IgG-Assisted Age-Dependent Clearance of Alzheimer’s Amyloid β Peptide by the Blood–Brain Barrier Neonatal Fc Receptor

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The role of blood–brain barrier (BBB) transport in clearance of amyloid β-peptide (Aβ) by Aβ immunotherapy is not fully understood. To address this issue, we studied the effects of peripherally and centrally administered Aβ-specific IgG on BBB influx of circulating Aβ and efflux of brain-derived Aβ in APPsw+/- mice, a model that develops Alzheimer’s disease-like amyloid pathology, and wild-type mice. Our data show that anti-Aβ IgG blocks the BBB influx of circulating Aβ in APPsw+/- mice and penetrates into the brain to sequester brain Aβ. In young mice, Aβ-anti-Aβ complexes were cleared from brain to blood by transcytosis across the BBB via the neonatal Fc receptor (FcRn) and the low-density lipoprotein receptor-related protein 1 (LRP), whereas in older mice, there was an age-dependent increase in FcRn-mediated IgG-assisted Aβ BBB efflux and a decrease in LRP-mediated clearance of Aβ-anti-Aβ complexes. Inhibition of the FcRn pathway in older APPsw+/- mice blocked clearance of endogenous Aβ40/42 by centrally administered Aβ immunotherapy. Moreover, deletion of the FcRn gene in wild-type mice inhibited clearance of endogenous mouse Aβ40/42 by systemically administered anti-Aβ. Our data suggest that the FcRn pathway at the BBB plays a crucial role in IgG-assisted Aβ removal from the aging brain.

Key words: antibody; amyloid β; Alzheimer’s disease; blood–brain barrier; amyloid; transport

Introduction

Although the amyloid hypothesis of Alzheimer’s disease (AD) remains controversial, many investigators consider accumulation of neurotoxic amyloid β peptide (Aβ) in the brain as a key pathogenic event contributing to neurodegeneration (Hardy and Selkoe, 2002). The levels of Aβ in the brain are controlled by its rates of production from the larger Aβ-precursor protein (APP) and the rates of clearance (Tanzi et al., 2004; Zlokovic, 2005). According to recent studies, the blood–brain barrier (BBB) transport of Aβ critically regulates the levels of brain Aβ and particularly the receptor for advanced glycation end products (RAGE)-mediated influx of circulating Aβ (Deane et al., 2003) and the low-density lipoprotein receptor-related protein 1 (LRP)-mediated efflux of brain-derived Aβ (Shibata et al., 2000; Deane et al., 2004). Under physiological conditions, the LRP-mediated brain efflux of Aβ prevails, whereas in animal models of AD and in AD brains, downregulation of LRP in concert with upregulation of RAGE may create an unfavorable Aβ gradient across the BBB, resulting in Aβ retention in the brain (Tanzi et al., 2004; Zlokovic, 2004).

Vaccination and passive immunization to Aβ lower brain Aβ (Schenk et al., 1999; Sigurdsson et al., 2001; Das et al., 2003) and improve behavior in animal models of AD (Janus et al., 2000; Morgan et al., 2000; Dodart et al., 2002). Although clinical Aβ immunization trials in AD patients were terminated because of adverse neuroinflammatory effect (Orgogozo et al., 2003), reduced Aβ brain deposition (Nicoll et al., 2003) and slower cognitive decline (Hock et al., 2003) have been reported. How anti-Aβ IgG clears brain Aβ remains debatable. The “sink” theory suggests that the interaction of Aβ with an Aβ-specific IgG in plasma creates a concentration gradient across the BBB that promotes efflux of brain Aβ into blood, as shown by passive and active immunization studies (DeMattos et al., 2001, 2002; Deane et al., 2003; Lemere et al., 2003). Conversely, some circulating anti-Aβ antibodies cross the BBB and activate microglia-mediated Fcy receptor-dependent (FcRγ) clearance of amyloid (Bard et al., 2000) and/or the FcRγ-independent clearance (Bacska et al., 2002; Das et al., 2003). Microglia clear amyloid slowly (Frautschy et al., 1992; Paresce et al., 1997; Bacska et al., 2002), whereas microglia-independent clearance of Aβ seems to be more rapid (DeMattos et al., 2001; Wilcock et al., 2003; Deane et al., 2004) and may require efflux of Aβ across the BBB (Shibata et al., 2000; Banks et al., 2003; Tanzi et al., 2004; Zlokovic, 2004).
Here, we show in APPsw+/− mice (Hsiao et al., 1996), a model that develops AD-like amyloid pathology, that an Aβ-specific IgG prevents RAGE-mediated transport of circulating Aβ across the BBB and enters into the brain to sequester brain Aβ and promote its vigorous clearance to blood. We demonstrated that the major histocompatibility complex class I-related neonatal Fc receptor (FcRn), which is functionally and structurally distinct from the Fcγ receptors (Borvak et al., 1998; Ravetch and Bolland, 2001; Roopenian et al., 2003; Ober et al., 2004; Yoshida et al., 2004), is critical for the IgG clearance from brain and for the elimination of Aβ–anti-Aβ complexes through the BBB. We also show that FcRn-mediated IgG-assisted Aβ efflux across the BBB remains active with aging, in contrast to LRP-mediated clearance of Aβ–anti-Aβ complexes, which decreases with aging. Finally, we demonstrate that inhibition of the FcRn pathway in old APPsw+/− mice (Hsiao et al., 1996) completely blocks rapid clearance of endogenous Aβ40/42 by centrally administered Aβ immunotherapy, whereas deletion of the FcRn gene in mice inhibits clearance of endogenous mouse brain Aβ by systemically administered anti-Aβ.

Materials and Methods

Animals. C57BL/6 and APPsw+/− (transgenic Tg2576) mice were from Taconic Farms (Germantown, NY), βM−/− mice (neonatal Fc receptor light chain β2-microglobulin null mice), FcRγ−/− mice (Fcγ receptor null mice), RAP−/− (receptor-associated protein null mice), and FcRn−/− mice (neonatal Fc receptor null mice) were from The Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized by ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). All procedures were according to National Institutes of Health guidelines approved by the University Committee on Animal Resources (University of Rochester).

Reagents. Human Aβ1–40 was synthesized at the W. M. Keck Facility (Yale University, New Haven, CT), using solid-phase N-terminal butyloxycarbonyl chemistry and purified by HPLC. We used human recombinant RAP (EMD Biosciences, San Diego, CA), monoclonal mouse antibody (ab) against C-terminal domain of human LRP β-chain, which cross reacts with mouse LRP (5A6, 1:350, 5 µg/ml; EMD Biosciences), rat anti-mouse CD31 antibody (1:2000; BD PharMingen, Lexington, KY), anti-RAGE IgG F(ab)2 (20 µg/ml; Dr. Shi Du Yan, Columbia University, New York, NY), rat anti-mouse F4/80, a microglial cell marker (1:100; Serotec, Indianapolis, IN), mouse anti-human GFAP (1: 100; DakoCytomation, Glostrup, Denmark), mouse monoclonal anti-phenoloxidase (1:2500; Sigma, St. Louis, MO), and monoclonal mouse anti-human Aβ (4G8; Signet Laboratories, Dedham, MA) against amino acids 17–24 of Aβ. Monoclonal anti-FcRn (1G3) was obtained as the condition medium of cultured mouse hybridoma cell line CRL-2434 (American Type Culture Collection, Manassas, VA) as described previously (Schlachetzki et al., 2002). The FcRn blocking antibody does not interact with 4G8 in vitro, as indicated by 4G8 ELISA (see below), which showed no change in 4G8 signal in the presence of the FcRn antibody. 4G8 F(ab)2 was prepared as described previously (Pierce, Rockford, IL) and diazylated overnight to remove elution buffer and digestion medium. The monoclonal antibody 1560, which recognizes Aβ epitope 1–17, was from Chemicon (Temecula, CA). All chemicals were obtained from Sigma, except protease inhibitor (Roche, Indianapolis, IN) and Triton X-100 (Electron Microscopy Sciences, Gibbstown, NJ). 125I-1Na and 14C-inulin were from Amersham Biosciences (Little, Chalfont, UK). 99mTc was obtained from Cardinal Health 414 (Rochester, NY).

Radio-iodinated ligands. Radio-iodination of the Aβ peptide (10 µg) was performed by mild “lactoperoxidase” method (Thorell and Johanson, 1971) using 2 mCi. 125I-1Na. The mono-iodinated nonoxidized form of Aβ was purified by reverse-phase HPLC separation (Shibata et al., 2000). Typically, the specific activities were in the range of 45–65 µCi/µg peptide. 4G8 (50 µg) and 4G8 F(ab)2 (10 µg) were radio-iodinated using IODO-BEADS or IODO-GEN (Pierce) and 0.5 and 0.7 mCi 125I-Na, respectively. Typical specific activity was 3–5 µCi/µg 4G8 or 4G8 F(ab)2. 125I-Aβ40–4G8 complex was prepared by incubating 125I-Aβ40 with 4G8 at 5:1 molar ratio for 3 h at 37°C, and excess 125I-Aβ was removed by ultrafiltration. Complex stability was confirmed by Tris-tricine native PAGE and autoradiography. 125I-Aβ–4G8 complex was prepared in a similar manner.

Brain perfusion technique. This method is used to determine influx of radiolabeled ligands across the BBB and has been described in detail previously (LaRue et al., 2004). Briefly, the right common carotid artery was cannulated with a polyethylene tubing (PE10), and the brains were perfused at 1.0 ml/min (Ranin peristaltic pump), with an artificial plasma solution as described previously (LaRue et al., 2004). Radiolabeled test ligands (e.g., 125I–Aβ40, 125I-4G8-Aβ40, and 125I-Aβ40–4G8) and the reference molecules (e.g., 14C-inulin and 99mTc-albumin) were infused simultaneously via a slow-drive syringe pump (Harvard Apparatus, Holliston, MA) at a rate of 0.1 ml/min. Influx of 125I-Aβ40 in APPsw+/− mice was determined at carrier concentrations corresponding to Aβ40 plasma levels in APPsw+/− mice at different ages (Kawarabayashi et al., 2001). The effect of RAGE-specific IgG F(ab)2, was tested at 20 µg/ml. After a timed perfusion, typically 5–10 min, the brain was rapidly removed, and the ipsilateral hemisphere was homogenized for radioactivity quantification. The perfusion fluid was centrifuged, and supernatant (plasma) was counted. 125I samples were subjected to TCA, SDS-PAGE, native PAGE, and/or HPLC analysis using the procedures we described previously (Shibata et al., 2000; Deane et al., 2003, 2004).

Brain clearance studies. Clearance of 125I-Aβ40, 125I-4G8, or 125I-Aβ–4G8 from brain interstitial fluid (ISF) was determined simultaneously with 14C-inulin (reference marker), using a procedure described previously (Shibata et al., 2000). Briefly, a stainless steel guide cannula was implanted stereotaxically into the right caudate–putamen with the cannula tip coordinates of 0.9 mm anterior and 1.9 mm lateral to bregma and 2.9 mm below the surface of the brain. Animals were recovered after surgery before tracer studies. The experiments were performed before substantial chronic processes occurred, as assessed by histological analysis of tissue, i.e., negative staining for astrocytes (glial fibrillar acidic protein) and activated microglia (anti-phenoloxidase), but allowing time for the BBB repair for large molecules, as reported previously (Cirrito et al., 2003; Deane et al., 2004). Isotope mixture (0.5 µl) containing 125I-labeled test molecule at 40 nM and 14C-inulin was injected over 5 min via an ultra micropump with a micro4-controller (World Precision Instruments, Sarasota, FL) into brain ISF. The recovery of both radiolabeled inulin and Aβ at zero time was 100%, indicating that 100% of injected material remains present for transport with no loss of tracers via tracking up the cannula. 4G8 was administered by two intraperitoneal injections at 200 µg at 0 and 48 h, and 125I-Aβ40 clearance was measured at 1 and 120 h of 4G8 administration in nontransgenic mice and at 120 h in 18- to 20-month-old APPsw+/− mice. The levels of 4G8 in plasma and brain capillaries at 120 h were determined by ELISA (see below). FcRn and molecular reagents [4G8 (0.5 and 2 µM), anti-FcRn (oFcRn, 60 µg/ml), RAP (0.5 and 5 µM), and fucoidan (1.5 µM)] were infused into brain ISF in control mice 30 min before radiolabeling ligands and then simultaneously with radioligands until the end of experiment. Clearance of 125I-4G8 or 125I-Aβ–4G8 was also studied in FcRn−/−, βM−/−, RAP−/−, or FcRγ−/− mice. In all studies, brains were sampled within 30 min after tracer injection and prepared for radioactive analysis. TCA and/or SDS-PAGE/immunoprecipitation analyses were determined to confirm the molecular forms of test tracers in brain and plasma (Deane et al., 2003, 2004).

Brain capillary uptake. Brain capillaries from wild-type and APPsw+/− mice were isolated as described previously (Wu et al., 2003) and incubated with 125I-4G8, 125I-Aβ40–4G8, or 125I-4G8–Aβ40 at 1 nM and 14C-inulin in mock CSF at 37°C for 1 min. The following potential inhibitors were used: 4G8 (0.5 and 2 µM), RAP (0.5 and 5 µM), Fc fragment (1 and 10 µM), and LRP-specific lgG (20 µg/ml). The capillary pellet was separated by centrifugation at 4°C, washed in ice-cold mock CSF, and prepared for radioactive analysis along with samples of the incubating medium.

Radioactivity measurements. 125I samples and 99mTc-albumin radioactivity analysis were determined by gamma counter analysis (Wallac Vizard Gamma Counter; PerkinElmer, Meriden, CT). 14C samples were solubilized in 0.5 ml of tissue solubilizer (PerkinElmer) overnight, fol-
lowed by addition of 5 ml of scintillation cocktail (Packard Ultima Gold; PerkinElmer) and analysis on a liquid scintillation counter (Packard Tri-Carb 2100TR Liquid Scintillation Counter; PerkinElmer).

Calculations. The BBB influx was determined as cerebrovascular permeability surface area product (PS) × Cpl, where Cpl was the concentration of the test molecule in plasma. The PS product of [125I]-labeled test molecule was calculated using 14C-inulin correction: PS × T = ([125I] cpm/g of brain tissue) × TCA-precipitable radioactivity ([125I] cpm/ml of arterial plasma inflow) × TCA-precipitable radioactivity (Dv) − 14C dpm/g of brain tissue)/[14C dpm/ml of arterial plasma inflow] (LaRue et al., 2004), where T is the infusion time and 14C-inulin was infused simultaneously with the test molecule. Influx was expressed per gram brain ISF, assuming the ISF space of 0.1 ml/g of brain (LaRue et al., 2004).

For brain clearance studies, calculations of clearance parameters were as reported previously (Shibata et al., 2000). The percentage of radioactivity of the test ligand and inulin was determined as follows: % recovery in brain = 100 × (Nf/N0), where, Nf is the radioactivity remaining in the brain at the end of the experiment, and the radioactivity injected into the brain ISF, i.e., the disintegrations per minute for 14C-inulin and the counts per minute for TCA-precipitable [125I]-radioactivity. The percentage of [125I]-labeled test ligands (i.e., 4G8, Aβ40 – Aβ40 complex in brain).

[125I]-Aβ40–4G8 complex in brain. After 120 h of the intraperitoneal injection of 4G8, 100 µl of ice-cold PBS by cardiac perfusion, and cerebral microvessels were isolated as described previously (Zlokovic et al., 1993). Protein homogenates (5–15 µg) were separated under reducing conditions (Ferritin or non-reducing conditions (GFAP and F4/80), electrophoresed blotted in Tris-glycine buffer on nitrocellulose membrane, and probed with anti-FcRn (1:1), anti-GFAP (1:100), or F4/80 (1:100). The signal was detected by enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

Western blotting. The cerebrovascular mouse system was washed with 100 ml of ice-cold PBS by cardiac perfusion, and cerebral microvessels were isolated as described previously (Zlokovic et al., 1993). Protein homogenates (5–15 µg) were separated under reducing conditions (Ferritin or non-reducing conditions (GFAP and F4/80), electrophoresed blotted in Tris-glycine buffer on nitrocellulose membrane, and probed with anti-FcRn (1:1), anti-GFAP (1:100), or F4/80 (1:100). The signal was detected by enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).
malized per total TBS-soluble brain protein to estimate the relative levels of oligomers.

The thioflavin S-positive amyloid load (Wilcock et al., 2003) was determined using the Image-Pro-Plus program (Media Cybernetics, Silver Spring, MD).

Mouse endogenous brain Aβ. Mouse endogenous Aβ40 and Aβ42 levels were determined by sandwich ELISA (Best et al., 2005). Cerebral cortex was homogenized in 2% SDS containing complete protease inhibitor cocktail (Roche) (Kawarabayashi et al., 2001). For mouse Aβ40-specific sandwich ELISA, the capturing and biotinylated detecting antibodies were mouse monoclonal mouse Aβ raised against amino acid residues 1–20 (AMB0062; BioSource International) and rabbit polyclonal anti-Aβ40 biotin conjugate (44-3489; BioSource International), respectively. For mouse Aβ42-specific sandwich ELISA, the capturing and detecting antibody were AMB0062 and rabbit polyclonal anti-Aβ42 biotin conjugate (44-3449; BioSource International), respectively. Murine synthetic Aβ40 and Aβ42 standards (American Peptide, Sunnyvale, CA) were prepared in the ELISA buffer (PBS, 0.05% Tween 20, 0.25% BSA, 0.05% sodium azide, and complete protease inhibitor cocktail). The capturing antibody was coated at 5 μg/ml overnight at 4°C in 50 mM carbonate buffer, pH 9.6, and 0.05% sodium azide on 96-well high-binding Stripwell immunoassay plates (Corning Life Sciences), blocked with 0.25% bovine serum albumin in PBS overnight at 4°C, and 100 μl of sample or standard was added and incubated overnight at 4°C. Aβ levels were obtained from the standard curve.

Statistical analysis. The results were compared by multifactorial analysis of variance and Student’s t test. The differences were considered to be significant at p < 0.05. All values are mean ± SEM.

Results

Figure 1A shows that 4G8, an Aβ-specific monoclonal IgG2b raised to 17–24 residues of Aβ, reduces by fivefold to sixfold influx of circulating 125I-Aβ40 across the BBB in APPsw+/−/− (Tg2576) mice at 2–3 and 4–6 months of age. At a later stage, 15- to 20-month-old APPsw+/−/− mice, the influx of circulating Aβ into brain ISF, although significantly higher than in controls, declines spontaneously. Still, 4G8 produced a significant inhibition of Aβ influx in 15- to 20-month-old and 24- to 36-month-old APPsw+/−/− mice (Fig. 1A).

In the cerebral arterial inflow, 125I-Aβ40 alone remains >98.5% in its free monoclonic form, as shown by the SDS-PAGE and HPLC analysis (Fig. 1B), as reported previously (Deane et al., 2003). However, in the presence of 4G8, Aβ forms stable complexes in plasma, as demonstrated by the SDS-PAGE and HPLC analysis within 30 min of the cerebral arterial infusion (Fig. 1C), which is longer than typical infusion times of 5–10 min used for influx calculations in Figure 1A. In the present study, the concentrations of 4G8 that block Aβ40 BBB influx (Fig. 1A) were by two orders of magnitude lower than previously reported anti-Aβ IgG plasma levels in actively (Das et al., 2003) or passively (DeMattos et al., 2002) immunized mice. Lowering the 4G8/Aβ40 plasma ratio from 80 to 1 resulted in comparable >95% inhibition of Aβ40 brain uptake (Fig. 1D). Aβ40–4G8 complexes labeled on either Aβ40 or 4G8 residues did not penetrate across the BBB, and their behavior was not influenced by an RAGE blocking antibody. In contrast, a significant transport of free, circulating Aβ was by >80% inhibited by the blockade of RAGE at the BBB (Fig. 1E), as reported previously (Deane et al., 2003; LaRue et al., 2004). These findings suggest that 4G8 effectively reduces RAGE-mediated increase in circulating Aβ influx across the BBB in APPsw+/−/− mice by sequestering circulating Aβ in plasma.

Figure 2, A and B, shows time-dependent 4G8 increases in plasma and brain ISF, respectively, within 120 h of two subsequent intraperitoneal injections of 4G8 (200 μg). By using a brain clearance method (Shibata et al., 2000), we showed that efflux of centrally administered 125I-Aβ40 from mouse brain ISF determined over 30 min was moderately but significantly increased by 15% at 1 h of peripheral 4G8 intraperitoneal administration (Fig. 2C). Because the levels of 4G8 in brain ISF were barely detectable after 1 h of peripheral 4G8 injections (Fig. 2B) and intracerebrally administered 125I-Aβ40 does not form complexes with plasma-derived 4G8 after 1 h of peripheral 4G8 administration, as indicated by the autoradiographic analysis of brain aqueous extracts (Fig. 2E), an increase in brain to blood clearance of centrally administered Aβ at this early time point is likely to be mediated by peripheral action of 4G8. Thus, an increased 125I-Aβ40 efflux at 1 h (Fig. 2C) probably reflects the sink effect of 4G8, similar to that reported for other peripheral Aβ sequestering agents by different groups (DeMattos et al., 2002; Deane et al., 2003; Lemere et al., 2003). Namely, after 1 h, plasma levels of 4G8 are already high and ~3–6 μM (Fig. 2A), which likely mopps up completely the endogenous free plasma Aβ (50–100 μM), alters the Aβ equilibration at the BBB in favor of Aβ efflux from brain. However, after 120 h of two subsequent 4G8 systemic injections, the BBB efflux of 125I-Aβ40 was substantially increased by 50% (Fig. 2D). Because at 120 h there was a significant increase in 4G8 levels in brain ISF (Fig. 2B), we hypothesized that enhanced clearance of centrally administered 125I-Aβ40 by peripheral administered 4G8 at 120 h (Fig. 2D) is not attributable to peripheral sink action (Fig. 2A). Indeed, the autoradiographic analysis of brain extracts indicated that intracerebrally administered 125I-Aβ40 forms within 30 min complexes with systemically administered 4G8 at 120 h of 4G8 administration (Fig. 2E). This result suggests that peripheral...
4G8 may enhance clearance of brain-borne αβ after its crossing into brain ISF. 125I-αβ40 clearance via the ISF bulk flow was not altered by 4G8.

To test whether αβ complexed to an αβ-specific IgG is cleared from brain, we measured the BBB efflux of centrally administered 4G8 alone and of αβ40–4G8 and αβ42–4G8 complexes in wildtype 2- to 3-month-old mice, using our clearance method (Shibata et al., 2000). The BBB clearance of 125I-4G8 was inhibited by the increasing concentrations of centrally administered unlabeled 4G8 (data not shown) and abolished by 2 μM 4G8 (Fig. 3A), suggesting a concentration-dependent IgG transcytosis at the BBB. RAP, which inhibits αβ BBB clearance via LRP (Shibata et al., 2000; Deane et al., 2004), or fucoidan, an inhibitor of the scavenger receptor on microglia, did not affect 4G8 clearance (Fig. 3A). Immunostaining for activated microglia was negative in the present model (data not shown), as reported previously (Cirrito et al., 2003; Deane et al., 2004).

Because IgG is carried across biological membranes by the FcRn (Roopenian et al., 2003; Ober et al., 2004; Yoshida et al., 2004) and FcRn is expressed in vascular endothelium and at the BBB (Borvak et al., 1998; Schlachetzki et al., 2002), we explored whether deletions of the FcRn gene and the FcRn light chain β2M gene affect 125I-4G8 BBB efflux. Figure 3A shows significantly reduced 4G8 clearance in FcRn−/− mice and β2M−/− mice by 68% and 63%, respectively, whereas deletions of the RAP gene (functional LRP knock-out) (Deane et al., 2004) or the FcRγ gene were without effect. Excess of 4G8 in brain ISF in FcRn−/− mice resulted in complete inhibition of 125I-4G8 BBB efflux (data not shown), consistent with the data demonstrating that unlabeled 4G8 inhibits 125I-4G8 clearance at the BBB in wild-type mice (Fig. 3A). After central administration of 125I-4G8, the radioactivity appearing in plasma was readily detectable and 100% TCA precipitable, suggesting transcytosis of intact 125I-4G8 from brain into blood (Fig. 3B), as confirmed by the autoradiography (data not shown). 125I-4G8 serum levels were significantly reduced (>90%) by central administration of unlabeled excess 4G8 and in FcRn−/− mice (70%) (Fig. 3B), indicating that the FcRn pathway is required for clearance of an anti-αβ IgG across the BBB.

Next, we showed that 125I-αβ40–4G8 was cleared across the BBB of 2- to 3-month-old wild-type mice at a rate 2.5-fold faster than 125I-4G8 (Fig. 3A). Unlike 4G8, clearance of αβ40–4G8 complexes was inhibited by central administration of either unlabeled 4G8 or RAP and was abolished by the combination of both, suggesting that in young mice 125I-αβ40–4G8 complexes can be cleared by an IgG-dependent mechanism and/or via a RAP-sensitive LRP mechanism (Shibata et al., 2000; Deane et al., 2004). That both the IgG-assisted and LRP-dependent transport are important for clearance of αβ40–4G8 complexes in young mice has been confirmed by demonstrating ~60% reductions in 125I-αβ40–4G8 BBB efflux in β2M−/− mice and RAP−/− mice (Fig. 3A). After central administration of 125I-αβ–4G8 complexes, the radioactivity appearing in plasma was 100% TCA precipitable (Fig. 3C), suggesting no degradation of 125I-αβ from the complex, as well as transcytosis of intact complexes, which has been confirmed by the autoradiography (data not shown). Serum levels of centrally infused 125I-αβ40–4G8 complexes were substantially
also demonstrated that Aβ40 and Aβ40–4G8 complexes. Consistent with a significant downregulation of LRP at the BBB with aging, and particularly in transgenic APPsw+/- mice (Deane et al., 2004), the BBB efflux of Aβ40 alone was significantly reduced in old APPsw+/- mice (Fig. 4B) compared with control young mice (Fig. 2C,D) or young APPsw+/- mice (data not shown). However, central infusion of 125I-Aβ40–4G8 complexes in 20- to 23-month-old APPsw+/- mice resulted in an approximately fivefold greater clearance than of 125I-Aβ40 alone (Fig. 4B), suggesting that IgG-assisted efflux of Aβ remains active in old APPsw+/- mice and is even somewhat higher than in young control wild-type mice (Fig. 3A).

In a next set of studies, we showed that both RAP and Fc fragment effectively block clearance of 4G8–Aβ complexes on isolated cerebral microvessels derived from younger, 3- to 5-month-old APPsw+/- mice (Fig. 4C). In contrast, RAP did not block clearance of 4G8–Aβ complexes on brain capillaries derived from 20- to 23-month-old APPsw+/- mice (Fig. 4C). The loss of inhibitory effect of RAP on capillary clearance in aged APPsw+/- mice is consistent with a significant loss of LRP expression in these animals at an older age (Deane et al., 2004). Conversely, the Fc fragment inhibited significantly by 67% clearance of complexes on aged APPsw+/- mouse brain capillaries (Fig. 4C), confirming that an IgG-dependent clearance of IgG–Aβ complexes remains functional with aging in APPsw+/- mice. These results are consistent with a significant FcRn expression in brain endothelium in older APPsw+/- mice and an age-dependent increase in the FcRn levels in brain capillaries of APPsw+/- mice (Fig. 4D,E). We also observed an age-dependent increase in the FcRn levels in control mice, although the levels of FcRn in APPsw+/- mice were substantially higher than in their corresponding age-matched littermate controls, at both 5–7 and 20–23 months of age, respectively (Fig. 4E). Studies on human tissue indicated an increase in FcRn-positive brain vascular profiles in AD compared with age-matched controls (Fig. 4F).

Next, we determined whether inhibition of the FcRn pathway affects intracerebral 4G8 immunotherapy in 24-month-old APPsw+/- mice. We limited studies to 24 h to minimize possible influence of the antibody on microglia-dependent clearance, because microglia are typically activated in older APPsw+/- mice after 24 h of an anti-AB antibody intracerebral injection (Wilcock et al., 2003), as confirmed in this study (data not shown). Our data corroborate findings by the previous studies (Wilcock et al., 2003; Oddo et al., 2004) by showing that centrally administered 2 μg of 4G8 significantly reduces Aβ immunostaining and the levels of human endogenous Aβ40 and Aβ42 in the hippocampus of 24-month-old APPsw+/- mice by 45, 47, and 25%, respectively (Fig. 5A,D–F). These results correlated well with the observed >35% reductions in the levels of TBS-insoluble/SDS-soluble human endogenous Aβ40 and Aβ42 by 4G8. It is noteworthy that the levels of TBS-soluble human Aβ oligomers were also reduced by 25% by 4G8, as demonstrated by an ELISA developed previously for determining the relative levels of Aβ oligomers (Levine, 2004; Yang et al., 2005) (Fig. 5G). However, 4G8 did not affect thioldavin S-positive amyloid load (Fig. 5H), consistent with our finding that 4G8 did not affect the levels of SDS-resistant/formic acid-soluble Aβ40 and Aβ42, using methods described previously (Kawarabayashi et al., 2001). This result corroborates findings by a previous study demonstrating that, within 24 h of intracerebral delivery, the Aβ-specific antibody does not reduce thioldavin S staining (Wilcock et al., 2003). Although 4G8 binds amyloid plaques on tissue sections ex vivo, its binding to plaques reduced by centrally administered unlabeled 4G8 and/or RAP, as well as by deletion of the B2-M gene and RAP gene (Fig. 3C). We also demonstrated that Aβ42–4G8 complexes are cleared by the FcRn pathway (Fig. 3A), as well as Aβ42 bound to anti-Aβ1560 (data not shown).

By using isolated cerebral microvessels positive for FcRn and negative for microglia and astrocytic markers (Fig. 3D), we corroborated our in vivo findings by showing that 125I-4G8 rapid brain capillary uptake at 37°C, which reflects its clearance by brain capillaries, in 2- to 3-month-old young mice was dose dependently (data not shown) and completely blocked by excess unlabeled 4G8 (Fig. 3E) but was not affected by RAP. Under the present experimental conditions, uptake of 125I-1-radiolabeled 4G8 and its complexes with Aβ occurred at the abluminal side of brain capillaries because short, 1 min exposure times precluded a significant diffusion of the studied tracers into the capillary lumen, which is required for the uptake at the luminal side (Deane et al., 2004). This has been confirmed by a negligible uptake of 14C-inulin, which does not interact with the cells and is taken up by the isolated capillaries only via passive diffusion into the lumen. Regarding 125I-labeled 4G8–Aβ40 complexes (with the label on either 4G8 or Aβ40 residues), both the unlabeled Fc fragment and RAP or an LRP-specific IgG blocked clearance of complexes on brain capillaries from 2- to 3-month-old young mice (Fig. 3E). As expected, Aβ40–4G8 F(ab')2 complexes lacking the Fc domain were cleared by an Fc-independent RAP-sensitive mechanism, confirming the involvement of LRP, as shown for Aβ-mediated clearance (Deane et al., 2004). That Aβ molecule in anti-Aβ–Aβ complexes is critical for interaction with LRP was shown by inhibition of brain capillary clearance of 125I-labeled 4G8–Aβ40 complexes (the label was on 4G8) by excess of unlabeled Aβ40 (data not shown).

Figure 4A shows that 4G8 systemic administration over 120 h increases by approximately threefold efflux of centrally administered 125I-Aβ40 in 18- to 20-month-old APPsw+/- mice (Fig. 4A). To test whether old APPsw+/- mice clear Aβ from brain through an IgG-assisted efflux, we compared clearance of Aβ40 alone and Aβ40–4G8 complexes. Consistent with a significant downregulation of LRP at the BBB with aging, and particularly in transgenic APPsw+/- mice (Deane et al., 2004), the BBB efflux of Aβ40 alone was significantly reduced in old APPsw+/- mice (Fig. 4B) compared with control young mice (Fig. 2C,D) or young APPsw+/- mice (data not shown). However, central infusion of 125I-Aβ40–4G8 complexes in 20- to 23-month-old APPsw+/- mice resulted in an approximately fivefold greater clearance than of 125I-Aβ40 alone (Fig. 4B), suggesting that IgG-assisted efflux of Aβ remains active in old APPsw+/- mice and is even somewhat higher than in young control wild-type mice (Fig. 3A).
Figure 5.  FcRn-dependent clearance of endogenous Aβ in APPsw+/− mice by centrally administered 4G8. A, Intrahippocampal 4G8 (2 μg/0.5 μl) reduces Aβ immunostaining 24 h after injection in 24-month-old Tg2576 mouse (top; ipsi) compared with nonimmune IgG-treated contralateral hippocampus (bottom; contra). B, 4G8 (2 μg/0.5 μl) was injected into the ipsilateral hippocampus in 24-month-old Tg2576 mouse 30 min after FcRn receptor antagonist (10 μg/0.5 μl) (top) or vehicle (contra). C, 4G8 F(ab′)2 (2 μg/0.5 μl) injected into the hippocampus in 24-month-old Tg2576 mouse (top) versus vehicle-treated contralateral hippocampus (bottom). Scale bar, 350 μm. D–F, Aβ load (D), Aβ40 (E), and Aβ42 (F) levels: the percentage change (% in the ipsilateral versus contralateral hippocampus in Tg2576 mice. *p < 0.05. G, H, TBS-soluble Aβ oligomers (G) and thioflavin S-positive amyloid load (H) in 4G8-treated (ipsilateral) versus vehicle-treated (contralateral) hippocampus in 24-month-old Tg2576 mice at 24 h. Mean ± SEM; n = 5 mice per group and 6–8 sections close to the injection site per mouse. I, 125I-4G8 F(ab′)2 (40 nm) and 125I-insulin brain retention 30 min after brain ISF microinjection of the tracer mixture. Mean ± SEM; n = 3. NS, Not significant.

Figure 6.  FcRn+/+ wild-type mice with systemically administered 4G8 results in significant 35 and 40% reductions in brain endogenous mouse Aβ40 and Aβ42, respectively (Fig. 6A, B). In contrast, there was a negligible 5–7% reduction in endogenous Aβ40 and Aβ42 brain levels in FcRn−/− mice (Fig. 6A, B). A reduction in endogenous mouse brain Aβ levels by systemic 4G8 in FcRn−/− mice compared with FcRn+/+ mice was associated with a corresponding significant increase in plasma Aβ, which was >97% G-protein precipitable (G-protein binds the Fc fragment of 4G8) (data not shown), indicating that the majority of Aβ in plasma after systemic therapy with the antibody is bound to 4G8, similarly as shown by previously passive and active immunization studies (DeMattos et al., 2001, 2002; Lemere et al., 2003).

Discussion
Our study shows that an anti-Aβ antibody regulates brain Aβ by exerting important peripheral and central actions on Aβ transport across the BBB. The mechanism of peripheral Aβ sequestration observed in the present study with 4G8 in APPsw+/− mice may be similar to that of other Aβ binding agents, i.e., apolipoproteins E2 and 3 (Martel et al., 1997), ganglioside M and gelsolin (Matsuoka et al., 2003), the soluble forms of RAGE (Deane et al., 2003) or LRP (Deane et al., 2004), and/or other antibodies to Aβ that reduce its influx across the BBB (Pan et al., 2002; Banks et al., 2005). This peripheral binding of Aβ may create a sink effect, providing that the activity of the LRP efflux pathway (Deane et al., 2004) is not lost or is sufficient to maintain elimination of brain Aβ and Aβ–IgG complexes. The central action of certain anti-Aβ IgGs may, however, critically depend on sequestering brain Aβ, followed by the FcRn-mediated rapid BBB clearance. Importantly, the FcRn IgG-dependent clearance for Aβ remains active at the BBB during normal aging and in APPsw+/− mice, and increased FcRn expression at the BBB in AD may likely be of a therapeutic value for Aβ immunotherapy.

Circulating IgG may enter the brain and have widespread extracellular distribution within the CNS and CSF from sites deficient in a BBB such as subarachnoid space pial surface in the Virchow-Robin spaces and subpial cortical gray matter and circumventricular organs (Balin et al., 1986; Kozlowski et al., 1990; Broadwell and Sofroniew, 1993). It has been reported that an antibody directed at 1–17 region of Aβ can use the extracellular pathways for its passive entry into the CNS (Banks et al., 2002). In addition, some IgG species are also transported slowly across the BBB via adsorptive-mediated transcytosis (Zlokovic et al., 1990; Partridge, 1991). Our results indicate that systemically administered 4G8 exerts a significant clearance effect on endogenous brain Aβ that is abolished in FcRn−/− mice at an age when LRP expression is substantially downregulated (Deane et al., 2004). Although 4G8 recognizes APP in addition to Aβ, it is unlikely that the Aβ-lowering effect of systemically administered 4G8 is attrib-
utable to inhibition of APP/αβ production because this effect is lost in FcRn−/− compared with FcRn+/+ mice. Inhibition of the FcRn pathway influences also the outcome of centrally administered αβ immunotherapy in old APPsw−/− mice. Although intracerebral immunotherapy has limitations regarding direct insight into what happens with peripheral passive or active approaches, studies using the intracerebral approach have been useful in delineating clearance pathways of αβ from brain (Tanzi et al., 2004; Zlokovic, 2005) and have been used extensively to demonstrate that antibody in the brain exerts a clearance action on brain αβ (Bacskai et al., 2001, 2002; Wilcock et al., 2003; Oddo et al., 2004). For example, it has been shown that intracerebral delivery of 2 μg of 4G8 or anti-αβ1560 (Oddo et al., 2004) and 2 μg of 6E10 (Wilcock et al., 2003), substantially reduces αβ load in 12-month-old triple-transgenic AD mice and in 20-month-old APPsw−/− mice, respectively. Our data suggest that the FcRn pathway is required for clearance of TBS-insoluble/SDS-soluble endogenous αβ40 and αβ42, which possibly represents αβ in diffuse plaques that is not yet converted into a hard-core thioflavin S-positive amyloid. Although we have not demonstrated FcRn-mediated clearance of thioflavin-S-positive amyloid, it is possible that, over longer periods of time (>24 h), centrally administered 4G8 will reduce thioflavin S-positive load from brain (Wilcock et al., 2003; Oddo et al., 2004), which, at least in part, could involve the FcRn pathway.

Topical administration of Fab fragments alone can reduce αβ immunostaining in APPsw−/− mice within 3 d (Bacskai et al., 2002). Our data show that this effect could be mediated via LRPI efflux system, which can clear αβ40–4G8 F(ab′)2, complexes. In contrast, Fab fragments alone are eliminated slowly from brain ISF at a rate comparable with that of a reference marker, inulin. However, we also demonstrated that 4G8 F(ab′)2 cannot exert a vigorous effect on αβ pathology within 24 h in 24-month-old APPsw−/− mice, which could be explained by substantial downregulation of LRPI levels at the BBB in old APPsw−/− mice (Deane et al., 2004).

FcRn plays a central role in delivering IgGs within and across the cells (Ober et al., 2004). Although FcRn was discovered as a developmental IgG receptor, recent studies suggest its expression in different cell types in adult rodents and humans, including polarized epithelia and endothelia, which are able to translocate the FcRn-bound cargo bidirectionally, from either the lumen or tissue spaces into the opposite pole of the cell (Roopenian et al., 2003; Yoshida et al., 2004). FcRn is directly involved in IgG exocytosis (Ober et al., 2004) and binds IgG best at an intracellular pH 6.0–6.5 (Roopenian et al., 2003). It is expressed at low levels in the cell membrane because of its rapid recycling after incomplete fusion with the plasma membrane, known as “kiss and run” (Ober et al., 2004). It is likely that the FcRn-mediated transcytosis of IgG across the BBB may require an additional binding step to another IgG receptor at the abluminal cell membrane, which will facilitate IgG endocytosis before its FcRn-mediated migration to the exocytotic site at the luminal membrane, analogous to the IgG transport across other biological membranes (Roopenian et al., 2003). This hypothesis is supported by our work in progress showing a single 55 kDa band (data not shown) after biotinyla-
tion of the abluminal mouse brain capillary membranes and 4G8 affinity purification. This band corresponded to a recently described IgG cell surface receptor in the placental endothelium (Gafencu et al., 2003). The exact cellular mechanisms involved in possible interaction between a putative 55 kDa IgG receptor and FcRn during IgG and αβ–anti-αβ complex internalization at the abluminal side of the BBB and exocytosis along the luminal side remain, however, to be investigated.

Based on the present work, we propose two possibilities for enhanced efficacy of immunization therapy directed at an improved clearance of αβ across the BBB: (1) to increase a peripheral sink of an anti-αβ antibody and facilitate brain efflux of αβ and αβ–IgG complexes by increasing the activity of the BBB LRPI efflux pathway at an older age; and (2) to optimize the FcRn pathway for enhanced IgG-mediated αβ clearance across the BBB in the aging brain.

References


