dusseldorf Medical School as follows: As immunogen, synthetic human Aβ1-16 was C-terminally crosslinked to key limpet hemocyanin by Eurogentech (Belgium). The immunogen (100µg) was suspended in 100µL PBS and mixed with an equal amount of RIBI adjuvant (Sigma, Germany) for immunizations. Immunizations of mice were performed at days 0, 21, 42 and 56 s.c. At day 61 and 62, 100 µg immunogen was injected intraperitoneally without adjuvant. Fusion was performed according to protocol with P3X63Ag8U.1 (ATCC 1597) myeloma cells [120]. IC16 was subcloned three times by limited dilutions and finally set to grow in serum free PFHM Medium (Invitrogen, USA), by gradually reducing the FCS content in weekly intervals from 10% to 5% to 2 % to 0%. IC16 was then grown in serum free medium in a bioreactor (Invitrogen, USA) yielding antibody concentrations between 1-2mg/mL. A first screen of the hybridoma supernatants was performed by ELISA, in which one mg HSA was C-terminally crosslinked to Aβ1-16 (Eurogentech, Belgium) and one µg HSA-Aβ1-16 was coated to Maxisorp ELISA plates (Nunc, Denmark) in sodium carbonate buffer (pH 10). Plates were blocked with 5% BSA and the hybridoma supernatants incubated for 2 hours at RT with 5% BSA plates as negative controls. In a second screen, the positives from the first

screen were tested for immunoreactivity against Aβ on a modified dot blot procedure [121]. Briefly, lyophilized Aβ1-40 was taken up in sterile water pH 5.5 and at a concentration of 200 ng/well dot-blotted on 0.45mM nitrocellulose using an ELIFA (Enzyme-Linked Immuno-Flow-Assay) apparatus (Pierce, USA). In the buffer, Aβ should be in monomeric, oligomeric and fibrillar conformations. After blocking with 5% milk, clones were incubated with the doted spots on the membrane within the ELIFA apparatus for 2 hours at RT. Finally, a secondary antibody against mouse IgG, crosslinked to horseradish peroxidase (Pierce, USA) was incubated with the NC membrane at 1:50.000 for 1 h at RT, and the Western blot was developed with ECL system (Invitrogen, USA). IC16 was found to react well with Aβ in both, the ELISA and the ELIFA assay.

Epitope mapping of VU-α-Aβ1-17 and IC16: ELISA

Wells of a microtiter plate (Costar 9018, Corning Incorporated NY USA) were coated overnight at room temperature (RT) with 100ng/well Aβ1-40 (BioSource, Nivelles, Belgium) in coatingbuffer (125mM $Na_2CO_3.10H_2O$, 125mM $NaHCO_3$, pH 9.6). The microtiter plate was washed once with phosphate buffered saline (PBS, 10mM, pH 7.2) and blocked with 2% low fat milk in PBS for 1 hour at RT. VU-α-Aβ1-17 and IC16 were diluted in assaybuffer (20mM Tris-HCl, pH 7.5, with 0,05% Tween20). Aβ peptides (aminoacids: 1-10, 6-15, 11-20, or 1-17) were added at 250, 25 and 2.5μg/mL end concentrations to VU-α-Aβ1-17 (final concentration 1.7ng/mL) or IC16 (100 ng/mL) and incubated for 5 minutes at RT. In one sample the peptides were omitted to determine the degree of binding to coated Aβ1-40, set as 100 %. After washing the microtiter plate (tree times) with assaybuffer, the samples were added to the wells and incubated (100μL/well) for 1 hour at RT and 500 rpm. Following three washes the plate was incubated with polyclonal goat anti mouse immunoglobulins conjugated with horseradish peroxidase (DakoCytomation, Glostrup, Denmark) for 1 hour at RT and 500 rpm, and after washing the plate was developed with TMB and the reaction stopped with 1M sulphuric acid.

Determination of the Isotype of VU-α-Aβ1-17 and IC16

To determine the (sub)class of mAb, a test strip (Hycult biotechnology b.v.) was used. VU-α-Aβ1-17 and IC16 were both identified as IgG2a subclass.

Immunohistochemistry

Paraffin sections (5μm) of 4% paraformaldehyde fixed (over night) brain specimens were mounted on Superfrost plus tissue slides (Menzel-Gläser, Germany) and dried over night at 37℃. For all immunostainings, sections were deparaffinized and subsequently immersed in 0.3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity. Subsequently sections were used directly or pre-treated with either FA, 10mM pH 6.0 sodium citrate buffer, or 10mM Tris/1mM EDTA buffer pH 9.0 buffer. FA treatment was for 15 minutes. For sodium citrate and Tris/EDTA buffer pre-treatment, sections were heated by autoclave during 10 minutes. Primary antibodies were dissolved in phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA, Boehringer Mannheim, Germany) and incubated over night at 4℃. Antibodies and dilutions used in this study are shown in table 1 and in the supplemental data. After washing with PBS, sections were incubated with EnVision-HRP (anti-Rabbit/Mouse) solution (undiluted, DAKO) for 30 minutes. Colour was developed with

3,3'-diaminobenzidine (EnVision Detection system/HRP, 1:50 dilution, DakoCytomation, Glostrup, Denmark, 10 minutes) as chromogen. Sections were counterstained with haematoxylin and mounted with DePeX (BDH Laboratories Supplies). VU-α-Aβ1-17 antibody was applied for double-immunohistochemistry with AT8 antibody which detects Tau phosphorylated at serine 202 (Innogenetics, Ghent, Belgium). Sections were deparaffinized and endogenous peroxidase activity was quenched. Subsequently sections were pre-treated with 10 mM pH 6.0 sodium citrate combined with autoclave heating for 10 min. After blocking with normal goat serum (1:10 dilution, DAKO) for 10 min sections were simultaneously incubated with VU-α-Aβ1-17 (1:800, IgG2a) and AT8 (1:50, IgG1) for 60 min. After washing with PBS sections were incubated with goat-anti-mouse-IgG1-HRP (dilution 1:100, Southern Biotech Associates) and goat anti-mouse IgG2a-AP (dilution 1:100, Southern Biotech Associates). Colour was developed using 3,3'-diaminobenzidine (DAB, DAKO) and Liquid Permanent Red (LPR, DAKO) as chromogens. Sections were counterstained with haematoxylin and mounted using aquamount (BDH Laboratories Supplies).

Scoring of Stainings

Since stainings with 4G8 and 6F/3D (with FA pre-treatment) are internationally most frequently used for diagnosis and research [104, 105, 119], we compared these stainings with the four different antibodies (VU-α-Aβ1-17, IC16, 6E10 and 3D6) on serial brain slices (four different pre-treatments) from five patients (see supplemental data). Brain slices immunostained with either antibodies 4G8, 6F/3D, VU-α-Aβ1-17, IC16, 6E10 and 3D6 were screened for six immunohistochemical characteristics: diffuse, compact or classic plaques (pl) [53], arteries (art) with cerebral Aβ angiopathy (CAA) [122, 123] or dyshoric angiopathy (DA) [124-126] or capillaries (cap) with DA [124-126] (see figure 3) with an 125x magnification. These six characteristics were scored (by the same persons; MMR and ESvH) and were graded as 1 (zero or one hit), 2 (two to ten hits), and 3 (more than ten hits). One representative area was assessed for each case at 125X magnification. Different pre-treatments and stainings were assessed in the same selected area.

Results

ELISA

To determine the epitopes recognized by antibodies VU-α-Aβ1-17 and IC16, ELISA wells were coated with Aβ1-40 and incubated with either VU-α-Aβ1-17 or IC16. In a dilution range the concentration resulting in ½ B max was determined. Next, in an inhibition experiment, the same concentration of either VU-α-Aβ1-17 or IC16 was pre-incubated with a 10-fold dilution range of small synthetic peptides corresponding to the Aβ-sequence 1-10, 6-15, 11-20, 1-17 (before detection of Aβ1-40). Pre-incubation with, Aβ1-10 (and 1-17), but not with 6-15 and 11-20, dose dependently inhibited the binding of VU-α-Aβ1-17 and IC16 to Aβ1-40, which indicates that the epitope of VU-α-Aβ1-17 and IC16 lies within the first 6 amino-acids of the Aβ peptide (see Figure 1 and 2).

Immunohistochemistry

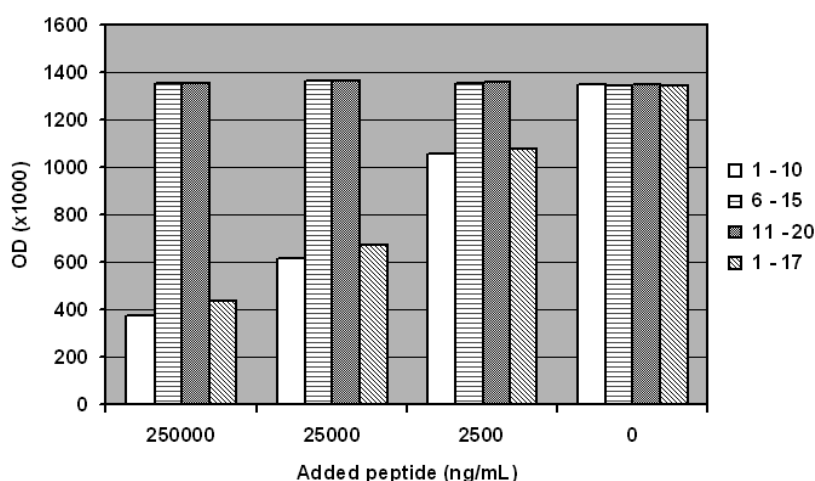
Serial paraffin sections of 5 AD cases with different Braak stage, but in which a large array

of Aβ deposits can be detected, were subjected to 4 different pre-treatment protocols before the specificity of the antibodies was tested. Staining characteristics, grouped as staining intensity of either art +CAA, art + DA, cap + CAA; diff pl, compact pl and classical pl, obtained with VU-α-Aβ1-17, IC16, 3D6 and 6E10 were compared to the results obtained with 6F/3D and 4G8 with FA pre-treatment (see supplemental data). Table 1 summarizes these results of the stainings performed with VU-α-Aβ1-17, IC16, 3D6, and 6E10 (with four different pre-treatments) in comparison to those of 6F/3D and 4G8 stainings performed with FA pre-treatment. VU-α-Aβ1-17 antibody performs well (low background) when brain slices are pre-treated with either FA or heating in citrate or Tris/EDTA. This antibody does not stain properly if no pre-treatment is performed. Different pre-treatments required different antibody concentrations for optimal detection of Aβ deposits. The optimal conditions for citrate pre-treatment are reached when this antibody (3.4mg/ml) is diluted 1:1600, for Tris/EDTA 1:3200 and for FA pre-treatment 1:12800. The six screened characteristics stained with VU-α-Aβ1-17 (pre-treated with citrate or Tris/EDTA or FA buffer), are at least as good as the stainings performed with 6F/3D and 4G8 performed with FA pre-treatment. Only diffuse plaques are slightly better observed with 6F/3D and 4G8. IC16 shows equal stainings when brain slices are not pre-treated and when these are pre-treated with citrate buffer or Tris/EDTA or FA. The optimal conditions are reached when this antibody (concentration between 1.0 and 2.0mg/ml) is diluted 1:800 if no pre-treatment is used, 1:1600 for citrate buffer, 1:50.000 for Tris/EDTA buffer and for FA 1:25.000. The six screened characteristics detected with IC16 (Tris/EDTA pre-treatment), are better observed than after detection with 6F/3D and 4G8. This is also the case for citrate and FA pre-treatment, when these immunostainings are only compared with 6F/3D performed with FA pre-treatment. When compared to 4G8 stainings performed with FA pre-treatment, IC16 shows comparable results concerning diffuse plaques. However classical plaques are better detected with 4G8 performed with FA pre-treatment. 3D6 shows an acceptable pattern when brain slices are pre-treated with citrate buffer, Tris/EDTA or FA. This antibody does not stain properly if no pre-treatment is performed. The optimal conditions are reached when this antibody, for citrate or Tris/EDTA pre-treatment, is diluted 1:400 and for FA 1:800. The six screened characteristics stained with 3D6 (pre-treated with citrate or Tris/EDTA or FA buffer), are at least as good as the stainings performed with 6F/3D. Only diffuse plaques are slightly better seen with 6F/3D. Compared to 4G8, 3D6 in general shows the same staining characteristics, except the classical plaques and diffuse plaques. Compared to the other stainings (VU-α-Aβ1-17, IC16 and 3D6), stainings with 6E10 show moderate staining results. Optimal dilutions can be found in the supplemental data. Compared to 6F/3D and 4G8, diffuse plaques, classical and compact plaques are less well detected by 6E10, irrespective the different pre-treatment protocols (see Table 1). In Figure 4, we see serial midtemporal cortex paraffin sections of 2 cases immunostained with four different mAbs and four different pre-treatments. A comparable staining pattern, showing all Aβ plaque types, was observed when serial sections of case 1 were immunostained with IC16 and VU- α-Aβ1-17 after FA as well as citrate and EDTA pre-treatment. As expected, upon immunostainings of serial sections of case 2 with 4G8 and 6F/3D, clear immunostaining of all plaque types is observed after FA pre-treatment. No immunostaining was observed with 6F/3D when no or other pre-treatments than FA were used. Although with 4G8 (case 2) a major part (especially larger sized fibrillar plaques) of the plaques was also detected after

pre-treatment with Tris/EDTA or citrate, also neuronal staining was observed. Because VU-α-Aβ1-17 and IC16 can be applied without FA pre-treatment these antibody are suitable for the detection of Aβ together with other antigens that are normally destroyed upon FA pre-treatment. In figure 5, we show an example of the application of VU-α-Aβ1-17 antibody to detect Aβ together with phosphorylated Tau using the AT8 antibody by double-immunohistochemistry. In summary, VU-α-Aβ1-17 (citrate pre-treatment) and IC16 (Tris/EDTA pre-treatment or citrate) show comparable results when compared with 6F/3D and 4G8 (both with FA acid pre-treatment). Except for diffuse plaques, 3D6 (citrate or Tris/EDTA pre-treatment) shows also results that can be compared with 6F/3D and 4G8 (both with FA pre-treatment). 6E10 shows moderate results in comparison to the other antibodies.

Figure 1

Detection of Aß40 with VU-a-Aß1-17
after incubation with different Aß-peptides



To determine the epitopes recognized by VU-α-Aβ1-17, ELISA wells were coated with Aβ1-40 and incubated for detection with VU-α-Aβ1-17.In an inhibition experiment, VU-α-Aβ1-17 was pre-incubated with a 10-fold dilution range of small synthetic peptides corresponding to the Aβ-sequence 1-10, 6-15, 11-20, 1-17 (before detection of Aβ1-40). Pre-incubation with, Aβ1-10 (and 1-17), but not with 6-15 and 11-20, dose dependently inhibited the binding of VU-α-Aβ1-17 to Aβ1-40, which indicates that the epitope of VU-α-Aβ1-17 lies within the first 6 amino-acids of the Aβ peptide.

Discussion

In this study immunostainings were performed with the use of two novel mAbs, VU-α-Aβ1-17 and IC16, and two well characterzed mAbs, 6E10 and 3D6. These immunostaining were compared with immunostaining performed on AD brain with 6F/3D and 4G8 (with FA pre-treatment), which are mAbs commonly used in AD standard diagnostics [104, 105, 119]. This comparison showed that VU-α-Aβ1-17 and IC16 can be applied for IHC detection of Aβ on paraffin embedded tissue without FA pre-treatment. The omission of FA pre-treatment gives

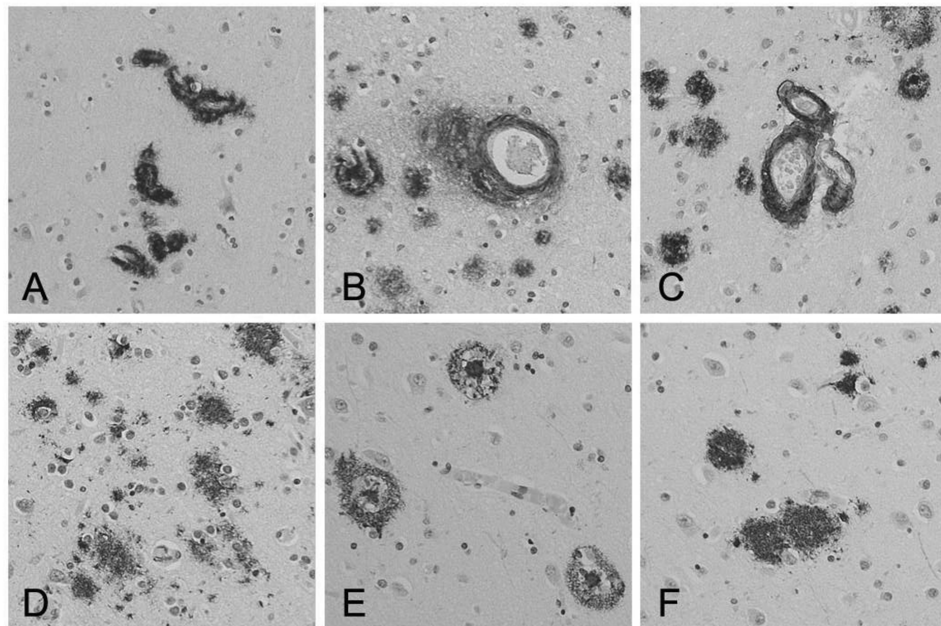


Figure 3

Overview of different subtypes of Aβ deposits. Shown examples of different Aβ deposits were taken from different AD cases immunostained with VU-α-Aβ1-17. Capillaries (A) and arteries (B) with dyshoric angiopathy are characterzed by the infiltration of fine radiating deposits of Aβ into the neuropil. Cerebral Aβ angiopathy (C) is a type of beta-amyloidosis in leptomeningeal and cortical vessels. Diffuse plaques (D) lack a central core, are negative positive for Congo red and degenerative neurites, and can be subdivided in irregular-shaped diffuse plaques, intermediate-sized, circumscribed and in small, stellate plaques. Classic plaques (E) have a dense Aβ core and are Congo red positive core which is compromized of fibrillar Aβ. Most of compact plaques (F) are round-shaped, lack a central core and show variable Congo red positivity.

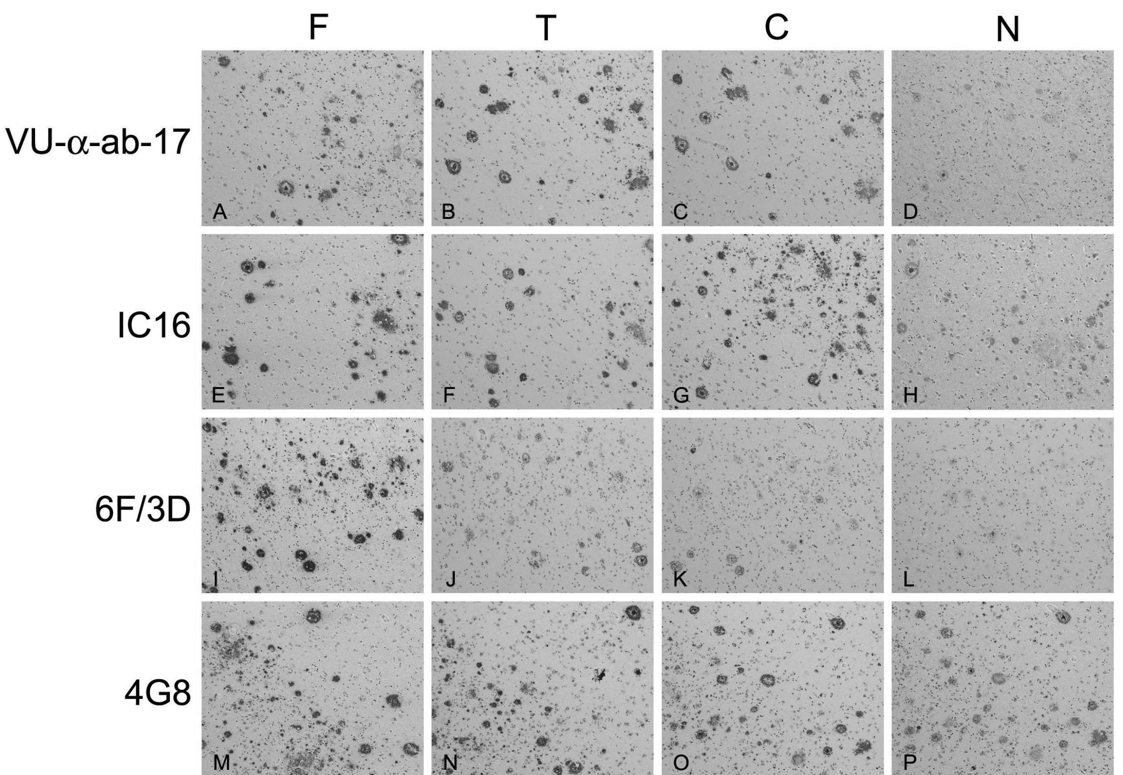
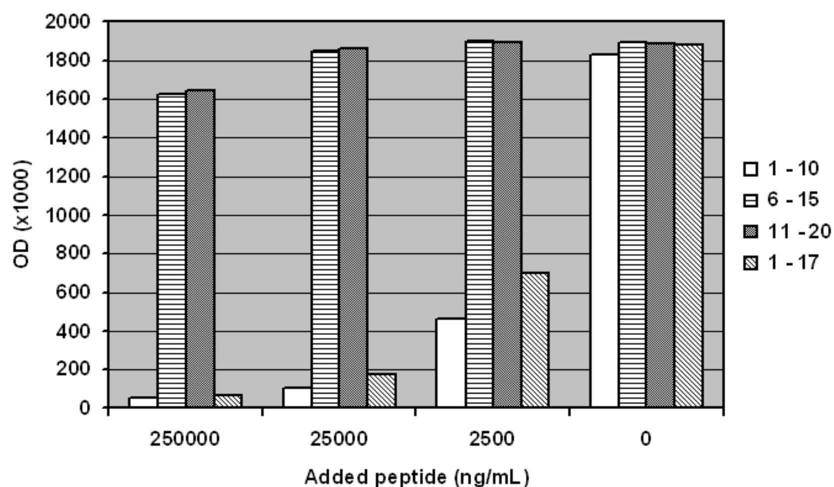


Figure 4

Overview of immunohistochemistry using different Aβ antibodies after different pre-treatments. Tissue derived from two cases which were applied for immunohistochemistry with different antibodies directed against Aβ. Different pre-treatment conditions were evaluated for each antibody. Sequential sections are shown for each antibody used with alternate pre-treatment methods (N no pre-treatment, T Tris/EDTA, C citrate, F formic acid). 6F/3D is diluted 1:25 (tris/EDTA pre-treatment) and 1:10 (citrate and no pre-treatment); 4G8 is diluted 1:1600 (tris/EDT A pre-treatment) and 1:800 (citrate and no pre-treatment). See table 1 for the other dilution factors and amount of concentration of the antibodies.

an important advantage, since double-/triple IHC performed with FA pre-treatment is difficult to interpret. FA pre-treatment induces changes in several peptides in the Aβ plaque [115-117]. These modifications can change the structure and the relation of different peptides of the Aβ plaque, making it difficult to investigate and to interpret the original composition of the Aβ plaque. Less harsh pre-treatment's, like citrate or Tris/EDTA pre-treatment in combination with microwave or autoclave, are preferable.

Figure 2



To determine the epitopes recognized by IC16, ELISA wells were coated with Aβ1-40 and incubated for detection with IC16. In an inhibition experiment, IC16 was pre-incubated with a 10-fold dilution range of small synthetic peptides corresponding to the Aβ-sequence 1-10, 6-15, 11-20, 1-17 (before detection of Aβ1-40). Pre-incubation with, Aβ1-10 (and 1-17), but not with 6-15 and 11-20, dose dependently inhibited the binding of IC16 to Aβ1-40, which indicates that the epitope of IC16 lies within the first 6 amino-acids of the Aβ peptide.

The target epitopes of 6F/3D and 4G8 are probably exposed upon FA treatment. 6F/3D and 4G8 recognize Aβ regions 8-17 and 17-24 [88, 118], respectively, which in Aβ plaques are hidden due to binding of Aβ associated factors [107-114] or due to the aggregation with other Aβ peptides [127]. This is probably not the case with the N-terminal part of the Aβ in the Aβ plaque, which makes it accessible for N-terminal antibodies without FA pre-treatment. A strength of the present study is that 4 different N-terminal antibodies have been used for immunodetection of Aβ, each under four different pre-treatment conditions. All these immunostainings were compared with two frequently used standard diagnostic immunostainings. In summary, two new antibodies VU-α-Aβ1-17 (citrate pre-treatment) and IC16 (Tris/EDTA pre-treatment or citrate) show comparable results when compared with 6F/3D and 4G8 (both FA pre-treatment). Omission of FA pre-treatment of paraffin embedded tissue make these antibodies suitable for investigating co-localization of Aβ with Aβ associated factors in various stages of plaque development and pathology.

Table 1

		Arteries with CAA		Arteries with DA		Capillaries with DA		Diffuse plaques		Classic plaques		Compact plaques	
		6F/3D	4G8	6F/3D	4G8	6F/3D	4G8	6F/3D	4G8	6F/3D	4G8	6F/3D	4G8
VU-α-ab-17	**N**	=	=	=	=	=	=	-	-	-	-	-	-
	T	=	=	=	=	=	=	-	-	=	=	+	+
	C	=	=	+	+	=	=	=	-	+	=	+	+
	F	=	=	+	+	=	=	+	=	=	-	=	+
IC16	**N**	=	=	+	+	=	=	+	+	=	-	-	-
	T	=	=	+	+	+	+	+	=	+	=	=	+
	C	=	=	+	+	+	+	+	=	=	-	=	+
	F	=	=	+	+	=	=	+	=	=	-	=	+
3D6	**N**	-	-	=	=	=	=	+	=	-	-	-	=
	T	=	=	+	+	=	=	+	=	=	-	+	+
	C	=	=	+	+	=	=	+	=	=	-	+	+
	F	=	=	+	+	=	=	+	=	=	-	=	+
6E10	**N**	=	=	+	+	=	=	+	+	-	-	-	-
	T	=	=	=	=	=	=	-	-	=	=	-	-
	C	=	=	+	+	=	=	+	+	-	-	-	-
	F	=	=	=	=	=	=	-	-	=	=	-	-

Summary: Comparison of the different staining characteristics.
Pre-treatment: N=No; T=Tris/EDTA; C=Citrate; F=Formic acid
=, + or - : equal, increased or decreased staining intensity, compared to either 6F/3D or 4G8 (with FA pre-treatment).
Stainings that are at least as good as or better than stainings performed with 6F/3D; 4G8) are marked in grey.

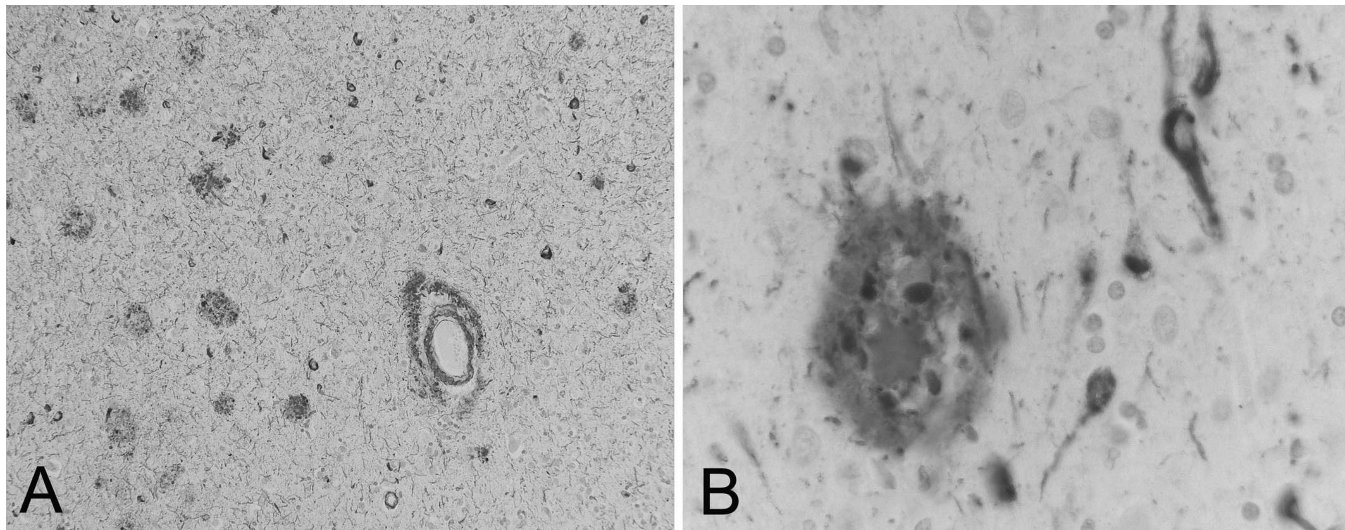


Figure 5

Doubleimmunostaining of VU-α-Aβ1-17 with AT8. VU-α-Aβ1-17 was used together with AT8 for simultaneous detection of Aβ deposits and phosphorylated Tau by doubleimmunohistochemistry (A, higher magnification shown in B). These antibodies were applied with citrate pre-treatment. AT8 was visualized by DAB (brown) and VU-α-Aβ1-17 by LPR (red). Nuclei were counterstained by haematoxylin.

Supplement

			Arteries with CAA					Arteries with DA					Capillaries with DA					Diffuse plaques					Classic plaques					Compact plaques				
	Pretreat.	**Dilution**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**
6F/3D, 0.19 mg/ml																																
Aβ Epitope 8-17	F	1:25	2	2	1	1	1	2	1	1	1	1	1	1	2	1	1	2	1	1	3	3	2	2	2	2	1	2	3	3	2	2
4G8, 1.0 mg/ml																																
Aβ Epitope 17-24	F	1:6400	2	2	1	1	1	1	2	1	1	1	1	1	2	1	1	2	2	1	3	3	2	3	2	2	1	2	2	3	2	2
VU-α-ab-17, 3.4 mg/ml																																
Aβ Epitope 1-5																																
	N	1:1600				1	1				1	1				1	1				1	1				1	1				1	1
	T	1:3200				1	1				1	1				1	1				3	2				2	1				2	3
	C	1:1600	2	2	1			2	2	1			1	1	2			2	1	1			2	3	2			3	3	3		
	F	1:12800	2	2	1			2	2	1			1	1	2			3	1	1			2	2	2			2	3	3		
IC16, 1.0– 2.0 mg/ml																																
Aβ Epitope 1-5	N	1:800	2	2	1			2	2	1			1	1	2			3	3	2			2	2	2			2	2	2		
	T	1:50000	2	2	1			2	2	1			2	1	2			3	1	1			2	2	3			2	3	3		
	C	1:1600	2	2	1			2	2	1			2	1	2			2	2	1			2	2	2			2	3	3		
	F	1:3200	2	2	1			2	2	1			1	1	2			3	1	1			2	2	2			2	3	3		
3D6, 0.7 mg/ml																																
Aβ Epitope 1-5	N	1:400	1	2	1			1	2	1			1	1	2			3	1	1			1	2	2			1	3	3		
	T	1:400	2	2	1			2	2	1			1	1	2			3	1	1			2	2	2			3	3	3		
	C	1:400	2	2	1			2	2	1			1	1	2			3	1	1			2	2	2			3	3	3		
	F	1:800	2	2	1			2	2	1			1	1	2			3	1	1			2	2	2			2	3	3		
6E10, 1.0 mg/ml																																
Aβ Epitope 3-8	N	1:800	2	2	1			2	2	1			1	1	2			3	2	1			1	2	2			1	2	2		
	T	1:6400				1	1				1	1				1	1				2	3				2	1				1	2
	C	1:400	2	2	1			2	2	1			1	1	2			3	3	1			1	1	1			1	1	2		
	F	1:6400				1	1				1	1				1	1				2	3				2	1				1	2

Results of the different stainings. A= male patient; 73 years; Braak 6C; B=male patient; 86 years; Braak 5C; C=male patient; 83 years; Braak 3C; D=male patient; 82 years; Braak 5C; E=male patient; 81 years; Braak 4C. Pre-treatment: N=No; T=Tris/EDTA; C=Citrate; F=Formic acid. Score (10xenlargement): 1=negative or one hit; 2=two to ten hits; 3 => ten hits. CAA=Cerebral Amyloid Angiopathy; DA=Dyshoric angiopathy.