The Second-Generation Active A β Immunotherapy CAD106 Reduces Amyloid Accumulation in APP Transgenic Mice While Minimizing Potential Side Effects

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Immunization against amyloid- β (A β) can reduce amyloid accumulation *in vivo* and is considered a potential therapeutic approach for Alzheimer's disease. However, it has been associated with meningoencephalitis thought to be mediated by inflammatory T-cells. With the aim of producing an immunogenic vaccine without this side effect, we designed CAD106 comprising A β 1–6 coupled to the virus-like particle Q β . Immunization with this vaccine did not activate A β -specific T-cells. In APP transgenic mice, CAD106 induced efficacious A β antibody titers of different IgG subclasses mainly recognizing the A β 3–6 epitope. CAD106 reduced brain amyloid accumulation in two APP transgenic mouse lines. Plaque number was a more sensitive readout than plaque area, followed by A β 42 and A β 40 levels. Studies with very strong overall amyloid reduction showed an increase in vascular A β , which atypically was nonfibrillar. The efficacy of A β immunotherapy depended on the A β levels and thus differed between animal models, brain regions, and stage of amyloid deposition. Therefore, animal studies may not quantitatively predict the effect in human Alzheimer's disease. Our studies provided no evidence for increased microhemorrhages or inflammatory reactions in amyloid-containing brain. In rhesus monkeys, CAD106 induced a similar antibody response as in mice. The antibodies stained amyloid deposits on tissue sections of mouse and human brain but did not label cellular structures containing APP. They reacted with A β monomers and oligomers and blocked A β toxicity in cell culture. We conclude that CAD106 immunization is suited to interfere with A β aggregation and its downstream detrimental effects.

Introduction

Amyloid- β (A β) immunotherapy has emerged as a potential therapy for Alzheimer's disease (AD) (Klaffki et al., 2006; Citron, 2010). Both active immunization against A β and passive transfer of monoclonal A β antibodies were shown to attenuate amyloid plaque formation in the brains of APP transgenic mouse models (Schenk et al., 1999; Bard et al., 2000). This treatment also diminished the amyloid-associated pathology (Lombardo et al., 2003; Oddo et al., 2004; Brendza et al., 2005; Buttini et al., 2005) and could improve learning deficits (Janus et al., 2000; Morgan et al., 2000). However, A β immunization of APP transgenic mice was shown to potentially change vascular amyloid and increase cere-

bral microhemorrhages (Pfeifer et al., 2002; Wilcock et al., 2004, 2007; Racke et al., 2005; Schroeter et al., 2008). Proposed mechanisms of A β immunotherapy include Fc receptor-mediated phagocytosis of amyloid (Bard et al., 2000), direct interference with A β aggregation (Bacskai et al., 2002; McLaurin et al., 2002) by antibodies in the brain, and peripheral sequestration of A β , enhancing its net cerebral efflux (DeMattos et al., 2001). Experimental data are not entirely consistent with these mechanisms (Levites et al., 2006; Winkler et al., 2010).

In humans, evidence for a reduction of the amyloid load by A β immunotherapy was obtained from AD patients immunized with aggregated A β 1–42 and the adjuvant QS21 (AN-1792). Histopathological examination showed neocortical areas devoid of amyloid plaques in antibody responders but not in control patients (Nicoll et al., 2003; Ferrer et al., 2004; Masliah et al., 2005; Holmes et al., 2008). However, cerebral amyloid angiopathy may have been (transiently) increased together with microhemorrhages (Boche et al., 2008). Neurofibrillary tangles were not reduced. Clinically, modest improvements were observed in some memory tests, while other measures did not change significantly after 1 year (Gilman et al., 2005). Recent reports of patients followed for several years are conflicting as they describe either no change in disease progression (Holmes et al., 2008) or a reduction in functional decline (Vellas et al., 2009). Studies with A β monoclonal antibody bapineuzumab indicated an amyloid reduction detected by PET imaging (Rinne et al., 2010) and possible bene-

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ficial cognitive and functional outcomes but also vasogenic edema in some patients (Salloway et al., 2009).

Early clinical trials involving immunization with A β 1–42 were stopped due to the development of aseptic meningoencephalitis in 6% of the treated patients (Orgogozo et al., 2003). Autopsy studies of affected patients demonstrated a T-cell-mediated autoimmune response (Nicoll et al., 2003; Ferrer et al., 2004) presumably due to the use of full-length A β , which contains T-cell epitopes in the C-terminal region of the molecule (Monsonogo et al., 2003). Consequently, different N-terminal A β peptides were used ending between amino acids 11 and 28 (Ghochikyan et al., 2006; Maier et al., 2006; Muhs et al., 2007; Petrushina et al., 2008).

In the present report, we describe the active A β immunotherapy CAD106 designed to avoid an activation of A β -specific T-cells. CAD106 contains only the short A β 1–6 peptide but was efficacious in reducing the amyloid accumulation in APP transgenic mice without evidence of unwanted side effects and is currently tested in AD patients (Phase II).

Materials and Methods

APP transgenic mice. The transgenic mice used express human APP751 under control of the murine Thy-1 promoter. In APP23 mice (Sturchler-Pierrat et al., 1997), transgenic APP contains the “Swedish” (K670N/M671L) mutation, whereas both the Swedish and the “London” (V717I) mutation are combined in APP24 (Abramowski et al., 2008). Expression is neuronal, largely brain-specific, and exceeds endogenous mouse APP expression by ~7-fold (APP23) or 3.5-fold (APP24). APP23 deposit mostly compact amyloid plaques starting at ~6 months of age in the neocortex, followed by hippocampus, thalamus (~9 months), and caudate–putamen (~17 months), but later also develop vascular amyloid deposits and microhemorrhages (Calhoun et al., 1998; Phinney et al., 1999; Winkler et al., 2001; Bondolfi et al., 2002). APP24 mice show an increased A β 42/A β 40 ratio due to the London mutation and develop mostly diffuse amyloid deposits starting at ~8 months in neocortex (hippocampus, ~10 months; thalamus, ~13 months; caudate–putamen, ~14 months). They contain less vascular amyloid than APP23. All mice were hemizygous for the transgene, on the C57BL/6 background, and female with the exception of study 3, in which males were used. The experiments were performed at Novartis Pharma AG (Basel, Switzerland) in compliance with protocols approved by the Animal Care and Use Committees of the Kanton Basel, Switzerland.

Immunization, bleeding, and tissue sampling of mice. Mice were immunized subcutaneously with 25 μ g of CAD106, 25 μ g of Q β , 100 μ g of A β 1–42 (Bachem AG) or PBS (vehicle) per mouse. For the dose–response study, female C57BL/6 mice (10 per group) were injected with 25, 75, 225 μ g of CAD106 per mouse. While CAD106 and Q β were administered in PBS, A β 1–42 was injected with complete Freund's adjuvant (first injection), incomplete Freund's adjuvant (second to fourth injections), or PBS (fifth to eighth injections). For passive immunization, each mouse received 500 μ g of β 1 antibody (Schrader-Fischer and Paganetti, 1996) intraperitoneally. To obtain sera, animals were bled via the tail vein before the first immunization (preimmune serum) and during the study. At the end of the experiment, mice were bled and brains were collected. One hemisphere was fixed in 4% formaldehyde solution. For biochemical analyses, the forebrain was prepared from the second hemisphere by removal of olfactory bulb, cerebellum, and brainstem, immediately frozen on dry ice, and stored at –80°C.

Determination of A β antibody titers. Antibody titers were measured by standard ELISA on plates coated with the A β 1–40 peptide (0.1 μ g/well). Serum dilutions from 1:100 to 1:10,000,000 were used and detected with goat anti-mouse IgG (H+L)-alkaline phosphatase (Jackson ImmunoResearch; 115-055-146) or rabbit anti-monkey IgG (whole molecule)-alkaline phosphatase (Sigma-Aldrich; A-1929) and *p*-nitro-phenylphosphate (Sigma-Aldrich; N-2765) as substrate. Mouse monoclonal antibody β 1 was used for the calibration curve. For monkey sera, a chimeric β 1 version with a

human Fc part was used. Titers were expressed in equivalents of micrograms per milliliter of the β 1 antibody. The lower limit of detection was 0.1 μ g/ml.

Cytokine determination by Elispot assay (T-cell activation). Female BALB/c mice (six per group) were immunized three times with A β 1–42/Freund's adjuvant as positive control (QS21 as in AN-1792 was not available) or nonadjuvanted CAD106 as used throughout the study. Spleen cells were prepared 10 d after final dose of vaccine. Plated cells were stimulated *in vitro* for cytokine release as described by Cribbs et al. (2003) using 20 μ g/ml A β 1–40, 40–1, 1–6-GGC, and 6–20 (Bachem AG). Positive spots were quantified by mouse IFN- γ Elispot kit (ALP) (Mabtech; 3321-2A) according to the manufacturer's recommendations.

Cytokine and chemokine quantification in brain. Supernatants (100,000 $\times$ g; 15 min; 4°C) from brain homogenates in TBS (Abramowski et al., 2008) were prepared and cytokine or chemokine protein levels measured using a multiplexed particle-based flow cytometric cytokine assay (Vignali, 2000). Kits were purchased from Bio-Rad and R&D Systems. The procedures closely followed the manufacturer's instructions. The analysis was conducted using a conventional flow cytometer (Guava EasyCyte Plus; Millipore).

Determination of A β in forebrain. Brain samples were homogenized, formic acid was extracted, and A β 40 and A β 42 were determined as described (Abramowski et al., 2008) using ELISAs and electrochemiluminescence-linked immunoassays (Meso Scale Discovery).

Immunohistochemistry. Brain histology and assessment of cerebral amyloid plaque load were done as described previously (Sturchler-Pierrat et al., 1997; Abramowski et al., 2008) using paraffin embedding and microwave-treated sections stained with A β antiserum NT12, biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories; BA1000), and the avidin–biotin peroxidase technique (ABC-Elite kit; Vector Laboratories; PK6100) with DAB (Roche Diagnostics; 11718096001) as substrate. To analyze different A β isoforms, fibrillar A β deposits, or blood vessel basement membranes, brain sections processed as above were stained with the following antibodies: rabbit polyclonal antibody to A β (NT12), rabbit antiserum to collagen type IV (Fitzgerald Industries; RDI-600-401-106), and mouse monoclonal antibodies to the C termini of A β 40 (25H10) and A β 42 (29C12; Nano-Tools). A β antigenicity was enhanced by microwave heating; sections for collagen staining were pretreated with 0.1% Pronase (20 min at 37°C). Indirect tyramide signal amplification was used for fluorescence visualization (TSA Plus kit NEL 741/TSA NEL 701; PerkinElmer). To test for lymphocytes, microwave-treated sections were stained with a rat anti-CD45R/B220 antibody (PharMingen; 550286) for B-cells followed by biotinylated anti-rat IgG (BA 4000; Vector Laboratories) and the ABC system. T-cells were labeled with a rat anti-CD3 antibody (Serotec; MCA1477), anti rat secondary antibody (DAKO; P0450), and the tyramide signal amplification system. Sagittal brain sections from three different anatomical levels, 100–150 μ m apart, were evaluated by microscopical inspection. The amyloid plaque load was quantified using an imaging system (Abramowski et al., 2008), while A β -positive neocortical vessels and T- or B-cells were counted manually. Microhemorrhages were evaluated after Perl's Berlin blue staining (Carson, 1996) using 12 sections per brain to visualize ferric iron in hemosiderin as previously described (Winkler et al., 2001; Pfeifer et al., 2002).

Immunization of rhesus monkeys. Four male rhesus monkeys (10–15 years) were initially immunized with 50 μ g of CAD106 subcutaneously, followed by immunizations with 25 μ g of CAD106 intramuscularly at days 28, 54, and 110. Blood was collected at various times for antibody titer determination. The study was conducted at the Novartis Forschungsinstitut (Vienna, Austria) in compliance with the Animal Care Committee-approved animal license and standard operation procedures. Antisera obtained after the third and fourth immunizations from the two monkeys with the highest titers were pooled and affinity-purified on a SulfoLink resin (Pierce; 20401) coupled with A β 1–6-GGC according to the manufacturer's instructions.

A β oligomer preparation and A β toxicity assay. A β oligomers were produced according to Lambert et al. (2001) from monomerized A β 1–42 (Bachem) and photochemically cross-linked as described by Bitan et al. (2001). Different dilutions of the centrifuged oligomer preparation and the A β antibodies were added into the culture medium of

PC12 cells. After 24 h, cell viability was assessed by MTT (Shearman et al., 1995).

Statistical analyses. Effects on amyloid plaque load and the corresponding total A β concentrations were analyzed with the nonparametric Mann–Whitney test as these data were not normally distributed. Student's *t* test was used to evaluate differences in APP, secreted APP (sAPP) or C-terminal fragments, A β cytotoxicity, and microhemorrhage number and severity, and the paired *t* test for T-cell stimulation assays. Animal number comparisons (microhemorrhages, T- or B-cells) were analyzed with Fisher's exact test. Two-tailed tests were used, and *p* < 0.05 was considered significant.

Results

To avoid activating A β -specific T-cells, we chose the A β 1–6 peptide (DAEFRH) as antigen, which is shorter than typical T-cell epitopes (Rammensee et al., 1993; Rötzschke and Falk, 1994; Rammensee, 1995) and resides outside the region of A β reacting with T-cells (Monsonogo et al., 2003). It was extended by a spacer (GGC) and covalently conjugated to the virus-like particle (VLP) derived from *Escherichia coli* RNA phage Q β . Each VLP contains ~350–550 A β peptides. The VLP carrier provides both a scaffold for ordered presentation of the antigen and T-helper cell epitopes (Bachmann et al., 1993). Screening of the A β 1–6 sequence including the spacer GGC against the SYFPEITHI database (<http://www.syfpeithi.de/>) comprising >4000 peptide sequences known to bind class I and class II MHC molecules did not identify any obvious or typical T-cell epitopes (data not shown).

Immunization with CAD106 does not activate A β -specific T-cells *in vivo*

A dose–response study conducted in old wild-type mice immunized three times with CAD106 indicated that saturation of the A β antibody response was achieved with dose levels between 75 and 225 μ g (data not shown). The 25 μ g dose was close to but not within the plateau and therefore was chosen for all further studies in mice.

The presence of activated A β -specific T-cells was checked for following three immunizations of mice with A β 1–42/Freund's adjuvant or CAD106. Ten days after the last injection, when all animals had developed A β antibody titers, isolated spleen cells were stimulated with different A β peptides and interferon- γ -secreting T-cell spots quantified (Fig. 1). All CAD106-immunized mice showed background values regardless of the peptide used for stimulation. In contrast, vaccination with A β 1–42 resulted in a threefold to fourfold increase in the number of A β -reactive T-cells after *in vitro* stimulation with A β 1–40 or A β 6–20, both of which contain T-cell epitopes (Cribbs et al., 2003; Monsonogo et al., 2003). As expected, no stimulation was found with the reverse peptide (A β 40–1) as well as the A β antigen of CAD106 (A β 1–6-GGC), confirming the lack of a T-cell epitope on this peptide. Immunization with CAD106 stimulated Q β -reactive T-cells, which is required for T-cell help (data not shown).

Generation of A β -specific antibodies in APP transgenic mice

Antibody responses were determined in all animals used for studies on amyloid deposition. Mice in the test groups received CAD106, while those in the control groups were administered PBS (Table 1). A β antibody titers in nonimmunized control animals were below the limit of detection. In contrast, all CAD106-immunized mice developed A β -specific IgG at least 10-fold above the detection limit. Median titers in the immunized groups were in the 10–110 μ g/ml range from the third injection onward and, as expected for active immunization, varied somewhat

Figure 1. Analysis of A β -specific T-cells in mice immunized with CAD106 or A β 1–42. Wild-type mice were immunized three times with CAD106 (*n* = 6) or A β 1–42/Freund's adjuvant (*n* = 6). Ten days thereafter, spleen cells were isolated and stimulated with DMSO (background) or A β peptides 1–40 (relevant antigen), 40–1 (reverse peptide), 1–6-GGC (A β epitope of CAD106), and 6–20 (A β fragment with T-cell epitope). Interferon- γ -secreting spots were quantified to determine T-cell stimulation. Mean number of spots $\pm$ SEM per 250,000 cells is given. **p* < 0.05 versus DMSO (paired *t* test).

Table 1. Efficacy studies with CAD106 in APP transgenic mice

Study	Transgenic mouse line	Treatment age (months)	Animals per group ^a	A β IgG (μ g/ml) ^b	Plaque number (%) / plaque area (%) / A β 42 (%) ^c
1 ^d	APP24	7.5–15.7	23/25	98	–80/–78/–46
2a ^e	APP24	9.5–19.5	16/9	73	–83/–80/–37
2b		13.5–19.5	15	67	–57/–32/–32
3 ^d	APP24	21.5–25.7	18	26	–15/ns/ns
4 ^d	APP23	2.9–18.5	15/14	109	–38/–33/–33
5 ^d	APP23	17.5–22.7	20/17	35	–32/ns/ns
6 ^e	APP23	16–22.2	27/21	78	–47/ns/ns

^aNumber of animals per group (CAD106/PBS) analyzed at the end of the study.

^bMedian serum A β IgG concentration at peak.

^cPlaque number and area in neocortex, A β 42 in forebrain, significant changes compared with control; ns, nonsignificant.

^dImmunization at weeks 0, 2, 4, and approximately every 4 weeks.

^eImmunization at weeks 0, 2, and approximately every 4 weeks.

throughout the studies. No difference in antibody response was apparent between the APP23 and APP24 transgenic mice and wild-type mice (Table 1) (data not shown).

In CSF, A β antibodies were either not measurable or just above the limit of detection (data not shown). As minor contaminations with blood during mouse CSF sampling cannot be excluded, these results indicate only a low level of A β antibodies in the CSF compartment.

The antibody response to CAD106 showed a classical IgM to IgG shift (data not shown). Thereafter, A β -specific anti-

Table 2. Plaque load reduction in different brain regions of APP transgenic mice after treatment with CAD106

	Hippocampus (%)	Thalamus (%)	Caudate–putamen (%)
Study 1 (APP24, 7.5–15.7 months) ^a			
Plaque number	–72	–84	–100
Plaque area	–80	–93	–100
Study 2a (APP24, 9.5–19.5 months) ^a			
Plaque number	–82	–93	–98
Plaque area	–89	–95	–98
Study 2b (APP24, 13.5–19.5 months) ^b			
Plaque number	–68	–71	–94
Plaque area	–61	–75	–92
Study 4 (APP23, 2.9–18.5 months) ^c			
Plaque number	–33	–28	–92
Plaque area	–20	–39	–97

^aTwo-tailed Mann–Whitney test, $p < 0.0001$ for all measures (CAD106 vs PBS).^bTwo-tailed Mann–Whitney test, $p < 0.002$ for all measures (CAD106 vs PBS).^cTwo-tailed Mann–Whitney test, $p < 0.05$ for all measures (CAD106 vs PBS).

bodies of all IgG subclasses were found with some variation in subclass ratio noted between animals. With few exceptions, the subclass pattern of each animal remained relatively constant with time (data not shown). The antibodies recognized A β amino acids 3–6, (E)FRH, as minimal epitope (data not shown).

Reduction of amyloid deposition after CAD106 immunization of APP24 mice

To first test CAD106 in a preventive setting, APP24 mice were immunized before the onset of plaque formation, at 7.5 months of age (study 1). At the end of the study (15.7 months), plaque number and relative area covered by plaques in the neocortex were reduced compared with PBS-treated animals by 80 and 78%, respectively (Table 1). Larger reductions up to 100% were found in thalamus and caudate–putamen (Table 2). For comparison, a group of mice was immunized in parallel with aggregated A β 1–42 in Freund's adjuvant. Tenfold lower A β antibody titers were obtained (data not shown) in agreement with a reduced response against A β 1–42 in C57BL/6 mice (Das et al., 2003), and amyloid deposition in the cerebral cortex and hippocampus was unaffected. However, despite the lower titers, there was still a remarkable reduction in plaque load in the thalamus (number, –62%; area, –79%) and caudate–putamen (number, –79%; area, –84%). These reductions were slightly lower than those observed with CAD106 in the corresponding regions.

In the second study, a first cohort of APP24 mice (study 2a) was treated from 9.5 until 19.5 months starting right after the onset of amyloid deposition in the neocortex. The reduction in plaque number and area was comparable with the first study (Tables 1, 2). This cohort also included a group of animals immunized with the carrier Q β in the absence of A β sequences. No effect on amyloid plaque deposition was found (Fig. 2). A second cohort (study 2b) received injections only from 13.5 months onward, at which time a considerable amount of amyloid has already been deposited in cortex and hippocampus, but was also terminated at 19.5 months. Treatment at this advanced stage of plaque formation led to highly significant reductions as well (Tables 1, 2), although the effects were considerably less pronounced. In the neocortex, a 57% reduction of plaque number and a 32% reduction of plaque area were found. Very strong effects were again observed in the caudate–putamen, where amyloid deposition starts latest: 92% reduction of the plaque area and 94% re-

Figure 2. Effect of CAD106 or the carrier Q β on amyloid plaque load in APP24 mice. Animals were immunized for 10 months (9.5–19.5 months of age; study 2a) with CAD106 or Q β VLP; controls received vehicle (PBS). **A**, Neocortical plaque number. **B**, Neocortical plaque area. Plaque number is expressed as plaques per square millimeter, and plaque area in percentage of total cortex area. The boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. *** $p < 0.001$ CAD106 versus PBS or Q β (Mann–Whitney rank sum test).

Figure 3. Amyloid deposition in neocortex of APP24 mice after CAD106 or vehicle treatment. Amyloid staining (A β antiserum NT12) of neocortex sections from mice treated between 9.5 and 19.5 months (study 2a) with PBS (**A**) or CAD106 (**B**). Note the decrease of diffuse parenchymal amyloid plaques, the compact appearance of most remaining plaques, and the increase of vascular amyloid in (**B**). Cerebral amyloid angiopathy in parenchymal vessels (arrows) and a single meningeal vessel (arrowhead) are shown. Scale bar, 100 μ m.

duction in plaque number. Visual inspection of the brain sections indicated that diffuse amyloid was affected to a larger extent than compact amyloid in all studies (Fig. 3).

In a further study (3), very old mice with a high amyloid load were treated from 21.5 to 25.7 months, which resulted in a borderline reduction in plaque number (–15%) but not plaque area in neocortex, indicating a small effect on newly formed deposits (Table 1).

CAD106 treatment significantly reduced total forebrain A β 42 (study 1, –46%; study 2a, –37%; study 2b, –32%) compared with control groups (Table 1). Smaller reductions (–6 to –20%), not always reaching significance, were found for A β 40 (data not shown). However, immunization of very old mice did not result in a significant reduction of either A β peptide (study 3).

Forebrain homogenates from study 2 were also analyzed for an effect of CAD106 immunization on APP and its metabolites. No significant change in the level of full-length APP or the

Figure 4. Effect of CAD106 or antibody $\beta 1$ on amyloid plaque load in aged APP23 mice. The animals were treated with CAD106, monoclonal $A\beta$ antibody $\beta 1$, or vehicle (PBS) from 17.5 to 22.5 months of age (study 5). Neocortical plaque number (**A**) and plaque area (**B**) are shown as described in Figure 2. *** $p < 0.001$, ** $p < 0.01$, # $p = 0.08$, CAD106 or $\beta 1$ versus PBS (Mann–Whitney rank sum test).

secreted N-terminal fragments sAPP α and sAPP β or the C-terminal fragments C99 and C83 was found (data not shown). This indicates that the antibodies induced by CAD106 did not alter APP metabolism *in vivo*, in contrast to their effect on amyloid deposition.

Amyloid lowering effect of CAD106 in APP23 mice

APP23 mice were immunized with CAD106 during preplaque to advanced stage of amyloid deposition (between 2.9 and 18.5 months; study 4). Compared with PBS controls, the number of amyloid plaques and plaque area were lower in all four brain regions examined (Tables 1, 2). Reductions in a similar range (20–40%) were found in the cerebral cortex, hippocampus, and thalamus. There was almost no amyloid deposition in caudate–putamen. In agreement with these histological findings, the overall $A\beta 42$ reduction in forebrain was 33%. No significant effect on $A\beta 40$ was found. As observed in APP24 mice, immunization with Q β did not affect any of the measures for $A\beta$ deposition (data not shown).

Old mice with very advanced amyloid plaque pathology were used to compare active and passive immunization approaches (study 5). Mice were immunized between 17.5 and 22.7 months of age with either CAD106 or the monoclonal antibody $\beta 1$ (500 μ g/mouse weekly, i.p.). With this dose, the $\beta 1$ group had approximately five times higher antibody titers until they had declined by study end. In neocortex, both CAD106 and $\beta 1$ reduced the number of plaques by 32 and 42% ($p < 0.00001$), respectively, compared with the control group (Fig. 4). The total cortical plaque area was slightly lower with both treatment regimens, amounting to –9% for CAD106 (nonsignificant, $p = 0.08$) and to –14% for $\beta 1$ ($p < 0.01$). Visual inspection indicated that small-sized plaques were reduced, while medium- and large-sized plaque numbers remained unchanged.

To confirm this, APP23 mice of similar age (16–22.2 months) were immunized with CAD106 (study 6). Consistent with the

Figure 5. Size distribution of amyloid plaques in CAD106 and vehicle-treated APP23 mice. Animals were immunized from 16 to 22.2 months of age (study 6). The number of plaques $\pm$ SEM in different size groups was determined in the cerebral cortex. Only plaques $> 1 \mu m^2$ were considered to assure a reliable determination. * $p < 0.05$, *** $p < 0.001$, CAD106 versus PBS (t test).

results of study 5, a 47% reduction in amyloid plaque number ($p < 0.001$) was found in the neocortex but the plaque area was not significantly reduced (Table 1). Quantification according to plaque size demonstrated a selective reduction of small $A\beta$ plaques (plaque area, 1–500 μm^2) (Fig. 5). These contribute relatively little to the total plaque area, which explains the discrepancy between the effects on plaque number and area. In line with this finding, treatment with CAD106 or $\beta 1$ did not significantly reduce the amount of $A\beta 40$ and 42 in these very old mice (Table 1).

Differential effect of CAD106 treatment on vascular $A\beta$

Inspection of brain sections from CAD106-treated animals indicated an overall lowering of amyloid deposits, while vascular amyloid remained constant or even increased relative to controls (Fig. 3). Quantitative analysis showed an elevation in number of $A\beta$ -containing blood vessel profiles in some but not all studies with CAD106-immunized APP24 mice (Fig. 6A). Only a very minor fraction of the blood vessels was associated with $A\beta$ in APP24 mice (data not shown). This low frequency of vascular $A\beta$ may explain the variation between these studies. Further characterization (study 1) indicated that CAD106 increased the deposition of $A\beta 42$ but not of $A\beta 40$ in the vasculature (Fig. 6B). Moreover, Congo red-stained vessels were not increased. The additional vascular $A\beta$ seemed mainly nonfibrillar, which differs from the fibrillar amyloid typically deposited in blood vessels.

No significant increase in amyloid-containing blood vessels was found in the CAD106-treated APP23 mice, although a non-significant elevation was observed after 2.9–18.5 months of treatment (data not shown).

No evidence of increased microhemorrhages following CAD106 immunization of APP24 and APP23 mice

Vascular amyloid is a risk factor for microhemorrhages and an increase has been observed after $A\beta$ immunization (Pfeifer et al., 2002; Wilcock et al., 2004, 2007; Racke et al., 2005). To investigate whether this occurred following immunization with CAD106, brains from the above studies were analyzed by hemosiderin staining and data from the same APP transgenic line were pooled to obtain sufficiently large groups. In APP24 mice, only a single microhemorrhage was found in a Q β control animal, which is in agreement with the rare occurrence of microbleeds in this line (data not shown). In contrast, aged APP23 mice had more microhemorrhages, but there was neither an increase in frequency

Figure 6. Effect of CAD106 immunization on vascular Aβ in APP24 mice. Aβ deposits in neocortical blood vessels were labeled as described below. The number of positive vessels (mean ± SEM) is given as percentage of vehicle (PBS). **A**, Total Aβ-containing blood vessels (NT12 antiserum) in different CAD106 studies (1–3) as indicated by the treatment period. **B**, Blood vessels stained for total Aβ (NT12), Aβ40 (25H10), Aβ42 (29C12), and fibrillar amyloid (Congo red) in animals from study 1. ** $p < 0.01$, * $p > 0.05$, CAD106 versus PBS (Mann–Whitney rank sum test).

nor severity in CAD106-treated animals (Table 3). Separate analysis of the studies also did not show any treatment-related increases.

Proinflammatory cytokines and chemokines

Brain homogenates were also analyzed for the proinflammatory cytokines TNFα and IL-12 (p40) as well as for the chemokines RANTES (regulated on activation normal T cell expressed and secreted) and KC (keratinocyte-derived chemokine)/GRO (growth-related oncogene). IL-1β and IL-6 were below the detection limit. Mice (8 per group) with antibody titers above the median were chosen from studies 1, 2a, 3, 4, and 6. Quantification did not show any changes the CAD106 as compared with the control groups (data not shown).

Evaluation of T-cell and B-cell infiltration

Brains from study animals were analyzed for T- and B-cells to identify potential inflammatory cell infiltration. Perivascular lymphocytes were only occasionally detected in aged, nontransgenic or APP transgenic mice, and immunization with CAD106 did not increase the incidence of these cells (data not shown). However, 1 of 116 CAD106-treated animals had frequent T- and B-cell infiltrates (study 2a). The infiltrates were present throughout the brain and not restricted to amyloid-containing regions. This is in contrast to the described meningoencephalitis associated with brain amyloid in an APP transgenic mouse passively

immunized with an Aβ antibody (Lee et al., 2005). The brain homogenate from the animal in our study was analyzed by PCR to identify an infectious agent but no positive result was obtained. Therefore, the relevance of this singular observation remains unclear.

CAD106-induced rhesus monkey antibodies react with different Aβ aggregation states and block its toxicity *in vitro*

Rhesus monkeys were immunized with CAD106 because they have the same Aβ sequence as humans and their immune system can be considered as closely related. All four immunized monkeys developed Aβ IgG antibodies. The reversibility of the Aβ-specific antibody response was evaluated after the final immunization and was found to decline in all animals with a half-life in the range of 2–4 weeks.

Reactivity of the monkey antibodies with amyloid plaques, APP, and other Aβ-containing polypeptides in brain was investigated by immunohistochemistry. Affinity-purified, pooled antibodies stained plaques on brain sections from APP23 mice and human AD patients (data not shown). No cross-reactivity with cellular brain structures was detected.

To further test reactivity against Aβ oligomers, CAD106-induced monkey Aβ antibodies were tested for their ability to recognize synthetic oligomers on Western blots (Fig. 7A). This analysis demonstrated binding of the antibodies to different higher-molecular-weight Aβ species representing oligomers. Reactivity with oligomers was independent of the method or the Aβ isoform (Aβ1–40 or 1–42) used for preparation (data not shown). Aβ monomers were also recognized.

The antibodies were also tested for neutralization of Aβ in an *in vitro* toxicity assay. As shown in Figure 7B, they blocked Aβ1–42 oligomer-induced toxicity to PC12 cells in a concentration-dependent manner. The IC₅₀ was equal to the antibody titer. Similar results were obtained when cytotoxicity was induced with fibrillar Aβ1–40 (data not shown).

Discussion

In this study, we have characterized the second-generation active Aβ immunotherapy CAD106, a vaccine designed to avoid undesired inflammatory side effects while inducing efficacious antibodies against Aβ. We demonstrated that the induced antibodies interfere with amyloid deposition in cortical and subcortical brain regions, bind to Aβ aggregates, and block their toxicity to cells.

All mice and rhesus monkeys immunized with CAD106 developed Aβ antibody titers regardless of their age. This finding is remarkable considering that Aβ1–6 is a small autoantigen in human APP transgenic mice and monkeys and that CAD106 was used without additional adjuvant. Immunogenicity of the vaccine is provided by its highly repetitive structure, provision of T-cell help, and optimal size for diffusion into the lymph node (Bachmann and Jennings, 2010). The antibodies recognized a short sequence, Aβ3–6, as minimal epitope. Because all main IgG subclasses were generated, the entire range of effector functions is possible for CAD106-induced Aβ antibodies. This seems advantageous as the mechanism of Aβ immunotherapy remains unclear and the IgG subclass requirements are controversial (Klaffki et al., 2006). When immunization was stopped, Aβ antibody titers declined regardless of the presence of endogenous Aβ, presumably due to the lack of T-cell help once the vaccine cleared. Discontinuation of CAD106 treatment thus leads to a decreased antibody exposure with a similar kinetic to passive immunotherapy.

Table 3. Microhemorrhage frequency and severity in APP23 mice immunized with CAD106

Treatment	Mice analyzed Number	Mice with microhemorrhages			Microhemorrhages			Severity grade			Mean severity	
		Number	%	<i>p</i> ^a	Number	Number/mouse	<i>p</i> ^b	I	II	III	Grade	<i>p</i> ^b
CAD106	62	18	29	0.39	22	0.35	0.84	7	12	3	1.8	0.92
PBS	52	11	21		20	0.38		9	7	4	1.8	

Animals from studies 4–6 were analyzed. From each brain, 12 sagittal sections were stained with Prussian blue, and hemosiderin-positive cell clusters were counted according to their grade (grade I, 1–3 positive cells; grade II, 4–10 positive cells; grade III, >10 positive cells). The numbers given refer to the counts in the 12 samples sections per mouse without back calculation to the entire brain.

^aFisher’s exact test (two-tailed).

^bt test (two-tailed).

Figure 7. Rhesus monkey antibodies induced by CAD106 react with A β oligomers and protect against A β toxicity. *A*, Western blot of SDS-stable A β 1–42 oligomers without (left lane) or with chemical cross-linking (right lane). CAD106-induced antibodies react with A β monomers (3.5 kDa) as well as oligomers of increasing size. No reactivity was observed with preimmune sera (data not shown). *B*, A β 1–42 oligomer toxicity assay using PC12 cells (control, non-treated). CAD106 antibodies from monkeys reverted the oligomer-induced toxicity in a concentration-dependent manner. The concentration of A β 1–42 oligomers was 0.1 μ M (normalized for monomeric A β). Error bars indicate SEM.

Immunization with the VLP Q β alone did not effect amyloid deposition. This finding together with the absence of A β -specific T-cell activation demonstrates that the efficacy of CAD106 is mediated by A β -specific antibodies. This conclusion is in accord with previous studies involving passive immunotherapy (Bard et al., 2000) and our data with monoclonal antibody β 1. In a direct comparison with CAD106, the higher A β antibody titers reached after β 1 infusion resulted in slightly larger group effects. Conversely, lower titers obtained with A β 1–42 immunization led to smaller effects suggesting a titer–response relationship. Nevertheless, a correlation between A β antibody titers and the effect on amyloid deposition could not be established. The high intrinsic variation of the amyloid deposition process together with the variation of titer over time did not allow establishing such a relationship.

Our efficacy studies further indicate that the reduction in amyloid accumulation after A β immunotherapy with CAD106 is inversely related to A β levels in the brain. First, larger effects were

generally observed in APP24 mice, which have lower soluble A β levels and deposit less amyloid than APP23 (Abramowski et al., 2008). Second, the magnitude of the effect on amyloid (plaque number and area) in both transgenic lines differed between the regions analyzed. The strongest reduction was in the caudate–putamen followed by thalamus, hippocampus, and cerebral cortex. Human APP expression and hence A β increase considerably in the reverse order (Sturchler-Pierrat et al., 1997).

A comparison of different readouts for amyloid reduction revealed the largest effect was measured for plaque number followed by plaque area, total forebrain A β 42, and total forebrain A β 40 levels. This rank order was most apparent when treatment started at plaque-bearing age, whereas differences vanished for preventive immunizations. Analyses in old, plaque-bearing mice demonstrated a reduction of small plaques, which contribute little to plaque area and A β peptide levels. It is conceivable that these small plaques are newly formed deposits and that A β immunotherapy with CAD106 or monoclonal antibody β 1 has a larger effect on the initiation of amyloid deposition. Further supporting this notion was the observation that larger effects were found when immunization started before amyloid deposition. Others have also described that immunotherapy used in a preventive mode rather than in a therapeutic setting is more efficacious (Das et al., 2001). Similar results were obtained following reduction of A β generation with a γ -secretase inhibitor (Abramowski et al., 2008), indicating that *de novo* amyloid deposition is more sensitive to lowering of free A β than its assembly onto preexisting deposits as observed during *in vitro* assembly studies.

Surprisingly, immunization with CAD106 or monoclonal β 1 reduced A β 42 more strongly than A β 40 even though the respective antibodies do not discriminate between either isoforms and the two APP transgenic lines used differ in their A β 42/A β 40 ratio. In line with this finding, we also showed diffuse amyloid deposits consisting mainly of A β 42 were principally removed. Additionally, the lack of effect on vascular A β may have contributed to this finding as it is dominated by A β 40.

Analysis of vascular amyloid showed increases in those APP24 mouse studies, in which a strong reduction of total amyloid accumulation was obtained. The relative increase (up to 2.5-fold) must be considered in relation to the rare occurrence of vascular amyloid in these animals. Studies in old APP24 and in APP23 mice, in which amyloid lowering was less, showed no elevation of vascular amyloid, suggesting an inverse relationship between overall amyloid removal and vascular deposition. A mechanistic link is supported by the observed increase of A β 42 but not A β 40 and its deposition in diffuse, nonfibrillar form, which is atypical for vascular amyloid. Similar deposition of vascular A β 42 was observed in humans during postmortem analysis of the active A β 1–42 immunotherapy study AN1792 (Boche et al., 2008). Both results contrast with the vascular amyloid reduction found after passive immunization with an N-terminal A β antibody in PDAPP mice (Schroeter et al., 2008). This transgenic APP line

deposits vascular amyloid primarily in meninges, while it was mostly parenchymal in our and the human studies. Differences in the models as well as antibody qualities may have contributed to these different outcomes.

Various APP transgenic mouse studies have demonstrated increases in microhemorrhages following A β immunotherapy (Pfeifer et al., 2002; Wilcock et al., 2004, 2007; Racke et al., 2005; Schroeter et al., 2008). These were associated with both, elevation (Pfeifer et al., 2002; Wilcock et al., 2004, 2007; Racke et al., 2005) or lowering (Schroeter et al., 2008) of vascular A β . The elevation of vascular A β in some CAD106 immunization studies did not lead to an increase in microhemorrhages. The nonfibrillar nature of the additional vascular A β , the slow antibody titer increases with active immunotherapy or antibody modifications (Wilcock et al., 2006) may have contributed to this result. In addition, no evidence was found for brain infiltration of lymphocytes or meningoencephalitis. Our expectation that immunization with CAD106 would not induce A β -specific T-cells because of the minimal length of the peptide antigen and lack of typical T-cell epitopes was confirmed by experimental analysis.

CAD106-induced antibodies selectively stained amyloid plaques on brain sections from human AD patients and APP transgenic mice. No cross-reactivity was observed with cellular APP or its metabolites. CAD106 treatment did not alter the steady-state level of full-length APP or the metabolites sAPP β and C99 containing the A β 1–6 sequence. These observations argue against a significant interaction of the antibodies with APP and its primary metabolites *in vivo*. Hence, the reduction of A β deposition is unlikely to be due to interference with APP processing as observed with a β 1 intrabody (Paganetti et al., 2005). Accordingly, our data demonstrate that CAD106-induced antibodies bind to different aggregation states of A β including toxic oligomers and fibrils. They further show the antibodies are able to neutralize toxicity induced by these assemblies in a cellular assay.

In conclusion, CAD106 induces efficacious A β antibody titers in animals while avoiding potential mechanism-related side effects. The antibodies effectively interfere with amyloid accumulation in brain and protect from A β toxicity. CAD106 is currently tested in AD patients as a potentially disease-modifying agent.

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