Antibodies to β-Amyloid Decrease the Blood-to-Brain Transfer of β-Amyloid Peptide[1]

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Amyloid-β peptides (Aβ) play an important role in the pathophysiology of dementia of the Alzheimer's type and in amyloid angiopathy. Aβ outside the CNS could contribute to plaque formation in the brain where its entry would involve interactions with the blood-brain barrier (BBB). Effective antibodies to Aβ have been developed in an effort to vaccinate against Alzheimer's disease. These antibodies could interact with Aβ in the peripheral blood, block the passage of Aβ across the BBB, or prevent Aβ deposition within the CNS. To determine whether the blocking antibodies act at the BBB level, we examined the influx of radiolabeled Aβ (^{125}I-$A\beta_{1-40}$) into the brain after ex-vivo incubation with the antibodies. Antibody mAb3D6 (élan Company) reduced the blood-to-brain influx of Aβ after iv bolus injection. It also significantly decreased the accumulation of Aβ in brain parenchyma. To confirm the in-vivo study and examine the specificity of mAb3D6, in-situ brain perfusion in serum-free buffer was performed after incubation of ^{125}I-$A\beta_{1-40}$ with another antibody mAbmc1 (DAKO Company). The presence of mAbmc1 also caused significant reduction of the influx of Aβ into the brain after perfusion. Therefore, effective antibodies to Aβ can reduce the influx of $A\beta_{1-40}$ into the brain. [Exp Biol Med Vol. 227(8):609–615, 2002]

Key words: β-amyloid peptides; antibody; blood-brain barrier; vaccine; Alzheimer's disease; mice

Deposition of the insoluble form of amyloid-β peptides (Aβ) with formation of senile plaques is one of the major pathological characteristics of Alzheimer's disease. Despite the clinical use of anticholinesterases and antioxidants, as well as trials of neurotrophic factors, there has not been an effective treatment that can arrest the irreversible neurodegeneration or restore premorbid function in patients with Alzheimer's disease. Therefore, vaccination by generation of antibodies to Aβ has been investigated *in vitro* and in mice in recent years in the hope of discovering a cure for Alzheimer's disease. Vaccination targeting Aβ can prevent the formation of Aβ plaques or cause regression of Aβ deposition (4, 14, 18, 19).

Aβ is produced not only in the brain but also in blood and other organs outside the central nervous system (CNS). After intravenous injection, iodinated $A\beta_{1-40}$ accumulates in the brain and can be recovered by HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the brain of mice (13). Peripheral Aβ probably has to negotiate the blood-brain barrier (BBB) to gain access to the CNS. The BBB is a selective barrier to peptides and polypeptides in that most hydrophilic molecules have very limited entry from blood to brain and spinal cord by simple diffusion. However, some bioactive peptides and polypeptides have facilitated influx by saturable transport systems at the BBB (2, 16).

The interactions of Aβ with the BBB could take many forms. Both $A\beta_{1-40}$ and the extracellular domain $A\beta_{1-28}$ (3) can cross the BBB to some extent (12, 13). While in the brain, $A\beta_{1-40}$ can be removed by low-density lipoprotein-receptor related protein-1, an efflux transporter at the BBB (20). Moreover, Aβ not only crosses the BBB itself, but at high concentrations, may increase the permeation of other substances such as the paracellular tracer polyethylene glycol (22). Intravenous (iv) infusion of $A\beta_{1-40}$ for 2 weeks induces IgG extravasation in brain as well as activation of glial cells (23), and intracarotid infusion of $A\beta_{1-42}$ dose dependently increases albumin content in the brain of rats (11). These studies suggest that Aβ not only crosses the BBB by itself, but also disrupts the BBB in certain circumstances.

An effective antibody to Aβ could bind to Aβ in the periphery, thereby preventing the entry and subsequent formation of insoluble forms of Aβ in the brain. It could also function by impeding the passage of Aβ across the BBB or by increasing the clearance of Aβ from the brain. The antibody m266, administered peripherally to PDAPP transgenic mice, is able to decrease the brain Aβ burden by altering CNS and plasma Aβ clearance (6).

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In the current study, we used two available antibodies: mAb3D6 from élan Pharmaceuticals (San Francisco, CA) and mAbmc1 from DAKO (Glostrup, Denmark). The monoclonal antibody 3D6 was raised against the N-terminal 1–5 domain of Aβ and is able to reduce plaque burden by 86% after weekly intraperitoneal (ip) injection in PDAPP transgenic mice for 6 months, an action independent of T cell immunity (4). The DAKO antibody; generated against the entire Aβ, is usually used for immunohistochemical studies, and is effective in reversing memory deficit in SAMP8 mice after intracerebroventricular or intraparenchymal delivery (15). The effects of these antibodies on the influx and brain distribution of Aβ are evaluated in this study.

Methods

The monoclonal IgG antibody 3D6 (mAb3D6) against $A\beta_{1-5}$ was provided by Dr. Dale Schenk at élan Pharmaceuticals (Lot 624, 0.76 mg/ml, molecular weight ~26 kD). The monoclonal mouse anti-human IgG antibody (mABmc1) was purchased from DAKO (Lot 029, Code M0749, 0.355 mg/ml). Human $A\beta_{1-40}$ (Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val, molecular weight of 4 kD) was purchased from Calbiochem (San Diego, CA) and was reconstituted in phosphate-buffered solution (PBS; 0.25 *M*) shortly before use. The iodobead method (Pierce, Rockford, IL) was used to radiolabel $A\beta_{1-40}$ with ^{125}I. The radiolabeled product (I-Aβ) was purified by elution on a column of Sephadex G-10 and had a specific activity of 88-100 Ci/g in various experiments in this study. The integrity of I-Aβ was confirmed by acid precipitation and HPLC.

Young adult male ICR mice (Charles River Laboratories, Wilmington, MA) weighing 25–30 g were used after urethane anesthesia. The effect of mAb3D6 in decreasing the influx of I-Aβ was studied after overnight incubation of the antibody with I-Aβ at 4°C before injection into the mice. The I-Ab:antibody ratio was 1:10, 1:100, 1:1000, and 1:10,000 in the individual groups with an additional control of I-Aβ only (n = 7/group). After incubation, I-Aβ, along with the antibody, was injected into the left jugular vein of the mouse (2×10^6 cpm in 200 μl of lactated Ringer's solution containing 1% albumin [LR/BSA]). At various times 2–30 min after iv injection, blood was collected from the right carotid artery, and the mouse was decapitated. The time course chosen was based on our previous HPLC and SDS-PAGE results showing that I-Aβ remains intact in blood during this period (13).

Multiple-time regression analysis (5, 17) was used to calculate the influx rate of I-Aβ. The whole brain with the exception of the pineal and pituitary glands was collected and weighed, and 50 μl of serum was obtained after centrifugation of the blood. The radioactivity for brain and serum was measured in a gamma counter. The one-phase decay of serum radioactivity (I-Aβ) was calculated, and exposure time (Expt) was obtained by the following equation:

$$\text{Expt} = [\int_0^t Cp(\tau)/d\tau]/Cpt$$

where Cp is counts per minute per milliliter of arterial serum, t is time in minutes, and Cpt is the counts per minute per milliliter in arterial serum at time t. Expt corrects for the decay of I-Aβ in serum and provides a theoretical steady-state value of time if the serum concentration of I-Aβ were constant. Therefore, when the ratio of brain/serum radioactivity of I-Aβ is plotted against Expt, the slope of the linear regression line represents the influx of I-Aβ from blood to brain (K_i). The influx rates (K_i) of I-Aβ among the groups studied at one time were compared by the GraphPad Prism statistical software (GraphPad, San Diego, CA). The values are expressed as means ± SE. For all experiments, ^{131}I-albumin, radiolabeled by the chloramine-T method and purified on a Sephadex G-10 column, was included in the same injection solution to function as a vascular control. The radioactivity of ^{125}I and ^{131}I could be measured simultaneously in a dual channel gamma counter (Wallac, Gaithersburg, MD) without crossover reading.

For *in situ* brain perfusion, the solution was diluted in modified Zlokovic's buffer (NaCl 7.19 g/l, KCl 0.3 g/l, $CaCl_2$ 0.28 g/l, $NaHCO_3$ 2.1 g/l, KH_2PO_4 0.16 g/l, $MgCl_2 \cdot 6H_2O$ 0.37 g/l, D-glucose 0.99 g/l, and 1% BSA, pH 7.4) at a concentration of 1000 cpm/μl of I-Aβ. The chest of the mouse was exposed, the abdominal aorta was clamped, and both jugular veins were cut. A prewash with 20 ml of perfusion buffer was made to clear the vasculature. Intracardial perfusion was performed at a rate of 2 ml/min from an insertion in the left ventricle. The perfusate was contained in a 60-ml syringe and was driven by a perfusion pump (Harvard Apparatus, Holliston, MA). Perfusion was stopped after 1–5 min for each mouse, followed by decapitation. Two groups of mice were studied: those with I-Aβ only and those with I-Aβ + mAbmc1 at a 1:1 *M* ratio (n = 11/group). The brain was collected, and the brain/perfusate ratio of radioactivity was calculated. Influx rate was derived from the slope of the regression line after the brain/perfusate ratio of I-Aβ was plotted against perfusion time.

Capillary depletion was performed in additional mice. ^{125}I-Aβ was incubated with mAb3D6 (n = 12) or PBS control (n = 10) overnight at 4°C. The mAb3D6 dilution was 1:10 (1 *M* I-Aβ:0.1 *M* mAb3D6). ^{131}I-albumin was added to the injection solution immediately before the iv study to serve as a vascular control. The endpoint of the study was 15 min after iv injection of I-Aβ and ^{131}I-albumin. Thus, for each mouse, blood was collected from the descending abdominal aorta 15 min after iv injection of I-Aβ (with or without preincubation with the antibody), and the mouse was decapitated immediately afterward. For each group, one-half of the mice received intracardial perfusion to wash out the I-Aβ in the cerebral vasculature immediately before decapitation. The cerebral cortex was isolated,

weighed, and homogenized in the capillary depletion buffer (24). It was reconstituted in 26% Dextran to yield a final concentration of slightly more than 14.5%, which is necessary for proper pelleting of mouse brain tissue (10). After Dextran density centrifugation at 5400*g* for 15 min at 4°C with a swinging-bucket rotor (TLS-55 rotor and TL-100 ultracentrifuge; Beckman Instruments, Fullerton, CA), the supernatant (composed of brain parenchyma) and the pellet (representing capillaries) were carefully separated. Effective separation with limited contamination has been previously shown by measurement of γ-glutamyl transpeptidase-specific activity, a vascular enzyme marker (10, 24). The radioactivity of I-Aβ and ^{131}I-albumin in each component was measured with a dual channel gamma counter, and the ratio of radioactivity in brain parenchyma/serum and capillary/serum was calculated for each mouse. Contamination of the supernatant by the vasculature, 1.9% of the level in the pellet from our previous study (10), was further corrected by subtraction of the volume of distribution of ^{131}I-albumin used in the same study. The group means were expressed with their SEs.

To determine whether the effect of I-Aβ crossing the BBB was unique to mAb3D6, additional studies were performed with mAbmc1 from DAKO. I-Aβ was incubated overnight at 4°C with an equal molar concentration of mAbmc1 or with PBS/BSA only (control). Protein A immobilized on crosslinked 4% beaded agarose (Sigma, St. Louis, MO) was used to determine the amount of I-Aβ associated with the antibody at the end of incubation. For this procedure, 10 μl of I-Aβ + Ab or I-Aβ in buffer only was incubated with 60 μl of protein A at 4°C for 4 h (n = 5/group). The protein A beads were then separated from the supernatant by microfuge at 4°C for 10 min. The percentage of radioactivity associated with protein A beads was calculated for each group.

Discontinuous native polyacrylamide gel of 10%–20% was used to further determine the association of I-Aβ with the antibodies. At the end of the overnight incubation, I-Aβ only, I-Aβ + mAbmc1, and I-Aβ + anti-TNFα antibody (control) were loaded onto the gel with an equal volume of nonreducing sampling buffer. Electrophoresis was performed at 120 V for 60 min. The gel was stained with Coomassie Brilliant Blue, and then fixed, destained, and dried before exposure to the phosphoimager plate reader.

Results

mAb3D6 Inhibited the Influx of I-Aβ across the BBB at High Doses. I-Aβ was incubated with mAb3D6 at 4°C overnight before the iv study. The amount of I-Aβ injected, estimated from the radioactivity and the specific

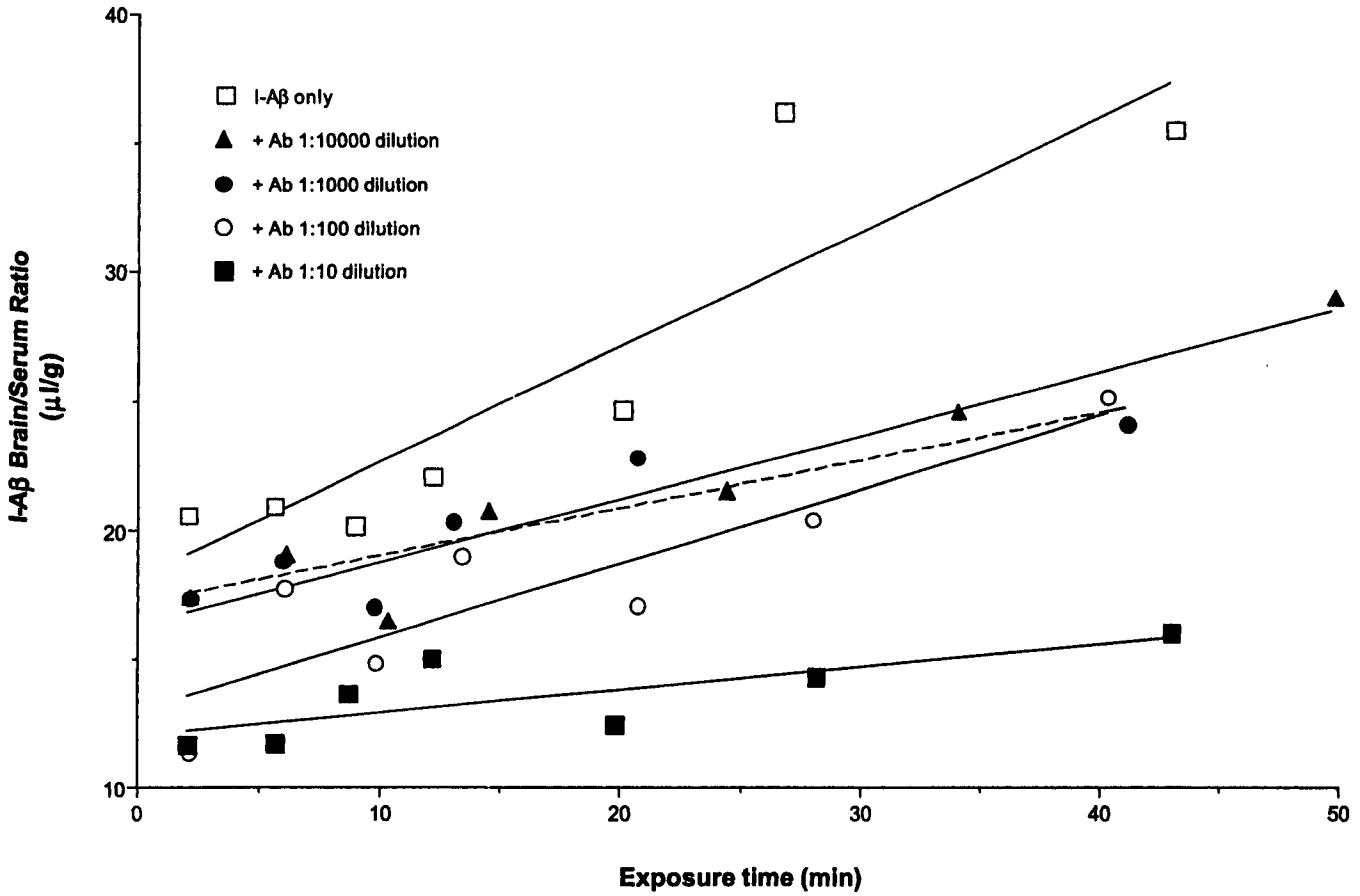

Figure 1. Incubation of mAb3D6 overnight at 4°C decreased the influx of ^{125}I-Aβ from blood to brain ($P < 0.001$ among all groups). A 1:10 M dilution of mAb3D6 almost completely abolished the influx of ^{125}I-Aβ, although groups with more diluted antibody (Ab) failed to show the same effect.

activity of the stock solution, was about 0.01 μg/200 μl/mouse. The groups studied were: I-Aβ with 1:10 dilution of mAb3D6 (i.e., 1 *M* I-Aβ:0.1 *M* antibody); I-Aβ with 1:100 dilution of antibody; I-Aβ with 1:1000 dilution of antibody; I-Aβ with 1:10,000 dilution of antibody; and I-Aβ only with no antibody (control group). Each group contained seven mice, each mouse being decapitated at a different time. The exposure time (Expt) thus derived (*x*-axis) was longer than the clock time (experimental time). Figure 1 shows that the difference of influx rate among the groups was statistically significant [$F(4,24) = 4.95$, $P < 0.001$]. When each group was compared with the control (K_i = 0.447 ± 0.095 μl/g/min), the antibody dilution of 1:10 (K_i = 0.088 ± 0.033 μl/g/min) significantly ($P < 0.01$) inhibited the influx of I-Aβ.

Influx of I-Aβ after *In Situ* Brain Perfusion and the Inhibition by mAbmc1. The group with I-Aβ only had an influx rate of 3.84 ± 0.54 μl/g/min ($r = 0.92$, $P < 0.0001$). The influx rate of I-Aβ after preincubation with the antibody was 1.22 ± 0.52 μl/g/min ($r = 0.62$, $P < 0.05$). The difference between the two regression lines could be explained by the difference of the slopes (influx rate) and was statistically significant [$F(1,18) = 12.3$, $P < 0.005$]. The difference between the initial volume of distribution of I-Aβ in the I-Aβ only group (6.21 ± 1.57 μl/g) and in the I-Aβ + Ab group (6.63 ± 1.57 μl/g) was not statistically significant (Fig. 2).

Distribution of I-Aβ in Brain Compartments in the Presence of mAb3D6. The volume of distribution of I-Aβ in the brain parenchyma (supernatant) and capillary (pellet), corrected for vascular contamination by subtraction of ^{131}I-albumin, was calculated for each group: control mice without mAb3D6 preincubation, receiving no intracardial perfusion; control mice without mAb3D6, but cleared of loosely adherent I-Aβ in the capillary lumen by intracardial perfusion; mice receiving I-Aβ + mAb3D6, but no washout perfusion; and mice receiving I-Aβ + mAb3D6 with washout perfusion.

For both control and mAb3D6 treatment groups, parenchymal uptake represents the amount of I-Aβ in the supernatant of mice receiving intracardial perfusion to wash out the vascular space, and capillary uptake represents the amount of I-Aβ in the pellet from the same mice. The total cortical value of I-Aβ, after subtraction for the vascular space reflected by ^{131}I-albumin, represents the sum of the volume of distribution in parenchyma and capillaries in

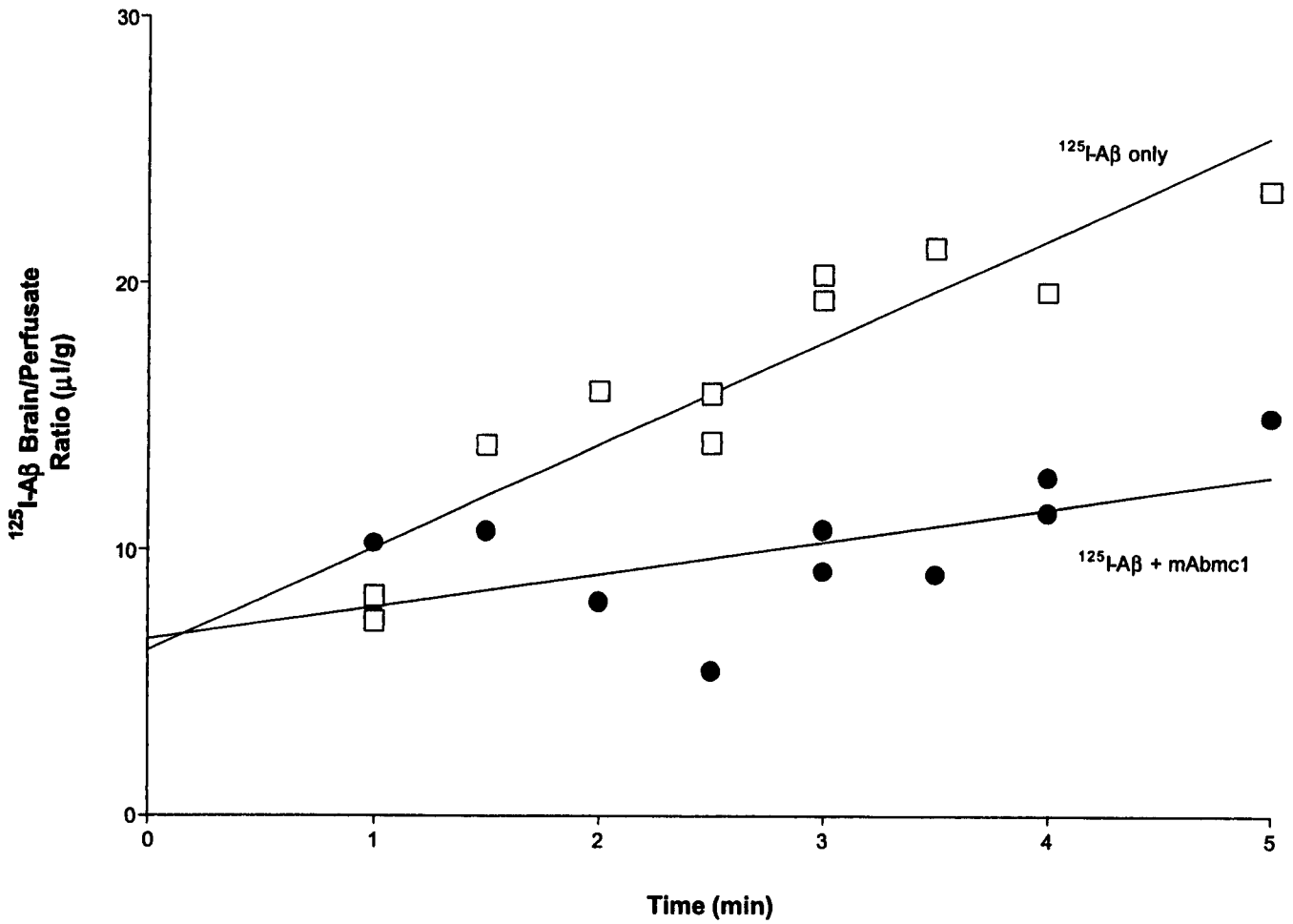

Figure 2. Reduction of the influx of ^{125}I-Aβ into the brain by mAbmc1 after *in situ* brain perfusion. The difference in the influx rate of the two groups was statistically significant ($P < 0.005$).

the nonperfused mice. The difference obtained by subtraction of the total cortical values of the nonperfused and perfused groups represents reversible vascular association. As seen in Fig. 3, the majority of I-Aβ entered brain parenchyma. mAb3D6 preincubation significantly reduced the uptake by brain parenchyma, thereby significantly reducing the uptake of I-Aβ in the whole cortex ($P < 0.0001$ compared with the control group). The presence of mAb3D6 did not significantly decrease the reversible binding of I-Aβ to microvessels.

Association of mAbmc1 with I-Aβ after Incubation. After overnight incubation, 61.15% ± 1.84% of radioactivity in the I-Aβ + Ab group was precipitated by protein A beads ($n = 5$). By contrast, 46.09% ± 5.31% of radioactivity was precipitated by protein A beads in the control group with I-Aβ only ($n = 4$). The difference between the two groups was statistically significant [$F(1,7) = 8.7$, $P < 0.05$], indicating that a significant amount of I-Aβ was still associated with the antibody. With native gel electrophoresis, multiple high-molecular-weight bands were present in the groups with antibody; however, the radioactivity in the high-molecular-weight bands was only detected in the group of I-Aβ + mAbmc1.

Discussion

We have shown that I-Aβ remains stable in blood during the study period, that it has a significant influx from blood to brain, and that the radioactivity measured in brain homogenate and CSF represents intact Aβ (13). In this study, I-Aβ was incubated with antibodies *ex vivo* before being administered to the mouse. The majority of I-Aβ was associated with the antibody at the end of incubation. mAb3D6 significantly decreased the influx of I-Aβ from blood to brain when administered in sufficiently large doses.

Capillary depletion studies were performed to evaluate the distribution of Aβ in the brain and whether the antibody affected its relative disposition in cerebral vasculature, endothelial cells, and brain parenchyma. mAb3D6 significantly decreased the amount of I-Aβ in brain parenchyma. This further suggests that the antibody is effective in reducing the entry of Aβ into brain parenchyma.

To determine whether the effect was unique to mAb3D6, I-Aβ was incubated with another antibody to Aβ (mAbmc1). During *in situ* brain perfusion in a blood-free environment, mAbmc1 significantly decreased the influx rate of I-Aβ. Therefore, mAbmc1 was also effective in re-

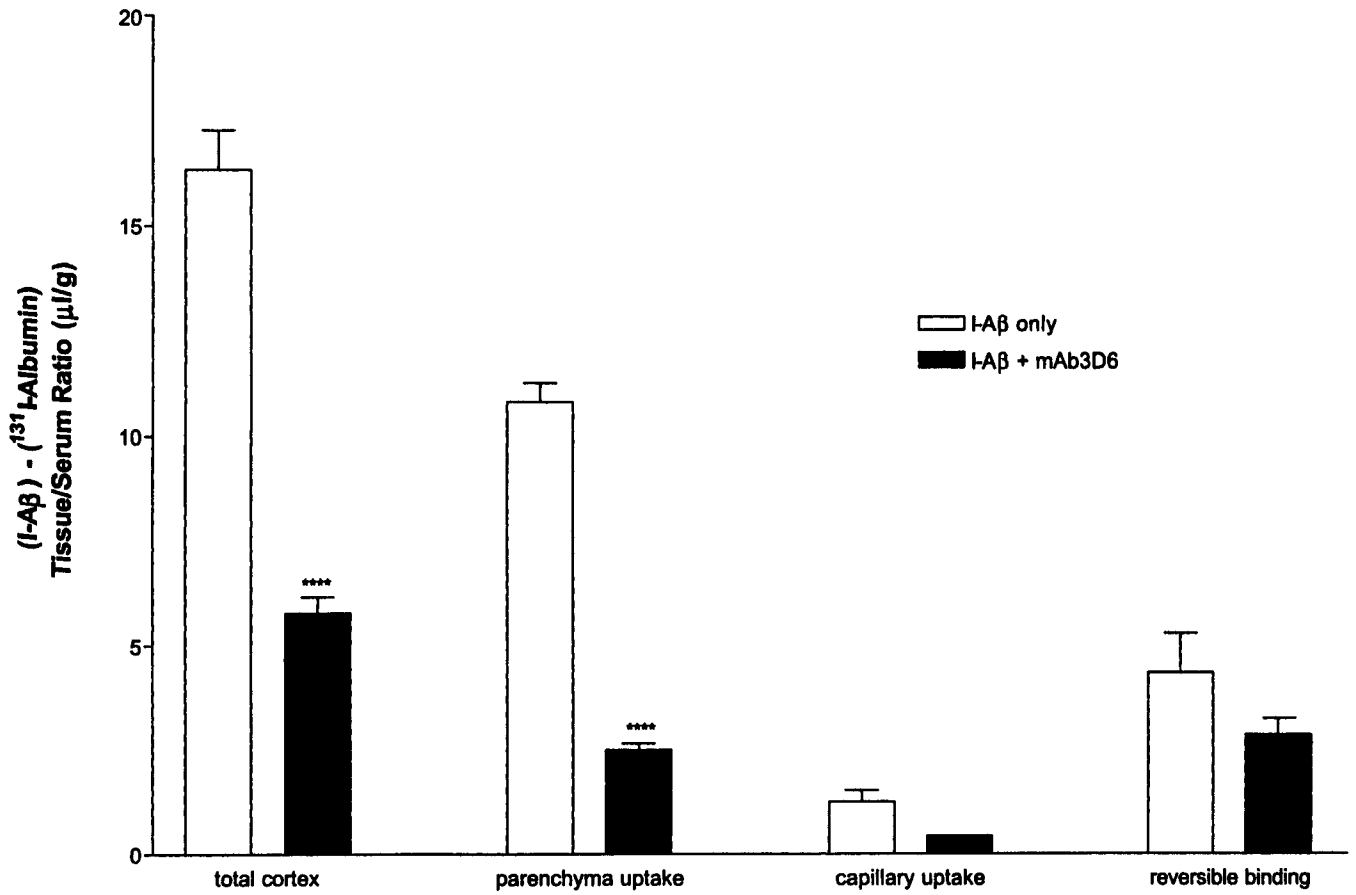

Figure 3. Effect of mAb3D6 on the compartmental distribution of ^{125}I-Aβ in the brain. ^{125}I-Aβ after incubation with mAb3D6 overnight at 4°C had significantly lower uptake by the cortex 15 min after iv injection, and the overall decrease was mainly attributed to the decrease in uptake by brain parenchyma. ****$P < 0.0001$ compared with the control.

ducing the penetration of I-Aβ across the BBB and did so in a different system.

Thus, we have shown that peripherally administered antibodies can decrease the availability of blood-borne Aβ to the brain. This does not rule out other routes of action, such as direct penetration of the antibody into the CNS or an influence on the solubility and CSF dynamics of Aβ. IgG has poor penetration of the intact BBB, but its perivascular distribution is immunolocalized in normal rat brain and is increased after traumatic head injury (1). Increased permeability of the BBB to antibodies may occur with pathological aging. The "decoration" of plaques in PDAPP mice by immunofluorescent 3D6 also suggests the availability of 3D6 at the perivascular space (4). This suggests that 3D6 could reach brain parenchyma. In addition, the N-terminal region (1–28) of Aβ is essential for aggregation (21) and the 3–6 sequential epitope EFRH (Glu-Phe-Arg-His) is particularly important (8, 9). mAb3D6 is directed to the 1–5 sequence and likely prevented the aggregation of Aβ.

No studies of the penetration of Aβ across the BBB have shown the self-inhibition characteristic of a saturable transport system (3, 13); however "stickiness" and self-aggregation (7, 25) might have prevented the identification of a transport system by classical methods. In addition, studies on brain microvascular endothelial cell monolayers show asymmetry in binding and Aβ transport, suggesting differential receptor function (12). If there is a specific transport system for Aβ across the BBB, not only the antibodies but also other potential modulators of the transport system will affect the influx of Aβ from blood to the brain.

Aβ deposition in the brain is related to Alzheimer's disease, whereas deposition in the cerebral vasculature leads to cerebral amyloid angiopathy, a major cause of hemorrhagic stroke in the elderly. Thus, our finding of the inhibitory effect of the antibodies on the penetration of Aβ across the BBB has exciting implications. It not only provides direct evidence for the CNS effect of the antibody, but it also indicates the potential for modification of the binding of Aβ at the BBB so as to reduce the influx of Aβ from blood to brain.

In summary, the antibodies mAb3D6 and mAbmc1 decreased the influx of I-Aβ into the brain after either iv injection or *in situ* brain perfusion. The reduction of the entry I-Aβ occurred mainly in the parenchyma of the brain. Our work provides the first direct evidence that antibodies can interact with Aβ at the BBB level.

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