European Journal of Neuroscience, Vol. 26, pp. 2458–2468, 2007 doi:10.1111/j.1460-9568.2007.05852.x

Deglycosylated anti-amyloid beta antibodies reduce microglial phagocytosis and cytokine production while retaining the capacity to induce amyloid beta sequestration

Kazuyuki Takata,[1] Chiho Hirata-Fukae,[2] Amanda G. Becker,[2] Saori Chishiro,[1] Audrey J. Gray,[2] Kouhei Nishitomi,[3] Andreas H. Franz,[4] Gaku Sakaguchi,[3] Akira Kato,[3] Mark P. Mattson,[5] Frank M. LaFerla,[6] Paul S. Aisen,[2] Yoshihisa Kitamura[1] and Yasuji Matsuoka[2]

[1]Department of Neurobiology, Kyoto Pharmaceutical University and 21st Century COE Program, Kyoto 607-8414, Japan
[2]Department of Neurology, Georgetown University Medical Center, Washington, DC 20057, USA
[3]Pain & Neurology, Discovery Research Laboratories, Shionogi Co. Ltd, Shiga 520-3423, Japan
[4]Department of Chemistry, University of the Pacific, Stockton, CA, USA
[5]Laboratory of Neurosciences, National Institute on Ageing Intramural Research Program, Baltimore, MD, USA
[6]Department. of Neurobiology & Behaviour, University of California, Irvine, CA, USA

Keywords: Alzheimer's disease, immunization, immunotherapy, transgenic mice

Abstract

Accumulation of amyloid beta (Abeta) is a pathological hallmark of Alzheimer's disease, and lowering Abeta is a promising therapeutic approach. Intact anti-Abeta antibodies reduce brain Abeta through two pathways: enhanced microglial phagocytosis and Abeta transfer from the brain to the periphery (Abeta sequestration). While activation of microglia, which is essential for microglial phagocytosis, is necessarily accompanied by undesired neuroinflammatory events, the capacity for sequestration does not seem to be linked to such effects. We and other groups have found that simple Abeta binding agents are sufficient to reduce brain Abeta through the sequestration pathway. In this study, we aimed to eliminate potentially deleterious immune activation from antibodies without affecting the ability to induce sequestration. The glycan portion of immunoglobulin is critically involved in interactions with immune effectors including the Fc receptor and complement c1q; deglycosylation eliminates these interactions, while antigen (Abeta)-binding affinity is maintained. In this study, we investigated whether deglycosylated anti-Abeta antibodies reduce microglial phagocytosis and neuroinflammation without altering the capacity to induce Abeta sequestration. Deglycosylated antibodies maintained Abeta binding affinity. Deglycosylated antibodies did not enhance Abeta phagocytosis or cytokine release in primary cultured microglia, whereas intact antibodies did so significantly. Intravenous injection of deglycosylated antibodies elevated plasma Abeta levels and induced Abeta sequestration to a similar or greater degree compared with intact antibodies in an Alzheimer's transgenic mouse model without or with Abeta plaque pathology. We conclude that deglycosylated antibodies effectively induced Abeta sequestration without provoking neuroinflammation; thus, these deglycosylated antibodies may be optimal for sequestration therapy for Alzheimer's disease.

Introduction

Accumulation of amyloid beta (Abeta) in the brain is a pathological hallmark of Alzheimer's disease (AD), and the reduction of Abeta has been proposed as a primary therapeutic target for AD (Hardy & Selkoe, 2002). Active immunization with Abeta peptides raised anti-Abeta antibodies and reduced brain Abeta and improved cognitive performance in AD mouse models (Schenk *et al*., 1999). One proposed mechanism of action is the enhancement of Abeta phagocytosis by microglia: antibodies enter the brain, accumulate surrounding the Abeta plaques and enhance microglial phagocytosis via Fc receptors (FcR) (Bard *et al*., 2000). In support of this mechanism, infusion of microglia reduced brain Abeta plaque load in AD model mice (Simard *et al*., 2006; Takata *et al*., 2007).

Correspondence: Dr Y. Matsuoka, as above.
E-mail: ym56@georgetown.edu

Received 24 April 2007, revised 27 July 2007, accepted 27 August 2007

In active immunization studies, plasma Abeta levels are significantly elevated; this is called 'Abeta sequestration' or 'peripheral sink' (DeMattos *et al*., 2001; Lemere *et al*., 2003). The mechanism is not yet fully clear, but it is believed that the presence of anti-Abeta antibodies in the blood enhances Abeta efflux from the brain (DeMattos *et al*., 2002a, b). We hypothesized that Abeta sequestration is sufficient to lower brain Abeta and found that simple Abeta binding agents, which do not evoke any immune reaction, reduced brain Abeta (Matsuoka *et al*., 2003). Other groups also found that various Abeta binding agents, heparin (Bergamaschini *et al*., 2004) and Nogo receptor (Park *et al*., 2006), reduced brain Abeta. In addition, Abeta active immunization reduced Abeta in an AD mouse model lacking FcR (Das *et al*., 2003).

Despite promising results in animal studies, a clinical trial of active immunization was terminated after meningoencephalitis occurred in some subjects (Orgogozo *et al*., 2003). The cause of the meningoencephalitis is not fully known, but immune activation is presumably involved, and T-cell infiltration to the central nervous system was

documented (Nicoll *et al*., 2003; Ferrer *et al*., 2004). Passive immunization does not involve T-cell activation, and thus may be safer than active immunization. However, T-cell activation is not the sole cause of neuroinflammation. In human AD brains, highly activated microglia accumulate surrounding the plaques and cytokines are up-regulated (Luterman *et al*., 2000). Passive immunization uses intact antibodies and enhances microglia phagocytosis of Abeta; presumably it enhances cytokine production and other neuroinflammatory events. Indeed, a Phase 1 study with a passive immunotherapy for AD noted the occurrence of focal cerebral edema in several treated patients (presented at the 9th International Geneva/Springfield Symposium), possibly an indication of neuroinflammation.

Glycosylation of immunoglobulin (Ig) is critically involved in binding to FcR (Radaev & Sun, 2001) and complement C1q (Winkelhake *et al*., 1980). While deglycosylated antibodies maintain binding affinity to their antigen, they have reduced interaction with FcR and C1q, suggesting that deglycosylated antibodies may cause less neuroinflammation. However, their effects on Abeta microglial phagocytosis and other neuroinflammatory events, and the relative potency of deglycosylated antibodies in Abeta sequestration remain unclear. In this study, we investigated the effects of deglycosylated antibodies on microglial Abeta phagocytosis, cytokine release and Abeta sequestration.

Materials and methods

Preparation and validation of deglycosylated antibodies

We used two mouse monoclonal anti-Abeta antibodies: clones 82E1 (Immuno-Biological Laboratories, Takasaki, Gunma, Japan) (Horikoshi *et al*., 2004) and 6E10 (Signet Laboratories, Dedham, MA, USA) (Kim *et al*., 1988). The subclass of these antibodies is IgG1 and the epitopes are amino acids 1–5 and 3–8 of Abeta, respectively. Preservative-free purified IgG was treated with peptide-N4-(acetyl-beta-glucosaminyl)-asparagine amidase, EC 3.5.1.52 (N-glycanase, 10 U/100 μg IgG, Prozyme, San Leandro, CA, USA) in phosphate-buffered saline (PBS), pH 7.4, for 18 h at 37 °C. N-glycanase was isolated from a strain of *Escherichia coli* expressing a cloned gene from *Chryseobacterium* [*Flavobacterium*] *meningosepticum* (Tarentino & Plummer., 1987). N-glycanase releases intact N-linked oligosaccharides from glycoproteins, in this case the Fc region of IgG (Krapp *et al*., 2003), and the site of enzyme cleavage is highly specific, with hydrolysis occurring between asparagines and the proximal N-acetyl-glycosamine of most oligomannose, hybrid- and complex-type N-glycans.

To validate deglycosylation of antibodies, intact and deglycosylated antibodies were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. A sample of 1 μL was mixed with 1 μL of 5 mg/mL sinapinic acid in ethanol and water (3 : 2 ratio) solution (Fluka, St Louis, MO, USA), dried at room temperature, and subjected to MALDI-TOF mass spectrometry (AXIMA-CFR mass spectrometer, Kratos-Shimadzu, Kyoto, Japan) using positive linear mode with nitrogen laser ($\lambda = 337$ nm) and 20 kV extraction potential. Mass spectra were calibrated using singly charged ions of a mixture of insulin, cytochrome c, apomyoglobin, aldolase and albumin, which have average $[M + H]^+$ $m/z = 5730.6$, 12 361, 16 952, 39 212 and 66 430, respectively. In parallel to the measurements, calibration was validated through desorption from three different MALDI target spots.

In addition, we examined the molecular weight shift due to deglycosylation via gel electrophoresis. Intact and deglycosylated antibodies were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% polyacrylamide gels) and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with skimmed milk, the membrane was probed with a horseradish peroxidase (HRP)-coupled sheep anti-mouse IgG antibody (1 : 1000, Amersham Pharmacia Biotech, Buckinghamshire, UK) and visualized using a chemiluminescence kit (Amersham Pharmacia Biotech).

Determination of the affinity of intact and deglycosylated antibodies for Abeta, and confirmation of Abeta binding capability

The affinity of the intact and deglycosylated antibodies, clones 6E10 and 82E1, was determined using a BIAcore 3000 biosensor (Biacore, Inc., Uppsala, Sweden). Testing antibodies were immobilized on CM5 sensor chips (Biacore) using 10 mM glycine buffer, pH 1.7, as a regeneration buffer. The K_D value was determined using various Abeta 1–40 at 0.8, 1.5, 2.9, 5.8, 11.5 and 23 nM in 0.01 M HEPES buffer consisting of 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20, pH 7.4, as a running buffer at a flow rate of 20 μL/min. Forty-microliter samples were injected. The dissociation curve was obtained while running buffer for 120 s. Data collected represent the value of the observed response units (RU) obtained in the sample cells subtracted from the RU obtained from a reference cell. The data were analysed using a BIAevaluation 4.1 software (Biacore).

To confirm that deglycosylated antibodies maintain the capacity to capture Abeta in liquid phase, we used immunoprecipitation. Five micrograms of intact or deglycosylated antibodies was mixed with 1 μg of synthetic human Abeta 1–42 (Anaspec, San Jose, CA, USA) in a final volume of 20 μL in 10 mM PBS, pH 7.4, for 2 h. Abeta and intact or deglycosylated antibody immunocomplex was isolated using protein G-conjugated sepharose (20 μL of 50% slurry; Amersham). Precipitated immunocomplex was dissociated in sample buffer consisting of 100 mM Tris-HCl (pH 6.8), 24% glycerol, 2% 2-mercaptoethanol, 1% SDS and 0.02% Coomassie Brilliant Blue, and subjected to SDS-PAGE (20% polyacrylamide gels in Tricine buffer). Proteins were transferred to a PVDF membrane, and it was then incubated with a mouse anti-Abeta monoclonal antibody (clone 6E10, 1 μg IgG/mL) following an HRP-coupled sheep anti-mouse IgG antibody (1 : 1000). Subsequently, Abeta bands were visualized using a chemiluminescence kit.

Abeta phagocytosis assay using primary cultured microglia

The primary culture experimental procedure was reviewed and approved by the Committee for Animal Research at Kyoto Pharmaceutical University. Primary cultured microglia (over 97% pure as determined by CD11b staining) were prepared from newborn rats as previously described (Kitamura *et al*., 2001). Mixed glial cells (a mixture of astrocytes and microglia) were prepared from newborn Wistar rats at postnatal day 1; rat pups were killed by decapitation and then brains were immediately isolated. Meninges were carefully removed and forebrains were minced and gently dissociated by trituration in Dulbecco's modified Eagle medium (DMEM). The tissue suspension was filtered through a 70-μm-diameter nylon mesh (cell strainers; Falcon, Franklin Lakes, NJ, USA) into 50-mL tubes, and cells were collected by centrifugation at 200***g*** for 10 min. Cells were resuspended in DMEM with 10% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin, and plated out onto 100-mm-diameter dishes at 37 °C in humidified 5% CO_2/95%

air. At 21 days after cultivation, we harvested floating microglia from mixed glial cultures and plated them out into new 24-well culture plates (3.0×10^5 cells per well). Purified microglia (> 97% pure as determined by CD11b immunostaining) were incubated overnight to allow attachment on dishes, and then subjected to treatment.

Synthetic human Abeta 1–42, hydrochloride form (≥ 95% pure as determined by HPLC; Anaspec) was dissolved in pyrogen-free distilled water in 1 mM aliquots, snap frozen and stored at −80 °C until use. Once Abeta was thawed, no Abeta was re-frozen to eliminate variance due to repeated freeze-thawing. Abeta, 1 μM, was preincubated with the intact or deglycosylated antibody (5 μg IgG/mL) in culture medium for 1 h prior to the treatment. Subsequently, the culture medium in the well was replaced.

For cytochemical examination, cells were rinsed with PBS and fixed with 4% paraformaldehyde in 100 mM PBS for 30 min. Cells were incubated with a rabbit polyclonal anti-Abeta antibody (1 : 2000, Chemicon International, Temecula, CA, USA) in PBS containing 0.3% Triton X-100, and visualized by fluorescein-labeled anti-rabbit IgG antibody (4 μg/mL; Molecular Probes, Eugene, OR, USA). The cells were also incubated with rhodamine-conjugated phalloidin (0.2 μg/mL; Molecular Probes) and Hoechst 33258 (6 μg/mL; Molecular Probes), which are markers for actin filaments and nuclei, respectively, to visualize the cellular structure. Fluorescence was detected using a laser scanning confocal microscope (LSM410; Carl Zeiss, Jena, Germany). Fluorescein, rhodamine and Hoechst 33258 were detected using Ar (excitation, 488 nm; emission filter, 515–540 nm), HeNe (excitation, 543 nm; emission filter, ≥ 570 nm) and ArUV (excitation, 364 nm; emission filter, ≤ 397 nm) lasers, respectively. Images (1024×1024 pixels) were obtained using a ×40 objective lens with ×4 digital zoom. For image analysis, the intensity of Abeta immunoreactivity in the intracellular space, i.e. the intensity of fluorescein within the phalloidin-visualized cellular structure, was determined after background subtraction using a fixed cut-off (≤ 95 out of a maximum brightness level of 255) (WinRoof, Mitani, Fukui, Japan).

Levels of Abeta phagocytosis were also determined by ELISAs. Culture medium was collected and snap frozen for cytokine assay (see below). Cells were rinsed with PBS and then collected in PBS containing 0.1% Triton X-100. Abeta levels in the cell lysate were determined by ELISA using antibodies against the middle region and the C terminus, clones 12B2 and 1C3 (epitope: Abeta 11–28 and 38–42, respectively), as previously described (Horikoshi *et al*., 2004). The epitopes of antibodies used for ELISA do not overlap with the study antibodies, 82E1 and 6E10 (epitope: Abeta 1–5 and 3–8, respectively).

Detection of internalized antibody in microglia

Primary cultured microglia were treated with Abeta and intact or deglycosylated antibody as described above. Internalization of intact and deglycosylated antibodies into the intracellular space was examined. Fixed cells were incubated with an Alexa 488-labeled goat anti-mouse IgG antibody (4 μg/mL; Molecular Probes) and then the fluorescence was observed using a laser scanning confocal microscope.

For biological examination, cells were collected after 12 h of treatment, and then levels of heavy and light chain in cell lysate were determined by immunoblotting using an HRP-coupled sheep anti-mouse IgG antibody (1 : 1000). Bands were visualized using a chemiluminescent kit and the bands were densitometrically analysed using NIH image 1.56 software.

Cytokine release in response to antibody treatment in primary cultured microglia

To determine cytokine release in response to antibody treatment, we collected culture medium after treatment (see above). Levels of tumor necrosis factor-alpha (TNFalpha) were determined using an ELISA kit (Biosource International, Camarillo, CA, USA).

Plasma Abeta elevation (Abeta sequestration) in vivo

All *in vivo* animal experimental procedures were reviewed and approved by the Animal Care and Use Committee of Georgetown University Medical Center. Triple transgenic mice expressing mutant amyloid precursor protein (APP), presenilin-1 and tau (Oddo *et al*., 2003) at 13 ± 1 weeks of age ($n = 5$ in each group) and 21 months ± 2 weeks of age ($n = 6$ in each group) were used. Mice at 13 weeks of age are free of Abeta plaques, while mice at 21 months of age have Abeta plaques in the hippocampus and cerebral cortex. Blood was collected from the tail vein, mixed with EDTA, and plasma was prepared after brief centrifugation. Blood was collected 3 days prior to the injection, and used to determine the baseline level of Abeta. Deglycosylated and intact antibodies (50 or 250 μg IgG per mouse for mice at 13 weeks and 21 months of age, respectively) were injected intravenously into the tail vein. Blood was collected at 24 and 48 h after the injection from the mice at prepathological stage, and at 24 h and 120 h (5 days) after the injection from the mice bearing Abeta plaques. EDTA-treated plasma was prepared and plasma Abeta levels were determined using the ELISA as described above.

Determination of antibody levels in the plasma

A 96-well Maxisorp plate (Nunc, Rochester, NY, USA) was coated with 500 ng Abeta per well, and non-specific binding was blocked with Block Ace (Serotec, Oxford, UK). The brain homogenate and plasma were incubated overnight. Known amounts of intact and deglycosylated anti-Abeta antibody (25 pg to 25 ng IgG per well) were mixed in non-transgenic mouse brain homogenate or plasma, and used to draw the standard curve. The captured anti-Abeta antibody was detected by HRP-coupled anti-mouse IgG, and visualized using TMB as a substrate (Pierce, Rockford, IL, USA).

Statistical analysis

Statistical significance of differences was determined by analysis of variance (ANOVA) followed by Bonferroni/Dunn *post-hoc* tests (StatView, Abacus Concepts, Berkeley, CA, USA).

Results

Deglycosylation was complete, and the deglycosylated antibodies retained affinity to Abeta

The molecular masses of the intact 82E1 and 6E10 antibodies were determined to be 151 492 and 149 491 Da, respectively (Fig. 1A and C, respectively). After deglycosylation, 82E1 and 6E10 IgG peaks were shifted to 147 653 and 146 877 Da, respectively (Fig. 1B and D, respectively). Doubly charged ions were observed around *m/z* 75 000 Da (half of the mass-to-charge ratio of the primary peaks). Gel electrophoresis indicated the molecular shift of heavy chain of both 82E1 and 6E10 antibodies after deglycosylation (Fig. 1E and F, respectively). After deglycosylation, an isolated peak of untreated intact antibody was not detectable. However, the ion

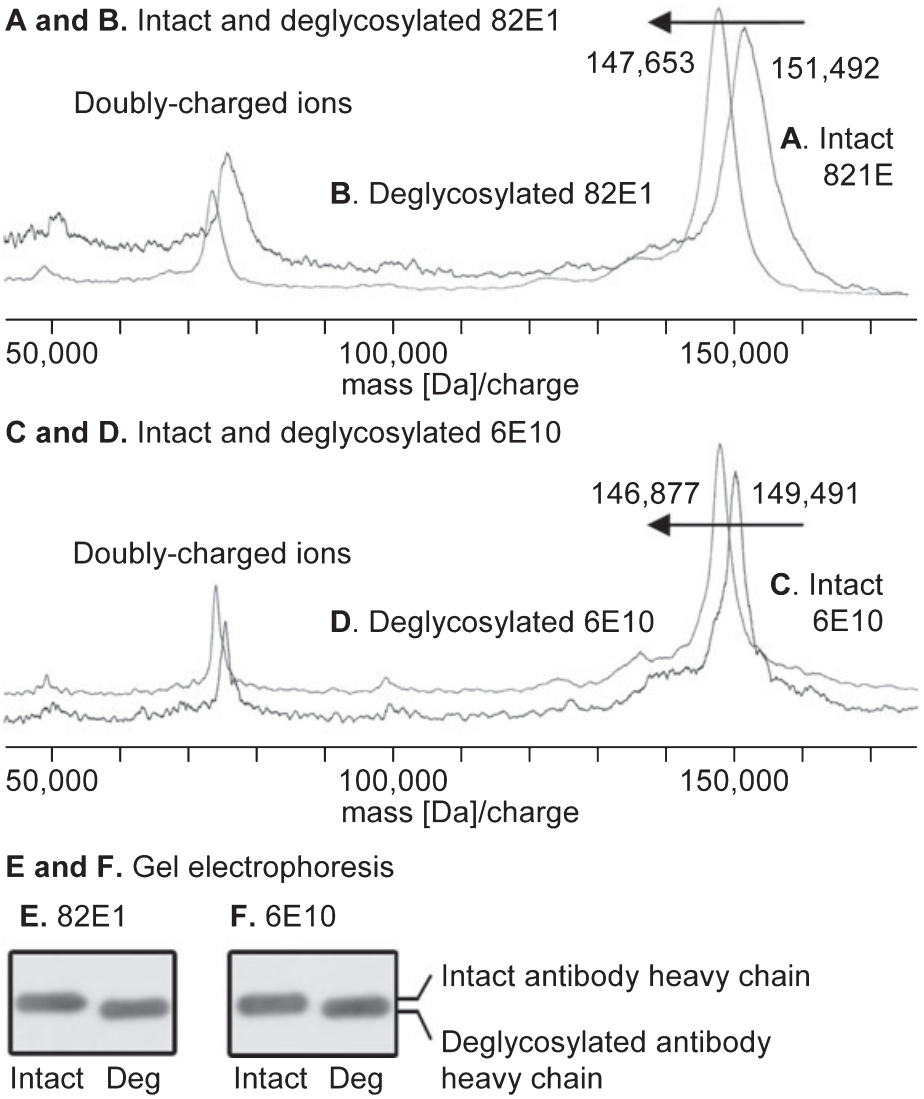


FIG. 1. Validation of deglycosylation. Two anti-Abeta antibodies, clones 82E1 (epitope: 1–5) and 6E10 (epitope: 3–8), were enzymatically deglycosylated and deglycosylation was validated by MALDI-TOF mass spectrometry (A/B and C/D, respectively) and immunoblotting (E and F, respectively). Mass spectra of the intact (A and C) and deglycosylated (B and D) antibodies are overlaid. IgG peaks, 151 492 Da (A, 82E1) and 149 491 Da (C, 6E10), were shifted toward lower molecular mass, 147 653 Da (B, 82E1) and 146 877 Da (D, 6E10), after deglycosylation. Doubly charged ions were observed around 75 kDa (half of the mass-to-charge ratio of the primary peaks). Immunoblotting of heavy chain confirmed that molecular mass was shifted lower after deglycosylation (E and F; Intact, intact antibody; Deg, deglycosylated antibody).

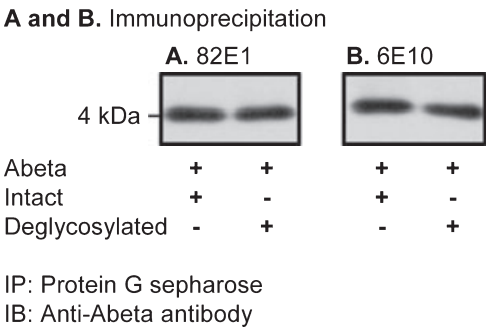
peak of the deglycosylated antibody displayed hydration shoulders that could mask relatively minor peaks of residual intact antibody. We estimate the maximum possible contamination of intact antibody to be less than 5%. More importantly, the effects of deglycosylated antibodies are similar to negative controls and significantly different from intact antibodies in functional assays (see Figs 3–6). Therefore, we conclude that contamination of untreated intact antibody is minimal. We did not investigate the residual sugar after deglycosylation in this study, but detailed analysis was previously done using N-glycanase and IgG1, the same subclass as clones 82E1 and 6E10 (Krapp *et al*., 2003).

To confirm that the deglycosylated antibodies retained their ability to bind Abeta, Abeta and intact or deglycosylated antibodies were mixed in PBS and precipitated the immunocomplex. Abeta peptide was co-precipitated with both intact and deglycosylated antibodies (Fig. 2A and B), i.e. deglycosylation of these antibodies did not inhibit their ability to bind Abeta.

We also determined the Abeta binding affinity of deglycosylated and intact antibodies using BIAcore. Both intact and deglycosylated antibodies showed comparable Abeta binding affinity (Fig. 2C). Thus, deglycosylation of antibodies did not affect their affinity and ability to capture Abeta.

In contrast to intact antibodies, deglycosylated antibodies had no effect on microglial phagocytosis of Abeta

We previously determined the forms of Abeta used in this study with immunoblotting and detected a mixture of oligomeric and fibrillar Abeta (Takata *et al*., 2003). We tested the effects of antibody deglycosylation on microglial phagocytosis using primary cultured microglia. We treated microglia with Abeta plus intact or deglycosylated antibody, and assessed microglial phagocytosis of Abeta. Microglia rapidly changed morphology when Abeta was added (Fig. 3A–E, 0 vs. 3 h). Microglial phagocytosis of Abeta started



C. BIAcore-determined affinity

	Affinity against Abeta [K_D]	
	Intact	Deglycosylated
82E1	0.9	1.0
6E10	7.4	9.2

FIG. 2. Deglycosylated antibodies retained the ability to bind to Abeta. Binding capacity of deglycosylated antibodies to Abeta was confirmed by immunoprecipitation (A and B), and the binding affinity was determined using BIAcore (C). Deglycosylated antibodies captured Abeta peptides as efficiently as intact antibodies (A and B). BIAcore analysis indicated that deglycosylation did not alter Abeta binding affinity (C).

gradually, becoming evident by 12 h (Fig. 3A at 12 h). Intact antibody significantly enhanced Abeta phagocytosis (Fig. 3B and D at 12 h), whereas deglycosylated antibody had no effect compared with vehicle treatment (Fig. 3C and E vs. Fig. 3A at any time point). To ensure that Abeta localizes within the intracellular space, we re-constructed three-dimensional images from the stacked confocal images, and found that Abeta immunoreactivity (visualized in green, Fig. 3G–I) was within the cellular structure outlined using an actin marker, phalloidin (visualized in red, Fig. 3G–I). In our previous study, we also generated a video and confirmed that Abeta was located in the intracellular space (Takata *et al.*, 2007, video as part of their supplementary data). Quantitation of these images demonstrated that intact antibodies significantly enhanced microglial phagocytosis of Abeta (Fig. 3J, $P < 0.001$ and $P < 0.05$ for 82E1 and 6E10, respectively); the phagocytic responses to intact and deglycosylated antibodies were significantly different (Fig. 3J, $P < 0.0001$ and $P < 0.05$ for 82E1 and 6E10, respectively). There was no difference in Abeta phagocytosis between vehicle and deglycosylated antibody treatment (Fig. 3J, open circles vs. open triangle).

We also determined Abeta levels in cell lysate after phagocytosis using ELISA. ELISA results indicated that intact antibodies, both clone 82E1 and clone 6E10, significantly enhanced Abeta phagocytosis (Fig. 4, $P < 0.001$ compared with vehicle control). In contrast, deglycosylated antibodies did not promote Abeta phagocytosis (Fig. 4, $P < 0.001$ and $P < 0.01$ compared with intact 82E1 and 6E10, respectively).

We were concerned that Abeta/anti-Abeta antibodies (82E1 and 6E10) complexes may have interfered with Abeta ELISA quantitation. In this study, we used our 12B2/1C3 ELISA (Horikoshi *et al.*, 2004). Abeta was captured by a C terminus antibody (1C3, epitope 38–42) and detected by HRP-coupled 12B2, which binds to an epitope within Abeta amino acid residues 17–28. The study antibodies, clones 6E10 and 82E1, bind to epitopes within 1–5 and 3–8 amino acid residues, respectively. Thus, the epitopes of the study antibodies and the ELISA antibodies do not overlap. ELISA data indicated Abeta levels of 100 ± 7% when various amounts of study antibodies were added to the Abeta peptide, confirming that Abeta quantitation using 12B2/1C3 ELISA was not compromised by the study antibodies.

Deglycosylated antibody/Abeta immunocomplex was significantly less internalized into microglia compared with intact antibody

To ensure that deglycosylation alters Fc receptor-mediated antigen (Abeta)/anti-Abeta antibody uptake, we examined levels of antibody in the intracellular space of microglia. Levels of intact antibody were gradually increased in the intracellular space after treatment (Fig. 5B), while levels of deglycosylated antibody in the intracellular space was significantly lower than intact antibody-treated cells (Fig. 5C).

We further examined the levels of internalized antibody in the cell lysate. SDS-PAGE dissociates heavy and light chains and two bands appeared on SDS-PAGE. While both heavy and light chains were evident in the cell lysate after treatment with intact antibodies, levels of heavy and light chains were significantly lower after treatment with deglycosylated antibodies (Fig. 5D and E).

In contrast to intact antibody, deglycosylated antibody had no effect on cytokine levels

We measured the effects of intact and deglycosylated antibodies on cytokine levels using primary cultured microglia. TNFalpha was detected in microglia culture medium even without Abeta stimulation. Treatment with the intact antibody significantly increased TNFalpha levels (Fig. 6A, $P < 0.001$). However, deglycosylated antibody did not affect TNFalpha levels (Fig. 6A, significant reduction compared with the intact antibody, $P < 0.001$).

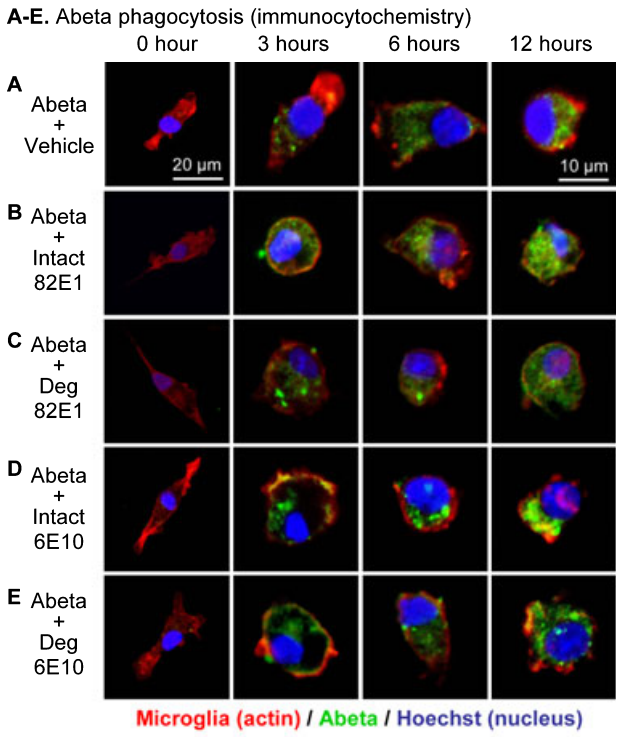
Treatment with Abeta increased TNFalpha levels (open columns in Fig. 6A vs. Fig. 6B). The intact antibody further enhanced TNFalpha levels (Fig. 6B, $P < 0.001$ compared with vehicle-treated controls). TNFalpha levels were significantly lower in deglycosylated antibody-treated microglia ($P < 0.001$ compared with treatment with intact antibody, and similar to control).

As the synthetic Abeta peptide used in this study was not manufactured under Good Manufacturing Practices (GMP), the manufacturer was unable to assure pyrogen-free status. Abeta peptide pre-incubated with polymyxin B, which traps lipolysaccharide (LPS) and blocks LPS-induced microglial reactions (Kakimura *et al.*, 2002), did not alter TNFalpha level (data not shown). Thus, the Abeta used in this study is pyrogen-free.

Deglycosylated antibodies elevate plasma Abeta levels to a similar or greater extent than intact antibodies in transgenic mice without and with Abeta plaque pathology

We investigated the potency of deglycosylated antibodies in Abeta sequestration. Deglycosylated and intact antibodies were intravenously administered to an AD mouse model and plasma Abeta level was determined (Fig. 7). We used mice at 13 weeks and 21 months of age (Fig. 7A and B, and Fig. 7C, respectively). At 13 weeks of age, no plaque was detectable. At 21 months of age, Abeta plaques

were widely detected in the cerebral cortex and hippocampus. Both intact and deglycosylated antibodies elevated plasma Abeta at both pathological stages ($P < 0.001$ compared with the baseline level). Although deglycosylated 82E1 antibody elevated plasma Abeta



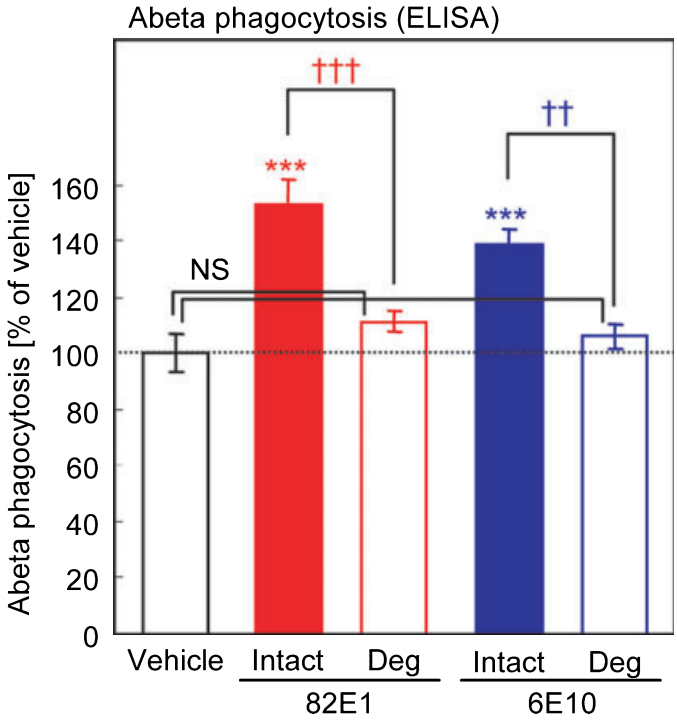


FIG. 4. Deglycosylated antibodies significantly reduced Abeta microglial phagocytosis (ELISA quantification). Abeta levels in the cell lysate, i.e. phagocytosed Abeta, were determined using an ELISA. The intact antibodies significantly enhanced Abeta phagocytosis (***$P < 0.001$ using ANOVA, compared with vehicle-treated control), while deglycosylated antibodies (Deg) significantly reduced Abeta phagocytosis ($^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$ using ANOVA, compared with intact antibodies). There was no difference between vehicle- and deglycosylated antibody-treated groups.

significantly more than the intact antibody at both pathological stages (Fig. 7A and C, $P < 0.05$ and < 0.01, respectively), intact and deglycosylated 6E10 antibodies yielded virtually identical results (Fig. 7B).

Deglycosylated antibodies have short-term pharmacokinetics similar to the intact antibodies

We tested whether deglycosylation of antibody affected the stability in blood or brain penetrations. The assay system we developed had a

FIG. 3. Deglycosylated antibodies did not evoke microglial phagocytosis in primary cultured microglia (cytochemical analysis). Effects of the intact and deglycosylated antibodies in microglial phagocytosis was determined using primary cultured microglia (> 97% pure as determined by CD11b immunostaining). (A–E) Primary cultured microglia were treated with Abeta and intact or deglycosylated (Deg) antibody, and immunostained with a rabbit polyclonal anti-Abeta antibody (green). Cellular structure was visualized using markers for actin (phalloidin, red) and the nucleus (Hoechst 33258, blue). Morphology of microglia rapidly changed after Abeta was added. Abeta was gradually internalized into the intracellular space of the microglia. Scale bars = 20 and 10 μm for 0, and 3, 6 and 12 h, respectively. (G–I) To ensure that visualized Abeta is within the intracellular space, we reconstructed three-dimensional data from the stacked confocal image (G). The constructed image was viewed from different angles (H and I). (J) Abeta uptake was quantitated by image analysis. While both 82E1 and 6E10 intact antibodies significantly enhanced Abeta phagocytosis (***$P < 0.001$, *$P < 0.05$, respectively, using ANOVA), microglial Abeta phagocytosis was significantly reduced by deglycosylated antibodies compared with intact antibodies ($^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger}P < 0.05$, respectively, using ANOVA). NB: In panel F, data points are slightly shifted along the *x*-axis at each time point to avoid overlap of points.

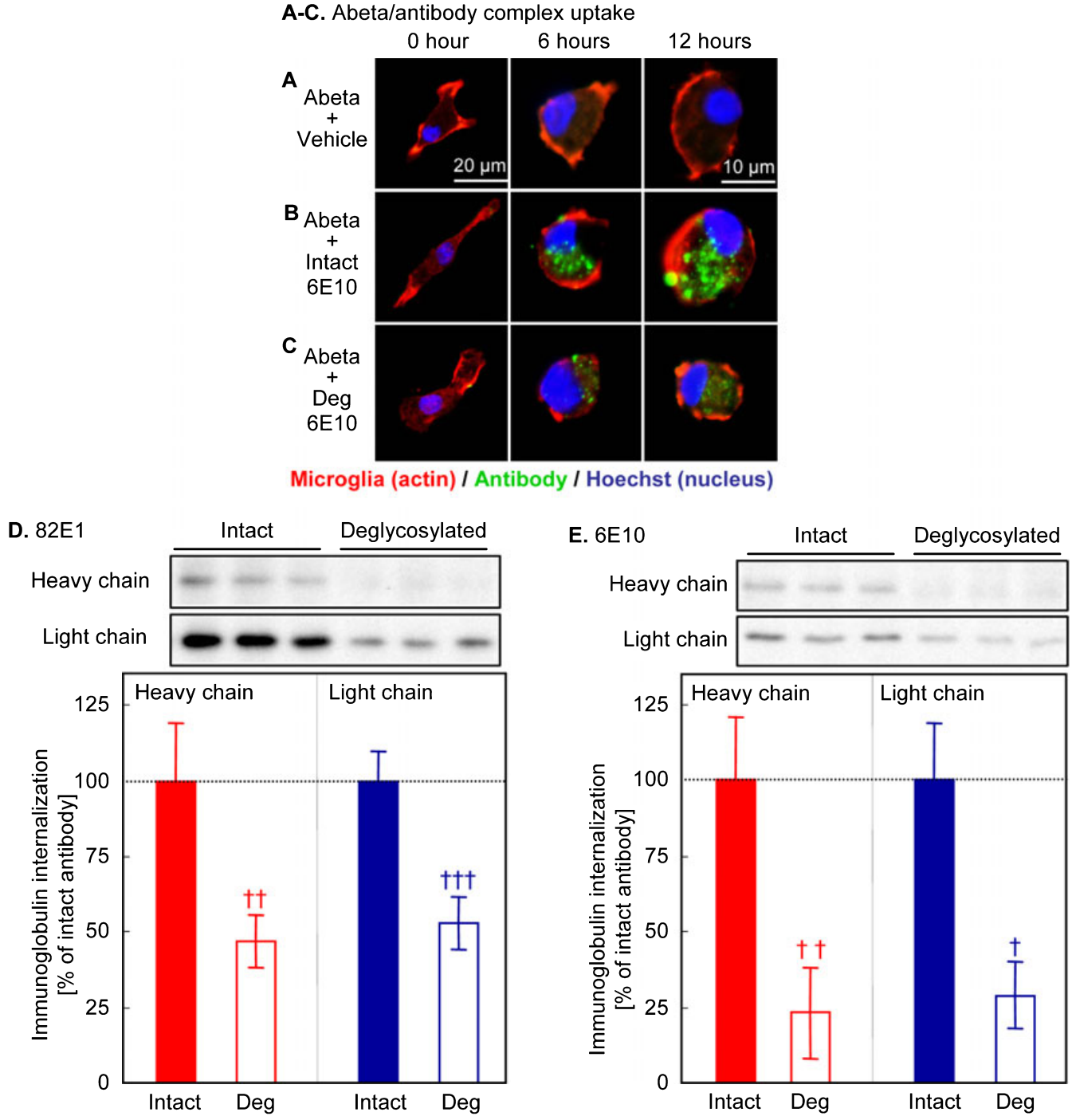


FIG. 5. Deglycosylated antibody/Abeta immunocomplex was significantly less internalized into the microglia compared with intact antibody/Abeta immunocomplex. Fc receptor-mediated Abeta/antibody complex uptake was examined. (A–C) Primary cultured microglia were treated with Abeta and intact or deglycosylated antibody, and intact and deglycosylated antibodies were visualized using anti-mouse IgG antibody (green). Cellular structure was visualized using markers for actin (phalloidin, red) and the nucleus (Hoechst 33258, blue). Intact antibody was detected significantly more in the intracellular space, compared with deglycosylated antibody (B vs. C at 6 and 12 h). (D and E) Levels of internalized antibodies were determined in the cell lysate. Cell lysate was run on a gel and levels of heavy and light chains were quantified using immunoblotting. Deglycosylated antibodies (Deg) were internalized less than intact antibodies ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ using ANOVA).

linear standard curve over the range 10 pg to 10 ng IgG per well of a 96-well microplate ($r^2 > 0.99$). Both intact and deglycosylation antibodies showed comparable kinetics up to 1 week after the injection, suggesting that deglycosylation does not alter antibody stability in the short term (Fig. 8). We also determined the presence of antibodies in the brain at the terminal study point (48 h after administration in mice without Abeta plaque pathology). The intact and deglycosylated antibodies showed similar brain entry, 0.06 and 0.10% in the case of intact and deglycosylated 82E1, and 0.05 and 0.08% in the case of intact and deglycosylated 6E10, respectively.

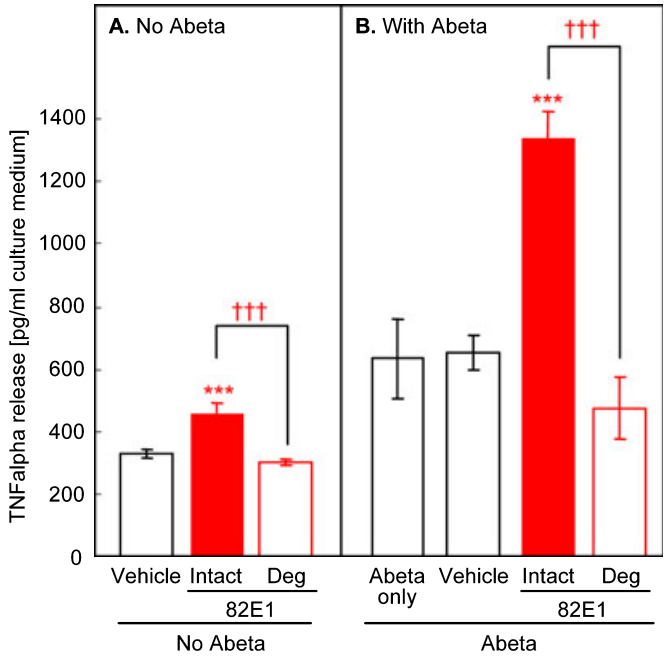


FIG. 6. Deglycosylated antibody does not enhance microglial cytokine production, while intact antibody does so significantly. Effects of intact and deglycosylated antibodies (Deg) on TNFalpha levels were examined using primary cultured microglia. Microglia were treated with intact or deglycosylated antibody, without or with Abeta (A and B, respectively), and the level of TNFalpha in culture medium was determined. (A) Intact antibody significantly increased TNFalpha levels (***$P < 0.001$), while deglycosylated antibody did not (significant reduction compared with treatment with intact antibody, $^{\dagger\dagger\dagger}P < 0.001$). (B) Abeta treatment elevated TNFalpha level, and intact antibody caused a further increase (***$P < 0.001$). TNFalpha level after treatment with deglycosylated antibody was slightly lower than the vehicle-treated control level (significant reduction compared with treatment with intact antibody, $^{\dagger\dagger\dagger}P < 0.001$). Statistical significance was determined by ANOVA.

Discussion

Abeta is generated from a parental molecule, APP, by sequential proteolytic cleavage at the N and C termini of the Abeta domain by beta and gamma secretases, respectively. Therefore, beta and gamma secretase inhibitors are aggressively being pursued as therapeutic targets (Pollack & Lewis, 2005). However, it is not yet certain that secretase inhibition will be sufficient to halt AD neurodegeneration, and combination therapy with other Abeta-lowering approaches may be required. Enhancement of Abeta metabolism and clearance represents an alternative approach to lowering brain Abeta level. Several enzymes, such as neprilysin, insulin-degrading enzyme and plasmin, are known to degrade Abeta (Selkoe, 2001). However, Abeta is not the sole substrate of these enzymes, and it may be difficult to enhance Abeta degradation specifically. Another plausible approach is the enhancement of Abeta clearance (Zlokovic, 2004). Antibodies present in the blood significantly enhance Abeta transfer from the brain to the periphery (Abeta sequestration) (DeMattos *et al.*, 2001; Lemere *et al.*, 2003). In addition, simple Abeta binding agents present in the blood reduce brain Abeta load without entering the brain (Matsuoka *et al.*, 2003; Bergamaschini *et al.*, 2004; Park *et al.*, 2006).

The ideal molecular properties for therapeutic agents based on Abeta sequestration are not yet known, but Abeta binding affinity is

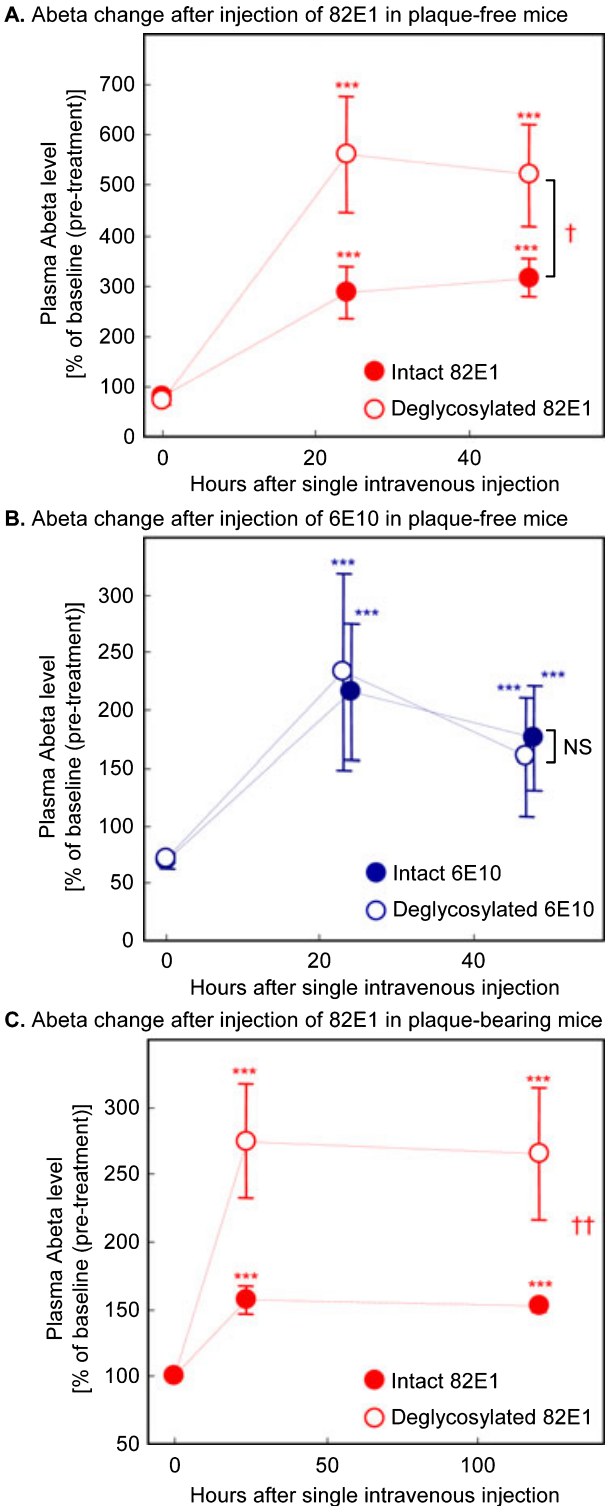


FIG. 7. Deglycosylated antibodies elevate plasma Abeta to a similar or greater level compared with intact antibodies in a transgenic mouse model of AD. Intact and deglycosylated antibodies were intravenously administered to an AD mouse model, triple transgenic mice (Oddo *et al.*, 2003). Mice without any Abeta plaque pathology, at 13 weeks of age (A and B), and mice bearing Abeta plaques, at 21 months of age (C), were used. Plasma Abeta change was determined using pretreatment level as the baseline. All antibodies significantly elevated plasma Abeta (***$P < 0.001$ compared with the baseline). In the case of 82E1, deglycosylated antibody elevated plasma Abeta significantly more at both pathological stages (A and C, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the intact antibody). Intact and deglycosylated 6E10 antibodies showed virtually identical change (B). NB: In panel B, data points are slightly shifted along the *x*-axis at each time point to avoid overlap of points.

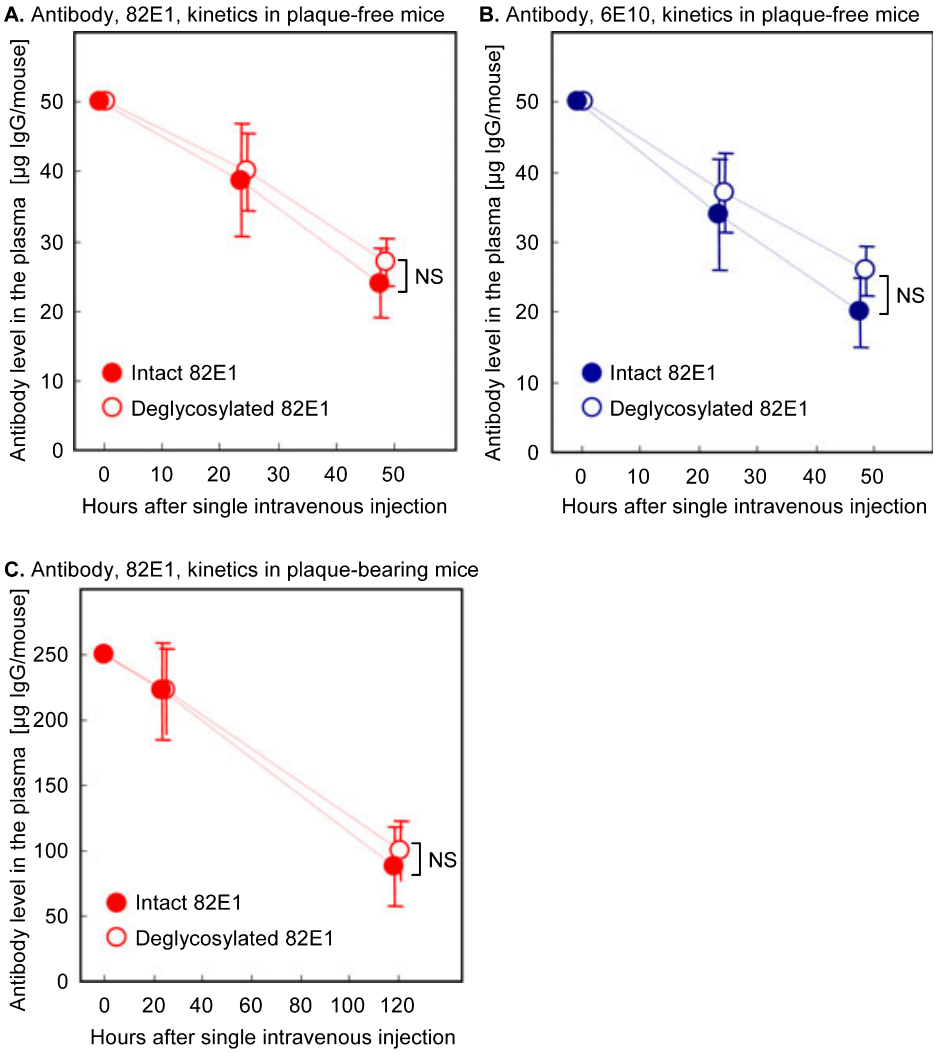


FIG. 8. Kinetics of intact and deglycosylated antibodies. Intact or deglycosylated antibodies were intravenously administered into mice without or with Abeta plaque pathology, and levels of antibodies in the blood were determined. NB: In panels A–C, data points are slightly shifted along the *x*-axis at each time point to avoid overlap of points.

presumably important. Good antibodies achieve subnanomolar affinity, but it is very challenging to develop low-molecular-weight drug candidates that interact with the primary structure of the peptide at similarly high affinity. Currently available Abeta binding agents, such as Congo red, thioflavin T and their derivatives, have limited affinity to non-aggregated Abeta, which is the target of Abeta sequestration; their potency to induce Abeta sequestration is limited (Matsuoka *et al.*, 2005). Intact antibody has high affinity, but evokes immune responses that may be undesirable. The glycan portion of IgG is critically involved in binding to immune response effectors including FcR (Heyman, 2000; Radaev & Sun, 2001), complement C1q (Winkelhake *et al.*, 1980) and others (Nose & Wigzell, 1983). Here, we confirmed that deglycosylation of anti-amyloid antibodies did not affect their binding affinity to Abeta.

Deglycosylated antibodies showed limited ability to activate immortalized microglia, BV-2, *in vitro* (Rebe & Solomon, 2005). In addition, chronic treatment with deglycosylated antibody against the C terminus of Abeta 40, in comparison with intact antibody, is associated with reduced accumulation of CD45-immunopositive activated microglia surrounding plaques and improved cognitive performance in an AD model mouse *in vivo* (Carty *et al.*, 2006; Wilcock *et al.*, 2006). These previous studies examined the effects of deglycosylation of antibody on microglial activation. However, studies of the activation of microglia may not fully evaluate Abeta phagocytosis and other neuroinflammatory events such as cytokine production. In this study, we quantitatively investigated the effects of deglycosylated antibodies against the Abeta N terminus on microglial phagocytosis and cytokine production using primary cultured microglia. We found that deglycosylated antibody did not increase Abeta phagocytosis or cytokine release above the control level. Brain immune responses are not involved in amyloid reduction via an Abeta sequestration approach, and immune inactive deglycosylated antibody therefore represents a promising therapeutic candidate. Although primary cultured microglia are the most physiologically relevant cultured cells, they are primed compared with resident microglia in the intact brain *in vivo* (Streit *et al.*, 1999). In response to Abeta treatment, cultured microglia rapidly change morphology from rod- to ameboid-type (Takata *et al.*, 2003), suggesting that primary cultured microglia maintain the ability to respond to insults. However, primary cultured microglia may be less sensitive to stimuli due to their primed status.

In addition to potent pharmacological effects, therapeutic candidates should have favorable pharmacokinetics. While brain penetration is a

major issue for central nervous system-acting drugs, Abeta sequestering agents act in the periphery. In the present study, we preliminarily found that deglycosylation of antibody did not alter kinetics in blood up to 1 week. Further study is required, but this result suggests that deglycosylated antibodies are likely to be effective in sequestering Abeta using dosing regimens similar to those of intact antibodies.

In Abeta sequestration, there is enhanced Abeta efflux from the brain to the periphery, where the sequestered Abeta forms complexes with the administered agent. Unbound Abeta (not complexed to an Abeta binding agent) is cleared from the body within 10 min (Kandimalla *et al*., 2005). Sequestration agents including anti-Abeta antibodies apparently stabilize Abeta in the blood by prolonging the clearance process. No adverse effects of high plasma Abeta have been reported, but this may be a potential problem with antibody-mediated sequestration.

In this study, we compared two anti-Abeta antibodies, clones 82E1 and 6E10. Both intact and deglycosylated 82E1 showed more significant Abeta elevation compared with clone 6E10. Clone 82E1 is specific for Abeta and not cross-reactive with uncleaved APP (Horikoshi *et al*., 2004), while clone 6E10 is fully cross-reactive. APP is more abundant than cleaved Abeta, and Abeta-specific (non-APP cross-reactive) antibodies are more efficient in Abeta sequestration. While plasma Abeta elevation in the mice treated with intact and deglycosylated 6E10 antibodies was virtually identical, deglycosylated 82E1 elevated plasma Abeta more than intact antibody in mice at two different pathological stages.

Although the active immunization clinical trial was terminated at an early stage, follow-up studies indicate that patients who received the Abeta immunization showed apparent reduction of Abeta plaque load (Nicoll *et al*., 2003), and subjects who had an immune response to the immunization showed slowing of cognitive decline (Hock *et al*., 2003). Because most generated antibodies remain in the periphery, presumably the therapeutic benefit was derived at least partially through Abeta sequestration. Although brain penetration is limited, peripherally administered antibodies do enter the brain. Transgenic mouse studies indicate 0.05–0.10% brain entry and clinical trials provide similar results in humans. Anti-Abeta antibodies enhanced microglial phagocytosis of Abeta in an *ex vivo* assay (Bard *et al*., 2000). After active immunization, an association of plaque clearance and microglial accumulation was documented (Nicoll *et al*., 2003). Furthermore, we and another group have recently demonstrated that transplantation of primary cultured microglia (Takata *et al*., 2007) and bone marrow-derived microglia cells (Simard *et al*., 2006) reduced brain Abeta plaque load in transgenic mice *in vivo*. Therefore, microglial Abeta phagocytosis may be a potent approach for Abeta clearance. However, overactivation of microglia occurs when Abeta pathology is overwhelmed, and may be accompanied by deleterious neuroinflammation, which needs to be carefully evaluated (McGeer & McGeer, 2001).

Here we have shown that deglycosylated antibody represents a simple Abeta binding agent that does not induce phagocytosis and cytokine production. Recently, chronic treatment with another deglycosylated anti-Abeta C terminus antibody reduced brain Abeta load and improved cognitive function (Carty *et al*., 2006; Wilcock *et al*., 2006). Mutations of presenilin-1, which are linked to familial AD, alter the ratio of Abeta 40/42, suggesting that the Abeta 40/42 ratio, rather than the absolute levels of Abeta 40 and 42, is critical to AD pathogenesis (Borchelt *et al*., 1996). Recent biomarker studies also suggest that the Abeta 40/42 ratio may be more important than the absolute levels (Graff-Radford *et al*., 2007). In our recent study, Abeta 40 antibody altered plasma Abeta 1–40 level specifically, with no effect on Abeta 1–42 level (Gray *et al*., 2007); thus Abeta 40-specific antibody likely alters the Abeta 40/42 ratio. In the present study, we found that deglycosylated Abeta N terminus antibody, which does not differentiate between Abeta 1–40 and Abeta 1–42 (and therefore should not alter the ratio of the peptides), also induces Abeta efflux from the brain (Abeta sequestration). Microhemorrhage, another critical adverse event associated with active immunization, was significantly reduced by deglycosylated antibody (Carty *et al*., 2006; Wilcock *et al*., 2006). Taken together, the evidence indicates that deglycosylated antibody against the N terminus of Abeta may be a potent therapeutic agent for AD with a good safety profile.

Acknowledgements

We thank Dr Bill Rebeck, Department of Neuroscience, Georgetown University, for critical reading of the manuscript. Antibodies used for Abeta ELISA were generously provided by Drs Yuko Horikoshi and Noriaki Kinoshita (Immuno-Biological Laboratories, Takasaki, Gunma, Japan). This study was supported by National Institute on Health grants AG026478 (Y.M.) and AG022455 (Y.M.), Alzheimer's Association grant IIRG-02-3815 (Y.M.), Japan Society for Promotion of Science grant (KAKENHI) (K.T., Y.K.), and National Institute on Ageing Intramural Research Program (M.P.M.).

Abbreviations

Abeta, amyloid beta; AD, Alzheimer's disease; APP, amyloid precursor protein; FcR, Fc receptor; HRP, horseradish peroxidase; Ig, immunoglobulin; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; TNFalpha, tumor necrosis factor-alpha.

References

Bard, F., Cannon, C., Barbour, R., Burke, R.L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Lieberburg, I., Motter, R., Nguyen, M., Soriano, F., Vasquez, N., Weiss, K., Welch, B., Seubert, P., Schenk, D. & Yednock, T. (2000) Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat. Med.*, **6**, 916–919.

Bergamaschini, L., Rossi, E., Storini, C., Pizzimenti, S., Distaso, M., Perego, C., De Luigi, A., Vergani, C. & Grazia, D.S. (2004) Peripheral treatment with enoxaparin, a low molecular weight heparin, reduces plaques and beta-amyloid accumulation in a mouse model of Alzheimer's disease. *J. Neurosci.*, **24**, 4181–4186.

Borchelt, D.R., Thinakaran, G., Eckman, C.B., Lee, M.K., Davenport, F., Ratovitsky, T., Prada, C.M., Kim, G., Seekins, S., Yager, D., Slunt, H.H., Wang, R., Seeger, M., Levey, A.I., Gandy, S.E., Copeland, N.G., Jenkins, N.A., Price, D.L., Younkin, S.G. & Sisodia, S.S. (1996) Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio in vitro and in vivo. *Neuron*, **17**, 1005–1013.

Carty, N.C., Wilcock, D.M., Rosenthal, A., Grimm, J., Pons, J., Ronan, V., Gottschall, P.E., Gordon, M.N. & Morgan, D. (2006) Intracranial administration of deglycosylated C-terminal-specific anti-Abeta antibody efficiently clears amyloid plaques without activating microglia in amyloid-depositing transgenic mice. *J. Neuroinflammation*, **2**, Act. 1.

Das, P., Howard, V., Loosbrock, N., Dickson, D., Murphy, M.P. & Golde, T.E. (2003) Amyloid-beta immunization effectively reduces amyloid deposition in FcRgamma-/- knock-out mice. *J. Neurosci.*, **23**, 8532–8538.

DeMattos, R.B., Bales, K.R., Cummins, D.J., Dodart, J.C., Paul, S.M. & Holtzman, D.M. (2001) Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. *Proc. Natl Acad. Sci. USA*, **98**, 8850–8855.

DeMattos, R.B., Bales, K.R., Cummins, D.J., Paul, S.M. & Holtzman, D.M. (2002a) Brain to plasma amyloid-beta efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. *Science*, **295**, 2264–2267.

DeMattos, R.B., Bales, K.R., Parsadanian, M., O'Dell, M.A., Foss, E.M., Paul, S.M. & Holtzman, D.M. (2002b) Plaque-associated disruption of CSF and plasma amyloid-beta (Abeta) equilibrium in a mouse model of Alzheimer's disease. *J. Neurochem.*, **81**, 229–236.

Ferrer, I., Boada, R.M., Sanchez Guerra, M.L., Rey, M.J. & Costa-Jussa, F. (2004) Neuropathology and pathogenesis of encephalitis following amyloid-beta immunization in Alzheimer's disease. *Brain Pathol.*, **14**, 11–20.

Graff-Radford, N.R., Crook, J.E., Lucas, J., Boeve, B.F., Knopman, D.S., Ivnik, R.J., Smith, G.E., Younkin, L.H., Petersen, R.C. & Younkin, S.G. (2007) Association of low plasma Abeta42/Abeta40 ratios with increased imminent risk for mild cognitive impairment and Alzheimer disease. *Arch. Neurol.*, **64**, 354–362.

Gray, A.J., Sakaguchi, G., Shiratori, C., Becker, A.G., LaFrancois, J., Aisen, P.S., Duff, K. & Matsuoka, Y. (2007) Antibody against C-terminal Abeta selectively elevates plasma Abeta. *Neuroreport*, **18**, 293–296.

Hardy, J. & Selkoe, D.J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*, **297**, 353–356.

Heyman, B. (2000) Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annu. Rev. Immunol.*, **18**, 709–737.

Hock, C., Konietzko, U., Streffer, J.R., Tracy, J., Signorell, A., Muller-Tillmanns, B., Lemke, U., Henke, K., Moritz, E., Garcia, E., Wollmer, M.A., Umbricht, D., de Quervain, D.J., Hofmann, M., Maddalena, A., Papassotiropoulos, A. & Nitsch, R.M. (2003) Antibodies against beta-amyloid slow cognitive decline in Alzheimer's disease. *Neuron*, **38**, 547–554.

Horikoshi, Y., Sakaguchi, G., Becker, A.G., Gray, A.J., Duff, K., Aisen, P.S., Yamaguchi, H., Maeda, M., Kinoshita, N. & Matsuoka, Y. (2004) Development of Abeta terminal end-specific antibodies and sensitive ELISA for Abeta variant. *Biochem. Biophys. Res. Commun.*, **319**, 733–737.

Kakimura, J., Kitamura, Y., Takata, K., Umeki, M., Suzuki, S., Shibagaki, K., Taniguchi, T., Nomura, Y., Gebicke-Haerter, P.J., Smith, M.A., Perry, G. & Shimohama, S. (2002) Microglial activation and amyloid-beta clearance induced by exogenous heat-shock proteins. *FASEB J.*, **16**, 601–603.

Kandimalla, K.K., Curran, G.L., Holasek, S.S., Gilles, E.J., Wengenack, T.M. & Poduslo, J.F. (2005) Pharmacokinetic analysis of the blood–brain barrier transport of 125I-amyloid beta protein 40 in wild-type and Alzheimer's disease transgenic mice (APP,PS1) and its implications for amyloid plaque formation. *J. Pharmacol. Exp. Ther.*, **313**, 1370–1378.

Kim, K.S., Miller, D.L., Sapienza, V.J., Chen, C.M.J., Bai, C., Grundke-Iqbal, I., Currie, J.R. & Wisniewski, H.M. (1988) Production and characterization of monoclonal antibodies reactive to synthetic cerebrovascular amyloid peptide. *Neurosci. Res. Commun.*, **2**, 121–130.

Kitamura, Y., Spleiss, O., Li, H., Taniguchi, T., Kimura, H., Nomura, Y. & Gebicke-Haerter, P.J. (2001) Lipopolysaccharide-induced switch between retinoid receptor (RXR) alpha and glucocorticoid attenuated response gene (GARG)-16 messenger RNAs in cultured rat microglia. *J. Neurosci. Res.*, **64**, 553–563.

Krapp, S., Mimura, Y., Jefferis, R., Huber, R. & Sondermann, P. (2003) Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. *J. Mol. Biol.*, **325**, 979–989.

Lemere, C.A., Spooner, E.T., LaFrancois, J., Malester, B., Mori, C., Leverone, J.F., Matsuoka, Y., Taylor, J.W., DeMattos, R.B., Holtzman, D.M., Clements, J.D., Selkoe, D.J. & Duff, K.E. (2003) Evidence for peripheral clearance of cerebral Abeta protein following chronic, active Abeta immunization in PSAPP mice. *Neurobiol. Dis.*, **14**, 10–18.

Luterman, J.D., Haroutunian, V., Yemul, S., Ho, L., Purohit, D., Aisen, P.S., Mohs, R. & Pasinetti, G.M. (2000) Cytokine gene expression as a function of the clinical progression of Alzheimer disease dementia. *Arch. Neurol.*, **57**, 1153–1160.

Matsuoka, Y., Saito, M., LaFrancois, J., Saito, M., Gaynor, K., Olm, V., Wang, L., Casey, E., Lu, Y., Shiratori, C., Lemere, C. & Duff, K. (2003) Novel therapeutic approach for the treatment of Alzheimer's disease by peripheral administration of agents with an affinity to beta-amyloid. *J. Neurosci.*, **23**, 29–33.

Matsuoka, Y., Shao, L., Debnath, M., LaFrancois, J., Becker, A., Gray, A., Aisen, P., Mathis, C., Klunk, W. & Duff, K. (2005) An Abeta sequestration approach using non-antibody Abeta binding agents. *Curr. Alzheimer Res.*, **2**, 265–268.

McGeer, P.L. & McGeer, E.G. (2001) Inflammation, autotoxicity and Alzheimer disease. *Neurobiol. Aging*, **22**, 799–809.

Nicoll, J.A., Wilkinson, D., Holmes, C., Steart, P., Markham, H. & Weller, R.O. (2003) Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. *Nat. Med.*, **9**, 448–452.

Nose, M. & Wigzell, H. (1983) Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl Acad. Sci. USA*, **80**, 6632–6636.

Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kayed, R., Metherate, R., Mattson, M.P., Akbari, Y. & LaFerla, F.M. (2003) Triple-transgenic model of Alzheimer's disease with plaques and tangles. Intracellular abeta and synaptic dysfunction. *Neuron*, **39**, 409–421.

Orgogozo, J.M., Gilman, S., Dartigues, J.F., Laurent, B., Puel, M., Kirby, L.C., Jouanny, P., Dubois, B., Eisner, L., Flitman, S., Michel, B.F., Boada, M., Frank, A. & Hock, C. (2003) Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization. *Neurology*, **61**, 46–54.

Park, J.H., Widi, G.A., Gimbel, D.A., Harel, N.Y., Lee, D.H. & Strittmatter, S.M. (2006) Subcutaneous Nogo receptor removes brain amyloid-beta and improves spatial memory in Alzheimer's transgenic mice. *J. Neurosci.*, **26**, 13279–13286.

Pollack, S.J. & Lewis, H. (2005) Secretase inhibitors for Alzheimer's disease: challenges of a promiscuous protease. *Curr. Opin. Invest. Drugs*, **6**, 35–47.

Radaev, S. & Sun, P.D. (2001) Recognition of IgG by Fcgamma receptor. The role of Fc glycosylation and the binding of peptide inhibitors. *J. Biol. Chem.*, **276**, 16478–16483.

Rebe, S. & Solomon, B. (2005) Deglycosylation of anti-beta amyloid antibodies inhibits microglia activation in BV-2 cellular model. *Am. J. Alzheimers. Dis. Other Demen.*, **20**, 303–313.

Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevert, C., Walker, S., Wogulis, M., Yednock, T., Games, D. & Seubert, P. (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature*, **400**, 173–177.

Selkoe, D.J. (2001) Clearing the brain's amyloid cobwebs. *Neuron*, **32**, 177–180.

Simard, A.R., Soulet, D., Gowing, G., Julien, J.P. & Rivest, S. (2006) Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron*, **49**, 489–502.

Streit, W.J., Walter, S.A. & Pennell, N.A. (1999) Reactive microgliosis. *Prog. Neurobiol.*, **57**, 563–581.

Takata, K., Kitamura, Y., Umeki, M., Tsuchiya, D., Kakimura, J., Taniguchi, T., Gebicke-Haerter, P.J. & Shimohama, S. (2003) Possible involvement of small oligomers of amyloid-beta peptides in 15-deoxy-delta 12,14 prostaglandin J2-sensitive microglial activation. *J. Pharmacol. Sci.*, **91**, 330–333.

Takata, K., Kitamura, Y., Yanagisawa, D., Morikawa, S., Morita, M., Inubushi, T., Tsuchiya, D., Chishiro, S., Saeki, M., Taniguchi, T., Shimohama, S. & Tooyama, I. (2007) Microglial transplantation increases amyloid-beta clearance in Alzheimer model rats. *FEBS Lett.*, **581**, 475–478.

Tarentino, A.L. & Plummer, T.H. Jr (1987) Peptide-N4-(N-acetyl-beta-glucosaminyl) asparagine amidase and endo-beta-N-acetylglucosaminidase from *Flavobacterium meningosepticum*. *Methods Enzymol.*, **138**, 770–778.

Wilcock, D.M., Alamed, J., Gottschall, P.E., Grimm, J., Rosenthal, A., Pons, J., Ronan, V., Symmonds, K., Gordon, M.N. & Morgan, D. (2006) Deglycosylated anti-amyloid-beta antibodies eliminate cognitive deficits and reduce parenchymal amyloid with minimal vascular consequences in aged amyloid precursor protein transgenic mice. *J. Neurosci.*, **26**, 5340–5346.

Winkelhake, J.L., Kunicki, T.J., Elcombe, B.M. & Aster, R.H. (1980) Effects of pH treatments and deglycosylation of rabbit immunoglobulin G on the binding of C1q. *J. Biol. Chem.*, **255**, 2822–2828.

Zlokovic, B.V. (2004) Clearing amyloid through the blood–brain barrier. *J. Neurochem.*, **89**, 807–811.