Non-Fc-Mediated Mechanisms Are Involved in Clearance of Amyloid-β In Vivo by Immunotherapy

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Transgenic (Tg) mouse models overexpressing amyloid precursor protein (APP) develop senile plaques similar to those found in Alzheimer’s disease in an age-dependent manner. Recent reports demonstrated that immunotherapy is effective at preventing or removing amyloid-β deposits in the mouse models. To characterize the mechanisms involved in clearance, we used antibodies of either IgG1 (10d5) or IgG2b (3d6) applied directly to the brains of 18-month-old Tg2576 or 20-month-old PDAPP mice. Both 10d5 and 3d6 led to clearance of 50% of diffuse amyloid deposits in both animal models within 3 d. Fc receptor-mediated clearance has been shown to be important in an ex vivo assay showing antibody-mediated clearance of plaques by microglia. We now show, using in vivo multiphoton microscopy, that FITC-labeled F(ab')2 fragments of 3d6 (which lack the Fc region of the antibody) also led to clearance of 45% of the deposits within 3 d, similar to the results obtained with full-length 3d6 antibody. This result suggests that direct disruption of plaques, in addition to Fc-dependent phagocytosis, is involved in the antibody-mediated clearance of amyloid-β deposits in vivo. Dense-core deposits that were not cleared were reduced in size by ~30% with full-length antibodies and F(ab')2 fragments 3 d after a topical treatment. Together, these results indicate that clearance of amyloid deposits in vivo may involve, in addition to Fc-dependent clearance, a non-Fc-mediated disruption of plaque structure.

Key words: amyloid; transgenic; Alzheimer; multiphoton; imaging; senile plaque; microglia; immunotherapy; antibody

Alzheimer’s disease (AD) is a debilitating neurodegenerative disease characterized by the presence of senile plaques in the brain (Hyman and Trojanowski, 1997; Marksbery, 1997; Powers, 1997). Amyloid-β, a 40–42 amino acid peptide, is the primary component of these plaques, and genetic causes of AD lead to dramatically increased amyloid-β plaque deposition (Selkoe, 1996; Rubinstein, 1997). Although the role of amyloid-β deposits in AD is unknown, senile plaques remain a primary target for drug development aimed at prevention or reversal of the disease. Transgenic (Tg) mouse models overexpressing amyloid precursor protein (APP) develop senile plaques in an age-dependent manner, similar to those found in the human disease (Games et al., 1995; Hsiao et al., 1996). The transgenic mice are valuable as a model system for evaluating therapeutic approaches toward removing or preventing formation of amyloid-β deposits.

Recently, an approach involving immunization of PDAPP mice with amyloid-β was shown to be effective at prevention of plaque deposition (Schenk et al., 1999). In these experiments, a peripheral immune response resulted in an alteration of amyloid-β deposition in the brain. A subsequent report demonstrated that anti-amyloid-β antibodies, given peripherally, were also effective at prevention of amyloid-β deposition in these mice (Bard et al., 2000). This result, using a “passive” immunotherapeutic approach, suggests that an active T-cell-mediated immune response is not necessary for alterations of amyloid-β deposits in the brain. Moreover, we showed recently that a single application of anti-amyloid-β antibodies to the surface of the brain in these mice led to clearance of existing senile plaques in the remarkably short time frame of 3–8 d (Bacskai et al., 2001). Together, these results demonstrated that immunotherapy prevents formation of new plaques in PDAPP mice and can lead to their clearance. The mechanism of this antibody-mediated clearance of plaques appears to be mediated, at least in part, by Fc receptor-mediated phagocytosis; however, additional mechanisms might also be involved.

Specifically, ex vivo experiments demonstrated that Fc receptor-mediated phagocytosis is at least one mechanism involved in removing the amyloid-β in the brain (Bard et al., 2000). F(ab')2 fragments were unable to stimulate microglial cells, and the process was blocked by anti-Fc receptor antibodies, demonstrating that clearance was mediated by Fc. The close association of microglial cells with dense-core plaques, as well as results showing activation of these cells after topical antibody treatment (Bacskai et al., 2001), supports this idea. Similarly, a recent report in double transgenics expressing APP and TGF-β demonstrated clearance of amyloid-β deposits via upregulation of microglia (Wyss-Coray et al., 2001). Together, clearance of amyloid-β deposits by an Fc-mediated mechanism involving active cellular removal seems likely. On the other hand, in vitro experiments have shown that antibodies binding to amyloid-β deposits may disrupt β-pleated sheet conformation and lead to plaque disaggregation directly (Solomon et al., 1997). Finally, an additional possibility for plaque clearance involves activation of complement systems that could enhance degradation or clearance. Our current study.
experiments used an in vivo approach to examine these possible mechanisms.

MATERIALS AND METHODS

Animal preparation. Eighteen-month-old Tg2576 mice (Hsiao et al., 1996) or 20-month-old homozygous PDAPP mice (Games et al., 1995) were used for in vivo imaging, as described previously (Bacskai et al., 2001). Briefly, the animals were anesthetized with avertin (1.3% tribromoethanol and 0.8% tert-pentylalcohol in distilled water; 250 mg/kg, i.p.), and immobilized in custom-built stage-mounted ear bars and a nosepiece, similar to a stereotaxic apparatus. A 2–3 cm incision was made between the ears, and the scalp was reflected to expose the skull. Four circular craniotomies (~1–1.2 mm in diameter; on either side of sagittal suture and just posterior to coronal suture) were made using a high-speed drill (Fine Science Tools, Foster City, CA) and a dissecting microscope (Leica, Wetzlar, Germany) for gross visualization. Heat and vibration artifacts were minimized during drilling by frequent application of artificial CSF (ACSF) (in mM: 125 NaCl, 26 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 1 MgCl2, 1 CaCl2, and 25 glucose). The dura was carefully removed from each site with fine forceps. An ACSF reservoir was created within the opened scalp over the open skull preparations to accommodate the long working distance, water immersion dipping objectives (Olympus Optical, Tokyo, Japan) of an Olympus Optical BX-50 microscope. Texas Red dextran [0.05 ml; 35 mg/ml; 70,000 molecular weight (MW); Molecular Probes, Eugene, OR] was injected into a lateral tail vein to create a fluorescent angiogram used to facilitate image alignment from session to session. Thioflavin S (thioS) (0.005% in ACSF; Sigma, St. Louis, MO) and 8 μl of 1 mg/ml fluorescein-labeled antibody was then applied to the surface of the brain for 20 min. Sites were washed with ACSF and imaged. After imaging, the animals were sutured and allowed to recover. Three days later, the animals were reanesthetized and prepared for imaging. Thioflavin S and Texas Red dextran were reapplied. A different, noncompeting labeled antibody was used in the second imaging session to ensure that the original epitopes of amyloid-β were not simply masked at the second session.

In vivo imaging. Two-photon fluorescence was generated with 750 nm excitation from a mode-locked Ti:Sapphire laser (Tsunami, Spectra-Physics, Mountain View, CA), mounted on a commercially available multiphoton imaging system (Bio-Rad 1024ES; Bio-Rad, Hercules, CA). Custom-built external detectors containing three photomultiplier tubes (Hamamatsu Photonics, Bridgewater, NJ) collected emitted light in the range of 380–480, 500–540, and 560–650 nm. Thioflavin S, FITC-labeled antibody, and Texas Red angiograms were separated spectrally into the three imaging channels. At the end of the experiment, the animal was killed, and the brain fixed in 4% paraformaldehyde.

Image analysis. Three-dimensional imaging volumes were analyzed using custom macros written for Scion Image (Scion, Frederick, MD) or rendered in three-dimension using Voxblast (VayTek, Fairfield, IA). Stacks of two-dimensional images were projected using maximal intensity and aligned from one imaging session to another using blood vessels as independent fiduciary markers. Thioflavin S-positive plaques were measured as described previously (Christie et al., 2001). Local image quality was assessed using the angiogram fluorescence as a positive control. A threshold was applied to the images, and diameters were obtained from the maximal cross-section of each plaque. Plaques were identified by thioflavin S staining and morphological appearance. Ambiguous deposits <5 μm in diameter were not counted. This technique allowed us to determine whether the thioflavin S deposit present in the original imaging session was cleared in the second imaging session. If the plaque was not cleared, the size of the identified plaque before and after treatment was compared. Diffuse deposits were analyzed by measuring antibody-positive, thioflavin S-negative amyloid-β in projections of the three-dimensional imaging volumes from each animal. Clearance was expressed as a percentage of amyloid burden within each site from each animal after 3 d.

RESULTS

Our previous report demonstrated antibody-mediated clearance of existing amyloid-β deposits (Bacskai et al., 2001) in PDAPP transgenic mice, which overexpress a minigene containing the human APPV717F mutation (Games et al., 1995). In vivo imaging of the brain in an intact animal is achieved with multiphoton microscopy, which offers significant advantages over other modes of fluorescence or confocal fluorescence, particularly in thick biological specimens (Denk et al., 1990; Christie et al., 2001). Multiphoton fluorescence microscopy achieves spatial resolution that is comparable with visible light confocal microscopy, i.e., on the order of 1 μm. This resolution is at least two orders of magnitude superior to other imaging modalities, such as magnetic resonance imaging or positron emission tomography scanning (Yang et al., 1998) and allows discrimination of single cells, as well as subcellular structures. This technology permits highly sensitive visualization of microscopic structures deep within biological tissue. Chronic imaging of the live mice with multiphoton microscopy showed that antibody application to the surface of the brain led to clearance of plaques within a few days. To generalize the efficacy of such a treatment in different transgenic mouse models, a similar approach was used in the Tg2576 mouse. This mouse overexpresses the Swedish mutation of the human APP695 gene driven by the hamster prion protein promoter (Hsiao et al., 1996). Mice were surgically prepared as described in Materials and Methods. A craniotomy was performed, and the dura was removed in up to four sites per animal. FITC-labeled antibody (10d5; Elan Pharmaceuticals, South San Francisco, CA) as well as thioS were applied to the exposed brain in ACSF for 20 min and then washed off. Texas red dextran (70,000 MW; Molecular Probes) was injected into a tail vein of each animal to permit simultaneous recording of a fluorescent angiogram. Three-dimensional image volumes of 615 × 615 μm and up to 200 μm deep to the surface of the brain were obtained in each of the cranial sites using a 20X water immersion objective (Olympus Optical; numerical aperture of 0.5). Dense-core plaques were defined as those plaques labeled with thioS (fluorescence in channel 1), whereas the labeled antibody (channel 2) binds to both dense-core and diffuse plaques. Both thioS and antibody label amyloid angiopathy. The angiogram (channel 3) is recorded to facilitate alignment of volumes from sequential imaging sessions within each cranial site, independent of plaque location. Figure 1 illustrates an example of antibody-mediated clearance of existing amyloid deposits in a Tg2576 mouse. The initial imaging session reveals dense-core and diffuse plaques, as well as amyloid angiopathy labeled with the fluorescent antibody. The subsequent imaging session of the same volume of brain obtained 3 d later
shows a marked diminution of diffuse deposits. Figure 2 compares the clearance of amyloid deposits with a single 10d5 application at 3 d in Tg2576 and PDAPP mice. This antibody is remarkably effective at mediating removal of plaques in both transgenic mouse models within 3 d. The Tg2576 and PDAPP mouse models differ in their expression of diffuse versus dense-core plaques. The Tg2576 mouse exhibits more frequent and larger dense-core plaques, with comparatively sparse diffuse amyloid deposits. The PDAPP mouse model develops ~10-fold more diffuse deposits than the Tg2576 mouse at comparable ages. Our imaging approach allows us to measure unidentified amyloid deposits within the same animal before and after treatment, permitting direct measurement of clearance of existing amyloid deposits regardless of their prevalence. These results demonstrate that clearance is independent of the genetic strain of animal, the specific mutation in the APP gene, or the promoter-driving expression of the transgene. These results are consistent with recent reports using different transgenic mouse models in which immunization with amyloid-β peptides prevented behavioral deficits (Janus et al., 2000; Morgan et al., 2000).

The determination of clearance of diffuse amyloid deposits in vivo depends on the addition of a second labeled antibody after treatment with the initial labeled antibody. When using 10d5 as the treatment antibody, 3d6 was used to detect remaining amyloid deposits that were not cleared. These antibodies have neighboring epitopes on amyloid-β but do not compete with each other at the concentrations used here, as described by Bacskai et al. (2001) and shown in Figure 3. In this experiment, paraformaldehyde-fixed tissue sections were first treated with FITC-labeled 10d5 at 1 mg/ml for 20 min, washed, and then subsequently incubated with rhodamine-labeled 3d6 at 1 mg/ml for 20 min. Confocal microscopy shows that both labeled antibodies are bound to amyloid deposits in these sections, and they colocalize identically. Similar results are obtained in cryostat sections and in vivo.

The following experiments were performed to determine whether the mechanism of amyloid-β clearance in vivo was specific to the 10d5 antibody. 3d6 monoclonal antibody, which recognizes amino acids 1–5 of amyloid-β, was compared with 10d5 antibody, which recognizes a close but distinct epitope of amyloid-β, residues 3–6. As can be seen in Figure 4, each of these antibodies was similarly effective after 3 d when applied topically to the PDAPP mouse. Diffuse amyloid burdens were measured in the identical volumes within each site before and after a single treatment. The amyloid burdens were decreased with 10d5 and 3d6 by 50 and 48%, respectively, with each antibody treatment. These results suggest that the mechanism of clearance is not dependent on a single antibody but can be generalized to other antibodies directed toward the N terminus of amyloid-β. Likewise, 10d5 is of the IgG1 isotype, whereas 3d6 is an IgG2b. Therefore, antibody-mediated clearance is not dependent on any
one specific IgG subtype. Because IgG1 antibodies do not activate complement in the mouse, this result demonstrates that activation of the complement cascade is not necessary for clearance of amyloid-β in vivo.

Previous reports have shown that amyloid-β clearance in an ex vivo assay proceeds through Fc-mediated phagocytosis by microglial cells, because F(ab′)2 fragments that lack the Fc portion of the antibody were ineffective (Bard et al., 2000). To evaluate this mechanism in vivo, we treated animals with FITC-labeled F(ab′)2 fragments of 3d6 and measured the clearance of amyloid-β deposits. The quantitative results are presented in Figure 5. F(ab′)2 fragments of 3d6 were as effective as full-length antibodies at clearing diffuse amyloid-β deposits in the PDAPP mice. The FITC-labeled F(ab′)2 fragments labeled amyloid-deposits in vivo, as shown in Figure 6, and, within 3 d, over one-half of the labeled deposits were cleared, similar to the results obtained with full-length 3d6 or 10d5. The F(ab′)2 preparation was examined by Western blot, and no Fc could be detected. Moreover, sections of animals treated with F(ab′)2 were immunostained for the presence of Fc on plaques, and none could be detected. Histological examination of the tissue after the experiments showed some activation of microglia in all cases, probably resulting from the surgical preparation, and no difference between F(ab′)2 and 16b5-treated animals. These results demonstrate that, in addition to mechanisms involving Fc-mediated phagocytosis of antibody-labeled amyloid deposits, an alternative and efficacious mechanism exists for clearance of amyloid deposits that does not depend on Fc. Direct biophysical interaction of antibodies with amyloid-β deposits may be responsible for clearance by direct disaggregation of the deposits. Prevention of fibril formation, disaggregation, and inhibition of toxicity with antibodies has been demonstrated in vitro (Solomon et al., 1996, 1997; Frenkel et al., 2000), and our current results indicate that antibody-mediated disaggregation may occur in vivo. The F(ab′)2 results demonstrate that a mechanism not requiring receptor-mediated cellular activation is involved in clearance by immunotherapy with topical application in vivo.

The results above focus on clearance of diffuse amyloid-β deposits. We also tested the ability of antibodies to lead specifically to the clearance of dense-core deposits in PDAPP mice. All plaques labeled with thioS were considered “dense-core” plaques.
within 3 d, in vivo and antibody 16b5. Error bars are mean ± SE for each group. *p < 0.05 indicates statistically significant by t test.

**DISCUSSION**

Transgenic mouse models that overexpress APP develop senile plaques over time that resemble those found in human Alzheimer’s disease. These animals are a valuable tool toward understanding the physiology and pathology of senile plaques in living tissue and are ideal for evaluating therapeutics aimed at clearance of amyloid-β deposits in the brain. With the recent success using immunotherapy for prevention of amyloid-β deposits in these animals (Schenk et al., 1999; Bard et al., 2000), as well as clearance of existing plaques (Bacskai et al., 2001), this treatment seems very promising. Recent reports have also indicated that immunotherapy may have positive effects on behavioral deficits exhibited in transgenic mouse models (Janus et al., 2000; Morgan et al., 2000). These findings are important for predicting whether anti-amylloid therapies will prove beneficial not just in arresting deposition of amyloid-β but also in prevention of the associated dementia. Microglial cells were implicated in the alterations of amyloid-β deposition by immunotherapy. Clearance of amyloid-β deposits in tissue sections by cultured microglia in an ex vivo system was shown to be Fc receptor dependent (Bard et al., 2000). However, additional or alternative mechanisms for clearance of amyloid-β peptide are possible in vivo. Activation of the complement cascade may play a role in clearance. Direct interaction of antibodies with amyloid-β may lead to disruption of aggregates, as has been shown in vitro (Solomon et al., 1997), and this process should not depend on Fc receptor activation. A combination of Fc-dependent and Fc-independent mechanisms may be involved.

We used in vivo imaging using multiphoton microscopy to study the mechanisms of immunotherapeutic approaches toward removal of amyloid-β deposits in the brains of transgenic mice overexpressing APP. Our current results extend our observations showing antibody-mediated clearance of plaques in PDAPP mice (Bacskai et al., 2001) in several ways. First, the direct demonstration of clearance of amyloid-β deposits in vivo with anti-amyloid-β antibodies in another mouse model, Tg2576, was shown. This result suggests that the previous work in PDAPP mice can be extended to other animal models and is not dependent on the strain of animal, specific mutation in APP, or the specific patterns of expression of the transgene; behavioral studies in other mouse models in which immunotherapy proved beneficial also support this conclusion (Janus et al., 2000; Morgan et al., 2000). Second, an additional mouse monoclonal antibody of a different IgG isotype also led to effective clearance of previously deposited amyloid-β plaques in vivo. This result complements the observation that both of these antibodies can prevent deposition of amyloid-β after passive administration over a 6 month period in PDAPP mice (Bard et al., 2000), as well as both of the efficacy of the antibodies for the targeted digestion of plaques in an ex vivo preparation (Bard et al., 2000). The direct demonstration of clearance of existing, identified plaques in vivo with different antibodies is important in determining the specificity of the response. IgG1s do not activate complement in the mouse and may exhibit other specific differences that provide insight into the mechanism of clearance, particularly in the short time frame of 3 d. The difference in isotype may contribute to the effectiveness of the two antibodies at clearing dense-core plaques in the mice. Although both 3d6 and 10d5 were equally effective at clearing diffuse deposits in the animals and both antibodies led to similar decreases in size of remaining, identified dense-core plaques, 10d5 was more effective at completely clearing dense-core plaques in 3 d. It is possible that this subtle difference in efficacy is dependent on either the dose or time course of the treatment. Although both antibodies were added at the same dose for the same duration, they may have unequal affinities or effective actions at this concentration. Nonetheless, it is possible that complement activation or other differences between IgG1 and IgG2b
Likewise, a recent report demonstrated that peripheral antibod-
...amyloid-Fc-mediated phagocytosis can be involved in the clearance of
...FC-mediated phagocytosis of amyloid-β by microglia. Previous
...work with primary cultures of microglial cells and frozen tissue
...sections has demonstrated that this Fc-mediated mechanism is
dominant in removal of amyloid-β deposits (Bard et al., 2000).
The current results suggest that an alternative mechanism besides
Fc-mediated phagocytosis can be involved in the clearance of
amyloid-β deposits after topical administration in vivo. Other
...cellular mechanisms include activation of scavenger receptors
(Huang et al., 1999; Bornemann et al., 2001) or receptors for
...advanced glycation end products (Munch et al., 1997; Tabaton et al.,
...2001; Bornemann et al., 2001). However, neither of these
...receptor-dependent mechanisms would be activated with F(ab’)_2
fragments. Direct biophysical interaction of antibodies with amyloid-β may have led to disaggregation and removal of deposits
in vivo, similar to results shown in vitro (Solomon et al., 1997).
Likewise, a recent report demonstrated that peripheral antibod-
...ies may act as a sink for amyloid-β, removing it from the CNS and
...preventing plaque deposition in the brain (DeMattos et al., 2001).
Clearance may depend on multiple mechanisms, one involving
direct interaction of antibodies [or F(ab’)_2 fragments] with the deposits resulting in disaggregation and the second involving
cell-mediated clearance, possibly involving Fc receptor activa-
...tion. The two mechanisms may occur independently, or may
...operate in tandem, with cellular removal of amyloid-β after
disaggregation. Passive redistribution of soluble amyloid-β in
...CSF or plasma after disaggregation may occur (DeMattos et al.,
...2001). Characterization of these mechanisms will lead to an
...optimized therapeutic plan for efficient clearance of amyloid-β
deposits.

REFERENCES

Christie RH, Bacsckai BJ, Zipfel WR, Williams RM, Kajdasz ST, Webb WW, Hyman BT (2001) Growth arrest of individual senile plaques in a model of Alzheimer’s disease observed by in vivo multiphoton mi-
Powers JM (1997) Diagnostic criteria for the neuropathologic assess-
Rubinszttein DC (1997) The genetics of Alzheimer’s disease. Prog Neuro-
...2:447–454.
Solomon B, Koppell R, Hanan E, Katzav T (1996) Monoclonal antibod-
...ies inhibit in vitro fibrillary aggregation of the Alzheimer beta-amyloid peptide. Proc Natl Acad Sci USA 93:452–455.
Yang X, Renken R, Hyder F, Siddeck M, Greer CA, Shepherd GM, Shulman RG (1998) Dynamic mapping at the laminar level of odor-