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Mechanisms of β -Amyloid Clearance by Anti-A β Antibody Therapy

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Mechanisms of β -Amyloid Clearance by Anti-A β Antibody Therapy

by

Donna Marie Wilcock

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Mechanisms of β -Amyloid Clearance by Anti-A β Antibody Therapy.

Donna Marie Wilcock

ABSTRACT

Alzheimer's disease (AD) is defined as a progressive neurodegenerative disorder that gradually destroys a person's memory and ability to learn. There are three pathological hallmarks of the disease which are necessary for diagnosis of AD, these are; extracellular amyloid plaques composed of β -amyloid (A β) protein, intracellular neurofibrillary tangles and neuronal loss. Amyloid plaques exist as both compact deposits which stain with Congo red and more numerous diffuse deposits. Active immunization against A β_{1-42} or passive immunization with monoclonal anti-A β antibodies reduces amyloid deposition and improves cognition in APP transgenic mice.

Over several studies of active immunization in APP+PS1 transgenic mice we showed a strong correlation between reduction of compact amyloid deposits and the degree of microglial activation suggesting a potential role of microglia in the removal of A β . Injection of anti-A β antibodies into the frontal cortex and hippocampus of aged APP transgenic mice revealed an early phase of A β removal which was removal of only diffuse amyloid deposits with no associated activation of microglia. A later phase was the removal of compact amyloid deposits. This was associated with significant activation of microglia. Prevention of this microglial activation by anti-A β F(ab')₂ fragments or its

inhibition by dexamethasone also precluded the removal of compact amyloid deposits but did not affect the removal of the diffuse deposits.

Systemic injection of anti-A β antibodies weekly over a period of 1, 2, 3 and 5 months transiently activated microglia associated with the removal of compact amyloid deposits and elevated plasma A β , suggesting a peripheral mechanism contributes to removal of brain A β . This systemic administration also dramatically improved cognitive performance in the Y-maze and in the radial-arm water maze. These studies also showed a significant increase in vascular amyloid dependent on the number of antibody injections the mice received. Associated with this increase in vascular amyloid was a dramatic increase in the numbers of microhemorrhages in the brain. Despite this pathology the mice showed cognitive improvement with the treatment. These effects could have major ramifications in humans and should be further investigated prior to any human clinical trials.

INTRODUCTION

Alzheimer's Disease:

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder characterized by a devastating mental decline. There are three pathological characteristics of AD. These are amyloid plaques, neurofibrillary tangles and neuron loss characterized by dystrophic neurites. The amyloid plaque is a microscopic focus of extracellular amyloid deposition. The plaque contains extracellular deposits of amyloid- β protein ($A\beta$) that occurs primarily in filamentous form. Much of the $A\beta$ found in the plaque is the 42 amino acid species ($A\beta_{1-42}$) which is slightly more hydrophobic than the shorter, 40 amino acid, species ($A\beta_{1-40}$). $A\beta_{1-40}$ is normally the more abundant form of $A\beta$ produced by cells and it does colocalize with $A\beta_{1-42}$ in the plaque. $A\beta$ can also be deposited as diffuse deposits, often thought to be an intermediate step in the formation of a compact amyloid plaque. Amyloid plaques are often termed neuritic plaques, due to the presence of dystrophic neurites within and immediately surrounding the plaque. The size of the deposit can vary greatly, ranging from 10 to more than 120 μ m (Selkoe, 2001).

Neurofibrillary tangles are intraneuronal inclusions of nonmembrane bound bundles of abnormal fibers consisting of pairs of helical filaments. The filaments consist of hyperphosphorylated microtubule-associated protein tau. It is unknown what causes this hyperphosphorylation although studies have implicated the cyclin-dependent kinase 5 (cdk5) (Patrick et al, 1999). Neuron loss is thought to result from toxicity of amyloid plaques, the inflammatory response which results in cytokine release and acute phase

protein release, oxidative injury which causes disruption of neuronal metabolic and ionic homeostasis, and impaired neuronal transport due to the presence of hyperphosphorylated tau filaments in the neuron.

Despite the many pathological characteristics of AD, the most favored hypothesis of the disease process is the amyloid hypothesis. This hypothesis suggests that deposition of A β as both diffuse and compact plaques can directly, and indirectly via an inflammatory cascade, result in progressive synaptic and neuritic injury. This injury is then thought to result in altered kinase/phosphatase activity which leads to hyperphosphorylation of tau and the formation of neurofibrillary tangles. This cascade of events is ultimately thought to result in widespread neuron dysfunction and loss which will cause the dementia characteristic of AD (Hardy et al, 2002). The problem with this hypothesis has been that researchers have been unable to show a strong correlation between neuron loss / dysfunction and levels of amyloid deposits, however, recent data suggests that it is actually small, soluble A β oligomers that cause the neurotoxicity in Alzheimer's disease (Lue et al, 1999, Klein et al, 2001). A β is a product of cleavage of larger precursor protein, the amyloid precursor protein (APP). APP is a single transmembrane polypeptide consisting of between 695 and 770 amino acid residues and is cleaved by enzymes named secretases. The three secretases have specific cleavage sites, using the 770 numbering α -secretase cleavage occurs at amino acid 687, β -secretase cleavage occurs at amino acid 671, while γ -secretase cleavage can occur at amino acid 711 or 713. If a β -secretase cleavage occurs along with a γ -secretase cleavage then A β will be produced, the length of the A β is dependent on whether the γ -secretase

cleaves at residue 711 ($A\beta_{1-40}$) or 713 ($A\beta_{1-42}$). Most mutations which have been found to cause early-onset familial AD are in the APP protein at the three secretase sites, primarily biasing cleavage to the β secretase site or C-terminal mutations increasing the length of $A\beta$ to the more fibrillogenic 42 length. Other mutations were found in the presenilin proteins 1 and 2, later it was discovered that these proteins alter APP metabolism and have a direct effect on γ -secretase by increasing the length of the $A\beta$ produced (Hardy 1997). It has recently been suggested by several studies that the presenilin protein is actually the γ -secretase.

Researchers continue to investigate genetic links to susceptibility for Alzheimer's disease since only 5% of all AD cases are linked to APP, PS1 or PS2 mutations and all are early-onset forms of the disease. Apolipoprotein E (ApoE) genotype has been found to be a significant risk factor for the development of AD. ApoE is a plasma protein involved in the transport of cholesterol and other lipids. ApoE has been shown to be present in amyloid deposits and neurofibrillary tangles and has been implicated in neuronal growth and regeneration during development and following injury. All humans carry two alleles for ApoE, of which there are three types; 2, 3 and 4. A person can be ApoE 4/4, ApoE 4/3, ApoE 3/3, ApoE 2/3 or, rarely, ApoE 2/2 (Soininen and Riekkinen Sr, 1996). It has been shown that onset of AD is earliest in those patients carrying both ApoE4 alleles while one ApoE4 allele is later but still earlier than other alleles with the rare ApoE2 allele possibly inferring protection from the disease. ApoE3 is the most common allele. Although only 16% of the population has ApoE4 as an allele approximately 40% of sporadic AD cases have been found to carry an ApoE4 allele (Strittmatter and Roses, 1995). The role of ApoE in the brain is not fully understood, nor

is the role ApoE4 plays in increasing risk of AD. In vitro it has been shown that ApoE2 and ApoE3 can bind to tau and microtubule associated protein 2c but ApoE4 cannot. This could suggest that binding of ApoE may stabilize the tau protein and possibly prevent the aggregation into neurofibrillary tangles (Strittmatter et al, 1994). However, this may be one of many functions of ApoE as it has been shown to be localized in neurons as well as the extracellular space of the brain and has been shown to have many metabolic functions. It is also important to note that an ApoE4/4 genotype does not guarantee that Alzheimer's disease will occur, only that the person has an increased risk for developing the disease. It is highly likely that many more genes like ApoE will be found in the coming years given that there are still many AD cases without an obvious genetic cause.

Cerebral Amyloid Angiopathy and AD:

Cerebral amyloid angiopathy (CAA) is a common term used to define the deposition of amyloid in the walls of blood vessels, primarily small and medium sized arteries and arterioles, of the brain. In humans, CAA primarily occurs in leptomeningeal and cortical vessels and is rarely observed in other brain regions such as the hippocampus or the striatum (Rensink et al, 2003). The protein accumulating in the vessels causing CAA has excessive β -pleated sheet folding and also has a tendency to form fibrils which are highly insoluble. In order to be defined as CAA the deposits must be stained by Congo red, a dye which stains fibrils comprised of β -pleated sheet folding. There are approximately seven proteins known to cause CAA, these are A β , cystatin C, transthyretin, Gelsolin, prion protein, Abri (familial British dementia) and ADan (familial Danish dementia) (Castellani et al, 2004). In AD the protein causing CAA is the A β protein.

The most severe consequence of CAA is cerebral hemorrhage, known as CAA-associated hemorrhage (CAAH). There are several types of hemorrhages occurring with CAA ranging from microhemorrhages (small leaks in the vessel wall) to aneurysm (a blood-filled dilation of the blood vessel) (McCarron and Nicoll, 2004). These hemorrhages can result in further cognitive decline or, if severe, even death.

CAA can occur alone or in conjunction with parenchymal amyloid deposits and neurofibrillary tangles in AD. When CAA occurs alone it can cause extensive dementia. There are several forms of hereditary CAA such as the Dutch type (HCHWA-D) (Natte et al, 2001) and the Iowa pedigree (D694N) (Grabowski et al, 2001), both mutations causing these hereditary CAAs lie in the APP molecule and result in A β formation. Unlike those mutations of APP occurring in some familial AD cases which produce excess A β ₁₋₄₂, mutations causing CAA result in excessive production of A β ₁₋₄₀ which appears to be excessively fibrillogenic in human cerebrovascular smooth muscle (HCSM) (Grabowski et al, 2001).

The role of CAA in AD is not yet fully known. The reported incidence of CAA in Alzheimer's cases has ranged from 78% to 98% (Kallaria and Ballard, 1999) which suggests an important role for CAA in the pathogenesis of AD although not necessary for diagnosis of AD. Of those cases, approximately 35-40% are associated with some form of hemorrhage (Jellinger et al, 2002). Support for the contribution of CAA to the cognitive decline observed in AD arises from observations that CAA produces ischemia and hemorrhage that in other disease processes is known to result in cognitive dysfunction (Cadavid et al, 2000) and also that the frequency and severity of CAA is increased in AD (Yamada, 2002). However, there is yet no evidence to suggest that the

rate of cognitive decline in AD patients without CAA is any different compared to those AD patients with CAA. Also, in non-demented individuals CAA appears to have no effect on cognitive ability (Castellani et al, 2004).

Transgenic mouse models of AD:

Transgenic mouse models for AD became an aim for researchers following the discovery of many genetic mutations in the APP and PS1 genes thought to cause early-onset familial AD. An ideal AD animal model would develop all pathological hallmarks of AD, as well as the cognitive and memory deficits characteristic of AD. This mouse model would then be the closest thing to an AD patient to allow testing of potential therapies. A mouse carrying the M146L mutation in the PS1 gene (methionine to leucine at 146) showed increased production of $A\beta_{1-42}/A\beta_{1-40}$ compared to wildtype littermates as measured by ELISA, however, these mice did not deposit amyloid, either as diffuse or compact plaques (Duff et al, 1996). Mice expressing mutations in the APP gene showed more promise and as a result several APP transgenic mice were produced. The PDAPP mouse carries the V717F mutation under the control of the platelet derived growth factor promoter and expresses APP695, APP751 and APP770. This mouse begins amyloid deposition between 4 and 6 months of age, accelerates rapidly at 7 to 9 months of age with significant numbers of both diffuse and compact amyloid deposits in the frontal cortex and hippocampus by 1 year of age. The Tg2576 mouse carries the Swedish mutation of KM670/671NL under the control of the hamster prion protein promoter and express APP695 (Hsiao et al, 1996). These mice have detectable diffuse and compact amyloid deposits by 6 months of age and continue to deposit in an age-dependent manner showing an acceleration between 8 and 12 months of age (Kawarabayashi et al, 2001).

Despite the ability to produce transgenic mice that develop amyloid deposits in an age-dependent manner, very few of these APP transgenics demonstrated reproducible cognitive and memory deficits. Crossing the M146L PS1 mouse with the Tg2576 APP mouse not only showed accelerated amyloid deposition (Holcomb et al, 1998, Gordon et al, 2002) but also developed cognitive and memory deficits detectable by several behavior paradigms which were reproducible at defined ages (Gordon et al, 2001). The benefits of this mouse model are that therapies can be tested to show not only their effect on amyloid deposition but also whether they may have any clinical benefit by showing whether the treatment improves cognitive function. Although this doubly transgenic mouse is a good model in which to test therapies, it does still lack two of the three pathological hallmarks of AD, those of neurofibrillary tangles and neuronal loss, despite the abundance of amyloid deposits.

Recently, there have been several tau transgenic mice developed, which provide a further step toward the ideal mouse model for AD. The P301L mutation on chromosome 17 expressed in mice results in development of hyperphosphorylated tau and neurofibrillary tangles detectable by Gallyas silver staining. The disadvantage to this mouse is that it also develops motor deficits due to expression of mutated tau in the brain stem and spinal cord, the animals are completely paralyzed by 12 months of age (Lewis et al, 2000). The mice also show differential expression between males and females, with females having 3 to 4 times more expression of the mutated tau than males. Despite the disadvantages of this mouse model it has been shown that crossing the P301L mouse with the Tg2576 mouse enhances forebrain neurofibrillary tangle formation, suggesting that the presence of A β influences the extent of neurofibrillary pathology (Lewis et al, 2001).

Transgenic mice expressing V337M mutant human tau show hyperphosphorylated tau and neurofibrillary tangles in the hippocampus resulting in behavioral abnormality (Tanemura et al, 2002).

The ideal mouse model would have all pathological hallmarks of AD; amyloid plaques, neurofibrillary tangles and neuron loss. The closest mouse model to date is a triple transgenic developed by Frank LaFerla and colleagues (Oddo et al, 2003). This group showed a mouse with the M146L PS1 mutation knock-in as well as mutant tau and APP which both have the Th1 promoter and both co-integrate at the same site, this produces a closer model of AD than had previously been shown. All three transgenes are expressed to homozygosity and are expressed at the same levels. The mice develop amyloid plaques and neurofibrillary tangles. Interestingly the mice develop the amyloid plaques prior to any tau pathology being observed. The mice also demonstrate age-dependent LTP impairment although this occurs prior to any AD-like pathology being present. At 6 months of age the mice have impaired long-term potentiation (LTP) suggesting that the mice may develop cognitive deficits. However, to this date no neuron loss has been reported in this transgenic model.

A better mouse model for AD may be possible thanks to a new mouse model of tau pathology. In a recent report from Peter Davies and colleagues a mouse, known as the htau mouse, undergoes age related accumulation of hyperphosphorylated tau like those observed in AD and the presence of neurofibrillary tangles (Andorfer et al, 2003). These htau mice are a cross of two existing mouse lines. One is a tau transgenic known as the 8c mouse which expresses all human tau isoforms but alone does not demonstrate any evidence of tau pathology (Duff et al, 2000). The other is a tau knockout mouse which

again, alone does not develop any tau pathology (Tucker et al, 2001). When crossed, a mouse with all human isoforms but no mouse tau is produced which demonstrates extensive and age-related tau pathology. This htau mouse has also importantly been shown to develop age related neuron loss in cortical and hippocampal areas. By the age of 18 months there is a 50% reduction of neurons in the piriform cortex. There is also an apparent shrinkage of the cortex and enlargement of the ventricles (Andorfer et al, 2004). Since this mouse appears to show extensive tau pathology and neuron loss, it is hoped that crossing this mouse with an APP transgenic mouse may yield a more perfect mouse model in which to test potential treatments for AD.

Behavioral Analyses of transgenic mice:

The major clinical symptom of AD is cognitive decline so therefore any effective clinical therapy must act to improve cognitive function of patients so it is critical that potential therapies are shown not only to affect the pathological characteristics of AD such as amyloid plaques or neurofibrillary tangles but must also affect memory. To test memory impairment in mice there have been several behavioral paradigms developed.

The Morris water maze was first described in 1982 by Richard Morris and colleagues (Morris et al, 1982) where he showed impairment in the task following hippocampal lesions. This task is consistently used to assess memory retention. It consists of a water pool with a hidden escape platform where the mouse must learn the location of the platform using either contextual or local cues. The mouse's aversion to water and swimming force it to look for an escape and therefore search out the platform. This task has been shown to be heavily hippocampal dependent, where lesions to the hippocampus or its cholinergic input significantly impair performance. Time taken to locate the

platform is measured which is known as escape latency. Also measured is the time spent in the quadrant where the platform was once removed, these trials are known as probe trials. A modification of the Morris water maze is the radial arm water maze (RAWM) which is a circular pool with six swim alleys (arms) radiated out from an open central area with a submerged escape platform located at the end of one of the arms which the animal must find. There are several spatial cues present on the walls and ceiling of the testing room. The platform remains in the same arm during testing for each mouse, however, the arm in which the mouse starts each time is different requiring the mouse to use the visual cues in order to remember where the platform is (Diamond et al, 1999). The number of wrong arms entered is measured as errors and also time to find the platform is measured. Again, this is a heavily hippocampal dependent task and performance has been shown to be impaired in some transgenic mouse models of AD (Arendash et al, 2001; Morgan et al, 2000).

Another memory task is contextual fear conditioning which uses an aversive stimulus coupled to sound. The animal learns to freeze when the sound is heard as it is associated with the aversive stimulus which is commonly a small electric shock. The amount of freezing is measured and lack of freezing is associated with impaired memory of the preceding events (Gerlai, 2001). This task is highly dependent upon the integrity of the amygdale however is also sensitive to disruptions in hippocampal function.

The Y-maze is not dependent upon learning a new behavior but depends upon the tendency of a mouse to explore new environments. The Y maze is a three arm maze with equal angles between all arms. Mice are initially placed within one arm and the sequence and number of arm entries is recorded for each mouse over set period of time (usually

between five and ten minutes). The percentage of triads in which all three arms are represented is recorded as an alternation to estimate short-term memory of the last arms entered. The total number of possible alternations is the number of arm entries minus two. Additionally, the number of arm entries serves as an indicator of activity.

Inflammation and AD:

Along with the three primary characteristics of AD there is also an extensive inflammatory response in the brain. Microglia and astrocytes are the two primary inflammatory cells in the brain and these respond to damage and foreign material in much the same way as do the immune cells of the periphery. Microglial cells are from the monocytic lineage and are the resident macrophage in the brain. They have the ability to produce complement proteins in vitro, potentially contributing to the complement cascade. They can produce and secrete IL-1, a cytokine with many immune functions. Microglial cells can also enter into a phagocytic state, at which point they are almost indistinguishable from a macrophage (Streit, 2002, Liu et al, 2003). Astrocytes are cells native to the CNS and have many normal functions such as inducing the blood brain barrier and contributing to the local homeostasis of the synapse by expressing reuptake proteins on their membrane. Astrocytes also have the ability to produce inflammatory mediators when activated and are thought to communicate with microglial cells through these mediators.

In AD activated microglia cluster at sites of amyloid deposition, surrounding the deposit. Activation can be initially detected by an increased expression of the leukocyte common antigen CD45 (Aloisi, 2001), a functional transmembrane protein-tyrosine phosphatase (Justement, 1997). In later stages of activation there is further increase in

expression of CD45 along with expression of the major histocompatibility complex class II (MHC-II).

Much of the literature in the past decade has focused on the deleterious consequences of microglial activation (Akiyama et al, 2000). Microglia are capable of producing many cytokines, reactive oxygen intermediates, excitatory amino acids and nitric oxide (NO) (Streit et al, 1999), all of which could significantly contribute to neuronal death seen in AD. In vitro, A β can stimulate release of IL-1, IL-6, TNF- α and superoxide free radicals from microglia (McGeer and McGeer, 2001). IL-1 has an autocrine induction in microglia and also enhances microglia proliferation, it causes direct neurotoxicity and apoptosis. IL-6 causes astrogliosis but can be both a survival factor and a neurotoxic factor depending on its levels. TNF- α can cause nitric oxide production and MHC-II expression in microglia (Wilson et al, 2002). All of this data led to the hypothesis that inflammation in the AD brain, particularly the activation of microglia, contributes negatively to the disease process, and inhibition of this inflammation was the target of AD therapies. It has been shown that the glucocorticoid anti-inflammatories are capable of inhibiting microglia activation as detected by nitric oxide production (Chang and Liu, 2000) and by measurement of the extent of proliferation (Tanaka et al, 1997). Epidemiological studies have shown a beneficial effect of NSAIDs in the prevention of AD. It was thought that this beneficial effect was due to inhibition of inflammation, however, a report in 2001 showed that the effects of non-steroidal anti-inflammatory drugs (NSAIDs) in AD may actually be independent of cyclooxygenase (COX) activity and may in fact be due to an effect at the γ -secretase enzyme. This study showed that in cultured cells following treatment with ibuprofen

there is a decrease in A β 42 production but an increase in A β (1-38), which is not amyloidogenic, although the concentrations of ibuprofen used in this study are much greater than those required for COX inhibition (Weggen et al, 2001). A novel non-steroidal anti-inflammatory drugs (NSAID) with a nitric oxide donor group and an antioxidant group showed an unexpected increase in microglial activation when administered to APP+PS1 mice, associated with a significant reduction in diffuse and compact amyloid deposits (Jantzen et al, 2002). Recent data suggests that inflammation in AD is much more complex than originally thought and that microglia may have a beneficial role to play in AD.

Microglia have been shown to phagocytose A β both in vitro and in vivo through several different mechanisms involving opsonization through the complement cascade (Rogers et al, 2002) or the scavenger receptor (Paresce et al, 1996). Curiously, however, 3D reconstruction of the microglia by electron microscopy in untreated transgenic mice was unable to detect intracellular amyloid despite amyloid fibrils being completely engulfed by microglia (Stalder et al, 2001). Interestingly, it has been shown that co-culture of microglia with astrocytes suppresses microglia phagocytosis of senile plaques (DeWitt et al, 1998). Removal of A β by microglia has led to the hypothesis that there exists a dynamic equilibrium between A β deposition and A β removal, and that inhibition of microglia activation may, in fact, result in greater deposition of A β and a more rapid progression of the disease. An interesting finding to support the beneficial role of microglia is that when APP transgenic mice express soluble complement receptor related protein y (sCrry), a complement inhibitor, a 2 to 3-fold increase in A β deposition is seen as well as a prominent accumulation of degenerating neurons not normally seen in the

APP transgenic mouse alone. Microglial activation is reduced significantly in this mouse model, suggesting that inhibiting complement, therefore inhibiting opsonization and phagocytosis, will have a detrimental effect on the disease process (Wyss-Coray et al, 2002). Another transgenic model that has supported the beneficial role of microglia is a mouse that overproduces transforming growth factor (TGF)- β 1 crossed with an APP transgenic mouse. In this model, the overexpression of TGF- β 1 results in a vigorous microglial activation along with a 50% reduction in A β deposition (Wyss-Coray et al, 2001). TGF- β 1 has also been shown to result in clearance of A β by microglial cells in culture. Further support for microglial removal of A β was shown when lipopolysaccharide (LPS) was intracranially injected into the hippocampus of APP+PS1 mice. One week following the injection, there was a significant removal of A β associated with significant microglial activation; however, compact plaques were not removed (DiCarlo et al, 2001; Herber et al, 2004).

Immunotherapy for AD:

Using the amyloid hypothesis as the basis for the development of AD therapies, Dale Schenk and colleagues at Elan pharmaceuticals reported the use of A β ₁₋₄₂, the amyloidogenic protein in AD, as an immunogen. They immunized PDAPP transgenic mice with A β ₁₋₄₂ in an aggregated / fibrillar preparation which is emulsified in Freund's adjuvant to increase the immune response to the antigen. Each mouse received 100 μ g A β . This was repeated 2 weeks later and then monthly thereafter. It was shown that immunization reduced and/or prevented A β accumulation in this mouse model and associated with this reduction was an activation of microglia suggesting that part of the

mechanism of clearance involved these inflammatory cells. Subsequent work by our group demonstrated that not only did A β immunization modestly reduce amyloid burden but more importantly it prevented cognitive impairment in the doubly transgenic APP + PS1 mice (Morgan et al, 2000). This finding was also shown by Janus and colleagues at the same time in a different mouse model, the TgCRND8 mouse which is transgenic for APP only (Janus et al, 2000). Following this our group conducted several more immunization studies which led to the finding that following immunization there is a strong correlation between microglial activation and reduction in the congophilic, compact amyloid deposits (Wilcock et al, 2001).

There has also been data to show that anti-A β antibodies can dissolve fibrils in vitro (Solomon et al, 1997). More recent data to support this dissolution of plaques showed that anti-A β F_{(ab')₂} fragments directly applied to the brains of Tg2576 or PDAPP mice results in reduction of A β and thioflavine-S comparable with the reduction seen when a whole anti-A β IgG is applied (Bacskai et al, 2002).

The vaccine, now known as AN1792, advanced to human clinical trials, however, during phase II trials there were several patients found to be suffering from cerebral inflammation and meningoencephalitis (Bowers and Federoff, 2002, Munch et al, 2002). It is important to note that the occurrence of the meningoencephalitis did not correlate at all with antibody titers in those patients (Orgogozo et al, 2003). Interestingly, it was shown that the antibodies generated by humans in response to the AN1792 immunization recognized both diffuse and compact amyloid deposits in transgenic mouse and human brain tissue, however, it did not cross react with full length APP or vascular A β (Hock et al, 2002).

Since the original report of meningoencephalitis in some participants of the AN1792 trial there have been several reports on pathology from some of the participants following their death. The first was from James Nicoll and colleagues (2003) who reported on a 72 year old female who had been clinically diagnosed with moderate AD and had developed meningoencephalitis following the fifth injection. Some features of AD were detected such as cortical atrophy, ventricle enlargement and the presence of neurofibrillary tangles and amyloid plaques. However, it was observed that this patient that amyloid plaques were sparse in comparison with control AD cases throughout most of the neocortex. Reactive microglia were also observed and appeared to colocalize with immunostaining for A β . Importantly, it was shown that vascular amyloid deposits persisted and appeared not to be reduced by immunization. In a report published a year later showing the pathology from another patient the findings were very similar (Ferrer et al, 2004). This report shows the brain pathology from a 76 year old male who developed meningoencephalitis following the second immunization. Tau pathology was comparable to that found in AD control brain, however, very low numbers of amyloid plaques were observed and remaining plaques were associated with high numbers of activated microglia. Also observed were severe small blood vessel disease and multiple cortical hemorrhages.

Hock et al (2003) reported that antibody titers measured by ELISA were not a good indicator of cognitive performance following immunization, however a new method for measuring antibody reactivity was developed called the tissue amyloid plaque immunoreactivity (TAPIR) assay. The TAPIR assay measures the binding capacity of the circulating antibodies to amyloid plaques in transgenic mouse tissue and human AD brain

tissue. Those patients showing strong TAPIR results also showed stabilization of cognitive performance while those patients without TAPIR reactivity results showed a normal cognitive decline for AD. Together with the pathology reports from two patients in the trial these data suggest that immunotherapy may be a promising approach to the treatment of AD if the meningoencephalitis can be avoided.

Subsequent to the initial studies of immunization using A β ₁₋₄₂ in 1999 and 2000, researchers began to look at passive immunization as a potentially safer approach to an immunotherapy since it had been shown that active immunization may be less effective in people with a significant amyloid burden. A study suggests that the vaccine may be much more effective at preventing amyloid deposition as opposed to removal of existing deposits (Das et al, 2001). It has also been found by work in our laboratory that the ability to produce sufficient antibody titers against A β ₁₋₄₂ may decline with age. The first report of passive immunization came in 2000 from Elan pharmaceuticals, who administered antibodies against A β via an intraperitoneal injection in PDAPP mice (Bard et al, 2000). The antibodies were administered weekly for a period of six months. This resulted in a significant reduction in plaque burden in both the cortex and hippocampus. Importantly they showed by immunohistochemical methods that the injected antibody does cross the blood-brain barrier and enter the brain. They also estimated the amount of IgG entering the brain to be 0.1% as calculated by examining endogenous IgG levels in brain parenchyma. The amount of IgG entering the CNS was confirmed by a study specifically aimed at detection of peripherally injected anti-A β IgG in the CNS of SAMP8 mice, which overexpress APP but do not deposit amyloid. The authors found that 0.11% of injected IgG enters the brain within 1 hour, IgG was still detectable in the

brain 72 hours following injection (Banks et al, 2002). In the Bard et al study (2000) the authors demonstrated that although the antibodies they had used for this immunization were capable of triggering microglial-mediated phagocytosis of amyloid in culture, $F_{(ab)2}$ fragments were unable to activate microglial removal despite retaining the full ability to bind to $A\beta$. This result suggests that clearance of fibrillar amyloid is via Fc receptor mediated phagocytosis. This proposed mechanism was further supported by a study involving direct imaging of amyloid deposits in living mice using multiphoton microscopy (Bacsikai et al, 2001). Three days following direct application of anti- $A\beta$ antibodies to the brain of PDAPP mice there was significant removal of amyloid deposits accompanied by activation of microglia surrounding the remaining deposits. Human postmortem microglia have been shown to phagocytose opsonized $A\beta$, which is inhibited in the presence of excess non-specific IgG, suggesting the phagocytosis is Fc receptor mediated (Lue et al, 2002).

More recently, studies involving passive immunization have suggested that the primary mechanism for $A\beta$ clearance is peripheral and is not due to the antibodies entering the CNS. It has been shown that following intraperitoneal injection of anti- $A\beta$ antibodies in the PDAPP mouse there is a rapid 1,000-fold increase in circulating plasma $A\beta$, suggesting that circulating $A\beta$ antibodies bind to plasma $A\beta$ and thus cause a disruption in the equilibrium between the brain and plasma removing $A\beta$ from the brain. This study importantly used antibodies that have been shown not to bind to plaques in the brain, so their effect on brain amyloid burden is not due to binding fibrils (DeMattos et al, 2001). This same group also demonstrated that administration of this same antibody to PDAPP mice is capable of reversing memory deficits in only one day without a reduction

in amyloid burden in the brain (Dodart et al, 2002). The authors suggest that this rapid reversal of cognitive deficits is due to removal of soluble A β from the CNS as opposed to reducing brain amyloid plaque burden. Cognitive improvement following passive immunization has also been shown in the Tg2576 mouse with an antibody recognizing A β 1-12 which did not reduce brain A β levels but did reverse memory deficits (Kotilinek et al, 2002). Application of anti-A β antibodies to the surface of the brain has been shown to not only reduce the size and number of amyloid deposits but also to recover dystrophic neuritis from a curvy, distorted appearance to a straighter, more normal appearance (Lombardo et al, 2003).

It appears that an important issue of passive immunization is the antibody isotype. It has been shown that IgG2a antibodies clear A β from PDAPP brain sections in an ex vivo assay much more effectively than either IgG1 or IgG2b antibodies despite all antibodies having the same epitope (Bard et al, 2003). This data also supports the hypothesis that microglia are responsible for the clearance of A β by immunotherapy since Fc γ RI and III bind with the greatest affinity to murine IgG2a antibodies (Radaev and Sun, 2001). The fact that IgG2a anti-A β antibodies appear to be the most effective indicate that A β clearance may be mediated through microglial Fc receptors. However, conflicting data suggests that effective clearance of A β by anti-A β antibodies can be obtained in the absence of Fc receptors. Das et al (2003) showed that when they actively immunized APP transgenic mice crossed with Fc receptor knockout mice they showed the same amount of A β reductions as immunized, age-matched APP transgenic mice.

A concerning effect of passive immunization is a report showing an increase in cerebral microhemorrhage in very old APP23 mice following passive anti-A β immunotherapy

(Pfeifer et al, 2002). This effect has not been shown in any other study, but will need to be investigated if passive immunization is to enter human trials.

To summarize, there are three main proposed mechanisms of action of immunotherapy for AD. The first is the binding of antibody to A β and resulting in Fc receptor mediated phagocytosis. The second is that antibodies binding to A β cause a disgregation of the plaque and result in a dissolution. The third is that the effects are primarily peripheral, with circulating antibodies binding to A β in plasma and causing a disruption in equilibrium between brain and plasma causing the A β to be “drawn out” of the brain. It would be naïve to think that only one of these could occur, as the data for all three is convincing. However, the main question is which mechanism is going to produce the most beneficial effect with the least adverse effects and whether immunotherapy can be manipulated to take advantage of this mechanism.

PAPER 1:

**INTRACRANIALY ADMINISTERED ANTI-A β ANTIBODIES REDUCE β -
AMYLOID DEPOSITION BY MECHANISMS BOTH INDEPENDENT OF AND
ASSOCIATED WITH MICROGLIAL ACTIVATION.**

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Abstract

Active immunization against A β ₁₋₄₂ with vaccines or passive immunization with systemic monoclonal anti-A β antibodies reduces amyloid deposition and improves cognition in APP transgenic mice . In this report, intracranial administration of anti-A β antibodies into frontal cortex and hippocampus of Tg2576 transgenic APP mice is described. The antibody injections initially results in a broad distribution of staining for the antibody which diminishes over 7 days. While no loss of immunostaining for deposited A β is apparent at 4 hours, a dramatic reduction in the A β load is discernable at 24 hours and maintained at 3 and 7 days. A reduction in thioflavine-S positive compact plaque load is delayed until 3 days, at which time microglial activation also becomes apparent. At one week after the injection, the microglial activation returns to control levels, while the A β and thioflavine-S staining remains reduced. The results from this study suggest a two phase mechanism of anti-A β antibody action. The first phase occurs between 4 and 24 hours, clears primarily diffuse A β deposits and is not associated with observable microglial activation. The second phase occurs between 1 and 3 days, is responsible for clearance of compact amyloid deposits and is associated with microglial activation. The results are discussed in the context of other studies identifying coincident microglial activation and amyloid removal in APP transgenic animals.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive deficits. There are several pathological characteristics to the disease process, including congophilic amyloid plaques containing the beta-amyloid peptide

(A β), and intracellular inclusions of neurofibrillary tangles consisting of hyperphosphorylated tau protein. Another characteristic of AD is the initiation and proliferation of a brain-specific inflammatory response consisting of activated microglia and astrocytes. Amyloid deposition is thought to be the key step in the pathogenesis of AD (Selkoe, 1991; Hardy and Selkoe, 2002); this is the reason why development of potential therapies focuses on clearance of amyloid.

Vaccination using A β_{1-42} was first described by Schenk et al. (1999). This report demonstrated that active immunization using A β_{1-42} in the PDAPP transgenic mouse dramatically reduced levels of A β deposits. This immunization protected APP+PS1 transgenic mice (Morgan et al., 2000) and TgCRND8 transgenic mice (Janus et al., 2000) from memory deficits. More recent studies showed that treatment with a passive immunization regimen consisting of anti-A β antibodies resulted in a dramatic reduction in A β (Bard et al., 2000; DeMattos et al., 2001) and reversal of memory deficits (Dodart et al., 2002; Kotilinek et al., 2002) in the PDAPP mouse.

In this experiment, we show that intracranially administered anti-A β antibodies have both an early, microglia independent, and a later, possibly microglia dependent mechanism of action. A β levels are dramatically reduced 24 hours following administration in the absence of microglial activation. However, 72 hours after antibody administration thioflavine-S positive compact plaques are reduced concomitant with a striking activation of microglia.

Materials and Methods.

Transgenic Tg2576 APP mice (Hsiao et al., 1996) were obtained following breeding of Tg2576 APP mice with line 5.1 PS1 mice (Duff et al., 1996) which yields four different genotypes; nontransgenic, transgenic APP, transgenic PS1 and doubly transgenic APP+PS1 mice. Animals were provided food and water *ad libitum* and were kept on a 12-hour light/dark cycle; they were housed in groups where possible until prior to the surgery when they were all singly housed until kill. We used 2 cohorts of mice in this study, the first cohort of 19 mo old APP mice (n=16) and the second cohort of 16 mo old APP mice (n=22).

Mice from the first cohort all received anti-A β antibodies (Biosource, Camarillo CA, mouse anti-A β IgG₁, recognizing AA 1-16). Mice from the second group were assigned to groups receiving either anti-A β antibodies, control antibody (anti-HIV, ID6, K. Ugen, Dept. Med. Micro. USF) (N=5), or vehicle (0.02% thimerosal in PBS, Sigma-Aldrich, St Louis, MO) (N=5). All mice were injected in both the frontal cortex and hippocampus of the right hemisphere while the left hemisphere remained untreated as an internal control. Those mice receiving anti-A β antibodies were assigned survival times of 4 (N=5), 24 (N=7), 72 (N=8) or 168 (N=6) hr. Mice receiving either control antibody or vehicle were examined after a 72 hr survival time. A third group of untreated 17 mo old APP mice (N=5) were killed uninjected and unmanipulated to assess differences between the right and left sides of the brain.

On the day of surgery the mice were weighed, anesthetized with isoflurane and placed in a stereotaxic apparatus (51603 dual manipulator lab standard, Stoelting, Wood

Dale, IL). A midsagittal incision was made to expose the cranium and two burr holes were drilled using a dental drill over the right frontal cortex and hippocampus to the following coordinates: Cortex: AP +1.5mm, L -2.0mm, hippocampus: AP -2.7mm, L -2.5mm, all taken from bregma. A 26 gauge needle attached to a 10 μ l Hamilton (Reno, NV) syringe was lowered 3mm ventral to bregma and a 2 μ l injection was made over a 2 minute period. The incision was cleaned with saline and closed with surgical staples.

On the day of kill the mice were overdosed with 100mg/kg of pentobarbital (Nembutal sodium solution, Abbott laboratories, North Chicago IL) and perfused intracardially with 25ml of 0.9% sodium chloride and 50ml of freshly prepared 4% paraformaldehyde (pH=7.4). The brains were collected and post fixed for 24 hours in 4% paraformaldehyde. The brains were then incubated for 24 hours in 10, 20 and 30% sucrose sequentially to cyroprotect them. Horizontal sections of 25 μ m thickness were then collected using a sliding microtome and stored at 4 $^{\circ}$ C in DPBS buffer with sodium azide to prevent microbial growth. Six to eight sections approximately 100 μ m apart were selected spanning the injection site and stained using free-floating immunohistochemistry methods for total A β (rabbit antiserum primarily reacting with the N-terminal of the A β peptide 1:10000), CD45 (Serotec, Raleigh NC, 1:3000) and major histocompatibility complex class II (MHC-II, BD Pharmingen, Palo Alto CA, 1:3000) as previously described (Gordon et al., 2002). For immunostaining, some sections were omitted from the primary antibody to assess non-specific immunohistochemical reactions. Also, immunohistochemical methods were used to stain for the injected antibody using anti-mouse IgG conjugated to horseradish peroxidase (Sigma-Aldrich, St Louis MO, 1:1000). Adjacent sections were mounted on slides and stained using 4% thioflavine-S (Sigma-

Aldrich, St Louis MO) for 10 minutes. Selected sections stained for CD45 were counterstained for Congo red (Sigma-Aldrich, St Louis MO) to detect amyloid deposits on these sections.

The immunohistochemical reaction product on all stained sections was measured using using a videometric V150 image analysis system (Oncor, San Diego, CA) in the injected area of cortex and hippocampus and corresponding regions on the contralateral side of the brain. Data are presented as the average ratio of injected side to non-injected side for A β , thioflavine-S and CD45, while data for MHC-II are expressed as area occupied by positive stain since many values on the contralateral side were close to zero.

To assess possible treatment-related differences, the measurement for either cortex or hippocampus of each subject were analyzed by ANOVA using StatView software version 5.0.1 (SAS Institute Inc., NC) followed by Fischer's LSD means comparisons.

Results

Immunohistochemistry against mouse IgG was performed to trace the diffusion of anti-A β antibodies after injection into the hilus of the dentate gyrus. The injected anti-A β antibody showed diffuse distribution throughout the entire hippocampus at 4 hours with a focal concentration in the outer molecular areas of the dentate and ammons' horn near the hippocampal fissure (Fig. 1A). By 24 hours, the diffuse pattern remained broad, but the focal concentration began shifting towards the granule cell layers of the dentate (Fig. 1B). At 72 hours, staining for the injected antibody was lighter and became concentrated at the granule cell layer of the dentate gyrus (Fig. 1C). Interestingly, by the one week time-

point the injected antibody staining has largely cleared with some residual staining in the outer molecular layer of the ventral (ventricular) blade of the dentate gyrus and the glial limitans. A similar time course of staining was seen in the frontal cortex (data not shown).

A β immunohistochemistry in APP transgenic mice resembled that reported by others and ourselves earlier (Hsiao et al., 1996; Gordon et al., 2002). In both cortex (Fig. 2A) and hippocampus (Fig. 2C) there were a few intensely stained deposits and a number of smaller, less intensely stained deposits. In prior work, we found the intensely stained A β deposits were usually also stained with thioflavine-S or Congo red (Holcomb et al., 1998; Gordon et al., 2001) indicating they were analogous to compact deposits containing fibrillar amyloid, while the less intense deposits were analogous to diffuse, nonfibrillar deposits commonly observed in AD tissue. While the deposits were fairly uniformly distributed within the cortex, in the hippocampus they were concentrated in the outer molecular layers of the dentate gyrus and ammon's horn (Fig. 2C). The subiculum also appeared more rich in deposits than other areas.

The injection of anti-A β antibody into brain did not result in a rapid loss of signal in postmortem immunohistochemical reactions since we did not observe a change in A β staining 4 hours post-injection in either cortex (Fig 3A) or hippocampus (Fig 3B). However, A β staining was reduced at the injection sites in frontal cortex and hippocampus 24 hours after administration of anti-A β antibody (Fig 2B and D respectively) and remained reduced to roughly the same extent through the one week time-point (Fig 3). The reduction in the frontal cortex was over 60% as compared to both the 4 hour time-points and the two control groups of vehicle and anti-HIV antibody (Fig

3A, $P < 0.001$). The reduction in the hippocampus was over 50% as compared to the 4 hour time-points and the control groups (Fig 3B, $P < 0.005$).

An interesting phenomena was that the ratio of A β staining on the right to left sides in untreated mice was greater than 1, indicating more A β deposition on the right side than the left (Figure 3). It appears that this pattern of A β deposition is a consistent property of the APP mice. The A β distribution seen in the mice administered control injections at 3 days and anti-A β antibody at the 4 hour time point is the typical distribution found in APP transgenic mice of this age.

As expected, the number of deposits stained with thioflavine-S were considerably fewer than those stained by A β immunohistochemistry. Nonetheless, the regional distribution of these deposits roughly paralleled that of A β positive deposits in the cortex and hippocampus (Fig. 4A and C). In contrast to the A β , thioflavine-S positive staining at the injection site was not reduced until 72 hours after administration of anti-A β antibody (Fig 4B and D) and remained reduced at the one week time point (Fig. 5). The reduction in frontal cortex was over 80% compared to the 4 and 24 hour time points as well as the control groups (Fig 5A, $P < 0.001$). The reduction in hippocampus was over 60% compared to both the 4 and 24 hour time points and the control groups (Fig 5B, $P < 0.005$).

In untreated mice, activated microglia stained with CD45 or MHC-II antibodies are found only in the immediate periphery of compacted plaques. In the injected control groups, some microglial activation was detected at the 72 hour survival time by CD45 antibodies and this was restricted primarily to the injection site (arrows, Fig 6A, C; quantified in Fig 7). It should be noted that very little staining for CD45 was detected on the uninjected side of the brain, leading to inflated R/L ratios with relatively small

increases in staining. MHC-II had a lower overall level of expression than that of CD45, and was largely unaffected in mice administered control injections (Fig 8A and C; quantified in Fig 9).

In contrast, 72 hours after the injection of anti-A β antibodies, the microglial activation detected with CD45 antibodies was more widespread, detected not only at the injection site but also away from the injection site in the frontal cortex (Fig 6B) and throughout the dentate gyrus, with a concentration within the granule cell layer at the 72 h time point (Fig 6D). MHC-II staining revealed a similar pattern, although not as extensive as that found with CD45 staining (Fig 8B, C).

Quantification of these results indicated that the injection of anti-A β antibodies significantly increased expression of the microglial marker CD45 only at the 72 hour time point as compared to all other time points and control groups in both cortex (Fig 7A; $P < 0.005$) and hippocampus (Fig 7B; $P < 0.005$). Also, the injection of anti-A β antibodies increased the expression of the microglial marker MHC-II at the 72 hour time-point as compared to all other time points and control groups in both cortex (Fig 9A; $P < 0.01$) and hippocampus (Fig 9B; $P < 0.005$). The expression of CD45 and MHC-II in the frontal cortex at the anti-A β injection site increased more than 8-fold over that of all other time points including one week and both of the control groups. The expression of CD45 in the hippocampus at the anti-A β injection site increased more than 2-fold while the increase in expression of MHC-II is over 8 fold. As in our prior work, there is considerable variability among samples with both microglial markers, however, all anti-A β injected animals were higher than the means for the control groups.

There are few remaining amyloid deposits near the injection sites in the anti-A β antibody injected mice at 72 hours (Fig 4 and 5). These residual deposits are relatively faint when stained with Congo red and are can be found contacted by rounded, CD45 positive microglial cells (Fig 10A). In contrast, the more abundant amyloid deposits on the contralateral side and in the control animals are contacted by microglia with long processes which are stained for CD45 while the cell body only stains faintly for this marker of microglial activation (Fig 10B).

Discussion

We report here that intracranial anti-A β antibody injections substantially reduce A β load in the vicinity of the injection in both anterior cortex and hippocampus over a 7 day time frame. By 4 hours after the injection there is a broad distribution of injected antibody filling a volume of roughly 0.5 mm³ as estimated from anti-IgG immunohistochemistry. In addition to the broad pattern of diffusion, the antibody is concentrated in the outer molecular layers of Ammon's horn and the dentate gyrus, a zone which largely overlaps with the distribution of A β staining in transgenic mice of this age (see fig 2C). Thus, it appears the injected antibody is binding to in situ A β at this early time point, but is also spread throughout the hippocampus. By 24 hours there is a reduction in the A β immunostaining in the vicinity of the antibody injection in both cortex and hippocampus. This reduction in A β load is unlikely to be an artifact caused by the injected antibody masking the epitope of the primary antibody used for immunohistochemistry because the reduced load was not detected 4 hours after administration, and by 24 hours the injected IgG appears to be concentrated closer to the

granule cell region than the outer molecular layer in the hippocampus. Furthermore, the stoichiometry of injected antibody (13 pmol) to A β in deposits (estimated at 250 pmol in 0.5 mg, Chapman et al., 1999) is likely too low to interfere substantially with the histochemical reaction. This early reduction in A β load occurs in the absence of the expression of microglial activation markers CD45 and MHC-II. Although this does not preclude some rapid response of the microglia, it does suggest that the role of microglia is qualitatively different at this early post-survival time than when markers of activation are being expressed.

Between 24 and 72 hours after injection of anti-A β antibodies, there were parallel reductions in fibrillar amyloid deposits detected by thioflavine-S and increases in microglial activation, evaluated by CD45 and MHC-II staining. Although the control injections of anti-HIV antibody and vehicle caused some elevation of the CD45 marker, the activation was restricted to the immediate vicinity of the injection site and likely caused by mechanical injury associated with the needle insertion and fluid compression of the tissue. Occasionally, in the anti-A β antibody injected mice, some remaining wisps of amyloid could be found in the vicinity of the antibody injection at 72 hours and these were in contact with rounded CD45 immunopositive cells suggestive of phagocytic microglia/macrophages. Also at the 72 hour time point, there is a concentration of staining for both the injected antibody and the microglia near the granule cell layer of the dentate gyrus. The temporal association of fibrillar amyloid loss with microglial activation suggests some causal role for microglial activation in this process. One possibility is that between 1 and 3 days activated microglia near the deposits in the outer molecular layer phagocytose opsonized amyloid via Fc receptor or complement mediated

mechanisms and migrate towards the granule cell layer. CD45 positive microglia can be readily detected in the outer molecular layer near the fissure at 3 days, although they are most heavily concentrated near the granule cell layer at this time point (Fig 6D). A second option is that after dissolution of the A β deposits, the antibodies diffuse to the granule cell region independent of the microglia. possibly, the fibrillar deposits simply require more time to dissolve than the more diffuse material. More detailed time course studies of the period between 1 and 3 days coupled with immunoelectron microscopy will likely be required to resolve between these options. Remarkably, the microglial activation is terminated rapidly, and returns to normal levels by the 1 week time point in parallel with a significant reduction in staining for the injected IgG and A β .

An accumulating body of evidence finds an association between microglial activation and amyloid reductions in transgenic mouse models of amyloid deposition. Schenk et al (1999) noted in the first study evaluating A β vaccines that the clearance of amyloid was associated with enhanced microglial activity around the remaining deposits. Wilcock et al (2001) largely confirmed this observation in a different transgenic model. Nakagawa et al (2000) unexpectedly found that fluid percussion injury activates microglia and results in reduced amyloid deposition as mice grow older. Lim et al (2001) noted that transgenic mice treated with curcumin had a reduced amyloid load, but an increase in the activation state of microglia surrounding plaques. Similarly, Jantzen et al (2002) found a reduced amyloid load in transgenic mice treated with a nitro-NSAID, NCX-2216, which was also associated with increased microglial activation. Wyss-Coray (2001) found that crossing APP transgenic mice with mice over-expressing TGF- β led to increased microglial activation and reduced amyloid loads. Conversely, these same

authors (Wyss-Coray et al, 2002) found that blocking complement activation with sCRRY overexpression diminished the microglial reaction in APP transgenic mice, and led to elevated amyloid loads. DiCarlo et al (2001) attempted to directly activate microglia by injecting LPS and found this was associated with clearance of A β in the vicinity of the injection. However, note that Qiao et al, (2001) injected LPS chronically into young transgenic mice prior to normal amyloid deposition and found it could stimulate A β deposition. It is also the case that careful serial section electron microscopy failed to detect internalized amyloid in microglia associated with amyloid deposits in untreated APP23 transgenic mice (Stalder et al, 2001), although mice treated to provoke microglial activation have yet to be examined. Nonetheless, there is a growing literature associating the activation of microglia with a reduction in A β deposition in the transgenic mouse models.

A number of studies have demonstrated that cultured microglial cells are capable of internalizing A β 1-42 aggregates (Paresce et al., 1996; Webster et al., 2001). A β can also be cleared from unfixed brain sections by anti-A β antibodies in a microglia dependent manner (Bard et al., 2000). Direct imaging of amyloid deposits *in vivo* by multiphoton microscopy has shown clearance of plaque following application of an anti-A β antibody in association with an upregulation of activated microglia (Bacsikai et al., 2001). Suggested alternative mechanisms to microglial phagocytosis include a physical interaction between antibody and A β resulting in disaggregation of deposits, which was demonstrated *in vitro* using monoclonal anti-A β antibodies (Solomon, 2001). Consistent with this idea, Bacsikai et al (2002) recently demonstrated that F_{(ab')₂} fragments, prepared from an anti-A β antibody reduce amyloid deposits as effectively as the intact antibody

when applied topically to the cortex of transgenic mice through a craniotomy. Although there is no measurement of microglial activation in this study, it is plausible this occurred in the absence of microglial involvement. This antibody mediated dissolution hypothesis is consistent with the early phase of A β reduction described here, and may still be found responsible for the second phase of fibrillar deposit reduction..

A major unresolved question is how this antibody mediated clearance of A β might apply to the human condition. Alzheimer's disease has increasingly been argued to involve inflammation as a component of its pathogenesis (McGeer and McGeer, 2001). The early stages of the A β vaccine trials resulted in a small fraction of patients developing adverse reactions consistent with inflammation of the central nervous system, presumably including microglial activation (Schenk et al, 2002; Hock et al., 2002). Although adverse reactions to immunotherapy have been rare in the transgenic models (Pfeifer et al, 2002), it remains the best experimental system in which to understand the different components of the immune reactions to vaccines and to identify those components causing adverse outcomes. Certainly identification of immunotherapies which avoid the problem of deleterious CNS inflammation will be necessary if this treatment approach is to ever find use in the clinic. Better understanding the mechanisms of antibody-mediated clearance of A β in the transgenic models of amyloid deposition should benefit this effort.

Figure 1: Time course of injected anti-A β antibody distribution in the hippocampus from 4 hours to seven days. Immunohistochemical staining for the injected antibody in the hippocampus at 4 hours (A), 24 hours (B), 72 hours (C) and 168 hours (D). Orientation and locations of hippocampal subregions as in figure 2 D. Magnification = 40X. Scale bar = 120 μ m.

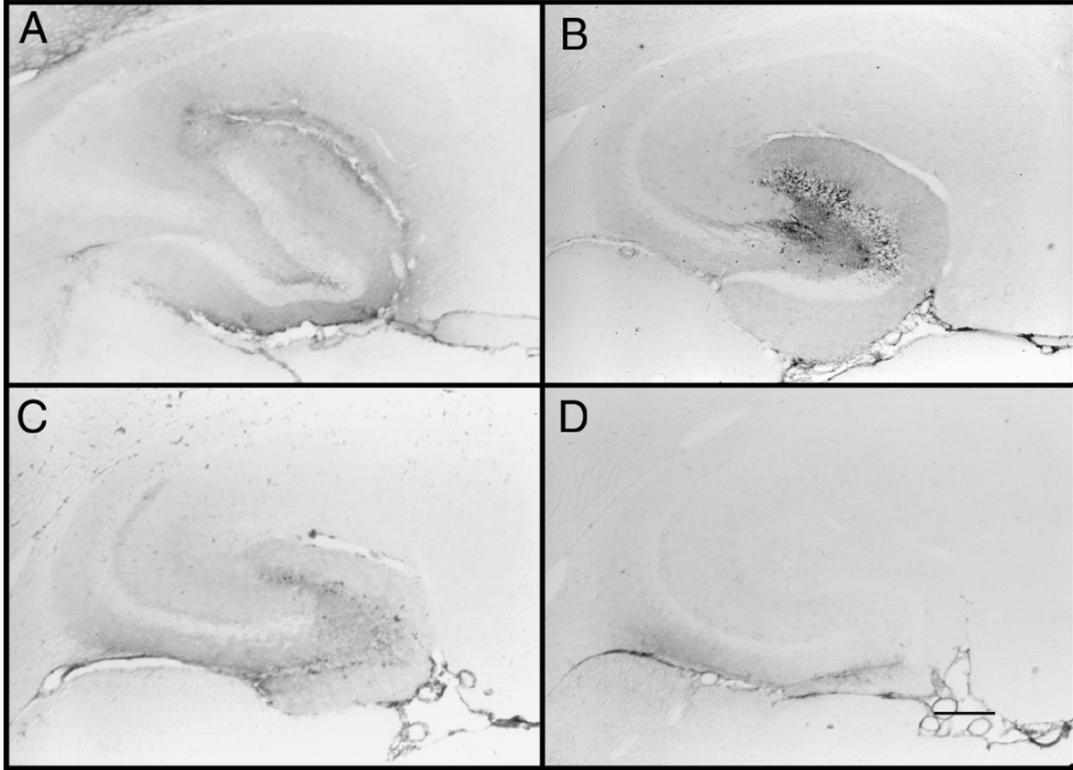


Figure 2: Reduction in A β immunohistochemistry one day after anti-A β antibody injections. Immunohistochemical staining is shown for A β in the frontal cortex (A and B) and hippocampus (C and D). A and C are from an animal injected with control antibody while B and D received the anti-A β antibody. Magnification = 40X. Scale bar = 120 μ m.

Panel B: FCX: frontal cortex, STR: striatum. Panel D: CA1: cornu ammonis 1, CA3: cornu ammonis 3, DG: dentate gyrus

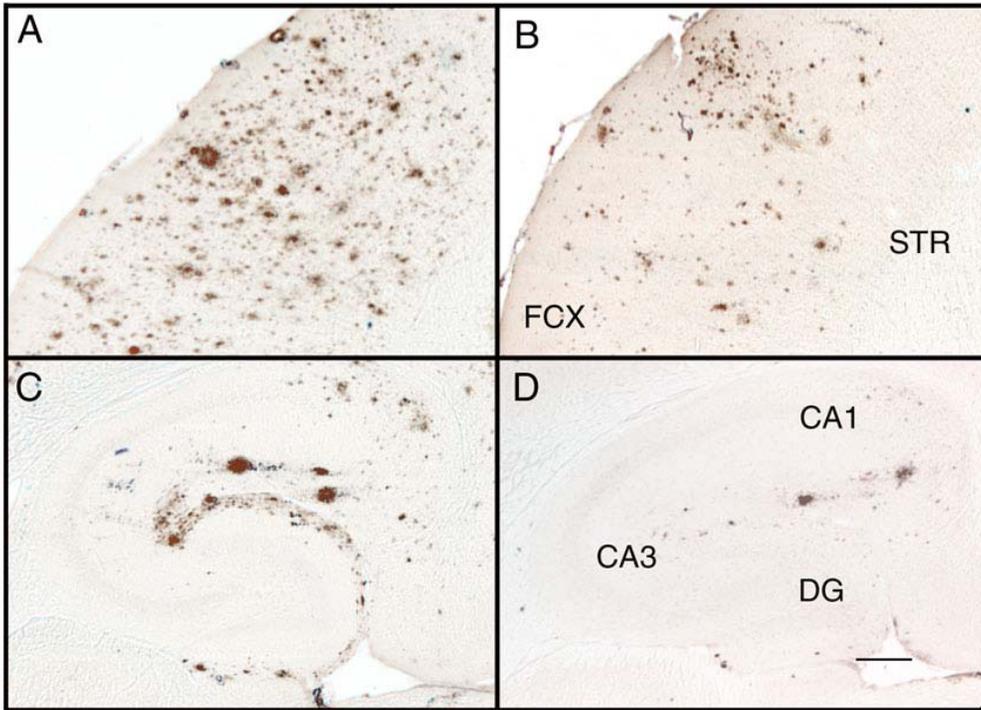


Figure 3: Quantification of reduced A β load after anti-A β antibody injections. Data are expressed as the ratio of A β staining in the injected hemisphere: control hemisphere. The three bars on the left indicate the A β load in the untreated group (none) and the vehicle (VEH) and anti-HIV antibody (Cont-Ab) groups at 72 hr. The line shows the ratio of A β immunohistochemical staining at 4, 24, 72 and 168 hr survival times. Reduced A β load was observed in the frontal cortex (panel A) and hippocampus (panel B) at 24, 72 and 168 hours compared with 4 hours and both control groups (** indicates P<0.005).

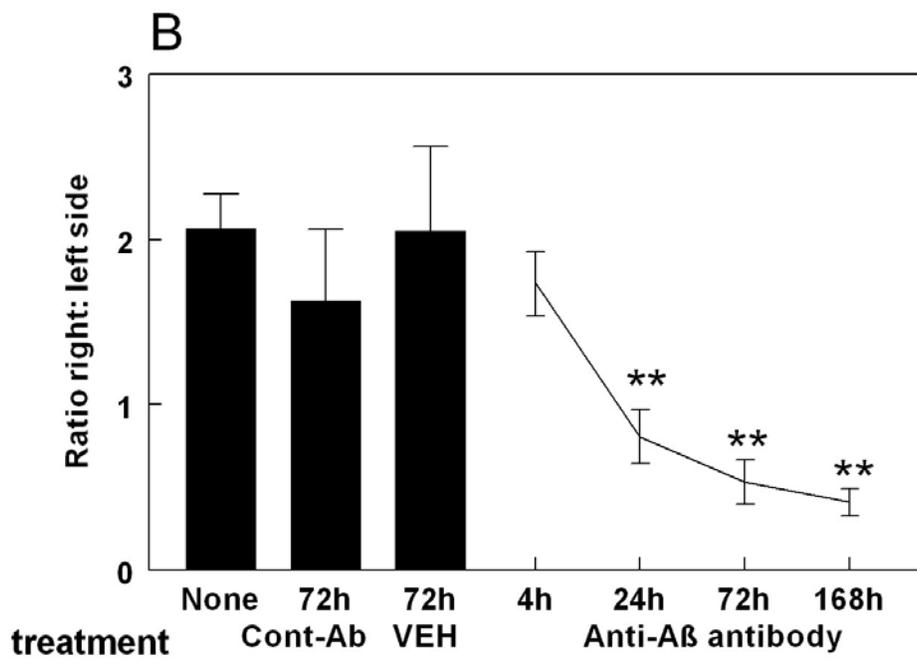
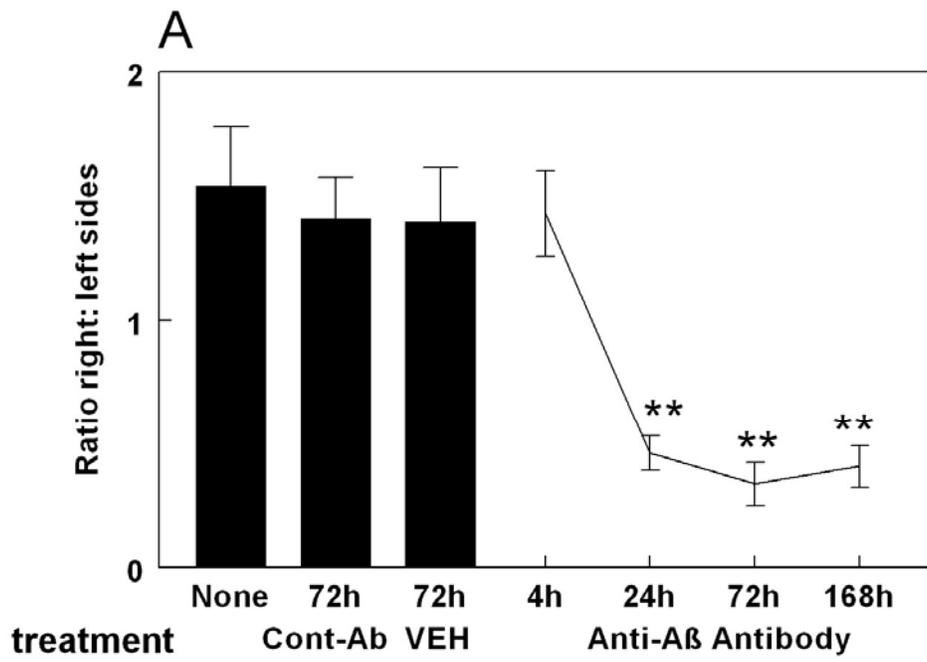


Figure 4: Reduction in thioflavine-S staining three days after anti-A β antibody injections. Thioflavine-S staining is shown in frontal cortex (A and B) and hippocampus (C and D). A and C received control antibody while B and D received anti-A β antibody. Magnification = 40X. Scale bar = 120 μ m. Orientation and locations of major subregions as in figure 2B and 2D.

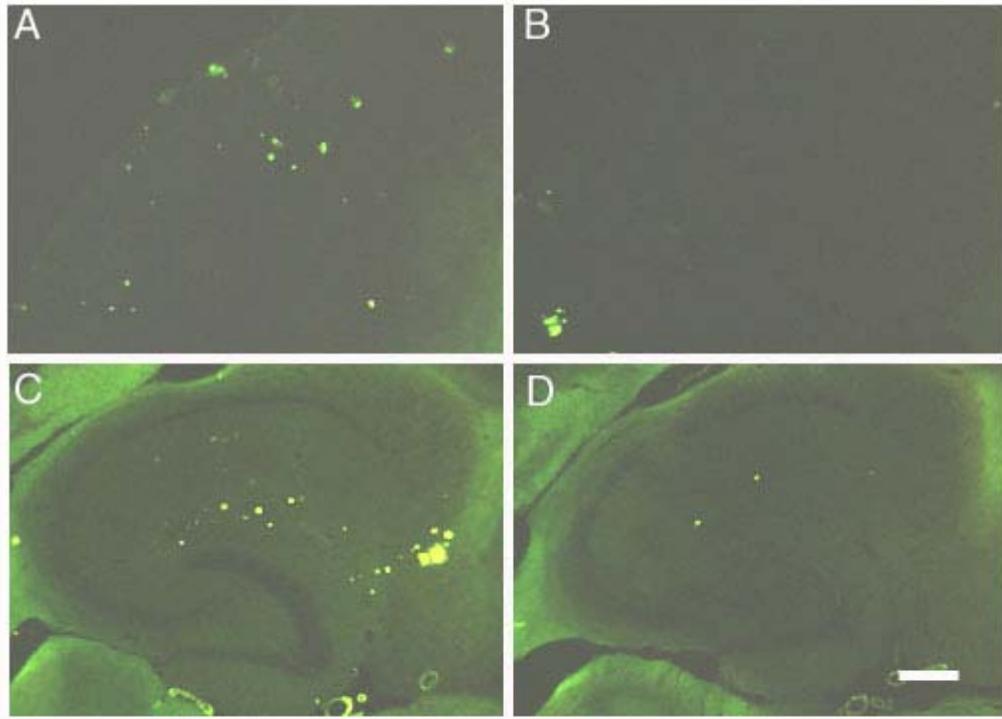


Figure 5: Anti-A β antibody injections results in a reduction of thioflavine-S positive plaques. Data are expressed as ratio of thioflavine-S staining in the injected hemisphere: control hemisphere. The three bars show the thioflavine-S positive staining in the untreated group (none) and the vehicle (VEH) and anti-HIV antibody (Cont-Ab) groups at 72hr. The line shows the ratio of thioflavine-S staining at 4, 24, 72 and 168 hour survival times. Reduced thioflavine-S staining was observed in the frontal cortex (panel A) and hippocampus (panel B) at 72 and 168 hours compared with 4 and 24 hours and both control groups (** indicates $P < 0.005$).

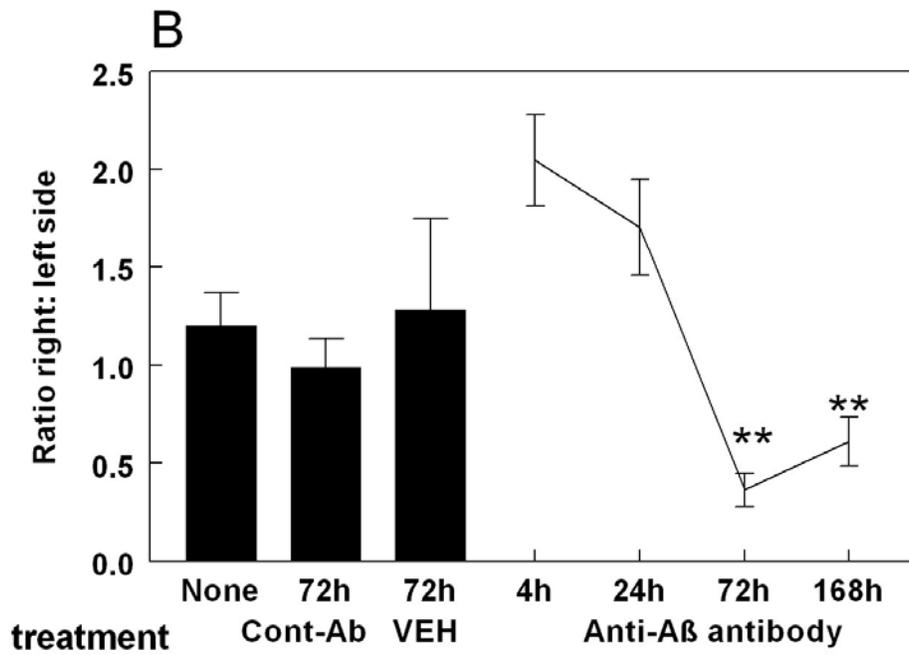
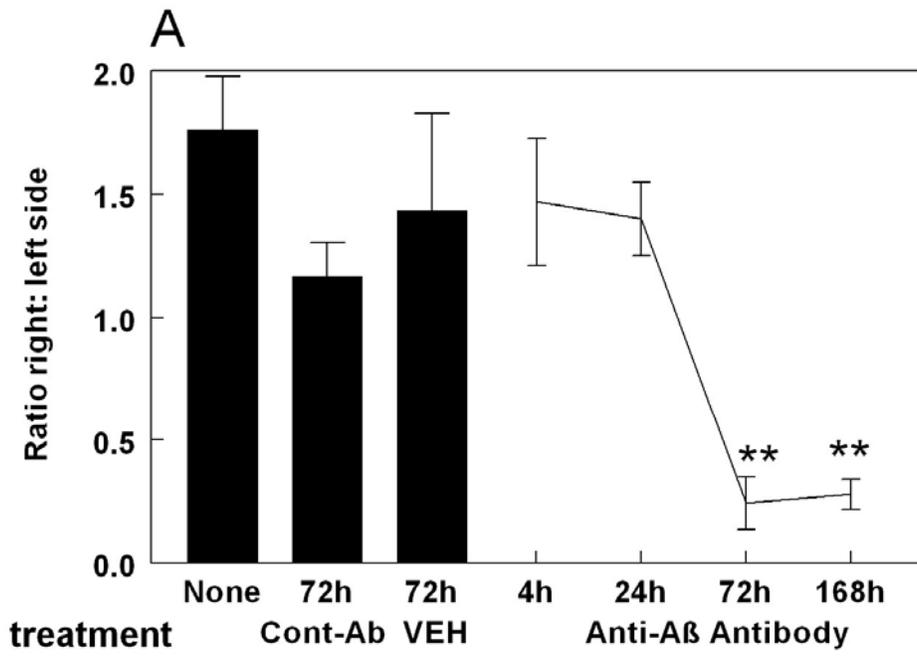


Figure 6: CD45 immunohistochemistry is increased three days following anti-A β antibody injections. CD45 immunohistochemistry is shown in frontal cortex (A and B) and hippocampus (C and D). A and C received control antibody while B and D received anti-A β antibody. Magnification = 40X. Scale bar = 120 μ m. Arrows indicate the site of injection identified from the needle tract.

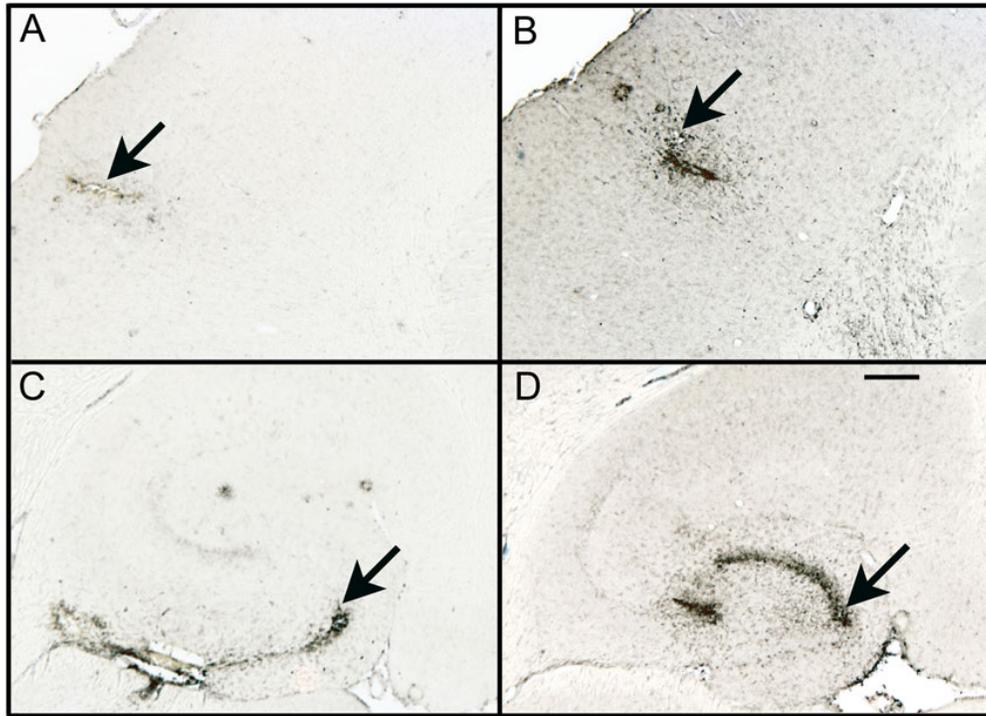


Figure 7: Anti-A β antibody injections results in an increased CD45 immunohistochemistry three days following injection. Data are expressed as the ratio of CD45 staining in the injected hemisphere: control hemisphere. The three bars indicate the CD45 expression in the untreated group (none) and the vehicle (VEH) and anti-HIV antibody (Cont-Ab) groups at 72hr. The line shows the ratio of CD45 staining at 4, 24, 72 and 168 hour survival times. Increased CD45 staining was observed in the frontal cortex (panel A) and hippocampus (panel B) at 72 hours compared with 4, 24 and 168 hours and both control groups (** indicates $P < 0.005$).

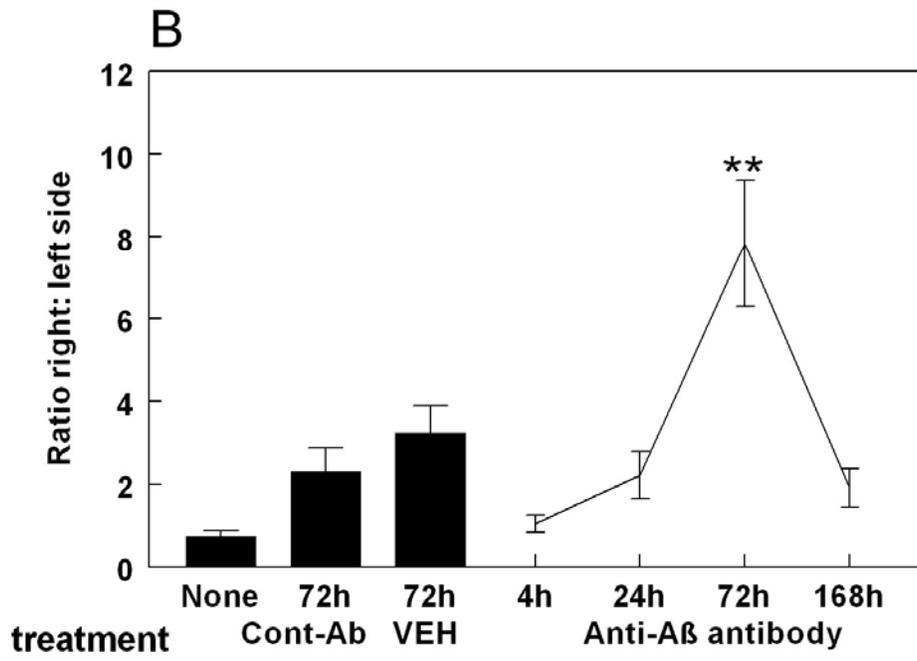
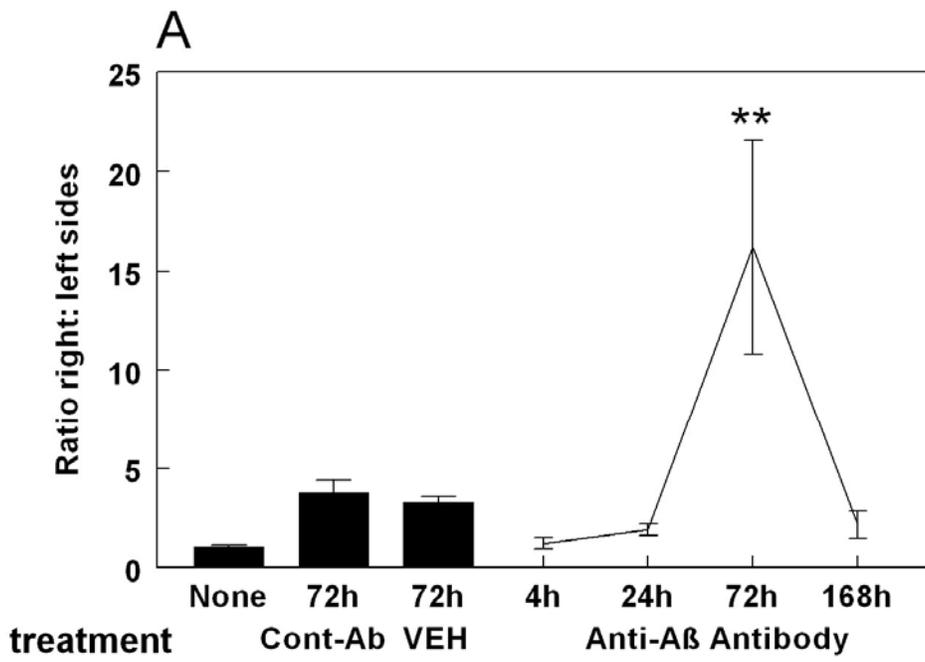


Figure 8: MHC-II immunohistochemistry is increased three days following anti-A β antibody injections. MHC-II immunohistochemistry is shown in frontal cortex (A and B) and hippocampus (C and D). A and C received control antibody, B and D received anti-A β antibody. Magnification = 40X. Scale bar = 120 μ m.

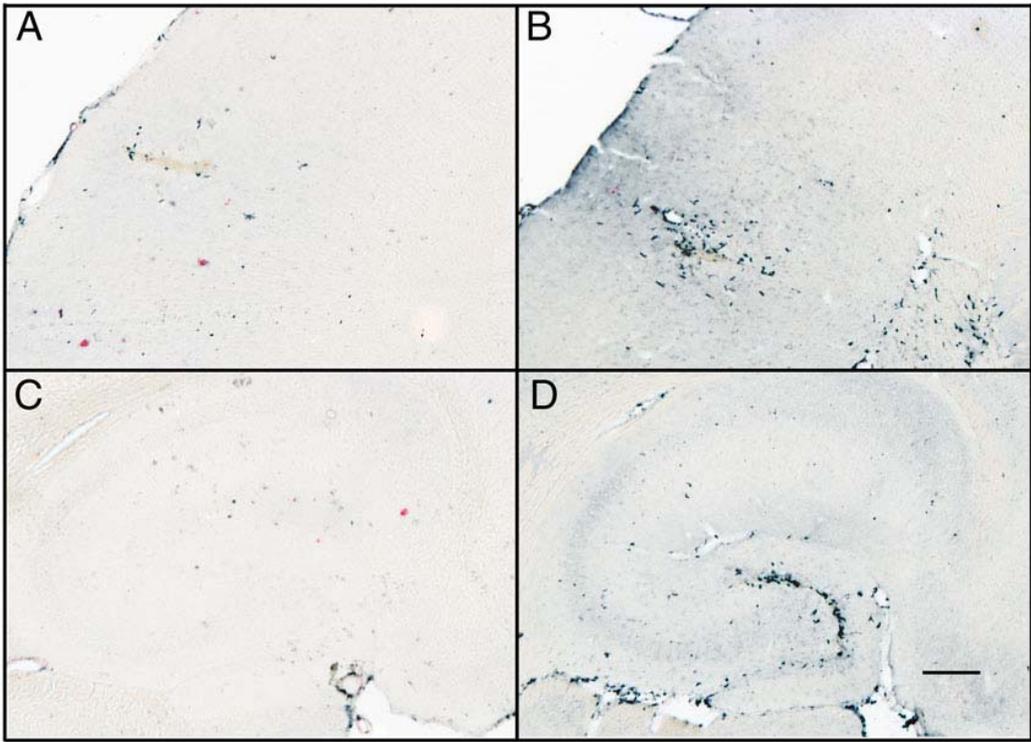


Figure 9: Anti-A β antibody injections results in an increase in MHC-II immunohistochemistry three days following injection. Data are expressed as percent area occupied by MHC-II positive staining in the injected hemisphere. The three bars indicate the MHC-II expression in the untreated (none) group and the vehicle (VEH) and anti-HIV antibody (Cont-Ab) groups at 72hr. The line shows the amount of MHC-II staining at 4, 24, 72 and 168 hour survival times. Increased MHC-II staining was observed in the frontal cortex (panel A) and hippocampus (panel B) at 72 hours compared with 4, 24 and 168 hours and both control groups (** indicates $P < 0.01$).

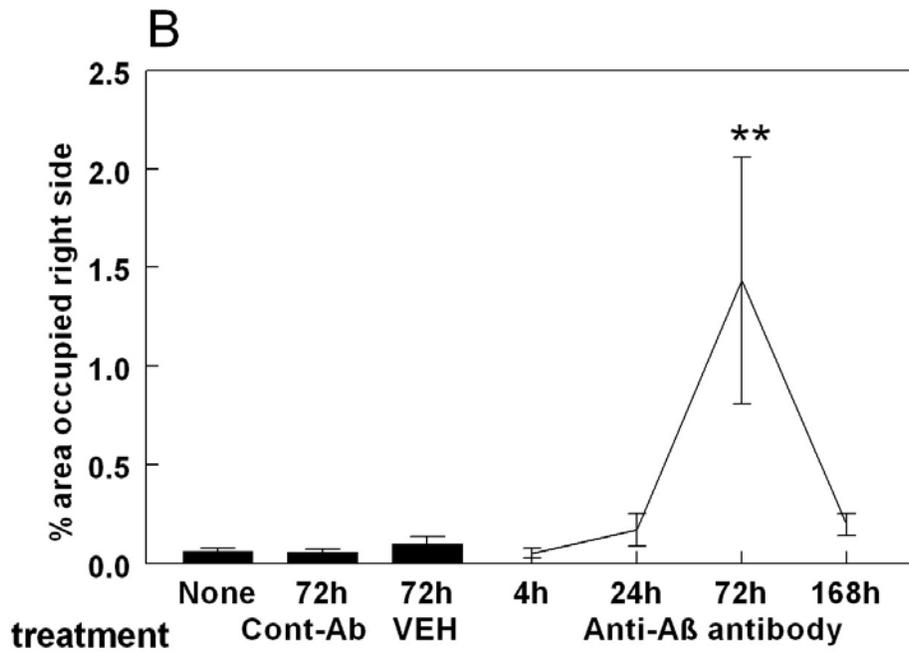
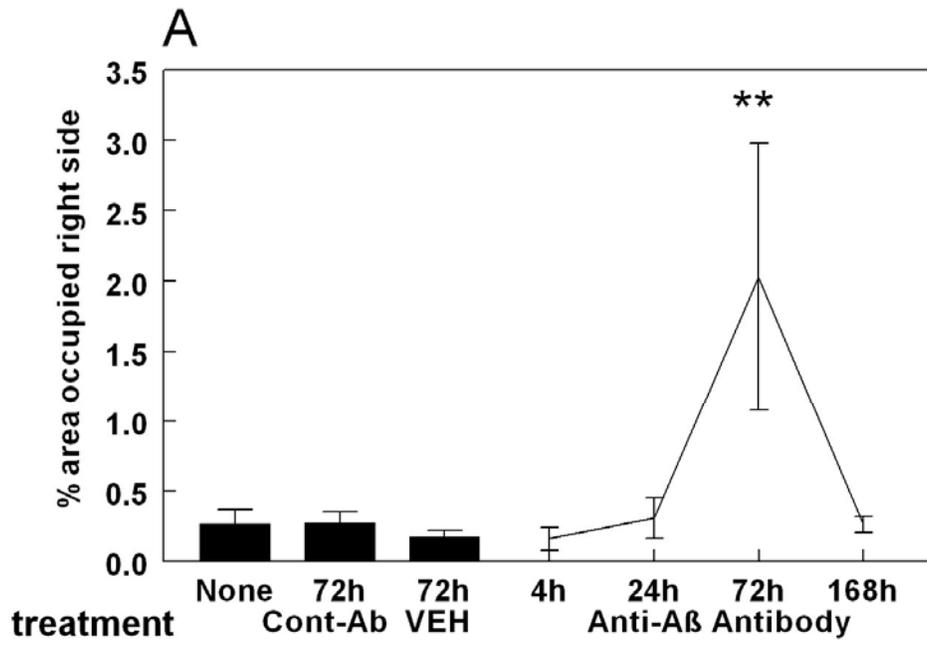
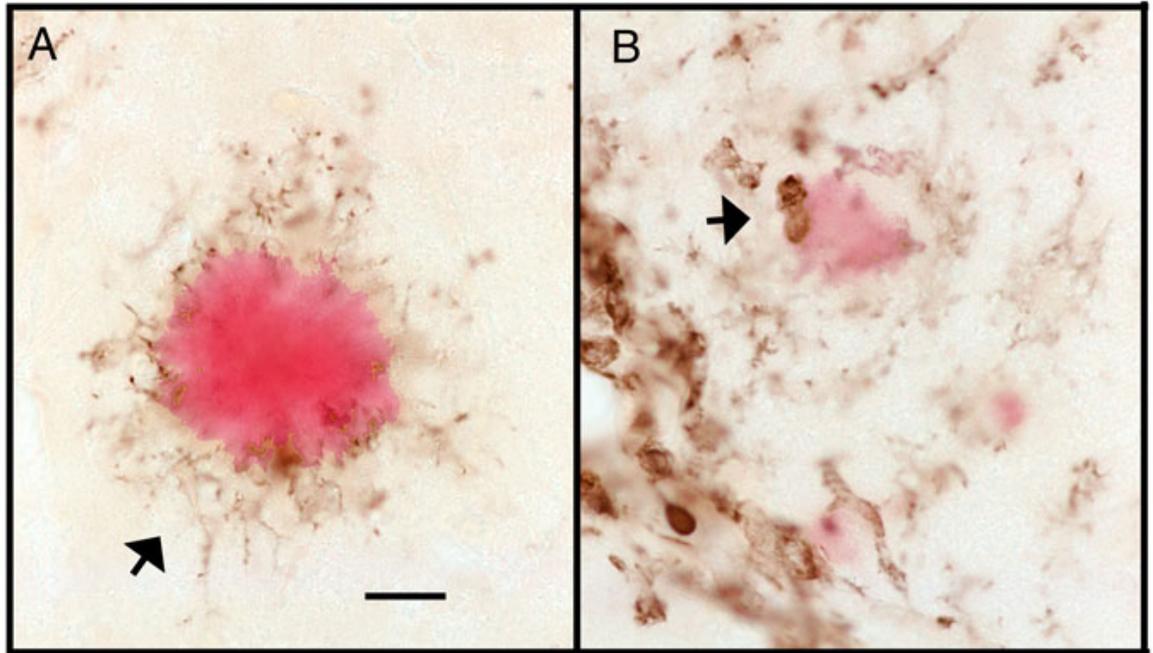


Figure 10: Anti-A β antibody injections results in rounded microglia in association with remaining Congophilic amyloid deposits three days after injection. CD45 immunostaining counterstained with Congo red is shown in the hippocampus at the 72 hour time-point. Panel A shows a typical intensely stained Congophilic deposit surrounded by CD45 immunostained microglial processes, with faintly stained somata (arrow). Panel B shows a faintly stained Congophilic deposit in the anti-A β antibody injected hippocampus. Note the two rounded intensely CD45 positive cells in contact with the faintly stained deposit (arrow). Magnification = 600X. Scale bar = 8.33 μ m



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PAPER 2:

**MICROGLIAL ACTIVATION FACILITATES A β PLAQUE REMOVAL
FOLLOWING INTRACRANIAL ANTI-A β ANTIBODY ADMINISTRATION.**

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Abstract

The mechanisms by which anti-A β antibodies clear amyloid plaques in A β depositing transgenic mice are unclear. In the current study we demonstrate that inhibition of anti-A β antibody-induced microglial activation with anti-inflammatory drugs, such as dexamethasone, inhibits removal of fibrillar amyloid deposits. We also show that anti-A β F(ab')₂ fragments fail to activate microglia and are less efficient in removing fibrillar amyloid than the corresponding complete IgG. Diffuse A β deposits are cleared by antibodies under all circumstances. These data suggest that microglial activation is necessary for efficient removal of compact amyloid deposits with immunotherapy. Inhibition of this activation may result in an impaired clinical response to vaccination against A β .

Introduction

Alzheimer's disease (AD) is characterized clinically by progressive cognitive decline and characterized pathologically by amyloid plaques, neurofibrillary tangles and neuron loss (Hardy and Selkoe, 2002). Another pathological event in AD is an inflammatory response which involves the activation and proliferation of microglia and astrocytes (Akiyama et al, 2000). The amyloid hypothesis has targeted the A β peptide as the primary focus for therapeutic interventions in AD (Hardy and Selkoe, 2002). Amyloid plaques consist of amyloid- β protein fibrils which are positively stained by Congo red and thioflavine-S. In addition, diffuse amyloid deposits can be identified using immunohistochemistry.

Vaccination using A β ₁₋₄₂ was first described by Schenk et al (Schenk et al, 1999). That report showed that immunization with A β ₁₋₄₂ in the PDAPP transgenic mouse dramatically reduced A β deposit accumulation, both diffuse and compact. The vaccination was later shown to prevent cognitive decline in APP+PS1 (Morgan et al, 2000) and TgCRND8 (Janus et al, 2000) transgenic mice. Passive immunization with anti-A β antibodies was also demonstrated to have benefit pathologically (Bard et al, 2000) and cognitively (Dodart et al, 2002 and Kotilinek et al, 2002). The A β vaccine advanced quickly to human clinical trials where, in Phase II, several patients developed cerebral inflammation, leading to a halt in further inoculations (Schenk 2002).

The exact mechanism by which immunotherapy reduces A β deposition remains unknown; suggested mechanisms include Fc receptor mediated phagocytosis via microglia (Schenk et al, 1999, Wilcock et al, 2001 and Wilcock et al, 2003), dissolution of amyloid fibrils (Solomon et al, 1997 and Frenkel et al, 1999) and sequestration of circulating A β resulting in an increased net efflux of A β from brain and plasma (DeMattos et al, 2001).

These competing hypotheses have led to disputes regarding the accessibility of circulating antibodies to the CNS, the role of systemic A β content in this process and the degree of requirement for specific A β epitopes to be targeted by the antibodies (Bard et al, 2003 and Holtzman et al, 2002). Moreover, in AD patients the blood-brain barrier is variably leaky (Hock et al, 2002). Bacskai et al (2001) were the first to demonstrate anti-A β antibody removal of amyloid deposits following direct application into the brain, therefore bypassing the blood-brain barrier. To identify the potential role of microglia in

antibody-mediated removal of A β deposits, we have opted to avoid some of the complications regarding brain penetration and apply antibodies directly to the CNS by intracranial injections.

We have recently reported that following intracranial anti-A β antibody administration there is a biphasic clearance of A β deposits (Wilcock et al, 2003). The first is a rapid removal of diffuse A β deposits occurring between 4 and 24 hours after injection. The second is the removal of compact, thioflavine-S positive amyloid deposits between 24 and 72 hours following injection. This removal of fibrillar deposits is associated with a transient activation of microglia, detectable at 72 hours, but not 7 days after the injection. Remarkably, by 7 days both diffuse and compacted A β deposits are largely cleared, the microglial reaction has resolved, and the injected anti-A β antibody is almost completely removed.

In the current study we further investigate the relationship between microglial activation and fibrillar amyloid removal. First, we test the capacity of several anti-inflammatory agents to impair the microglial response and monitor their effect on A β clearance. We also investigate whether antibody fragments lacking the Fc domain can clear the fibrillar deposits, and monitor the effects on microglial activation. The results are consistent with the argument that microglial activation and Fc receptor mediated phagocytosis are important steps in the rapid clearance of A β deposits by intracranially administered anti-A β antibodies.

Materials and Methods

Anti-inflammatory drug study

Singly transgenic APP Tg 2576 mice were obtained from our breeding program at USF started in 1996 (Holcomb et al, 1998). In the first experiment, 39 APP transgenic mice aged 16 months were assigned to one of 5 experimental groups. Four of these groups received intracranial anti-A β antibody injections (44-352; Mouse monoclonal anti-human A β ₁₋₁₆ IgG1; Biosource, Camarillo, CA) into the frontal cortex and hippocampus at a concentration of 2 μ g/2 μ l in each region. The remaining group received intracranial anti-HIV monoclonal antibody directed against gp120 (from Ken Ugen, Univ. South Florida) into frontal cortex and hippocampus at a concentration of 2 μ g/2 μ l in each region (N=7) as a control for potential nonspecific activity associated with injecting IgG into the brain. Of the 4 groups receiving anti-A β antibody one group received no further treatment (N=8), one group received twice daily intraperitoneal injections of dexamethasone (Sigma-Aldrich, St Louis MS) at a dose of 5mg/kg (N=9), one group received twice daily intraperitoneal injections of minocycline (Sigma-Aldrich, St Louis MS) at a dose of 45mg/kg (N=7) and one group received once daily subcutaneous injections of NCX-2216 (nitro-ferulo-flurbiprofen; NiCox, S.A., Sophia-Antipolis, France) at a dose of 7.5mg/kg (N=8). All treatments following the intracranial injection were commenced immediately following a 30 minute recovery from surgery. All mice were killed 72 hours following surgery and treatments were continued through the morning of kill.

Antibody Fragment Study

Twenty Tg2576 APP transgenic mice aged 19.5 months were assigned to one of four groups, all groups received intracranial injections into the frontal cortex and hippocampus. The first group received anti-A β antibody (2286; Mouse monoclonal anti-human A β_{28-40} IgG1; Rinat Neurosciences, Palo Alto, CA) at a concentration of 2 μ g /2 μ l in each region. The second group received anti-A β F(ab')₂ fragments prepared from the anti-A β antibody at 2.2 μ g /2 μ l in each region. The third group received IgG directed against drosophila amnesiac protein (Rinat neurosciences, Palo Alto, CA) as a control for nonspecific aspects of intact IgG injection. The final group received control F(ab')₂ fragments prepared from the IgG directed against drosophila amnesiac protein to control for nonspecific effects of F(ab')₂ injection. All mice survived for 72 hours after surgery.

Preparation of F(ab')₂ fragments

The Immunopure IgG1 Fab and F(ab')₂ preparation kit (Pierce Biotechnology, Rockford, IL) was used to prepare the F(ab')₂ fragments from the anti-A β IgG and the control IgG against drosophila protein. The instructions provided with the kit were followed (<http://www.piercenet.com/files/0465jm5.pdf>). Briefly, 0.5ml of 1mg/ml IgG was added to 0.5ml mouse IgG1 mild elution buffer. This was applied to an equilibrated immobilized ficin column, allowed to enter the column and digested at 37°C for 20 hours. A 4ml elution was obtained and applied to an equilibrated immobilized protein A column for separation of the F(ab')₂ from Fc fragments and undigested IgG. Four 1ml fractions of product were obtained. As determined by running a gel electrophoresis only the 2nd and

3rd elutions were found to contain F(ab')₂ fragments and appeared of similar intensities on the gel. The two elutions containing F(ab')₂ fragments were pooled and concentrated using centricon centrifugal filter devices (Millipore Corp. Bedford, MA) to a volume of approximately 200 µl. Preliminary experiments found that injections of the F(ab')₂ fractions concentrated directly from the column caused seizures when injected into some mice. Thus the initial concentrate was diluted in 4 ml of fresh PBS and reconcentrated to dilute residual proprietary elution buffer components which may cause seizures. No seizures or neurotoxicity were found in the mice included here. The concentrated product was run on an SDS-PAGE. A Bradford assay was also performed to establish concentrations of the F(ab')₂ fragments using Bradford protein assay reagent concentrate (Bio-Rad, Hercules, CA).

Surgical procedure

On the day of surgery the mice were weighed, anesthetized with isoflurane and placed in a stereotaxic apparatus (51603 dual manipulator lab standard, Stoelting, Wood Dale, IL). A midsagittal incision was made to expose the cranium and two burr holes were drilled using a dental drill over the right frontal cortex and hippocampus to the following coordinates: Cortex: AP +1.5mm, L -2.0mm, hippocampus: AP -2.7mm, L -2.5mm, all taken from bregma. A 26 gauge needle attached to a 10µl Hamilton (Reno, NV) syringe was lowered 3mm ventral to bregma and a 2µl injection was made over a 2 minute period. The incision was cleaned with saline and closed with surgical staples.

Tissue Preparation

On the day of kill mice were weighed, overdosed with 100mg/kg pentobarbital (Nembutal sodium solution, Abbott laboratories, North Chicago IL) and intracardially

perfused with 25ml 0.9% sodium chloride followed by 50ml freshly prepared 4% paraformaldehyde (pH=7.4). Brains were rapidly removed and immersion fixed for 24 hours in freshly prepared 4% paraformaldehyde. The brains were then incubated for 24 hours in 10, 20 and 30% sucrose sequentially to cyroprotect them. Horizontal sections of 25 μ m thickness were then collected using a sliding microtome and stored at 4°C in DPBS buffer with sodium azide to prevent microbial growth.

Immunohistochemical methods

Six to eight sections approximately 100 μ m apart were selected spanning the injection site and stained using free-floating immunohistochemistry methods for total A β (rabbit antiserum primarily reacting with the N-terminal of the A β peptide 1:10000) and CD45 (Serotec, Raleigh NC, 1:3000) as previously described (Gordon et al, 2002). For immunostaining, some sections were omitted from the primary antibody to assess non-specific immunohistochemical reactions. Adjacent sections were mounted on slides and stained using 4% thioflavine-S (Sigma-Aldrich, St Louis MO) for 10 minutes. It should be noted that there are a limited number of sections that include the injection volume. We have opted to measure a few markers reliably rather than a larger number of markers with fewer sections each.

Data analysis

The immunohistochemical reaction product on all stained sections was measured using using a videometric V150 image analysis system (Oncor, San Diego, CA) in the injected area of cortex and hippocampus and corresponding regions on the contralateral side of the brain. Data are presented as the ratio of injected side to non-injected side for A β , thioflavine-S and CD45. Normalizing each injection site to the corresponding

contralateral site diminishes the influence of inter-animal variability and permits reliable measurements of drug effects with a smaller number of mice. Importantly, there is no injected antibody detectable in the contralateral side. To assess possible treatment-related differences, the ratio values for each treatment group were analyzed by ANOVA using StatView software version 5.0.1 (SAS Institute Inc., NC) followed by Fischer's LSD means comparisons.

Results

Following intracranial injection of anti-A β antibody 44-352 into the hippocampus and frontal cortex there was a significant activation of microglia detectable by CD45 immunohistochemistry. In the hippocampus, the most intense area of activation appeared in the granule cell layer of the dentate gyrus close to the site of injection within the hilus/CA4 region. However, there was a much more diffuse activation which filled the remainder of the dentate gyrus (Fig. 1A). In the frontal cortex, the activation formed a gradient surrounding the injection site without a clear laminar profile (not shown). Following the intracranial injection of anti-A β antibody, treatment with the steroidal anti-inflammatory agent dexamethasone completely inhibited the microglial activation with only several small cells faintly stained for CD45 in hippocampus (Fig 1B; $P < 0.05$ Fig 2A) and in frontal cortex (Fig 2A, $P < 0.001$). The staining pattern and values observed in this group matched that of the group administered a control IgG directed against an HIV protein in both brain regions (Fig. 1E, Fig. 2A).

Minocycline, a drug previously shown to inhibit microglial activation in several CNS inflammation models, appeared relatively ineffective at inhibiting the microglial

activation observed as a result of intracranial anti-A β antibody administration. In the hippocampus the intense area of activation in the granule cell layer was still present, as was the more diffuse activation in the remainder of the dentate gyrus (Fig 1C). In the frontal cortex, although there was a significant difference between the minocycline-treated mice and the untreated mice ($P < 0.01$), the microglial activation in the minocycline treated mice was still significantly greater than in the dexamethasone treated mice ($p < 0.05$; Fig 2A).

NCX-2216 combines a nitric oxide generating moiety with the typical NSAID drug flurbiprofen. In the hippocampus, NCX-2216 treatment following the intracranial injection of anti-A β antibody partially inhibited the activation of microglia (Fig 1D). This drug did not inhibit the intense activation observed in the granule cell layer of the dentate gyrus but did diminish the more diffuse activation. The quantification from the frontal cortex found a significant inhibition of microglial activation ($P < 0.01$; Fig 2A). Thus, with respect to inhibiting microglial activation following anti-A β antibody injection, dexamethasone was the most effective drug with NCX-2216 having a partial inhibition followed by an even weaker inhibition caused by minocycline.

Total A β immunohistochemistry in mice administered the control antibody directed against human immunodeficiency virus (HIV) protein gp120 was similar to that described previously in the APP transgenic mouse (Hsiao et al, 1996 and Gordon et al, 2002). The ratio of A β in the right: left sides was also the same as that observed previously in unmanipulated APP transgenic mice (Wilcock et al, 2003). The A β immunohistochemistry showed a few large, intensely stained deposits, which are normally also stained by Congo red or thioflavine-S, indicating fibrillar compact amyloid

deposits. There were also a large number of smaller, less intensely stained deposits analogous to diffuse amyloid deposits observed in human AD brain tissue. In the hippocampus, A β deposition was localized primarily to the molecular layers of the dentate gyrus and Ammon's horn adjacent to the hippocampal fissure, as well as a large concentration in the subiculum (Fig. 1J).

Anti-A β antibody administration into frontal cortex and hippocampus resulted in a reduction of total A β immunohistochemistry 72 hours following injection (Fig 1F). This reduction was approximately 80% in frontal cortex and 65% in hippocampus compared to APP transgenic mice administered HIV antibody (Fig. 2B). The anti-inflammatory agents dexamethasone, NCX-2216 and minocycline had no effect on the removal of this largely diffuse A β staining (Fig. 1G, H and I and Fig 2B).

Thioflavine-S staining showed a different response to anti-inflammatory drug treatment than A β immunostaining. Although fewer in number, the subregional distribution of thioflavine-S positive plaques matched that observed with A β immunohistochemistry in APP transgenic mice administered control IgG (Fig 1O). Anti-A β antibody injected into the frontal cortex and hippocampus resulted in a virtually complete removal of thioflavine-S positive plaques 72 hours following injection, reaching 90% in frontal cortex and 85% in the hippocampus (Fig 1K; Fig 2C). Administration of dexamethasone resulted in complete arrest of anti-A β antibody mediated clearance of thioflavine-S compact amyloid deposits in the hippocampus (Fig. 1L) or the frontal cortex, with values equivalent to those mice given anti-HIV control antibody (Fig. 2C). In contrast, administration of minocycline had no detectable effects

on anti-A β antibody mediated clearance of thioflavine-S deposits in either frontal cortex (Fig. 2C) or hippocampus (Fig. 1M, Fig. 2C). NCX-2216 treatment had a partial effect on thioflavine-S reduction following intracranial anti-A β antibodies. In the frontal cortex the levels of thioflavine-S were almost equivalent to those seen following anti-HIV control antibody administration, and were significantly different from the levels observed following anti-A β antibody administration alone (Fig. 2C). The levels of thioflavine-S in the hippocampus were not significantly different from mice administered anti-A β antibody or mice administered anti-HIV control antibody, and their values were in between these two groups (Fig. 2C), suggesting a partial impairment of antibody mediated clearance by this drug.

A second series of studies investigated the potential role of the Fc domain of the antibody in microglial activation and amyloid clearance. F(ab')₂ fragments prepared from anti-A β monoclonal antibody 2286, and a control monoclonal antibody directed against the drosophila protein *amnesiac* were analyzed via SDS-polyacrylamide-gel electrophoresis (PAGE). The gel showed very pure product, with a single band at approximately 105kDa, the molecular weight for F(ab')₂ fragments. The intact IgG molecule produced one intense band at approximately 150kDa, the correct molecular weight for IgG molecules and a less intense band at approximately 110 kDa. Following confirmation of purity via SDS-PAGE we then performed a Bradford assay to assess the recovery of F(ab')₂ in the purified fraction. Because we dissolved the anti-A β F(ab')₂ fragments in a smaller volume than was used for the starting material the concentration of F(ab')₂ fragments injected intracranially was 1.2 μ g/ μ l, while the complete IgG

concentration was 1 $\mu\text{g}/\mu\text{l}$, resulting in an excess of anti-A β Fv domains in the F(ab')₂ solutions.

The only antibody which activated microglia 72 hours following intracranial injection into frontal cortex and hippocampus was the intact anti-A β antibody. The frontal cortex shows a greater degree of activation than the hippocampus, however, in both regions the activation is significantly greater than that in the groups receiving control anti-amnesiac protein IgG, F(ab')₂, or anti-A β F(ab')₂ (Fig 3A, C and D, Fig. 4A; $P < 0.01$ or greater in all comparisons). The pattern of activation in the hippocampus following the anti-A β antibody 2286 injection resembled that shown in Fig 1A when using the anti-A β antibody 44-352. There is a very intense area of activation in the granule cell layer of the dentate gyrus, with a much more diffuse activation filling the remainder of the dentate gyrus (Fig. 3A). Interestingly, the anti-A β F(ab')₂ fragments produced no microglial activation in either the frontal cortex and hippocampus (Fig. 3B, Fig. 4A).

A β immunohistochemistry in the two anti-amnesiac protein control groups shows the typical staining pattern observed in APP transgenic mice at 19.5 months (Fig. 3G and H). This pattern was qualitatively the same as observed at 16 months (Fig 1J), although quantitatively greater as the mice were 3.5 months older. Both the anti-A β antibody and the anti-A β F(ab')₂ groups significantly reduced total A β immunohistochemistry to a similar extent 72 hours following injection into frontal cortex and hippocampus. In the frontal cortex there was a reduction of approximately 60% (Fig. 4B). In the hippocampus the reduction was approximately 65% (Fig. 3E and F, Fig. 4B).

Thioflavine-S staining detects only compact fibrillar amyloid deposits. The mice receiving intracranial injections of either control anti-amnesiac protein IgG or control F(ab')₂ resembled the typical staining observed in the APP transgenic mouse at this age. In the hippocampus the majority of thioflavine-S positive plaques were located in the outer molecular layer of Ammon's horn and the dentate gyrus near the hippocampal fissure (Fig. 3K and L). Anti-A β antibody IgG significantly reduced thioflavine-S positive compact plaque by approximately 90% in the frontal cortex and hippocampus (Fig. 4C). There were no, or very few, remaining thioflavine-S positive deposits in the hippocampus (Fig. 3I). In contrast, the anti-A β F(ab')₂ fragments did not remove compact amyloid plaques as effectively as the whole IgG molecule. In the frontal cortex there was no significant reduction in thioflavine-S staining when compared to either control antibody group (Fig. 4C). In the hippocampus there was a significant difference between the anti-A β F(ab')₂ group and the control groups ($P < 0.05$), however, this reduction was also significantly less than the reduction observed with the whole IgG molecule (Fig. 3J, Fig. 4C; $P < 0.02$ or greater).

Discussion

The data presented here support the argument that activation of microglia in APP transgenic mice facilitates the removal of compact amyloid plaques. The first experiment, using several anti-inflammatory agents to regulate the microglial response, showed that the extent of fibrillar amyloid removal roughly corresponds to the extent of microglial activation 3 days after intracranially applied anti-A β antibody. The second study identified that anti-A β F(ab')₂ fragments were less capable of activating microglia

(presumably because they lacked the Fc domain) and were significantly less effective than the corresponding whole IgG in removing fibrillar A β , despite the presence of excess anti-A β Fv in the F(ab')₂ injections.

Our earlier work in this system demonstrated that intracranial administration of anti-A β antibodies into APP Tg2576 mice resulted in diffusion of the antibody throughout most of the hippocampus by 4 hours, however, no specific localization to amyloid plaques was noted. This caused a rapid removal of diffuse A β deposits between 4 hours (when no reduction is detected) and 24 hours (when removal of diffuse deposits appeared complete; Wilcock et al, 2003). This removal was not associated with any apparent activation of microglia using markers such as MHC-II or CD45 at 24 hours, nor was there any reduction of the fibrillar amyloid deposits measured with thioflavine-S staining. However, by 72 hours following the injection there was a dramatic reduction of thioflavine-S positive compact amyloid deposits associated with a florid microglial activation as detected by CD45 immunohistochemistry. One week following the anti-A β antibody injection, the injection site remained devoid of most forms of amyloid, the microglial reaction had terminated and the injected antibody had been fully cleared from the area.

An issue of concern with these studies is whether the A β epitope utilized for immunohistochemistry is masked by the injected antibody. This issue was addressed in our previous work where it was shown that although there is a broad distribution of the injected antibody 4 hours following injection, no reduction in A β immunohistochemistry is apparent (Wilcock et al, 2003). Also, if the reduction observed is simply an artifact of

masking the A β epitope, a reduction in thioflavine-S staining would not be observed since this is a conformation dependent stain and not epitope dependent. Further, the stoichiometry of injected antibody (13 pmol) to A β in deposits (estimated at 250 pmol in 0.5 mg, Chapman et al., 1999) is likely too low to interfere substantially with the histochemical reaction. Finally, 7 days after injection, the injected antibody is no longer detectable, yet the A β immunostaining remains absent.

The present report further investigated the relationship between activation of microglia and the clearance of the fibrillar amyloid plaques associated with anti-A β antibody injections. We used several distinct pharmacological agents in an attempt to inhibit the microglia activation observed 72 hours following intracranial injection of anti-A β antibodies. Dexamethasone is a glucocorticosteroid which inhibits the cyclooxygenase and lipoxygenase inflammatory pathways as well as inducing a general state of immunosuppression. It has been shown that microglia respond differently to mineralocorticoid and glucocorticoid receptor stimulation. Mineralocorticoid receptor activation stimulates the microglia while glucocorticoid receptor activation inhibits microglia (Tanaka et al, 1997). All pharmacological glucocorticosteroids possess some degree of mineralocorticoid action also. For the present study we selected dexamethasone as it has the maximum glucocorticoid receptor activity with the minimum mineralocorticoid receptor activity detectable among all available pharmacological glucocorticosteroids (Schimmer and Parker, 2001). It was found that dexamethasone was the most efficacious compound for the inhibition of microglial activation among those used in this study. Dexamethasone administered immediately following intracranial anti-A β antibody administration completely inhibited the microglial activation caused by the

injection. Associated with this profound arrest of microglial activation was a complete inhibition of the antibody's ability to remove compact amyloid deposits detected with thioflavine-S staining, strongly suggesting a role for microglial involvement in the removal of compact amyloid deposits.

The novel non-steroidal anti-inflammatory (NSAID) NCX-2216, which is a flurbiprofen molecule conjugated to an antioxidant and a nitric oxide releasing group, was moderately effective at inhibiting microglial activation. Interestingly, this compound has previously been shown to cause the activation of microglia and removal of amyloid from the brains of otherwise untreated doubly transgenic APP+PS1 mice (Jantzen et al, 2002). NCX-2216 has also been shown to inhibit the microglial activation caused by intracranial infusion of lipopolysaccharide (LPS), a proinflammatory agent in young rats, but to increase microglial activation in old rats (Haus-Wegrzyniak et al, 1999). In the present study, NCX-2216 partially reduced the activation of microglia caused by antibody injection. Associated with this partial inhibition of microglial activation is also a partial impairment of the anti-A β antibody's capacity to remove the compact amyloid plaques. The discrepancy between the effects observed in the current study and the effects previously observed by Jantzen et al (2002) may be explained by the fact that NCX-2216 essentially is three drugs. In a situation where there is intense, local microglial activation the anti-inflammatory properties of the drug appears to dominate. In a situation where there is diffuse microglial activation it appears that maybe the nitric oxide release dominates to enhance microglial activation and aid in the clearance of A β . Although not quantified, the microglial reaction immediately adjacent to amyloid deposits appeared more intense in NCX-2216 treated mice in regions distant from the

injection (e.g. contralateral anterior cortex). A similar bidirectional effect of this drug was found by Hauss-Wegrzyniak et al, 1999, who found reduced microglial activation in young rats, but enhanced activation in old rats treated with the NCX-2216 relative, nitroflurbiprofen.

Minocycline is a tetracycline derivative which has been shown to have a novel action independent of its antibiotic property. This agent has been shown to inhibit microglial activation following excitotoxicity (Tikka et al, 2001), ischemia (Yrjanheikki et al, 1998) and 6-hydroxydopamine lesions (He et al, 2001). In the present study we demonstrate that minocycline is capable of a modest inhibition of microglial activation following antibody injection. Associated with this is no difference in compact plaque removal in either the frontal cortex or hippocampus.

To further investigate whether the activation of microglia, which appears to be specific to the anti-A β antibody, is due to Fc receptor activation we administered F(ab')₂ fragments intracranially into APP transgenic mice as well as the whole IgG, control IgG and F(ab')₂ fragments from the control IgG. We found that of the four experimental groups only the animals receiving anti-A β IgG showed significant activation of microglia. The fact that anti-A β F(ab')₂ fragments were unable to activate microglia, strongly suggests that Fc receptor activation is required for the significant activation of microglia following intracranial administration of anti-A β antibodies.

Associated with this inability to activate microglia was a significantly impaired capacity to remove fibrillar amyloid deposits. Still, at least in hippocampus, there was some residual capacity for fibrillar amyloid removal using the F(ab')₂ fraction. However, with respect to diffuse A β clearance, the F(ab')₂ are just as effective as the corresponding

intact IgG. These results suggest that there may be an equilibrium between the fibrillar deposits and the diffuse deposits, and the F(ab')₂ antibody fragments can whittle away at the fibrillar plaques without requiring Fc receptor mediated phagocytosis. This is also the likely explanation why Bacskai et al (2002) found topically applied F(ab')₂ fragments equally effective to intact antibodies in the clearance of thioflavine-S labeled material. It is plausible that had we injected a greater amount of F(ab')₂ fragments, or extended the post-injection interval, we also might have found complete clearance of fibrillar amyloid with the F(ab')₂ material. However, even though it may be capable of clearing fibrillar amyloid, the results presented here demonstrate F(ab')₂ fragments were much less efficient than the intact IgG molecule in mediating clearance associated with microglial activation. It is plausible that this also explains the recent observations from Bard et al (2003) that the ability of different monoclonal anti-A β antibodies to clear brain amyloid when administered systemically was correlated better with their capacity to bind Fc receptors than with their affinity for A β . The amounts of antibody entering the brain are roughly 0.1% of the injected amount per hour (Banks et al, 2002). Thus, a 500 μ g injection of a monoclonal antibody should result in 0.5 μ g entering the CNS within 60 minutes, somewhat less than the amounts injected directly in our system (2 μ g). Therefore, with systemically injected antibodies, the facilitation of amyloid removal by Fc receptor mediated phagocytosis is likely to be even greater than that observed here with intracranially administered antibodies.

Certainly, it will be useful to measure A β content by means other than histochemistry. Increasingly complex methods of fractionating homogenates are being used to identify A β pools linked most closely to neuropathology and cognitive disruption

(Lue et al, 1999 and Golde et al, 2000) but consensus regarding the specific fractions corresponding to the most toxic form of amyloid has not yet been achieved (Walsh et al, 2002 and Kaye et al, 2003). Additionally, the relatively small portions of the brain affected by the intracranial injections and difficulty dissecting these regions consistently limits the ability to use solution methods to evaluate A β loads in our studies. As consensus emerges regarding the relationships between soluble, oligomeric and fibrillary forms of A β in the solution domain and the histochemical domain, and larger portions of the brain become involved with either intraventricular or systemic antibody injections, we will investigate the effects of anti-A β antibodies on these different A β pools in both domains.

It has been shown previously that vaccination using A β_{1-42} results in activation of microglia, which is associated with a reduction in A β accumulation in PDAPP transgenic mice (Schenk et al, 1999) and APP+PS1 transgenic mice (Wilcock et al, 2001). It has also been shown that following direct application of anti-A β antibodies to the brains of PDAPP mice there is an activation of microglia and a reduction in amyloid deposits (Bacskai et al, 2001). In vitro studies using F(ab')₂ fragments demonstrated that they were unable to activate microglia despite retaining full ability to bind to A β (Bard et al, 2000). These fragments also failed to remove fibrillar A β in an ex vivo assay. Human postmortem microglia have been shown to phagocytose opsonized A β , which is inhibited by excess non-specific IgG, suggesting this phagocytosis is Fc receptor mediated (Lue et al, 2002). All of these data suggest that one likely mechanism of antibody action in removing amyloid deposits from transgenic mouse brains is via binding to microglial Fc

receptors and triggering activation of the microglia, possibly including phagocytosis of the opsonized amyloid. This by no means precludes other possible mechanisms, such as catalytic dissolution of amyloid fibrils (Solomon et al, 1997), or sequestration of A β in the periphery, effectively drawing A β out of the brain (DeMattos et al, 2001).

Recently, the first pathology report from a patient receiving the A β_{1-42} vaccination (AN1792) was published (Nicoll et al, 2003). This report showed that the patient had considerably fewer amyloid deposits than would have been predicted from other AD cases. Interestingly, it is reported that in those regions devoid of amyloid plaques, the remaining A β -immunoreactivity was associated with activated microglia. This patient did develop meningoencephalitis and other symptoms of CNS inflammation, as did several others in the trial. The treatment chosen for the CNS inflammation in this case was dexamethasone.

Assuming that the mechanisms of A β vaccination in clearing amyloid is similar to that demonstrated in the present work, it might be anticipated that dexamethasone would counter the amyloid removing effects of the vaccine. Although the case described by Nicoll et al above did show evidence of removal, the patient had received 5 inoculations before developing adverse reactions and being administered dexamethasone. It was also not indicated what the antibody titer was in the patient, nor how long the dexamethasone treatment had continued. The data presented here suggest that administration of glucocorticoids to vaccinated patients may counteract any benefit the vaccine has with respect to amyloid clearance and possibly cognitive function. It is also conceivable that removal of only the soluble and diffuse A β may provide cognitive improvement and inhibition of microglial activation by anti-inflammatory drugs or administration of

F(ab')₂ fragments may avoid some of the inflammatory adverse effects observed in the human clinical trial (Orgogozo et al, 2003). The inhibition of microglial mediated amyloid clearance may also have been a factor in the failure of the prednisone clinical trial for AD (Aisen et al, 2000).

The adverse reactions in the human vaccine trial demonstrates a need to more fully investigate the mechanisms involved in beneficial and detrimental effects of immunotherapy. Encouraging data recently published by Hock et al (2003) showed that the a subset of patients administered the A β vaccine remained cognitively stable for one year after treatment while the control patients declined at a normal rate, some vaccinated patients actually improved. While the immunotherapeutic approach may hold promise for the treatment of AD, it would appear very important to better understand both the mechanisms of vaccine action, and how the tools to effectively modulate the immune reaction interact with these mechanisms. The data presented here make some headway toward determining what effects modulation of the immunotherapeutic approach mechanisms would have pathologically. Future studies will extend these investigations to a more clinically relevant, systemically administered, passive immunization regimen to determine the importance of the mechanisms discussed in the current study.

Figure 1: Anti-inflammatory drugs impaired fibrillar amyloid removal to roughly the same extent as they decreased microglial activation following anti-A β antibody injections. Panels A-E show CD45 immunohistochemistry in the hippocampus. Panels F-J show A β immunohistochemistry in the hippocampus. Panels K-O show thioflavine-S staining in hippocampus. Mice were injected intracranially with anti-A β antibody followed by no treatment (A, F, K), dexamethasone treatment (B, G and L), minocycline treatment (C, H and M) or NCX-2216 treatment (D, I and N). Mice shown in panels E, J, and O were injected with anti-HIV antibody as a control for nonspecific effects of IgG injection. Magnification = 40X. Scale bar = 120 μ m.

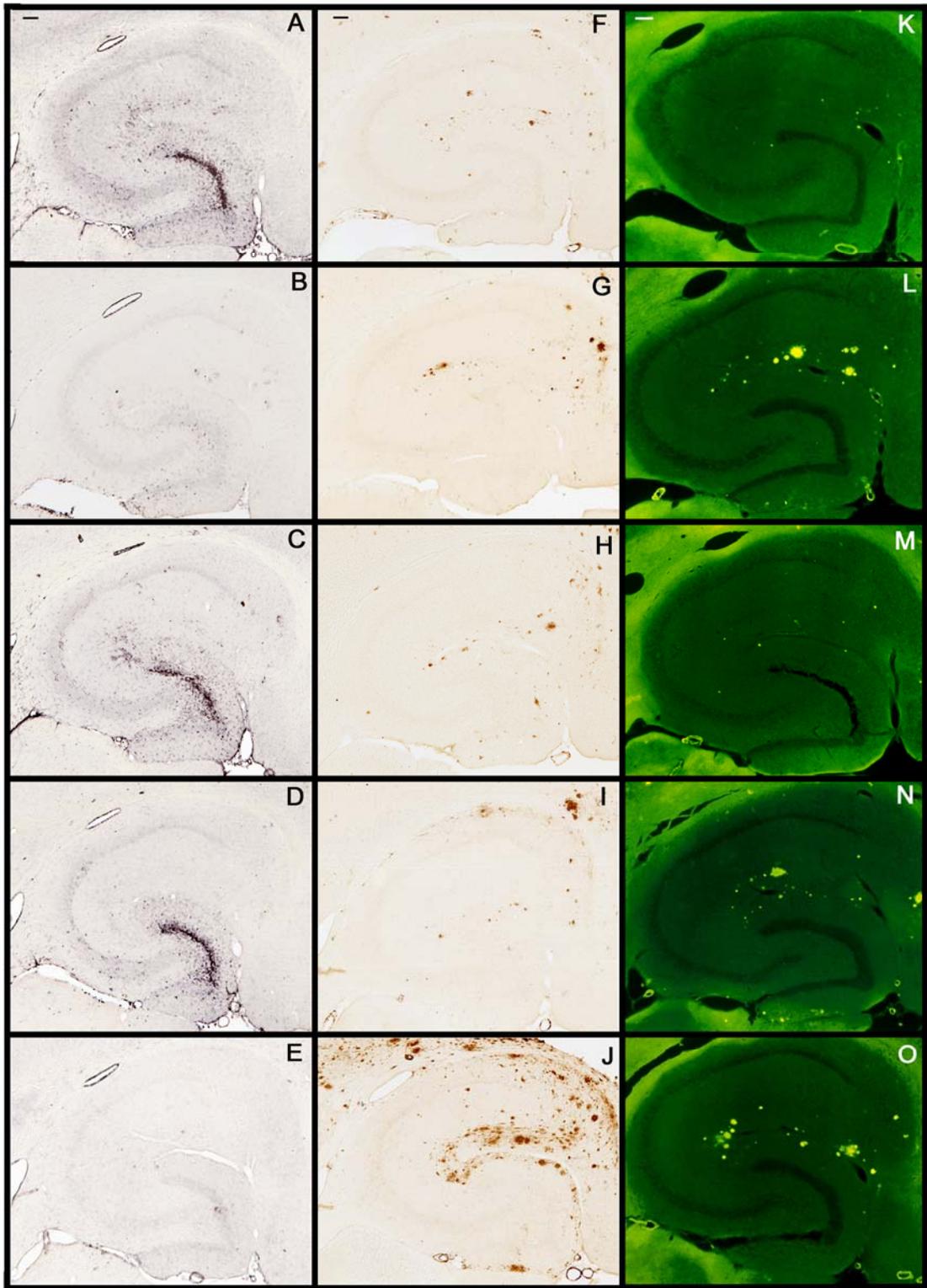


Figure 2: Quantification of CD45, total A β and thioflavine-S following inhibition of microglial activation by anti-inflammatory compounds. Panel A shows the ratio of right to left sides for CD45 immunohistochemistry. Panel B shows the ratio of right to left sides for total A β immunohistochemistry. Panel C shows the ratio of right to left sides for thioflavine-S staining. The solid bars indicate values for frontal cortex, the open bars indicate values for hippocampus. On the x-axis the type of antibody injected (anti-A β antibody; Abeta, or control antibody; HIV) is shown. The post-injection treatment the mice received is also shown; Dex: dexamethasone, Min: minocycline treatment, NCX: NCX-2216 treatment. *** indicates $P < 0.001$, ** indicates $P < 0.01$, * indicates $P < 0.05$ as compared to the anti-A β antibody alone.

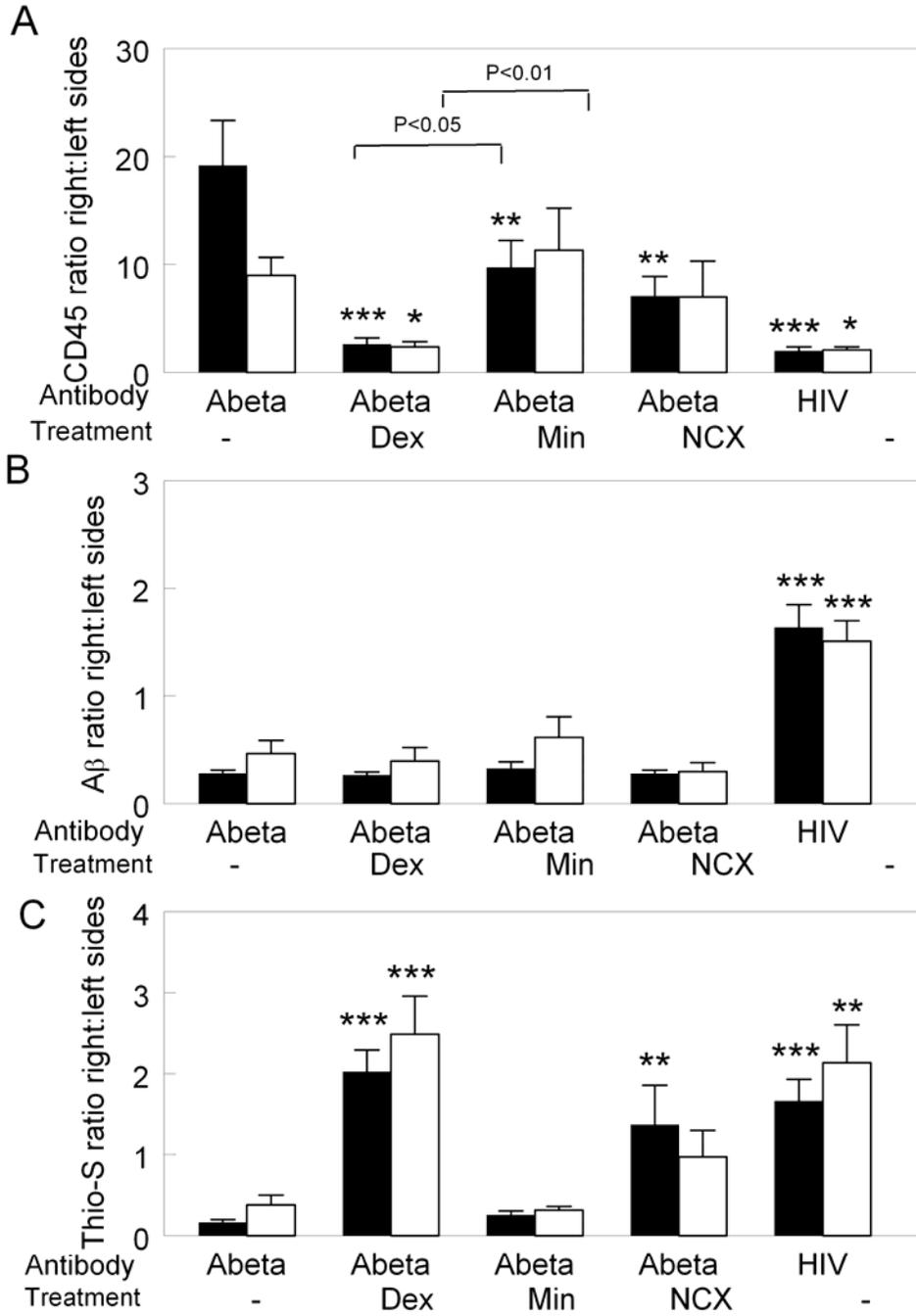


Figure 3: Anti-A β F(ab')₂ fragments do not activate microglia, nor do they remove compact amyloid deposits as effectively as the complete anti-A β IgG. Panels A-D show CD45 immunohistochemistry in the hippocampus. Panels E-H show total A β immunohistochemistry in the hippocampus. Panels I-L show thioflavine-S staining in the hippocampus. Mice were injected with intact anti-A β IgG (A, E and I), anti-A β F(ab')₂ fragments (B, F and J), control (anti-amnesiac) IgG (C, G and K), or control (anti-amnesiac) F(ab')₂ fragments (D, H and L). Magnification = 40X. Scale bar=120 μ m.

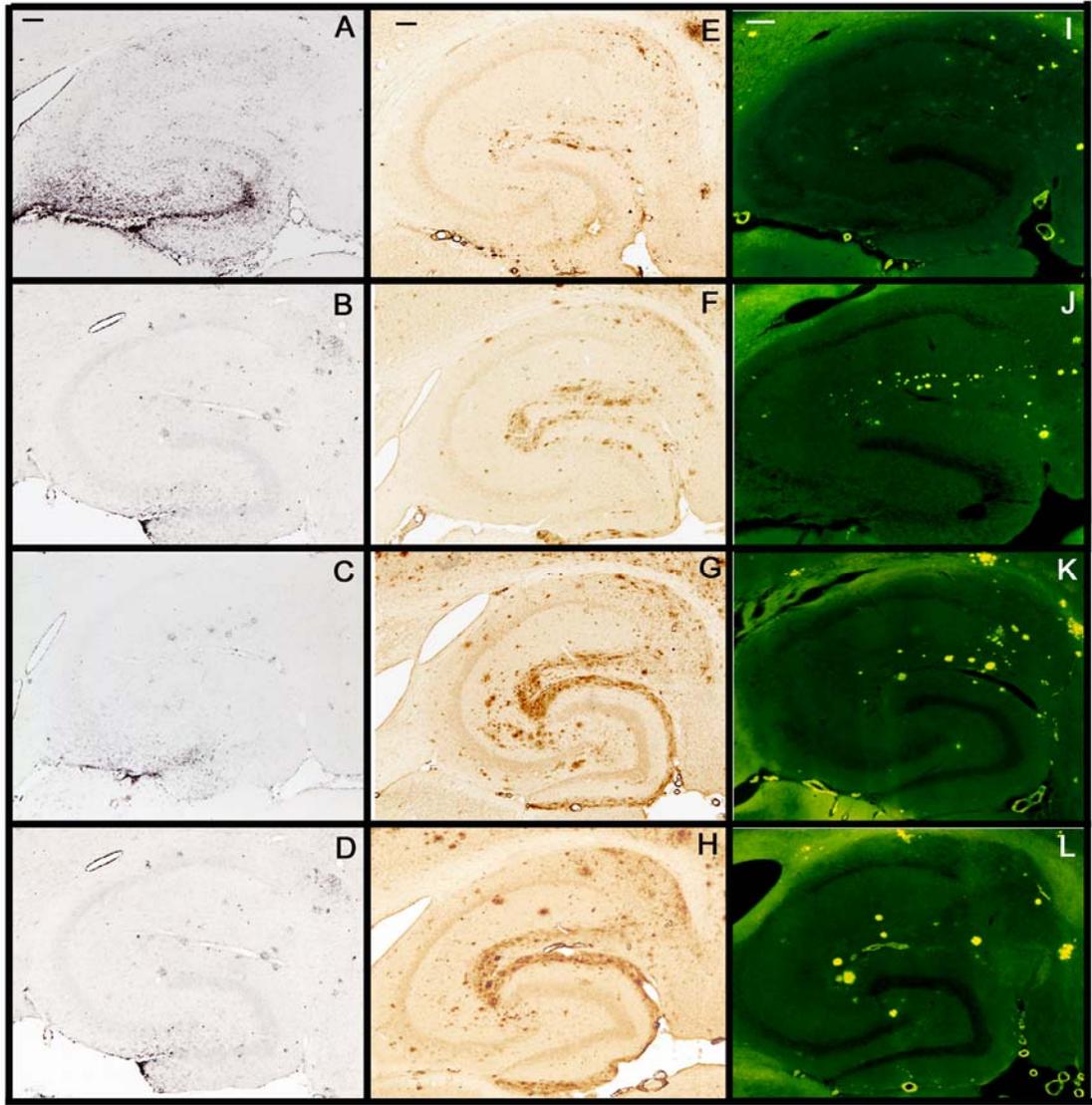
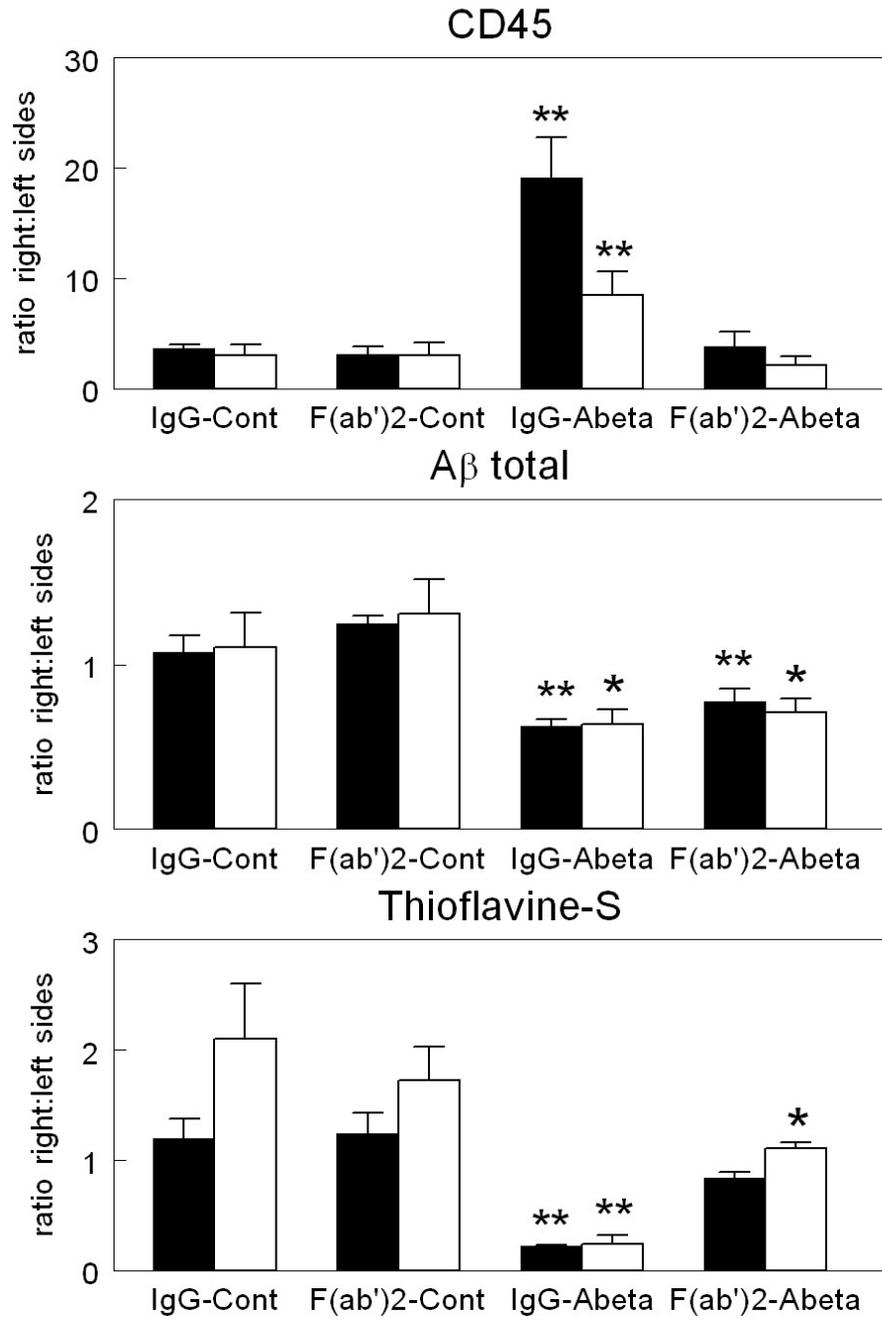


Figure 4: Quantification of CD45 and total A β immunohistochemistry and thioflavine-S staining following intracranial injection of anti-A β antibodies and anti-A β F(ab')₂ fragments. Panel A shows the ratio of right to left sides for CD45 immunohistochemistry. Panel B shows the ratio of right to left sides for total A β immunohistochemistry. Panel C shows the ratio of right to left sides for thioflavine-S staining. The solid bars indicate values for frontal cortex, the open bars indicate values for hippocampus. On the x-axis IgG-Cont= control (anti-amnesiac) intact IgG,; F(ab')₂-Cont = Control (anti-amnesiac) F(ab')₂ fragments; IgG-Abeta = anti-A β intact IgG; F(ab')₂-Abeta= anti-A β F(ab')₂ fragments.*** indicates P<0.001, * indicates P<0.05 as compared to both control antibody groups. Lines over bars indicates P values for comparisons between the specific pair of groups indicated.



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PAPER 3:

**PASSIVE AMYLOID IMMUNOTHERAPY CLEARS AMYLOID AND
TRANSIENTLY ACTIVATES MICROGLIA IN A TRANSGENIC MOUSE
MODEL OF AMYLOID DEPOSITION.**

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Abstract

The role of microglia in the removal of amyloid deposits following systemically administered anti-A β antibodies remains unclear. In the current study we injected Tg2576 APP transgenic mice weekly with anti-A β antibody for a period of one, two or three months such that all mice were 22 months at the end of the study. In mice immunized for three months we found an improvement in alternation performance in the Y maze. Histologically, we were able to detect mouse IgG bound to congophilic amyloid deposits in those mice treated with anti-A β antibody but not in those treated with control antibody. We found that Fc γ receptor expression on microglia was increased following one month of treatment while CD45 was increased following two months of treatment. Associated with these microglial changes was a reduction in both diffuse and compact amyloid deposits following two months of treatment. Interestingly, the microglia markers were reduced to control levels following three months of treatment while amyloid levels remained reduced. Serum A β levels and anti A β antibody levels were elevated to similar levels at all three survival times in mice given anti-A β injections rather than control antibody injections. These data show that antibody is able to enter the brain and bind to the amyloid deposits, likely opsonizing the A β and resulting in Fc γ receptor mediated phagocytosis. Together with our earlier work, our data argue that all proposed mechanisms of anti-A β antibody mediated amyloid removal can be simultaneously active.

Introduction

Reduction of brain amyloid following anti-A β immunotherapy was first demonstrated by Schenk and colleagues (1999). Their report showed that vaccination with A β_{1-42} in the PDAPP transgenic mouse model of Alzheimer's disease dramatically reduced levels of A β deposits in the brain. Later it was shown that using the same vaccination protocol in APP+PS1 doubly transgenic mice (Morgan et al, 2000) and in TgCRND8 transgenic mice (Janus et al, 2000) not only reduced A β levels in the brain but also protected these mice from memory deficits. More recent studies have demonstrated that passive immunization consisting of direct anti-A β antibody injections not only results in dramatic reduction of A β levels (Bard et al, 2000; DeMattos et al, 2001) in the brain but also reverses memory deficits in transgenic mouse models of AD (Dodart et al, 2002; Kotilinek et al, 2002).

The mechanism(s) by which immunotherapy acts remain unclear. Suggested mechanisms include microglial mediated phagocytosis (Schenk et al, 1999, Wilcock et al, 2001, 2003, 2004), disaggregation of amyloid deposits (Solomon 1997, Wilcock et al, 2003, 2004), and removal of A β from the brain by binding of circulating A β in plasma with the anti-A β antibodies, resulting in a concentration gradient from brain to plasma. This latter mechanism is also known as the peripheral sink hypothesis (DeMattos et al, 2001, Dodart et al, 2002, Lemere et al, 2003, Das et al, 2003).

We have previously reported that following intracranial anti-A β antibody injections into APP transgenic mice there is a rapid removal of diffuse amyloid deposits

apparently independent of microglial activation and also a later removal of compact amyloid deposits which appears to require microglial activation (Wilcock et al, 2003). In fact, in a later study using the same model, administration of dexamethasone, which suppresses microglial activation, anti-A β antibody administration inhibits the removal of compact, thioflavine-S positive, amyloid deposits (Wilcock et al, 2004).

In this report we show that weekly systemic administration of anti-A β antibodies for a period of one, two or three months results in a dramatic reduction of both diffuse and compact amyloid deposits. Associated with this reduction is a behavioral improvement using the Y-maze task. Following one month of treatment there is a large induction of Fc γ receptor expression on microglia and following two months of administration there is an increase in CD45 expression indicative of microglial activation. We have detected antibody binding to congophilic plaque in APP transgenic mice treated with anti-A β antibody. We also observe a dramatic increase in circulating A β levels following one month of administration. Two months following administration we observe a dramatic reduction in compact and diffuse deposits. After three months of administration the microglia markers are down to control levels whilst the compact and diffuse amyloid deposits remain reduced. These results demonstrate systemically administered anti-A β antibodies are accessing the brain, binding to amyloid deposits and activating microglia. The data also show an increase in circulating A β in plasma, consistent with the peripheral sink hypothesis.

Materials and Methods.

Experiment design:

Singly transgenic APP Tg 2576 mice were obtained from our breeding program at USF started in 1996 (Holcomb et al, 1998). Twenty two APP transgenic mice aged 19 months were assigned to one of four experimental groups. The first three groups received weekly intraperitoneal anti-A β antibody injections (antibody 2286; Mouse monoclonal anti-human A β_{28-40} IgG1; Rinat Neurosciences, Palo Alto, CA) for 1 month (n=6), 2 months (n=9) or 3 months (n=4). The fourth group received weekly intraperitoneal anti-AMN antibody injections (2906; Mouse monoclonal anti-drosophila amnesiac protein IgG1; (Rinat Neurosciences, Palo Alto, CA) for 3 months (n=3). Twelve nontransgenic mice were assigned to one of two experimental groups. The first group received intraperitoneal anti-A β antibody injections for 3 months (n=4). The second group received no treatment (n=3). Treatment of 1 month and 2 month groups was delayed to insure the mice were killed at the same age (22mo). One week prior to kill and one day following the 5th, 9th or 13th injection mice were tested behaviorally using the Y maze task.

Behavioral testing:

The Y maze is a three arm maze with equal angles between all arms. Mice were initially placed within one arm and the sequence and number of arm entries was recorded for each mouse over an 8 minute period. The percentage of triads in which all three arms were represented (ABC, CAB or BCA but not BAB) was recorded as an alternation to estimate short-term memory of the last arms entered. The total number of possible

alternations is the number of arm entries minus two. Additionally, the number of arm entries serves as an indicator of activity.

Antibody Purification:

Antibody 2286 (mouse monoclonal anti-human A β ₂₈₋₄₀ IgG1) and the antibody 2906 (mouse monoclonal anti-drosophila amnesiac protein IgG1) was purified from mouse ascites on AKTA instrumentation using protein A beads (MabSelect, Amersham Biosciences). Briefly, ascites was filtered in pyrogen-free .22 μ m filter system (Corning) and applied to a 20ml bed volume in a XK16/20 column (Amersham Biosciences) after equilibrating the beads with 5 vol of binding buffer (0.6M NaCl, 0.3M glycine, pH 8.0). The column was washed with 3 vol of binding buffer and the antibody was eluted in 4 vol of elution buffer (0.1M Na Citrate pH 3.0), and held at low pH for 30 min for viral inactivation. The resulting eluant was neutralized with 1/10th vol of 1.0M Tris, pH 9.5. The antibody was dialyzed into sterile PBS, pH 7.4 and the concentration was determined by reading absorbance at 280. All buffers were made in pyrogen-free water.

Tissue preparation:

On the day of kill mice were weighed, overdosed with 100mg/kg pentobarbital (Nembutal sodium solution, Abbott laboratories, North Chicago IL). Blood was collected and allowed to coagulate at 4°C for at least one hour before being centrifuged and the serum removed and stored at -80°C until required. The mice were then intracardially perfused with 25ml of 0.9% sodium chloride. Brains were rapidly removed and the right half of the brain was dissected and frozen for biochemistry while the left half of the brain was immersion fixed for 24 hours in freshly prepared 4% paraformaldehyde in 100mM PO₄ (pH 7.2) for histopathology. The latter hemibrains were then incubated for 24 hours

in 10, 20 and 30% sucrose sequentially to cyroprotect them. Horizontal sections of 25 μ m thickness were collected using a sliding microtome and stored at 4°C in Dulbecco's phosphate buffered saline with sodium azide (pH 7.2) to prevent microbial growth.

ELISA methods; A β and anti-A β antibody:

For the A β assay serum was diluted and incubated in 96-well microtiter plates (NUNC MaxiSorp, Denmark), which were pre-coated with antibody 6E10 (Signet, Dedham, MA) at 5 μ g/ml in PBS buffer, pH 7.4. The secondary antibody was biotinylated 4G8 (Signet, Dedham, MA) at 1:5000 dilution. Detection was done using streptavidin horseradish peroxidase conjugate (Amersham Biosciences), followed by TMB substrate (KPL, Maryland). Standard curves of A β ₄₀ (Global Peptide, Ft. Collins, CO) scaling from 400 - 6pm were used.

Anti-A β antibody was dissociated from endogenous A β in serum as described previously (Li et al, 2004). Briefly, serum was diluted in dissociation buffer (0.2M glycine HCl, 1.5% BSA pH 2.5) and incubated at room temperature for 20 min. The sera were pipetted into the sample reservoir of Microcon centrifugal device, YM-10 (10,000 MW cut-off, Millipore) and centrifuged at 8,000 x g for 20 min. at RT. The sample reservoir was then separated from the flow through, placed inverted into a second tube and centrifuged at 1000 x g for 3 min. The collected solution containing the antibody dissociated from the A β peptide was neutralized to pH 7.0 with 1M Tris buffer pH 9.5. The dissociated sera were assayed by ELISA for antibody titer. A β ₁₋₄₀ (Global Peptide, Ft. Collins, CO) coated 96-well microtiter plates (NUNC MaxiSorp, Denmark) were incubated with dissociated serum samples. A biotinylated goat-anti mouse IgG (H+L) (Vector, Burlingame, CA) at

1:5000 dilution, followed by peroxidase -conjugated streptavidin (Amersham Biosciences) was used to detect serum anti-A β binding activity.

Immunohistochemical methods:

A series of eight equally spaced tissue sections 2.4mm apart were randomly selected spanning the entire brain and stained using free-floating immunohistochemistry methods for total A β (rabbit polyclonal anti-pan A β Bisource, Camarillo, CA, 1:10000), CD45 (rat anti-mouse CD45, Serotec, Raleigh NC, 1:3000), Fc γ receptors II /III (rat anti-mouse CD16/ CD32, BD Pharmingen, San Diego, CA, 1:3000) as previously described (Gordon et al, 2002). Briefly, tissue was incubated in primary antibody overnight at room temperature. Sections were then washed and incubated in the appropriate biotinylated secondary antibody (for A β : goat anti-rabbit 1:3000; for CD45 and Fc γ R: goat anti-rat 1:1000. All Vector Laboratories, Berlingame, CA) for two hours. Following multiple washes tissue was incubated in ABC (Vector Laboratories, Berlingame, CA) for a period of one hour. Color development was performed using 3,3'-Diaminobenzidine (DAB, Sigma-Aldrich, St Louis, MO) enhanced with nickelous ammonium sulfate (J.T. Baker Chemical Co., Phillipsburg, NJ) for CD45 and Fc γ R, or without enhancement for A β . For immunostaining, some sections were omitted from the primary antibody to assess non-specific immunohistochemical reactions.

Additional sections were also stained for mouse IgG using immunohistochemical methods similar to that described above. Briefly, sections were incubated overnight in a 1:3000 concentration of anti-mouse IgG conjugated to horseradish peroxidase (Sigma-Aldrich, St Louis, MO). The sections were then washed and incubated for 5 minutes in 100ml TBS (tris-buffered saline) containing 50mg DAB (3,3'-diaminobenzidine, Sigma-

Aldrich, St Louis, MO), 500mg nickelous ammonium sulfate (J.T. Baker Chemical Co. Phillipsburg, NJ) and 100µl 30% hydrogen peroxide to produce a purple/ black color reaction product. The sections were then mounted on slides and counterstained with a 0.2% Congo red solution in 80% ethanol to assess the localization of positive mouse IgG stain with compact amyloid deposits.

A set of sections also mounted and stained using 0.2% Congo red solution in NaCl saturated 80% ethanol. Another set of sections was also mounted and stained using 4% thioflavine-S (Sigma-Aldrich, St Louis MO) for 10 minutes.

The immunohistochemical reaction product on all sections was measured using the Image-Pro Plus version 4.5 software (Media Cybernetics, Silver Spring, MD). One region of the frontal cortex for all sections from each animal was analyzed and the average of 6-7 sections was taken to give a value for each animal. Three regions of the hippocampus were analyzed on approximately 4-5 sections where hippocampus was present; the CA1, CA3 and dentate gyrus. These regions were analyzed both individually to yield an average per region and also combined to give an overall value for hippocampus for each animal. This ensured that there was no regional bias in the hippocampal values. These same analysis methods were used to evaluate the Congo red stain also. To assess possible treatment-related differences, the values for each treatment group were analyzed by one-way ANOVA followed by Fischer's LSD means comparisons. Nontransgenic mice showed no treatment related differences in any histological analyses and so these groups were pooled.

Data analysis:

Percent alternation and arm entry numbers from the Y-maze behavior task were analyzed using a one-way analysis of variance (ANOVA) followed by Fischer's LSD means comparisons using StatView software version 5.0.1 (SAS Institute Inc., NC). Nontransgenic mice showed no treatment related differences in any behavioral analyses and so these groups were pooled. ELISA values for serum A β levels and circulating antibody levels were analyzed using a one- ANOVA followed by Fischer's LSD means.

Results.

Transgenic APP mice given control antibody injections showed significantly reduced Y-maze alternation when compared to the nontransgenic mice (Fig. 1A). This reduced alternation was reversed in the APP transgenic mice receiving weekly anti-A β antibody injections for three months. This group of mice was indistinguishable from the nontransgenic animals and showed significantly increased alternation compared to the APP transgenic mice receiving control antibody (Fig. 1A). The APP transgenic mice given weekly anti-A β antibody injections for either one or two months were intermediate between nontransgenic and transgenic mice given control antibodies and not significantly different from either group. Nontransgenic mice also made significantly fewer arm entries than the APP transgenic mice receiving control antibody injections indicating hyperactivity in the APP transgenic mice. The APP transgenic mice receiving anti-A β antibody injections for two and three months did not exhibit this hyperactivity and were not significantly different from any other treatment groups (Fig 1B).

One day following anti-A β antibody administration anti-A β antibodies were detected in serum at high levels (400nM) following one month of administration. This level of antibody in the serum was the same after two or three months of administration with no apparent accumulation of antibody (Fig 2A). Associated with high anti-A β antibody levels in serum at one month was a dramatic increase in circulating A β levels in serum. APP transgenic mice receiving control antibody had only 1.5nM circulating A β in plasma compared to APP transgenic mice receiving A β antibody for one month which had 130nM circulating A β in plasma; an almost 100-fold increase (Fig 2B). Despite similar levels of anti-A β antibody at one, two or three months of administration, circulating A β levels declined between one and two month. They also showed a slight decline between two and three months of administration although with both two and three months of administration circulating A β levels were still significantly elevated compared to APP transgenic mice receiving control antibody (Fig 2B).

Following systemic administration of anti-A β antibodies weekly for one month staining for mouse IgG could be detected on plaques throughout the brains of APP transgenic mice (Fig 3B). The staining was the most intense where plaque load is greatest; the hippocampus and frontal cortex. This staining was not observed in APP transgenic mice receiving control antibody (Fig 3A). It should be noted that staining with higher concentrations of anti-mouse IgG-HRP did show staining of plaques in both control treated and anti-A β treated APP transgenic mice. Staining for mouse IgG was still present, and slightly more intense, around the plaques that remain following two (Fig. 3C) and three (Fig. 3D) months of treatment.

Total A β immunohistochemistry in the APP transgenic mice receiving control antibody (Fig. 4A) showed a few intensely stained deposits suggesting compacted amyloid deposits along with more numerous diffuse deposits. There was a concentration of deposits around the hilus of the hippocampus as well as the molecular layers of Ammon's horn. This was a typical amount and distribution of A β for APP transgenic mice of this age, as previously described (Hsiao et al, 1996 and Gordon et al, 2002). Following one month of weekly anti-A β antibody injections there appeared to be a slight reduction in A β immunohistochemistry in the hippocampus (Fig 4B) although this was not statistically significant (Fig 4E). The reduction appeared to be primarily diffuse deposits, with most of the compact amyloid deposits remaining (Fig 4B). After two months of weekly anti-A β antibody injections we observed a dramatic reduction in A β immunohistochemistry which appeared to be both compact and diffuse amyloid deposits from the hilus and dentate gyrus regions of the hippocampus as well as the pyramidal cell regions, with only a few deposits remaining, often in the vicinity of the hippocampal fissure and outer molecular layers (Fig 4C). This reduction in A β load at two months was approximately 60% in the hippocampus and approximately 55% in the frontal cortex (Fig 4E, hippocampus $P < 0.001$, frontal cortex $P < 0.005$). Total A β levels remained reduced after three months of treatment but did not appear to decrease any further (Fig 4D and E).

Congo red staining detects only compact amyloid deposits in the beta pleated sheet structure. There were far fewer Congo red positive amyloid deposits than A β deposits detected by total A β immunohistochemistry. Congo red positive deposits were located primarily along the fissure of the hippocampus as well as the CA1/ subiculum

region in APP transgenic mice receiving control antibody (Fig 5A). There was no reduction in Congoophilic deposits 1 month following treatment in either the hippocampus (Fig. 5B and E) or the frontal cortex (Fig 5E). Following two months of treatment there was a significant reduction in both number and size of congophilic deposits in both the hippocampus (Fig. 5C and E) and frontal cortex (Fig. 5E). This reduction was approximately 60% in the frontal cortex and approximately 50% in the hippocampus (Fig. 5E, $P < 0.005$ frontal cortex $P < 0.01$ hippocampus,). There was a small further reduction between two months and three months which is approximately 30% in hippocampus and frontal cortex (Fig. 5E). Thioflavine-S staining was also measured and confirmed the Congo red data showing the same reductions in stained area as did Congo red (data not shown).

Immunohistochemical staining for Fc γ receptors II and III in APP transgenic mice receiving control antibody treatment for three months showed only very faint staining of microglia in close association with amyloid deposits (Fig 6A). Following one month of anti-A β antibody administration there was a dramatic induction of Fc γ receptors II and III on microglia. The microglia expressing the Fc γ receptors after 1 month of treatment were not only associated with amyloid deposits but are also diffusely distributed (Fig 6B). This induction averaged 100-fold in the hippocampus (Fig. 6B and E, $P < 0.05$) and frontal cortex (Fig. 6E, $P < 0.05$). Fc γ receptor expression levels fell only slightly between one month and two months of treatment although this expression was once again concentrated on microglia around remaining amyloid deposits (Fig. 6D). Induction remained approximately 100-fold in hippocampus (Fig. 6E, $P < 0.05$) and frontal cortex (Fig. 6E, $P < 0.05$). Following three months of treatment Fc γ receptor expression was reduced to

levels observed in APP transgenic mice receiving control antibody (Fig. 6E) although it appeared to be increased in microglia around the few remaining amyloid deposits (Fig. 6D).

CD45, a protein tyrosine phosphatase, is normally moderately expressed on microglia around amyloid deposits in aged APP transgenic mice and is a commonly used marker for microglial activation. This moderate expression was observed in the APP transgenic mice receiving control antibody treatment for three months (Fig 7A and E). Following one month of treatment we observed an increase in CD45 expression on microglia surrounding amyloid deposits in both the hippocampus (Fig. 7B, F and I) and frontal cortex (Fig. 7I). While the expression in hippocampus was approximately 2.5 times that observed in control treated APP transgenic mice in the hippocampus (Fig. 7I, not significant) and twice the values found in the frontal cortex of control animals, the elevation was not statistically significant (Fig. 7I, not significant). Following two months of anti-A β antibody treatment there was a further increase in CD45 expression on microglia not only surrounding the amyloid deposits but also diffusely distributed throughout the amyloid containing brain regions (Fig. 7C and G). It is possible that this more widespread activation is in association with diffuse amyloid deposits although we cannot confirm this. The increased expression was approximately 3.5 times that observed in control treated mice in the hippocampus (Fig. 7I, $P < 0.05$) and 3 times in the frontal cortex (Fig. 7I, $P < 0.01$). After three months of anti-A β antibody treatment CD45 expression remained at the same levels as that observed after one month of treatment (Fig 7I), however, the microglia were still diffusely distributed with fewer microglia around deposits compared to one or two months of treatment (Fig 7D and H).

Discussion.

The data presented here suggest that peripherally administered anti-A β antibodies entered the brain, bound to congophilic amyloid plaques, and led to removal of deposited amyloid. In support of the argument that anti-A β antibodies entered the brain, we found mouse IgG decorating the remaining congophilic amyloid plaques of APP transgenic mice administered anti-A β antibody, but no IgG in APP transgenic mice administered control antibody. This difference was best discerned when low titers of the anti-mouse IgG-HRP were used. Lemere et al (2004) also reported immunohistochemical labeling of amyloid deposits for mouse IgG after passive immunization, but detected signals in both immunized and non-immunized mice. It is unclear whether lower anti-mouse IgG concentrations might have revealed selective staining in anti-A β treated animals. These data confirm in paraformaldehyde fixed tissue the observations of Bard et al (2000), who used unfixed cryostat sections.

Associated with the presence of antibody in the brain after one month of treatment was a dramatic activation of Fc γ receptor expression on microglia, further arguing that anti-A β antibodies entered the brain and opsonized the amyloid deposits. Later, following two months of treatment, we observed an increase in CD45 expression on microglia, indicating activation of these cells beyond the level normally associated with amyloid deposits. It has previously been shown that following active immunization with A β ₁₋₄₂ in humans that anti-A β antibodies are present in cerebrospinal fluid, in some instance equal to serum concentration, suggesting some penetration into the brain from the periphery

(Hock et al, 2002). It has also been shown that 0.1% of an intravenous injection of radiolabeled anti-A β antibody crosses the blood-brain barrier of SAMP8 mice (Banks et al, 2002). Thus, accumulating data indicates that circulating antibodies can access the brain parenchyma, which has important implications not only for the use of immunotherapy in Alzheimer's disease but also for other diseases in which immunotherapy is being pursued such as Creutzfeldt-Jakob disease (Manuelidis, 1998; Sigurdsson et al, 2003) and neural infections associated with human immunodeficiency virus (McMichael and Hanke, 2003).

Associated with the changes in microglial markers was a significant reduction in both compact and diffuse amyloid deposits following two months of treatment, these remained reduced following three months of treatment. Removal of A β deposits from the brain appeared to be a gradual process. We did not observe significant reductions in either diffuse or compact amyloid deposits following one month of weekly anti-A β antibody treatment. Following two months of treatment there was a dramatic reduction in total A β immunohistochemistry, Congo red staining and thioflavine-S staining, suggesting removal of both diffuse and compact amyloid deposits. There appeared to be no accumulation of the injected antibody, since serum anti-A β antibody levels were the same regardless of duration of treatment. This would suggest that this time-dependent removal of amyloid deposits was not occurring because of increasing antibody levels, rather, it appears that some mobilization of removal mechanisms must be present for some time before significant removal is apparent.

An early feature we observed was the increase in Fc γ receptors II and III (CD16 / CD32) expression on microglia, which was apparent following one and two months of

treatment. The murine Fc γ receptors II and III share a high affinity for IgG1 antibodies (the isotype used in the current study) as well as IgG2a (Gessner et al, 1998). Following this increased Fc γ receptor expression was an increase in CD45 expression on microglia following two months of treatment. CD45 is a protein tyrosine phosphatase which is elevated with microglial activation. In this study, it appears that the increase in CD45 expression represents a further activation step from that seen after one month of treatment where we observe the increased Fc γ receptor expression. Following three months of treatment, both Fc γ receptor and CD45 expression on microglia were reduced to control levels, possibly due to the substantial reduction in amyloid deposits. It is important to note that if we had looked at only the three month time-point we would not have detected the activation of the microglia by CD45 or, likely by other markers such as Mac-1 (Das et al, 2003).

We have previously observed a similar loss of microglia activation following intracranial antibody administration (Wilcock et al, 2003) and active immunization (Wilcock et al, 2001). Three days following a single injection of anti-A β antibody in the frontal cortex and hippocampus we observed an increase in CD45 expression, however, seven days following injection the CD45 expression was reduced to control levels, in parallel with clearance of the A β deposits (Wilcock et al, 2003). This suggests that the reduced microglial activation could possibly be due to the clearance of most amyloid plaques. It is also conceivable that the microglia could be undergoing apoptosis due to the robust activation as has been described previously by Liu et al (2001) when microglia are overactivated by LPS. An alternative explanation could be tolerance of the microglia to antibody opsonized A β . We have previously shown a reduction in microglial reaction in

an active immunization model using doubly transgenic APP +PS1 mice. Following five monthly inoculations we observed a significant increase in CD45 expression, however, following nine monthly inoculations, CD45 levels were comparable to control animals despite continued high antibody titer levels (Wilcock et al, 2001).

Due to the inflammatory adverse effects seen in the human clinical trial of the active immunization by Elan pharmaceuticals it could be suggested that the microglial activation observed in this study was due to an immune response unrelated to opsonization of A β by the antibody. To evaluate whether this was the case we examined the thalamus and cerebellum, which do not contain any amyloid deposits, for any increase in CD45 or Fc γ receptor expression and did not observe any such increase. Thus it appears that the microglial activation is specific to amyloid containing brain regions and is likely a specific response to opsonized A β as opposed to a general non-specific inflammatory reaction.

The data presented here extend our earlier observations of the benefits of active anti-A β immunization on learning and memory (Morgan et al, 2000). We show that passive immunization with anti-A β antibodies for a period of three months reduced amyloid deposits and improved behavioral performance as indicated by a significant increase in alternation in the y-maze as well as a decrease in the number of arm entries. The arm entry data suggests that there is not a complete reversal of the increased activity. There is a trend towards some improvement in alternation at the 1 month time point (although not significant) despite no reduction in total A β immunohistochemistry. Such improvements may reflect rapid reductions of an A β pool (oligomeric?) closely linked to memory impairments yet not easily detected by immunohistochemistry. This phenomena

was previously described by Dodart et al (2002) and Kotilinek et al (2002) who showed rapid reversal of memory deficits in transgenic mice following passive immunization without significant reduction in brain A β .

The results described above indicating entry of anti-A β antibody into brain and activation of microglia suggests that some opsonization of A β is likely stimulating microglial involvement in the clearance of A β deposits. This is consistent with the phagocytosis mechanisms of amyloid removal put forward by the Elan group (Schenk et al, 1999; Bard et al, 2000; Bard et al, 2003). Our earlier work with direct injection of anti-A β antibody into brain suggests two mechanisms; one not requiring an Fc component nor activation of microglia which can clear diffuse A β , and a second that requires the Fc domain and activation of microglia (Wilcock et al, 2003; Wilcock et al, 2004). It is conceivable that the first non-Fc requiring mechanism is analogous to the catalytic dissolution mechanism described by Solomon et al (1996). The diffuse material, whatever its state of oligomerization may be more accessible to this action of anti-A β antibodies. Finally, at all durations of antibody exposure we observe a dramatic increase in circulating A β levels in plasma. This is consistent with a role for the peripheral sink mechanism (DeMattos et al, 2001, Dodart et al, 2002; Lemere 2003) in the reduction of CNS A β after passive immunization.

We conclude that our studies using antibody 2286, in aggregate, provide support for all three major proposed mechanisms of anti-A β antibody action in lowering brain amyloid. It is essential to recognize that these mechanisms are not mutually exclusive, and are likely to be synergistic if multiple mechanisms are elicited by a single

antibody/serum. It is also important to recognize that not all monoclonal antibodies need work via all three mechanisms. Both isotype and epitope selectivity could regulate which anti-A β action is dominant for a specific antibody. These studies also do not speak towards other immune system related actions that might underlie the benefits (or adverse effects) of active immunization. Nonetheless, given the preliminary data that anti-A β immunotherapy may stabilize cognitive function in Alzheimer patients (Hock et al, 2003) and the consistent reversal of the phenotype found in APP transgenic mice by such approaches, these results support further development of the optimal strategies for using anti-A β immunotherapy as a treatment for Alzheimer's dementia.

Figure 1: Y-maze behavioral improvement after systemic anti-A β antibody administration. Panel A shows percent alternation for nontransgenic (NTg) mice receiving no treatment (\emptyset), APP transgenic mice (APP) receiving control antibody (Cont) for three months and APP transgenic mice (APP) receiving anti-A β (A β) antibody for 1, 2 or 3 months. * indicates P<0.05 when compared to nontransgenic untreated mice and APP transgenic mice receiving anti-A β antibody for 3 months. Panel B shows number of arm entries for nontransgenic (NTg) mice receiving no treatment (\emptyset), APP transgenic mice (APP) receiving control antibody (Cont) for three months and APP transgenic mice (APP) receiving anti-A β (A β) antibody for 1, 2 or 3 months. * indicates P<0.05 when compared to nontransgenic untreated mice.

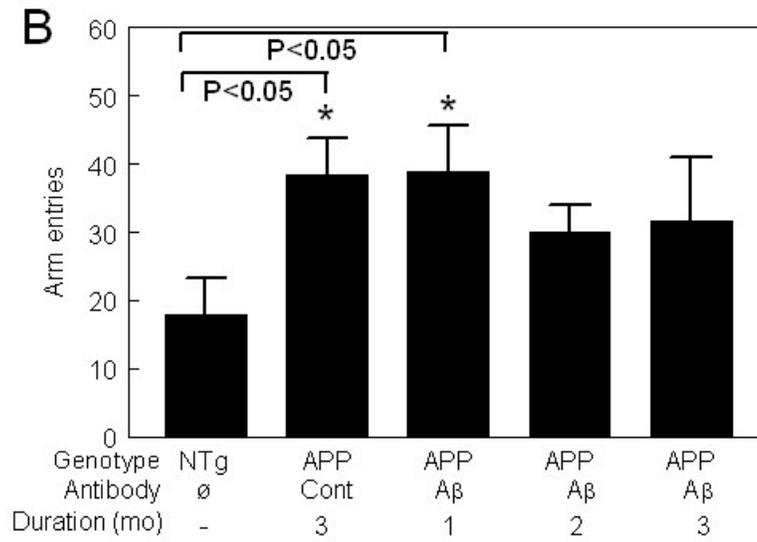
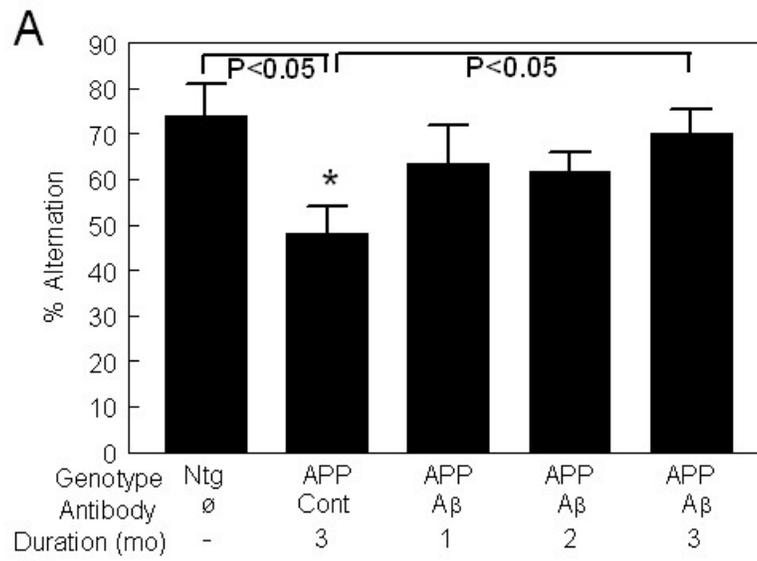


Figure 2: Increased serum levels of anti-A β antibody and A β after anti-A β antibody administration. Panel A shows amounts of circulating anti-A β antibodies in APP transgenic mice (APP) receiving either control antibody (Cont) for 3 months or anti-A β antibody (A β) for 1, 2 or 3 months, nontransgenic (NTg) mice receiving either control antibody (Cont) or anti-A β antibody (A β) for 3 months and nontransgenic mice receiving no treatment. ** indicates P<0.001 compared to APP mice given control antibody injections. Panel B shows amounts of circulating A β in sera in APP transgenic mice (APP) receiving either control antibody (Cont) for 3 months or anti-A β antibody (A β) for 1, 2 or 3 months, nontransgenic (NTg) mice receiving either control antibody (Cont) or anti-A β antibody (A β) for 3 months and nontransgenic mice receiving no treatment. ** indicates P<0.01, * indicates P<0.05.

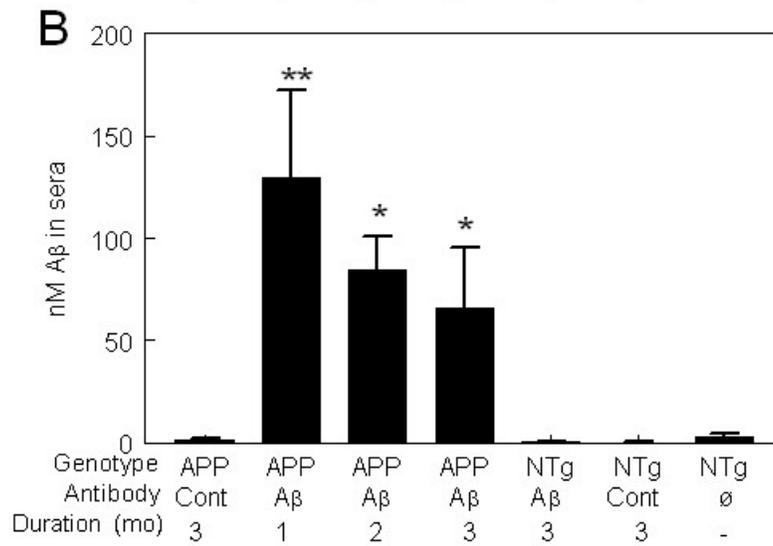
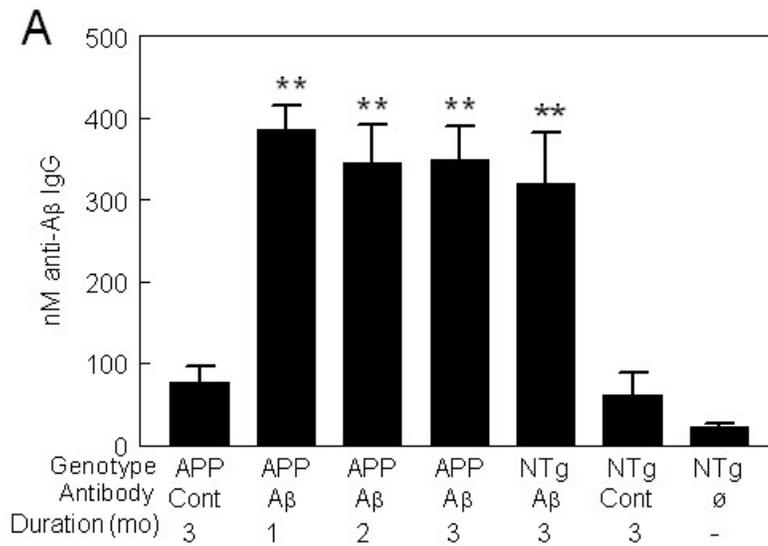


Figure 3: Mouse IgG immunohistochemistry shows antibody binding to congophilic plaques in anti-A β antibody treated mice but not control antibody treated mice. Panels A-D show anti-mouse IgG-HRP immunohistochemistry counterstained with Congo red to detect compact amyloid deposits. Panel A shows a representative amyloid deposit and associated anti-mouse IgG immunostaining (black) in the hippocampus of a mouse injected with control antibody for three months. Panels B-D shows a representative amyloid deposit (red) associated with anti-mouse IgG immunostaining (black) in the hippocampus of a mouse injected with anti-A β antibody for one month (B), two months (C) or three months (D). Magnification = 200X, scale bar = 25 μ m.

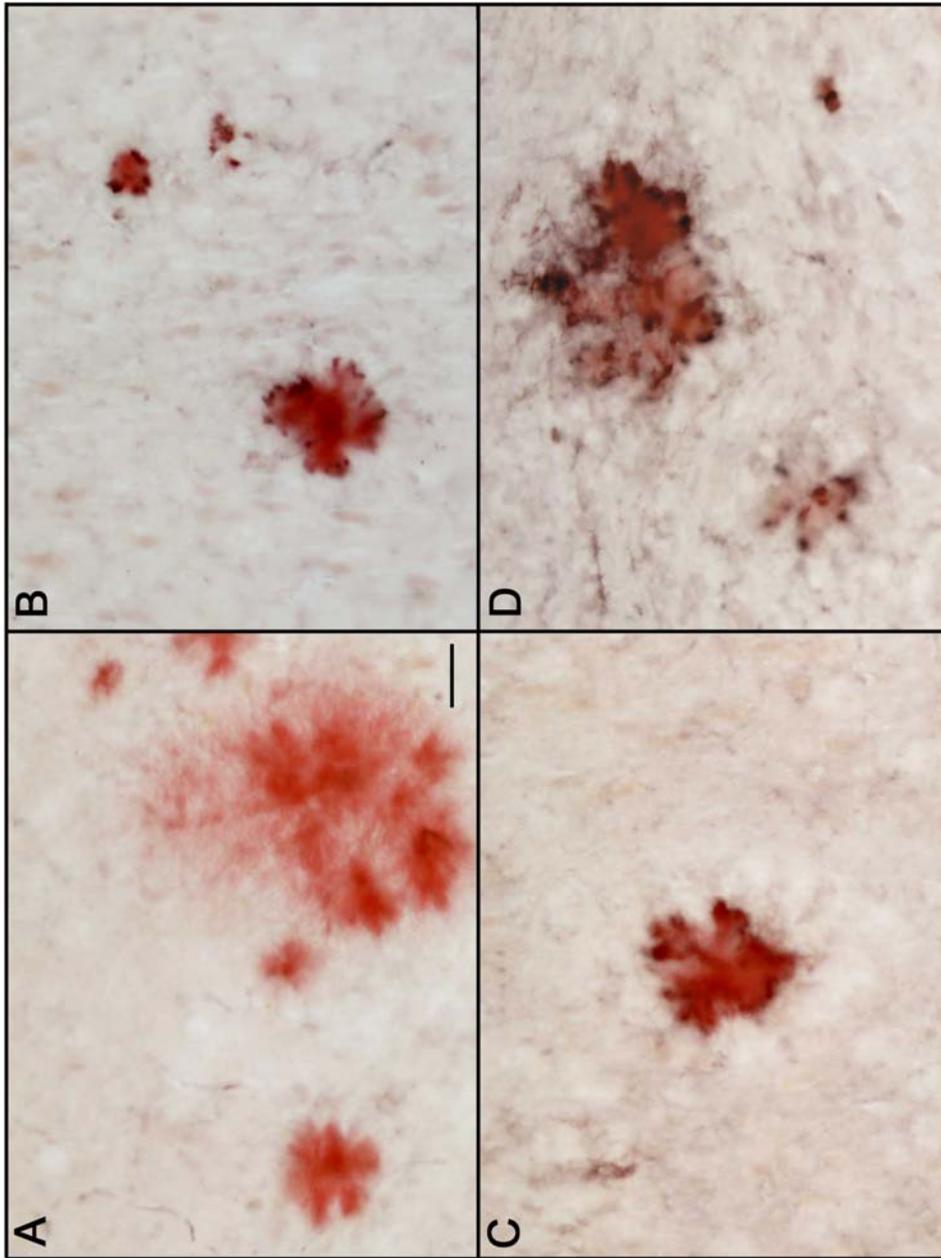


Figure 4: Total A β immunohistochemistry is reduced following two months of systemic anti-A β antibody administration. Panels A-D show total A β immunohistochemistry in the hippocampus of APP transgenic mice receiving control antibody for three months (Panel A. Percent area for this section was 9.12%), anti-A β antibody for one month (Panel B. Percent area for this section was 6.84%), anti-A β antibody for two months (Panel C. Percent area for this section was 3.23%) or anti-A β antibody for three months (Panel D. Percent area for this section was 2.49%). Magnification = 40X, scale bar = 120 μ m. In panel D, CA1 indicates cornu ammonis 1, CA3 indicates cornu ammonis 3, F indicates the hippocampal fissure and DG indicates the dentate gyrus. Panel E shows quantification of the percent area occupied by A β positive stain in the frontal cortex and hippocampus. The single bar shows the value for APP transgenic mice receiving control antibody for three months. The line shows the values for APP transgenic mice receiving anti-A β antibody for a period of one, two and three months. ** indicates P<0.01.

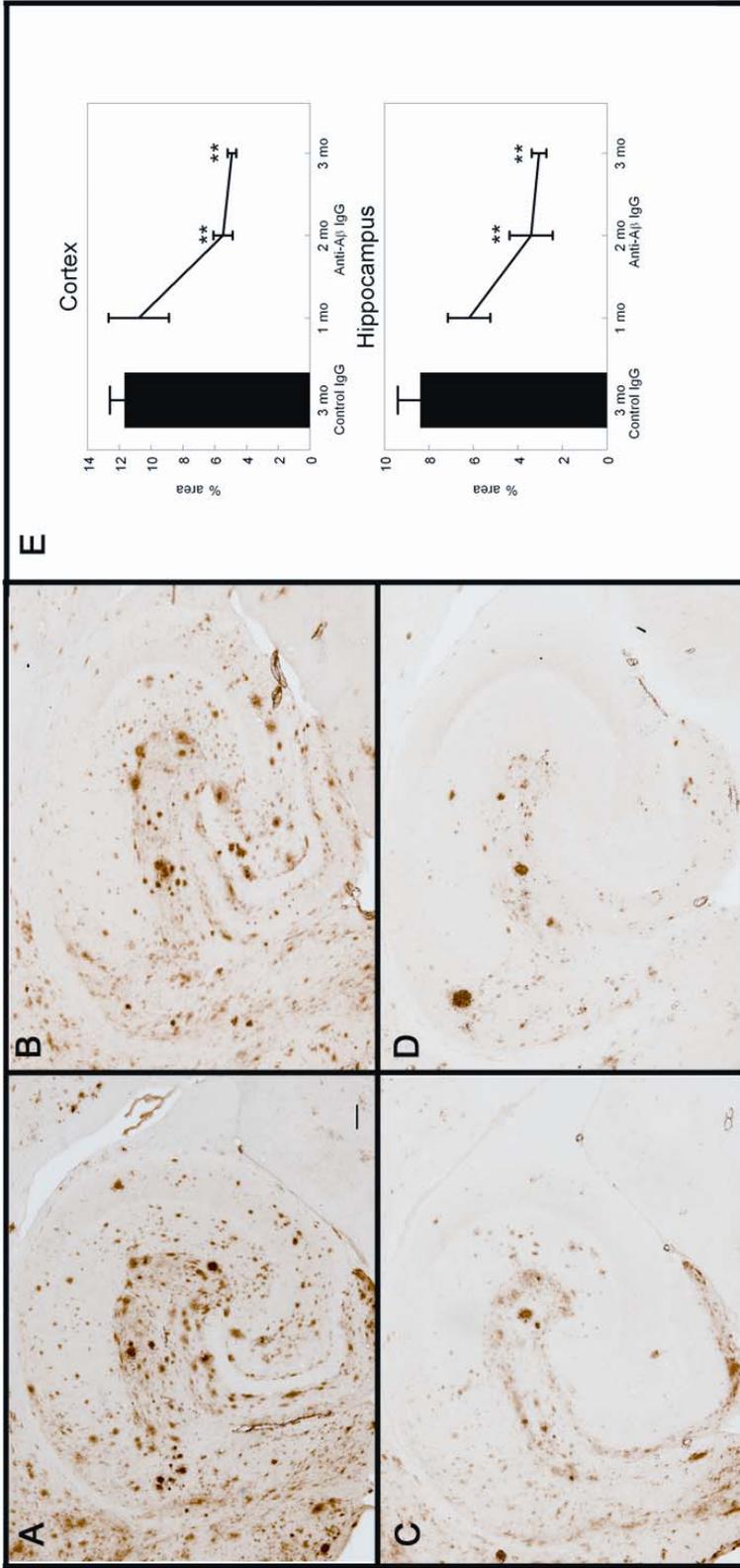


Figure 5: Congophilic compact amyloid plaques are reduced following two months of anti-A β antibody administration. Panels A-D show Congo red staining in the hippocampus of APP transgenic mice receiving control antibody for three months (Panel A), anti-A β antibody for one month (Panel B), anti-A β antibody for two months (Panel C) or anti-A β antibody for three months (Panel D). Magnification = 40X, scale bar = 120 μ m. In panel D, CA1 indicates cornu ammonis 1, CA3 indicates cornu ammonis 3, F indicates the hippocampal fissure and DG indicates the dentate gyrus. Panel E shows quantification of the percent area occupied by Congo red positive stain in the frontal cortex and hippocampus. The single bar shows the value for APP transgenic mice receiving control antibody for three months. The line shows the values for APP transgenic mice receiving anti-A β antibody for a period of one, two and three months. ** indicates P<0.01.

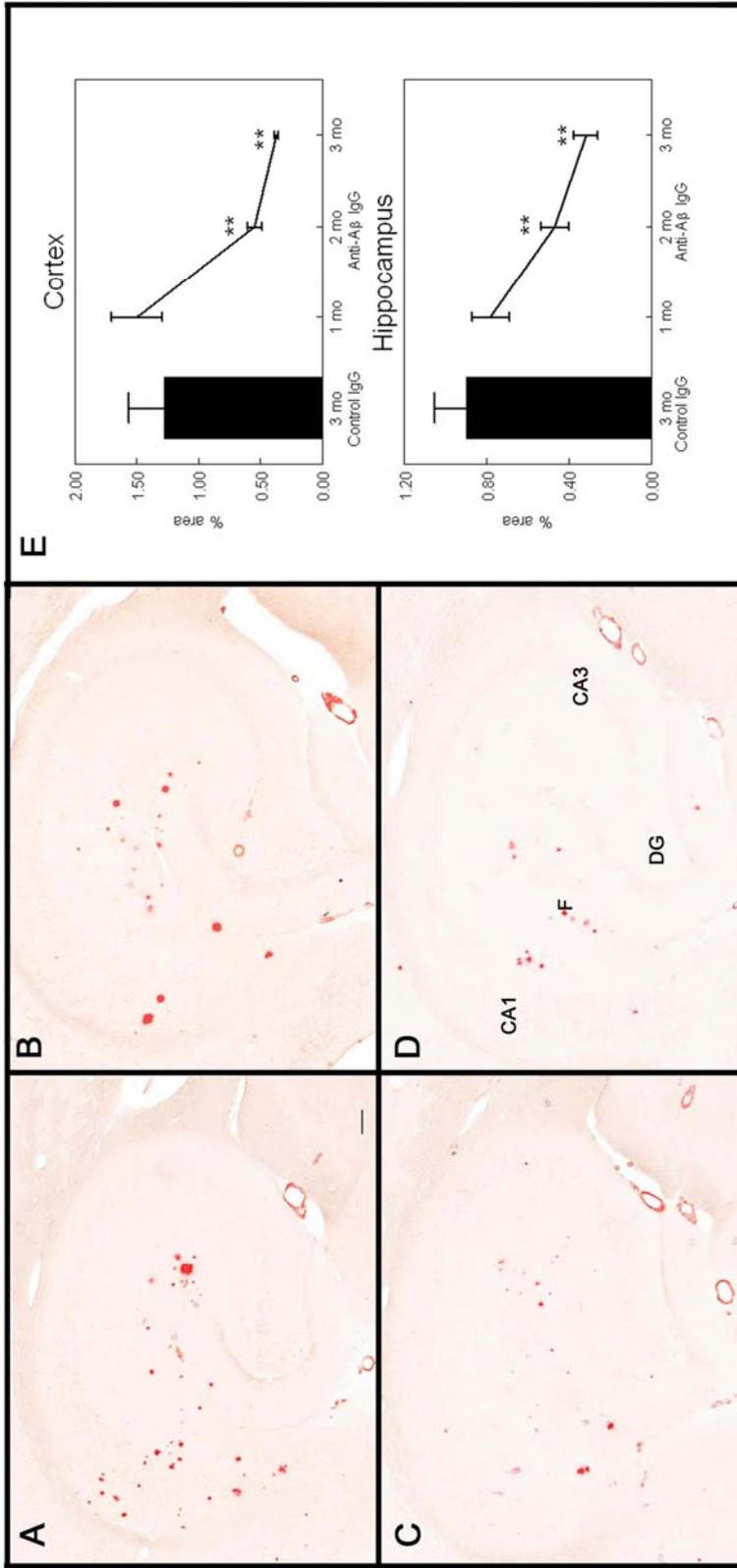


Figure 6: Fc γ receptor expression on microglia is increased following one month of anti-A β antibody treatment and remains increased following two months of treatment. Panels A-D show Fc γ receptor immunohistochemistry in the hippocampus of APP transgenic mice receiving control antibody for three months (Panel A), anti-A β antibody for one month (Panel B), anti-A β antibody for two months (Panel C) or anti-A β antibody for three months (Panel D). In panel A, F indicates the hippocampal fissure, DG indicates the dentate gyrus. Magnification = 100X, scale bar = 50 μ m. Panel E shows quantification of the percent area occupied by Fc γ receptor positive stain in the frontal cortex and hippocampus. The single bar shows the value for APP transgenic mice receiving control antibody for three months. The line shows the values for APP transgenic mice receiving anti-A β antibody for a period of one, two and three months. * indicates P<0.05.

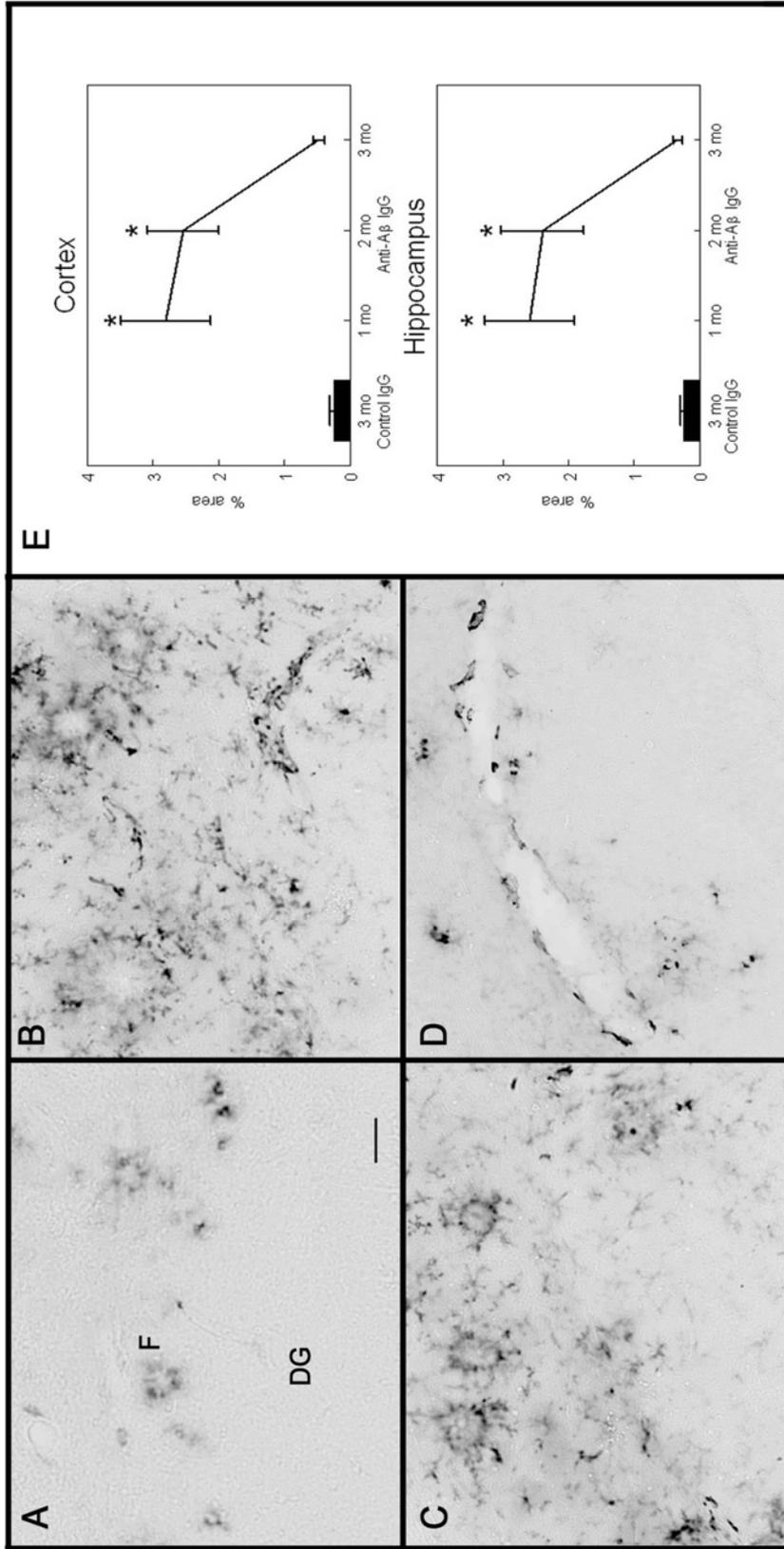
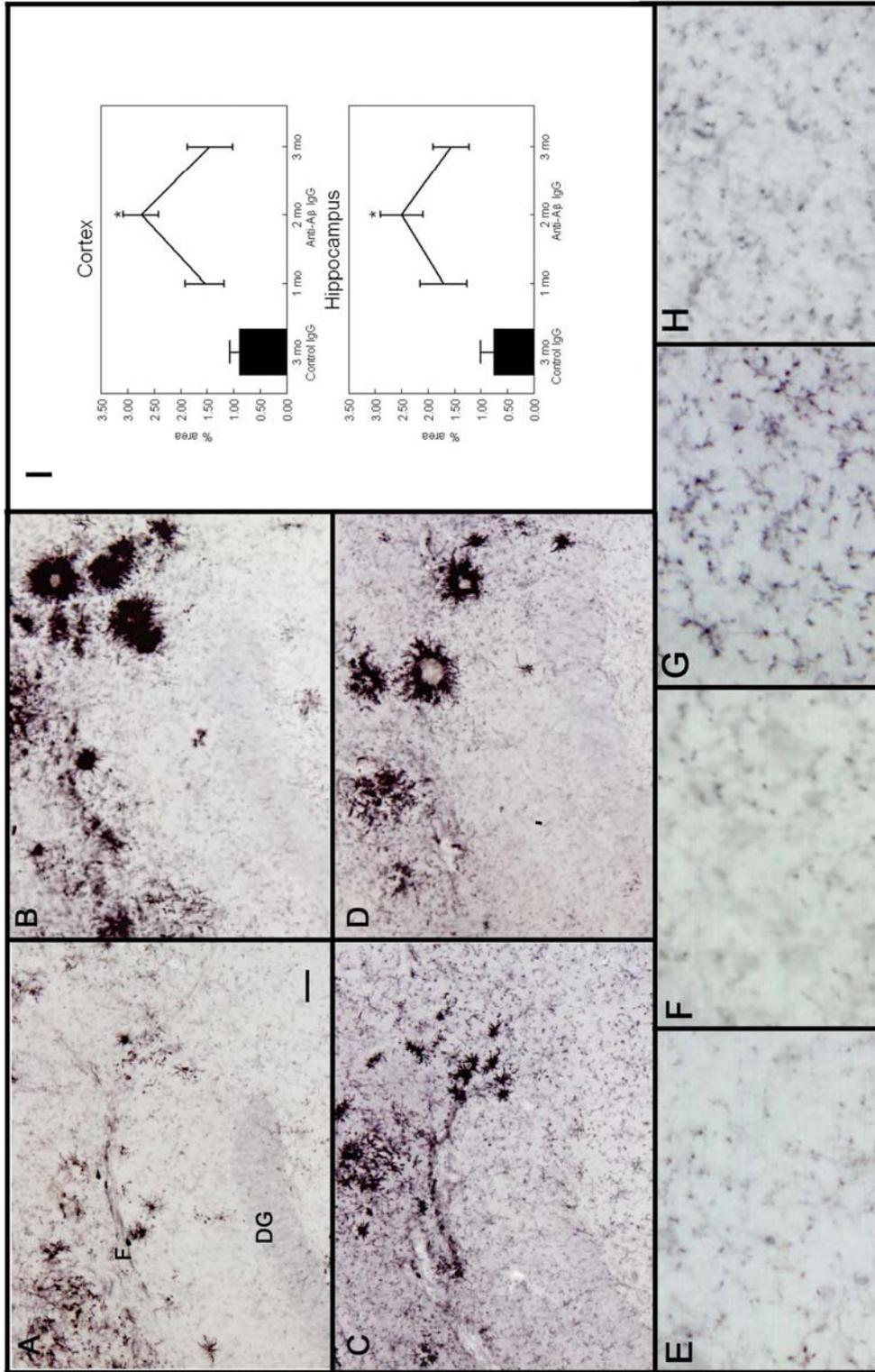


Figure 7: CD45 expression on microglia is increased following two months of anti-A β antibody treatment. Panels A-D show CD45 immunohistochemistry in the hippocampus of APP transgenic mice receiving control antibody for three months (Panel A), anti-A β antibody for one month (Panel B), anti-A β antibody for two months (Panel C) or anti-A β antibody for three months (Panel D). In panel A, F indicates the hippocampal fissure, DG indicates the dentate gyrus. Magnification = 100X, scale bar = 50 μ m. Panels E-H are magnified images of non-amyloid containing areas from panels A-D. Panels E-H show CD45 immunohistochemistry in the hippocampus of APP transgenic mice receiving control antibody for three months (Panel E), anti-A β antibody for one month (Panel F), anti-A β antibody for two months (Panel G) or anti-A β antibody for three months (Panel H). Panel I shows quantification of the percent area occupied by CD45 positive stain in the frontal cortex and hippocampus. The single bar shows the value for APP transgenic mice receiving control antibody for three months. The line shows the values for APP transgenic mice receiving anti-A β antibody for a period of one, two and three months. * indicates P<0.05.



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PAPER 4:

PASSIVE IMMUNOTHERAPY AGAINST A β IN AGED APP TRANSGENIC MICE REVERSES COGNITIVE DEFICITS AND DEPLETES PARENCHYMAL AMYLOID DEPOSITS IN SPITE OF INCREASED VASCULAR AMYLOID AND MICROHEMORRHAGE.

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Abstract

Background:

Anti-A β immunotherapy in transgenic mice reduces both diffuse and compact amyloid deposits, improves memory function and clears early-stage phospho-tau aggregates. As most AD cases occur well past midlife, the current study examined adoptive transfer of anti-A β antibodies to 19 and 23 month old APP transgenic mice.

Results:

After three months of weekly injections, this passive immunization protocol completely reversed learning and memory deficits in these mice, a benefit which was undiminished after 5 months of treatment. Dramatic reductions of diffuse A β immunostaining and parenchymal Congoophilic amyloid deposits were observed after 5 months, indicating even well established amyloid deposits are susceptible to immunotherapy. However, cerebral amyloid angiopathy increased substantially with immunotherapy and some deposits were associated with microhemorrhage. Reanalysis of results collected from an earlier time course study demonstrated that these increases in vascular deposits were dependent on the duration of immunotherapy.

Conclusions:

The cognitive benefits of passive immunotherapy persist in spite of the presence of vascular amyloid and small hemorrhages. These data suggest that clinical trials evaluating such treatments will require precautions to minimize potential adverse events associated with microhemorrhage.

Introduction

Alzheimer's disease (AD) is characterized not only by the presence of parenchymal amyloid deposits and intracellular tangles but also by the presence of amyloid deposits in the vasculature, a condition referred to as cerebral amyloid angiopathy (CAA). The CAA observed in both Alzheimer's disease patients (Iwatsubo et al, 1994) and some of the transgenic mouse models (Gordon et al, 2002) is primarily composed of the shorter form of amyloid beta (A β), A β ₁₋₄₀, while the majority of amyloid deposits in the parenchyma are composed of A β ₁₋₄₂, although the compact amyloid deposits also contain A β ₁₋₄₀.

Anti-A β immunotherapy has been considered as a potential treatment for AD for some time (Solomon et al, 1996; Schenk et al, 1999). Active immunization with a vaccine including A β ₁₋₄₂ fibrils progressed to human clinical trials where its administration was suspended due to meningoencephalitis in a subset of patients (Orgogozo et al, 2003). To date there have been pathology reports on two patients who participated in the trial and subsequently died (Nicoll et al, 2003, Ferrer et al, 2004). Both reports note that while the numbers of parenchymal amyloid deposits appeared lower than expected in these cases, the CAA in these patients did not appear outside the normal range. In addition, one report mentioned multiple cortical hemorrhages and the presence of hemosiderin around the CAA vessels (Ferrer et al, 2004).

Given the adverse reactions to the active immunization, the irreversibility of such procedures and the variable antibody response to vaccines in older individuals (Weksler et al, 1997), passive immunization against the A β peptide emerged as an alternative immunotherapeutic strategy. Studies in young and middle aged APP transgenic mice

have reported significant amyloid reductions with passive immunization (DeMattos et al, 2001, Bard et al, 2000, Wilcock et al, 2003). Such treatments also demonstrate rapid improvements of memory function in APP mice, sometimes without detectable reductions in amyloid (Dodart et al, 2002, Kotilinek et al, 2002, Wilcock et al, 2004b). Most recently, intracranial administration of anti-A β antibodies has been shown to not only remove A β but also clear early-stage hyperphosphorylated tau aggregates (Oddo et al, 2004). Importantly, in the only prior study evaluating adoptive antibody transfer in older APP mice, Pfeifer et al (2002) reported a doubling of cerebral microhemorrhages associated with significant reductions in amyloid burden after administration of an N-terminal specific anti-A β antibody.

Materials and Methods:

Experiment design:

Mice derived from APP Tg2576 mice were obtained from our breeding program at USF started in 1996 (Holcomb et al, 1998). For the five month treatment study thirteen APP transgenic mice aged 23 months were assigned to one of two groups. The first group received weekly intraperitoneal anti-A β antibody injections (antibody 2286; Mouse monoclonal anti-human A β ₂₈₋₄₀ IgG1; Rinat Neurosciences, Palo Alto, CA) for a period of five months ($n=6$). The second group received weekly intraperitoneal anti-AMN antibody (2906; Mouse monoclonal anti-drosophila amnesiac protein IgG1; (Rinat Neurosciences, Palo Alto, CA) injections for a period of five months ($n=7$). Seven nontransgenic mice were also assigned to one of two groups. The first group received weekly intraperitoneal anti-A β antibody injections for a period of five months ($n=4$). The

second group received weekly intraperitoneal anti-AMN antibody injections for a period of five months ($n=3$).

For the time course study of 1, 2 or 3 mo treatment, twenty two APP transgenic mice aged 19 months were assigned to one of four experimental groups, as described previously (Wilcock et al, 2004b). The first three groups received weekly intraperitoneal anti-A β antibody injections for 3 months, 2 months or 1 month, ending when all mice were 22 mo of age. The fourth group received weekly intraperitoneal anti-AMN antibody injections for 3 months.

Behavioral analysis:

Following three and five months of treatment the mice from the 5 month study were subjected to a two day radial-arm water maze paradigm. The apparatus was a 6 arm maze as described previously (Morgan et al, 2000). On day 1, 15 trials were run in 3 blocks of five. A cohort of 4 mice were run sequentially for each block (i.e. each of 4 mice get trial one, then the same mice get trial two, etc). After each 5 trial block, a second cohort of mice were run permitting an extended rest period before mice were exposed to the second block of 5 trials. The goal arm was different for each mouse in a cohort to minimize odor cues. The start arm was varied for each trial, with the goal arm remaining constant for both days. For the first 11 trials, the platform was alternately visible then hidden (hidden for the last 4 trials). On day two, the mice were run in exactly the same manner as day 1 except that the platform was hidden for all trials. The number of errors (incorrect arm entries) were measured in a one minute time frame. As in prior studies, mice failing to make an arm choice in 20 seconds were assigned 1 error (no mice in this

study had to be assigned an error in this manner). The same individual administered the antibody treatments and placed mice in the radial arm water maze. Due to the numbers of mice in the study the researcher was unaware of treatment group identity of each mouse. Also, the dependent measures in the radial-arm water maze task are quantitative, not evaluative, so the potential for tester bias is reduced. In order to minimize the influence of individual trial variability, each mouse's errors for three consecutive trials were averaged producing 5 data points for each day which were analyzed statistically by ANOVA using StatView (SAS Institute Inc., NC).

Tissue preparation and histology:

On the day of kill mice were weighed, overdosed with 100mg/kg Nembutal sodium solution (Abbott laboratories, North Chicago IL). The mice were then intracardially perfused with 25ml of 0.9% sodium chloride. Brains were rapidly removed and the left half of the brain was immersion fixed for 24 hours in freshly prepared 4% paraformaldehyde in 100mM KPO₄ (pH 7.2) for histopathology. The hemibrains were then incubated for 24 hours in 10, 20 and 30% sucrose sequentially to cyroprotect them. Horizontal sections of 25µm thickness were collected using a sliding microtome and stored at 4°C in Dulbecco's phosphate-buffered saline with sodium azide (pH 7.2) to prevent microbial growth. A series of eight equally spaced tissue sections 0600µm apart were randomly selected spanning the entire brain and stained using free-floating immunohistochemistry for total Aβ (rabbit polyclonal anti-pan Aβ Bisource, Camarillo, CA, 1:10000) as previously described (Gordon et al, 2002, Wilcock et al, 2004b). A second series of tissue sections 0.6mm apart were stained using 0.2% Congo red solution in NaCl saturated 80% ethanol. Another set of sections were also mounted and stained for

hemosiderin using 2% potassium ferrocyanide in 2% hydrochloric acid for 15 minutes followed by a counterstain in a 1% neutral red solution for 10 minutes. Quantification of Congo red staining and A β immunohistochemistry was performed using the Image-Pro Plus (Media Cybernetics, Silver Spring, MD) to analyze the percent area occupied by positive stain. One region of the frontal cortex and three regions of the hippocampus were analyzed (to ensure that there was no regional bias in the hippocampal values). The initial analysis of Congo red was performed to give a total value. A second analysis was performed after manually editing out all of the parenchymal amyloid deposits to yield a percent area restricted to vascular Congo red staining. To estimate the parenchymal area of Congo red we subtracted the vascular amyloid values from the total percentage. For the hemosiderin stain the number of Prussian blue positive sites were counted on all sections and the average number of sites per section calculated. Looking at the sections at a low magnification we were able to observe a qualitative difference between animals, however, the percent area was so low that many fields contained no positive stain. Eight equally spaced sections were examined and number of positive profiles were counted and averaged to a per section value. To assess possible treatment-related differences, the values for each treatment group were analyzed by one-way ANOVA followed by Fischer's LSD means comparisons.

Results

Reversal of cognitive deficits by passive amyloid immunotherapy:

The radial-arm water maze task detects spatial learning and memory deficits in transgenic mouse models (Morgan et al, 2000; Gordon et al, 2001). We treated 23 mo old mice for 5 mo with anti-A β antibody 2286 or control antibody 2906 (against a

drospohila-specific protein) and tested them for spatial navigation learning in a 2 day version of the radial arm water maze after 3 mo of treatment and, using a new platform location, again after 5 mo of treatment. At both testing times we found that APP mice treated with the control antibody failed to learn platform location over two days of testing and were significantly impaired compared to the nontransgenic mice treated with either antibody (Fig 1). However, APP mice administered the anti-A β antibodies demonstrated a complete reversal of the impairment observed in the control APP transgenic mice, ending day 2 with performance near 0.5 errors per trial (Fig. 1). Although learning at the later time point, when the mice were 28 mo of age, may have been slightly slower for all groups, there was no impairment of the anti-A β antibody treated APP.

Passive amyloid immunotherapy clears parenchymal A β deposits, but increases vascular amyloid:

In a prior experiment examining the effects of passive anti-A β immunotherapy for 1, 2 or 3 mo in APP mice killed at 21 mo of age (Wilcock et al, 2004b), we found a time dependent reduction of both A β immunostaining of diffuse and fibrillar deposits and Congo red staining of fibrillar amyloid deposits. In the current study we found a similar reduction in both A β immunostaining (Table 1) and total Congo red staining (Fig 2A, left panel; $P < 0.001$ frontal cortex and $P < 0.01$ hippocampus) after 5 months of immunotherapy. We noted that the bulk of what remained was vascular amyloid. We then separately analyzed vascular and parenchymal deposits which revealed a near 90% reduction in parenchymal deposits ($P < 0.001$), but a 3-4 fold elevation of vascular Congo red staining ($P < 0.0001$; Fig 2A, center and right panels respectively). We also separately analyzed vascular and parenchymal Congo red staining on mice from our earlier study

(14) treated passively for 1, 2 or 3 months with anti-A β or control antibody, and found a similar result. There was a graded reduction in overall Congo red staining nearing 75% as duration of antibody exposure increased (as reported previously; Fig 2B). However, when separated into vascular Congo red deposits and parenchymal deposits, there was an antibody exposure time dependent increase in vascular deposition in both hippocampus and frontal cortex (Fig 2C; P<0.05 frontal cortex and hippocampus) and a corresponding near 90% decrease in parenchymal deposits (Fig 2D; P<0.001 in frontal cortex and hippocampus).

These differences were readily observed examining micrographs of sections from these mice. Mice treated with control antibodies revealed occasional cortical vascular amyloid deposits (22 mo Fig 3A, 28 mo Fig 3C), while mice administered anti-A β antibodies had increased amounts of vascular amyloid staining (3 mo treatment Fig 3B; 5 mo treatment Fig 3D). Those vessels containing amyloid following treatment with anti-A β antibody also exhibited apparent increases in microglial activation as measured by CD45 expression (Fig 3F) compared to mice treated with control antibody (Fig 3E). Unfortunately, the shifting numbers and sizes of vascular and parenchymal deposits caused by the antibody therapy greatly complicated measurement of microglial activation per vascular deposit area. This apparent increase in staining intensity could not be quantified accurately.

Passive amyloid immunotherapy causes increased microhemorrhage:

We used the Prussian blue histological stain to label hemosiderin, a ferric oxide material produced in the breakdown of hemoglobin. Extravenous blood in the brain leads to microglial phagocytosis of the erythrocytes and breakdown of hemoglobin within

them. These ferric oxide containing microglia are thus markers of past hemorrhage. In untreated, aged APP transgenic mice we observed very few profiles positive for Prussian blue staining in the frontal cortex (section counterstained with neutral red; Fig. 4A). However, following anti-A β antibody treatment for five months we observed an increase in the number of Prussian blue profiles in the frontal cortex which were readily detectable at a low power in the microscope (Fig 4B). In the absence of anti-A β treatment, or even when treated with antibody for 1 month, most vessels did not stain with Prussian blue, and could be identified only using the red counterstain (Fig 4C). However, even with 3 months of anti-A β antibody treatment we observed frequent vessels with associated Prussian blue staining (Fig 4D) Using adjacent sections stained for Congo red, we confirmed that all vessels showing microhemorrhage contained amyloid (Fig 4E and 4F; we were unable to double-label Prussian blue with either Congo red or thioflavine-S). However, only a minority of vessels containing amyloid demonstrated hemorrhage.

When we counted the number of Prussian blue positive profiles in those animals receiving control antibody there was an average of one profile per every 2 sections (Fig 5) and this number remained the same in both control groups (aged 22 or 28 mo). Following treatment with anti-A β antibody for a period of two months we observed a striking increase in Prussian blue staining, approximately five times that observed in either the control group or the mice immunized for one month (Fig 5, $P < 0.001$). Following this initial increase in Prussian blue staining we observed a linear increase in staining associated with increasing duration of anti-A β antibody treatment (Fig 5). Five months of anti-A β antibody treatment demonstrated a 6-fold increase in Prussian blue staining when compared the control groups (Fig 5).

Discussion

Earlier studies with vaccines against the A β peptide demonstrated protection from the learning and memory deficits associated with amyloid accumulation in APP transgenic mice (Morgan et al, 2000, Janus et al, 2000). Passive immunization protocols with anti-A β antibodies also produced cognitive benefits, in some cases even in the absence of significant reduction in amyloid burden (Dodart et al, 2002, Kotilinek et al, 2002). Our recent work found that 3 months of anti-A β treatment of 18 mo old APP mice improved spontaneous alternation performance on the Y-maze (Wilcock et al, 2004b). In the present work we confirmed that passive anti-amyloid immunotherapy can reverse spatial learning deficits in APP transgenic mice and that this benefit of immunotherapy is retained even in aged mice (26 and 28 mo at testing) with long established amyloid pathology.

Additionally, we describe a more rapid means of testing spatial reference memory to reveal learning and memory deficits in APP transgenic mice. This 2 day version of the radial arm water maze included greater spacing of individual trials (mice spent time in their home cage after every trial), combined with less spacing of aggregate trials (15 trials per day rather than 4 or 5) to facilitate learning of platform location in the nontransgenic mice with a clear absence of learning in the age-matched transgenic mice.

A substantial reduction in total Congoophilic amyloid deposits was observed in old APP mice treated with anti-A β antibodies for 2 or more months. This measurement of total Congo red staining included both parenchymal and vascular amyloid staining. When we analyzed the sections for only CAA we found that this measure was significantly increased following two, three and five months of anti-A β antibody treatment. The

remaining parenchymal amyloid load was almost completely eliminated with this antibody approach. Clearly, since total amyloid load is significantly reduced not all amyloid was shifted into the vessels but it appears that at least some of the Congoophilic material was redistributed to the vasculature. At the present time the mechanism for this redistribution is unclear. However, one possibility is that the microglia associated with the antibody-opsonized amyloid, either by phagocytosis or surface binding, and transported the material to the vasculature, possibly in an attempt to expel it. We and others have shown evidence for microglial involvement in the removal of amyloid using both intracranial anti-A β antibody injections (Wilcock et al, 2003, 2004a) and systemically administered anti-A β antibody treatment (Wilcock et al, 2004b) as well as ex vivo studies (Bard et al, 2000, 2003). Here we also report our impression that microglia surrounding CAA vessels in immunized mice expressed more CD45 than control transgenic mice. This increased expression could be due to either increased expression in the same number of microglial cells or an increased number of microglial cells in these animals. It is feasible that this microglial activation was simply in reaction to the presence of increased amyloid in the blood vessels. However, it is equally likely that microglia activated by the opsonized material migrated to the vessels for disposal of the amyloid.

CAA is defined as the deposition of Congoophilic material in meningeal and cerebral arteries and arterioles (capillaries and veins can also show CAA but less frequently) and it occurs to some extent in nearly all Alzheimer's disease patients (Jellinger, 2002). Severe CAA affecting about 15% of cases, can be associated with both infarction and hemorrhagic injury (Olichney et al, 1997, Maurino et al, 2001). It has also

been shown that the severity of CAA can be directly linked to the severity of dementia in Alzheimer's disease patients (Thal et al, 2003).

In the current study we show a significantly increased number of microhemorrhages in the brain as detected by Prussian blue staining associated with the increase in CAA following passive immunization. Another transgenic mouse model of amyloid deposition, the APP23 mice, have been shown to deposit amyloid in both brain parenchyma and blood vessels and show a CAA associated increase in spontaneous cerebral hemorrhages (Winkler et al, 2001). Moreover, Pfeifer et al (2002) showed that these spontaneous hemorrhages were significantly increased following 5 months of passive immunization of 21 mo old APP23 mice using an anti-A β antibody with an N-terminal epitope, similar to those typically developed against active immunization with vaccines (Schenk et al, 1999, Dickey et al, 2001, McLaurin et al, 2002) When young mice (6 mo) were immunized following the same protocol no hemorrhages were observed. More recently DeMattos et al (9th International Conference on Alzheimer's disease and related disorders, 2004) showed that passive immunization with an N-terminal antibody (3D6: directed against aa 1-5 of A β) of PDAPP transgenic mice also resulted in significantly increased microhemorrhage. They were unable to detect increased microhemorrhage with a mid-domain antibody (266: directed against aa 13-28 of A β). Notably, antibody 266 fails to bind A β deposited in CAA vessels or amyloid plaques (9th International Conference on Alzheimer's disease and related disorders, 2004). Importantly, Ferrer et al (2004) noted the presence of CAA and microhemorrhage in the brain of one patient that participated in the A β vaccine trial, even though the parenchymal amyloid appeared lower than expected. Also, Nicoll et al [6] noted that

CAA appeared unaffected in the brain of another patient that participated in the A β vaccine trial.

It remains to be determined whether these observations regarding increased CAA and microhemorrhage in transgenic mice are relevant to trials of passive immunotherapy in humans. It should be noted that in spite of extending the period of immunotherapy to 5 months, there was no discernable loss of the cognitive benefits of immunotherapy in the transgenic mice, all of whom showed increased microhemorrhage. While the observation that antibody 266 does not result in vascular leakage encourages testing of this idiomotype, data from the Zurich cohort of the A β vaccine trial argue that brain reactive antibodies may be important for cognitive benefits (Hock et al, 2003). Our opinion is that these results suggest that passive immunotherapy against A β should proceed with appropriate precautions taken to minimize the risk of hemorrhage (e.g. by excluding patients taking anticoagulants), and instituting measures to detect such hemorrhages if they do occur irrespective of the antibody specificity or proclivity for microhemorrhage in aged APP transgenic mice.

Author contributions.

DMW treated the mice, performed the behavioral analysis, processed the tissue and performed pathological analyses, and drafted the manuscript. AR evaluated slides and provided expert opinion regarding CAA and microhemorrhage. AR and SS developed, produced and purified the antibodies used in the studies. KS aided in histological processing of the tissue. NW was responsible for animal husbandry during the study. MJF performed DNA extraction and PCR for genotyping of the mice. MNG oversees the breeding colony generating mice for the studies, collected samples from the mice and assisted in editing the manuscript. DM conceived the design of the study, guided data interpretation and assisted in editing the manuscript.

Figure 1: Spatial learning deficits in APP transgenic mice were reversed following three and five months of immunization. Mice were tested in a 2 day version of the radial arm water maze. Solid lines represent APP transgenic mice while dashed lines represent nontransgenic mice. Open symbols indicate anti-AMN control antibody treatment (○: APP control antibody, △: Nontransgenic control antibody) while closed symbols indicate anti-A β antibody treatment (●: APP A β antibody, ▲: Nontransgenic A β antibody). Panel A shows mean number of errors made over the two day trial period following three months of immunization. Each datapoint is the average of three trials. Panel B shows the mean number of errors made over the two day trial period following five months of immunization. For both graphs * indicates $P < 0.05$, ** indicates $P < 0.001$ when the APP mice receiving control antibody are compared with the remaining groups.

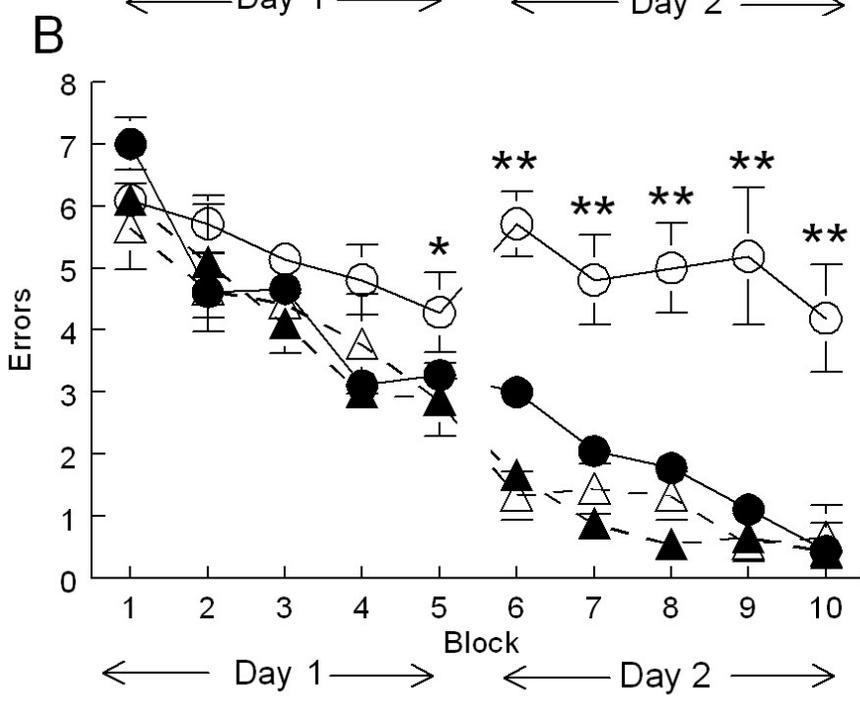
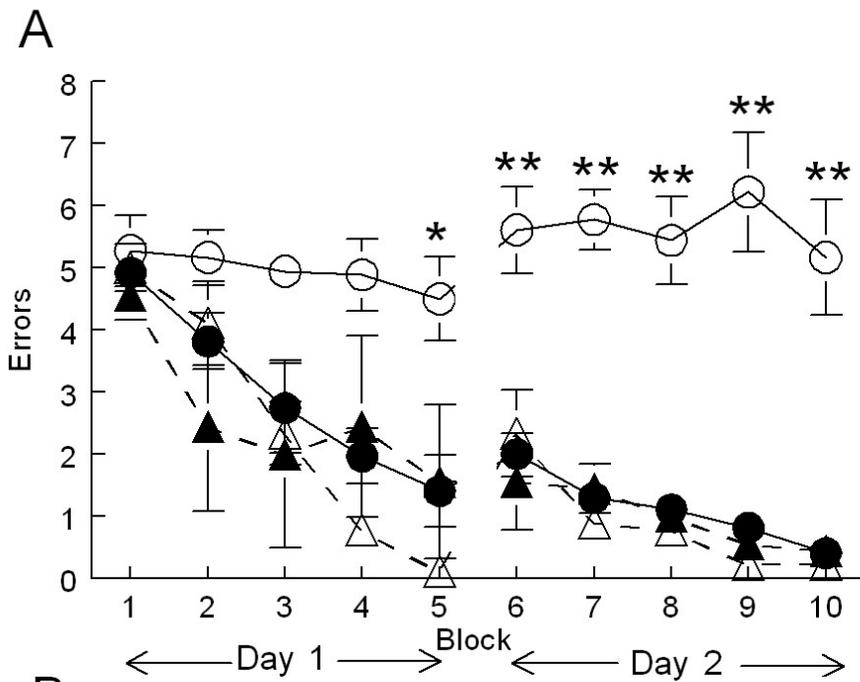


Figure 2: Passive immunization with anti-A β antibodies decreases total and parenchymal amyloid loads while increasing vascular amyloid in frontal cortex and hippocampus of APP transgenic mice. Panel A shows total amyloid load measured with Congo red, vascular amyloid load and parenchymal amyloid load from APP transgenic mice administered control IgG (C) or anti-A β IgG (A β) for a period of five months. Panels B-D show total amyloid load (Panel B), vascular amyloid load (Panel C) and parenchymal amyloid load (Panel D) from APP transgenic mice administered control IgG for 3 months (Cont IgG) or anti-A β IgG for a period of one, two or three months (Anti-A β IgG). For all panels, the solid bar and solid line represent values from the frontal cortex while the open bar and dashed line represent values from the hippocampus. ** P<0.01.

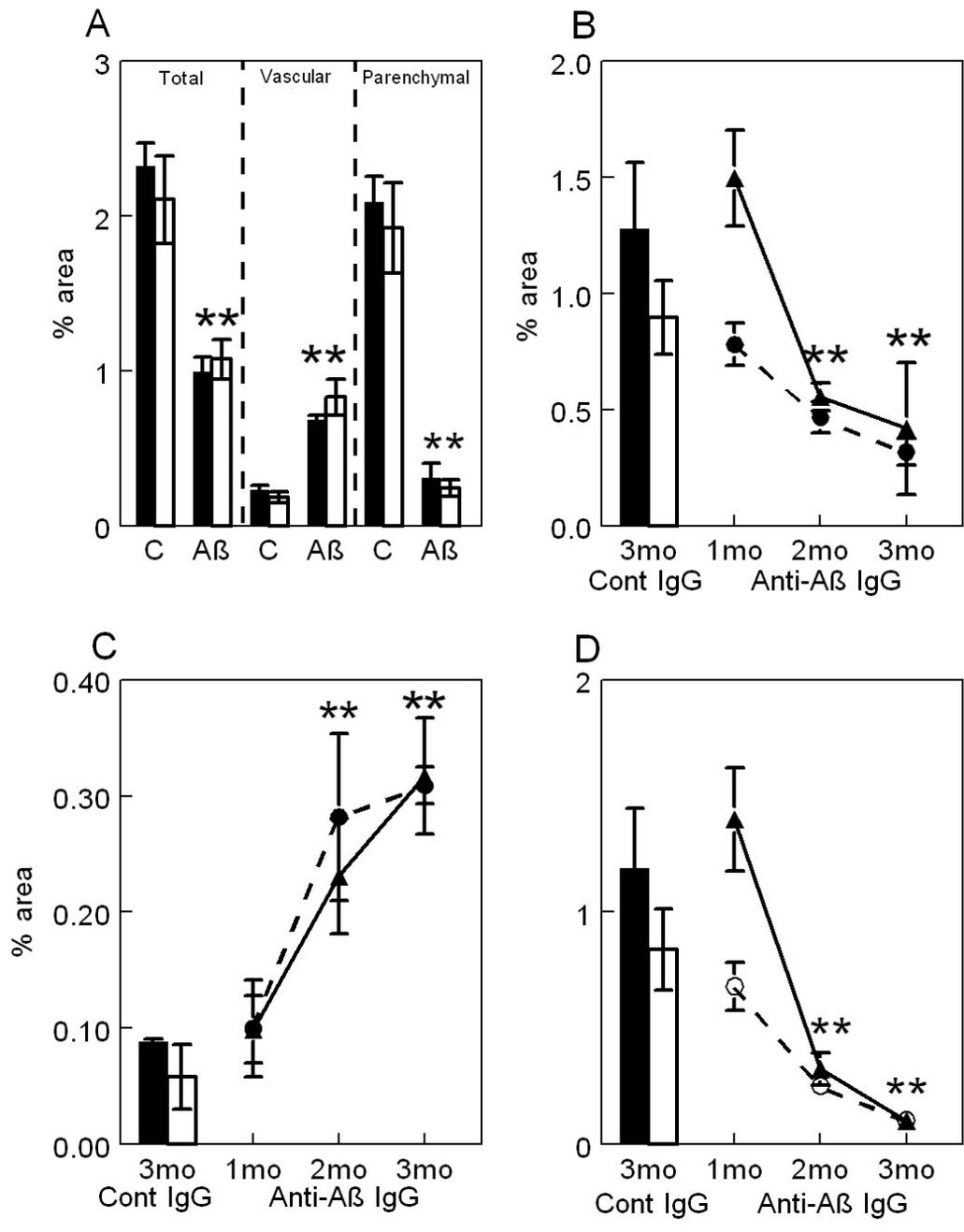


Figure 3: Increased Congo red staining of blood vessels following anti-A β antibody administration is associated with activated microglia. Panels A and B are from the frontal cortex of 22 month old APP transgenic mice immunized for 3 months with either control antibody (3A) or anti-A β antibody (3B). Panels C and D are from the frontal cortex of 28 month old APP transgenic mice immunized for five months with either control antibody (3C) or anti-A β antibody (3D). Panels E and F show a high magnification image of CD45 immunohistochemistry (black) counterstained with Congo red (red) from 28 month old APP transgenic mice immunized for five months with either control antibody (Panel E) or anti-A β antibody (Panel F). Panels A-D magnification = 100X. Scale bar in Panel B = 50 μ m for panels A-D. Panels E-F magnification = 200X. Scale bar in Panel E = 25 μ m for panels E-F.

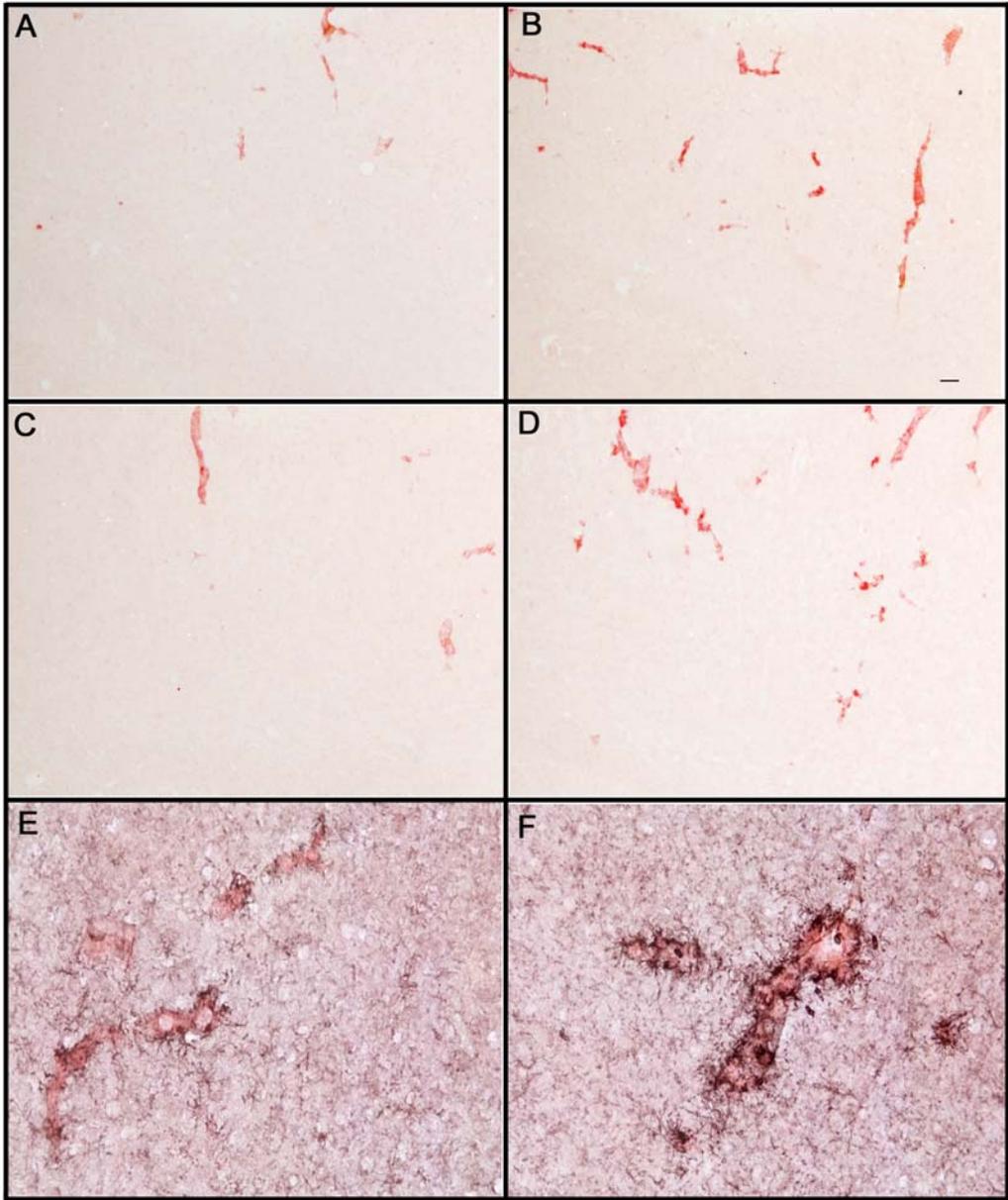


Figure 4: Microhemorrhage associated with CAA following systemic administration of anti-A β antibodies. Panels A and B are low magnification images of the frontal cortex of APP transgenic mice receiving either control antibodies (Panel A) or anti-A β antibodies (Panel B) for a period of five months. Panels C and D show representative images of amyloid containing vessels stained for Prussian blue (blue) counterstained with neutral red (red) from APP transgenic mice receiving either control antibodies (Panel C) or anti-A β antibodies (Panel D) for a period of three months. Panel E shows a blood vessel in the frontal cortex stained for Prussian blue (blue) counterstained with neutral red from an APP transgenic mouse administered anti-A β antibodies for five months. Panel F shows the same blood vessel on an adjacent section stained for Congo red indicating that the blood vessel does in fact contain amyloid. Scale bar panel A = 120 μ m for panels A-B. Scale bar panel C = 25 μ m for panels C-D. Scale bar in panel F = 25 μ m for panels E-F.

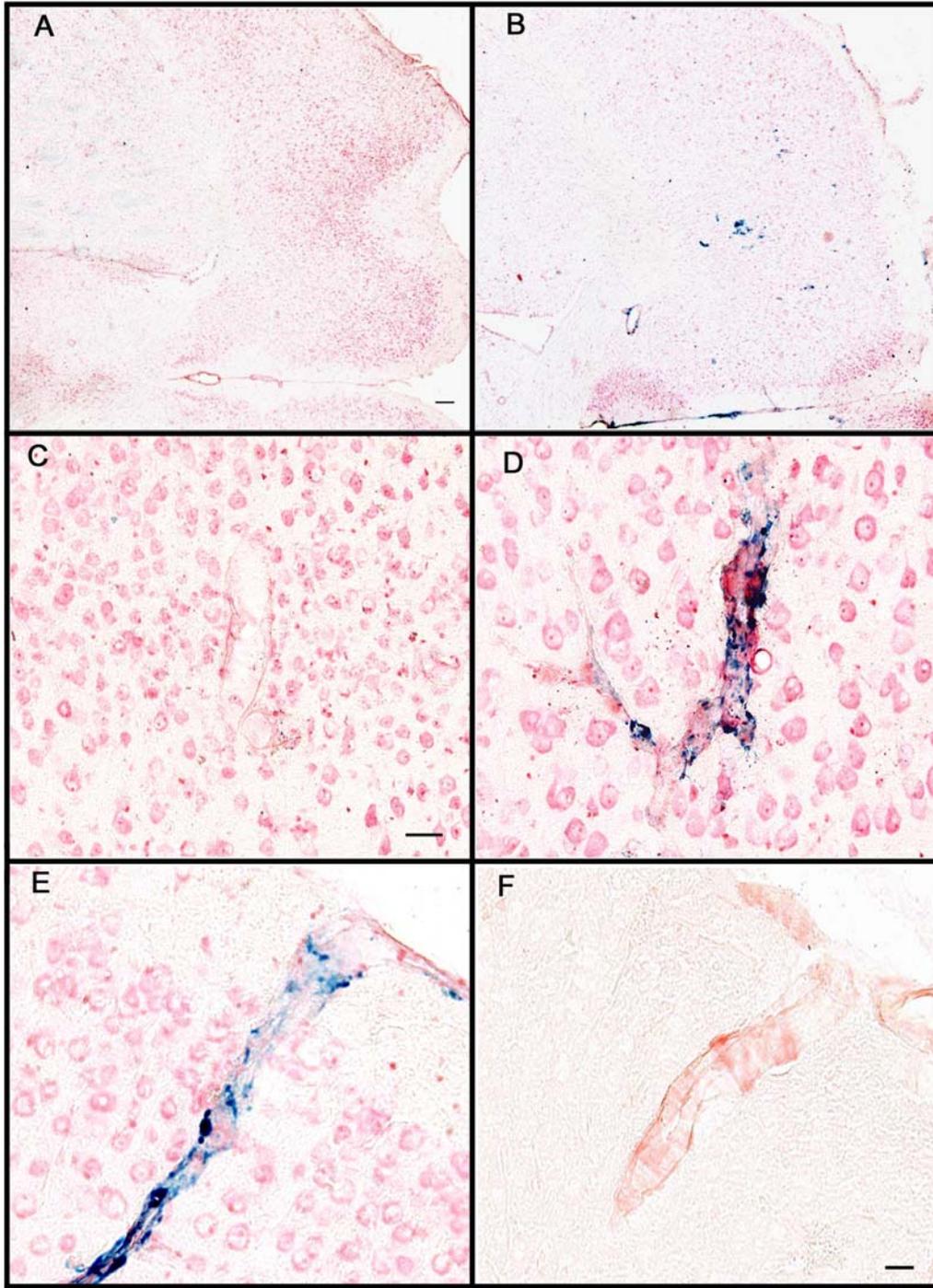


Figure 5: Number of Prussian blue positive profiles increases with duration of anti-A β antibody exposure. The graph shows quantification of the average number of Prussian blue positive profiles per section from mice administered control IgG for 3 or 5 months (Control) or anti-A β IgG for 1, 2, 3 or 5 months (anti-A β). ** P<0.01.

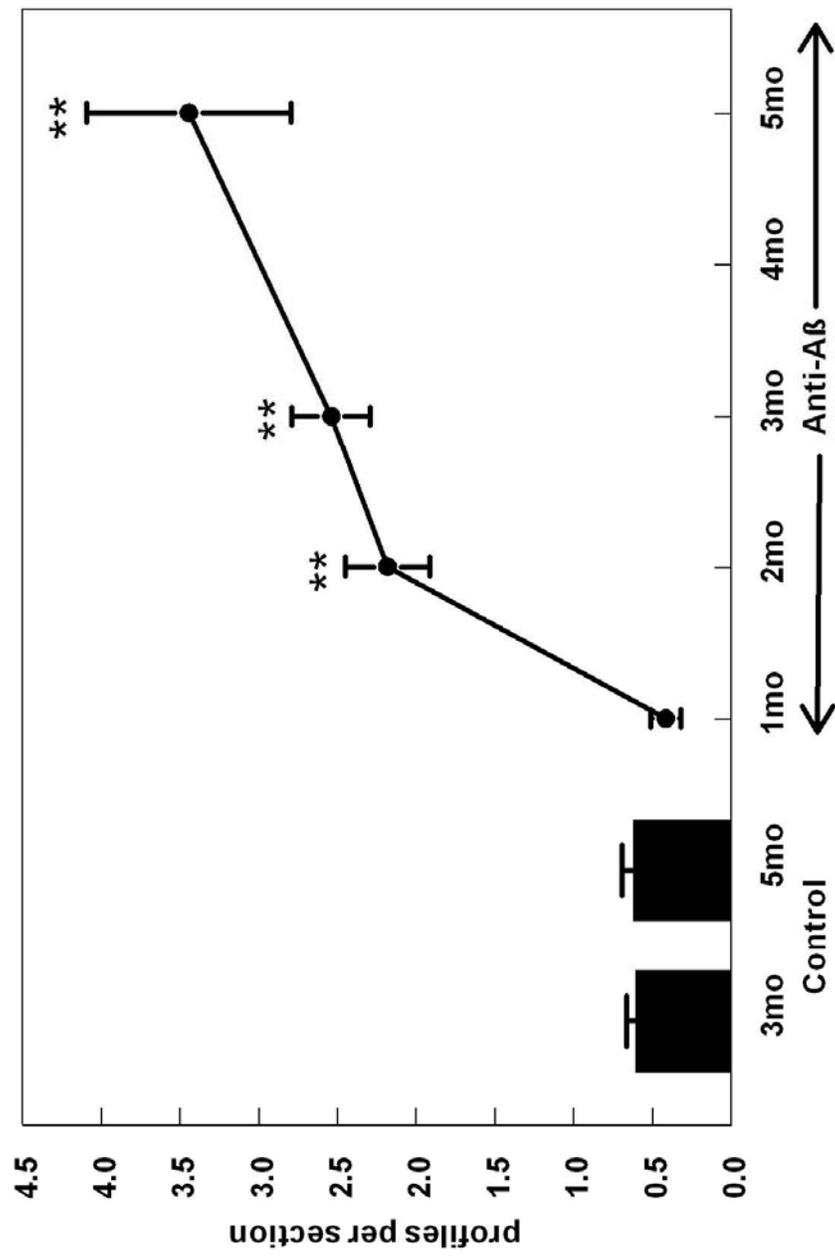


Table 1. A β Loads after 5 months of Immunotherapy

Region	% area control antibody treated	% area anti-A β antibody treated	% reduction following anti-A β antibody treatment
Frontal Cortex	34.855 \pm 2.265	9.681 \pm 0.754	72
Hippocampus	23.994 \pm 0.985	8.212 \pm 0.596	66

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CONCLUSIONS

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that slowly robs sufferers of their ability to remember, reason, make judgements and carry out daily activities; it is the most common cause of dementia with a duration of anywhere from 3 to 20 years. It is estimated that in 2004 there are currently 4.5 million Americans have the disease, with a projected number of 11.6 to 16 million sufferers by the year 2050 (Hebert et al, 2003). Several genes have been found to cause AD in humans; these are the amyloid precursor protein (APP) and presenilin 1 and 2 (PS1 and PS2). Mutations in these genes result in a rare, aggressive form of the disease known as familial early onset Alzheimer's disease (FAD) with the disease commonly occurring by age 60 and sometimes in the 30s and 40s. The discovery of these mutations led to the development of transgenic mouse models, the first of which carried normal beta-APP751 with no mutations and demonstrated some extracellular A β deposits (Quon D et al, 1991).

The first mouse overexpressing mutated human APP which developed extensive amyloid pathology but no tau pathology or neuron loss was the PDAPP transgenic mouse (Games et al, 1995). The Tg2576 APP transgenic mouse was the first transgenic model to show learning and memory deficits associated with the amyloid pathology (Hsiao et al, 1996). Mice overexpressing human PS1 mutations were less successful models on their own, not developing any amyloid deposits (Duff et al, 1996). However, it was shown that crossing an APP transgenic mouse with a PS1 transgenic mouse (APP+PS1) results in an accelerated model of amyloid deposition (Borchelt et al, 1997; Citron et al, 1998;

Holcomb et al, 1998). It was also later shown that an APP+PS1 transgenic mouse model developed reliable cognitive dysfunction which correlated with the extent of amyloid pathology (Gordon et al, 2001). These transgenic mouse models of amyloid pathology also develop another pathological characteristic of AD which is activation of inflammatory cells in the brain; namely microglia and astrocytes, which has been shown to be associated with the extent of amyloid deposition (Gordon et al, 2002).

The amyloid hypothesis for AD is currently the most favored hypothesis for the cause of the disease which states that the precipitating event in the development of AD is the over-production and deposition of A β in the form of diffuse and compact amyloid deposits which in turn results in hyperphosphorylation of tau and neuronal death (Hardy et al, 1992). This hypothesis has been the focus of therapeutic intervention in AD including the development of immunotherapy as a potential treatment for AD.

Immunotherapy was first shown in 1999 by Schenk et al (1999) of Elan pharmaceuticals. In this report the authors showed that immunization of young PDAPP transgenic mice with A β ₁₋₄₂ fibrils in Freund's adjuvant over a period of 11 months prevented amyloid deposition while immunization of older PDAPP mice for 4 and 7 months resulted in significant reductions in amyloid burden. Following this initial report it was later shown that immunization not only ameliorated amyloid pathology but also in resulted in improved cognition in both APP+PS1 transgenic mice (Morgan et al, 2000) and TgCRND8 APP transgenic mice (Janus et al, 2000). We later reported that over a series of several immunization studies in APP+PS1 mice that the degree of microglial activation strongly correlated with the reduction in compact, Congophilic amyloid deposits (Appendix A) suggesting a critical role of microglia in the removal of amyloid

by this active immunization approach. Since the original report in 1999 there has been a plethora of data published regarding the use of both active and passive immunization for the treatment of AD. Following the failure of active immunization in phase II clinical trials we decided that discovery of the mechanism(s) by which immunotherapy acts to reduce amyloid pathology would be critical if immunotherapy was to be successful in humans.

Here we show evidence for three mechanisms for A β removal by immunotherapy as well as some potentially unwanted effects of passive immunization which will likely need to be overcome if immunotherapy is to be successful in humans. By 2001 there had been three suggested mechanisms of action. The first was microglial phagocytosis via the Fc γ receptor which was first suggested by Schenk et al (1999) in their active immunization report and later by Bard et al (2000) in a report showing the benefits of passive immunization in the PDAPP mouse. We also show evidence for this mechanism in Appendix A where we observed a correlation between microglial activation and amyloid reduction. Another mechanism was shown before immunotherapy for AD was suggested; this showed that anti-A β antibodies are capable of inhibiting amyloid fibril formation (Solomon et al, 1996). The same group also showed that anti-A β antibodies could disaggregate already formed amyloid fibrils (Solomon et al, 1997). A third suggested mechanism stated that anti-A β antibodies need not enter the CNS but act peripherally by binding to circulating A β in the plasma and therefore resulting in a shift in the concentration gradient between CNS and plasma causing A β to exit the brain and enter the serum where it would be bound by antibodies and removed. This was evidenced by a rapid and dramatic increase in circulating A β levels hours following anti-A β

antibody administration. Importantly, this study used an anti-A β antibody shown not to bind to amyloid deposits in the brain (DeMattos et al, 2001).

To approach the question of which mechanism was important for A β removal by immunotherapy we decided to bypass the issue of blood-brain barrier penetration and work on finding out what happens when antibody is in the brain. To answer this question we injected anti-A β antibodies (anti-A β ₁₋₁₆) into the right frontal cortex and hippocampus of aged Tg2576 APP transgenic mice with significant amyloid burdens, leaving the left side of the brain untreated as an internal control for each mouse. We then examined what effects the antibody had after 4, 24, 72 and 168 hours. We found that diffuse amyloid deposits, stained immunohistochemically for A β but not stained by thioflavine-S, were significantly reduced following 24 hours of treatment (Paper 1; Figures 2 and 3) but there was no associated microglial reaction or any reduction in compact, thioflavine-S positive, amyloid deposits. These compact deposits were found to be significantly reduced 72 hours following injection of anti-A β antibodies (Paper 1; Figures 4 and 5) and this was associated with a significant activation of microglia as detected by CD45 (Paper 1; Figures 6 and 7) and MHC-II (Paper 1; Figures 8 and 9) immunohistochemistry. 168 hours following injection we found that both diffuse and compact amyloid deposits remained reduced with no further reduction and microglial activation had returned to control levels. These data suggest two phases of removal of A β by anti-A β antibodies which may be occurring through two different mechanisms. The first phase is the removal of diffuse amyloid deposits by a mechanism independent of microglial activation, possibly via a direct dissolution of the deposits. The second phase is the removal of compact amyloid deposits associated with activation of microglia.

Since the time-course data was performed using an anti-A β antibody recognizing the 1-16 portion of the A β peptide we decided to examine the differences in anti-A β epitopes on removal of A β from the brains of APP transgenic mice seven days following intrahippocampal and intracortical injections. We injected antibodies recognizing 1-16, 12-28 and 28-40 portions of the A β peptide as well as a control antibody directed against drosophila amnesiac protein. We found that both total A β and compact amyloid deposits were removed equally as effectively by all three antibodies directed against A β (Appendix B; Figures 1 and 2). We also found that anti-A β ₂₈₋₄₀ was slightly more efficacious in microglial activation than either anti-A β ₁₋₁₆ or anti-A β ₁₂₋₂₈ (Appendix B; Figure 3) although no microglial activation would have been expected given that the mice were killed seven days following injection and the time-course data indicated that microglial activation peaks at three days and is over by seven days.

Additional evidence for for microglial involvement in removal of compact amyloid deposits was found when we injected anti-A β ₁₋₁₆ antibodies into the frontal cortex and hippocampus of aged Tg2576 APP transgenic mice again but this time some mice were treated with anti-inflammatory drugs for the three days post-injection while others remained untreated. We treated mice with NCX-2216, minocycline or dexamethasone immediately following surgery and continued treatment until the morning of killing. NCX-2216 is the non-steroidal anti-inflammatory drug (NSAID) flurbiprofen with a nitric oxide donor group and a ferulic acid group attached. In APP+PS1 transgenic mice this drug has been shown to cause an activation of microglia and reduction of amyloid burden (Jantzen et al, 2002) but has also been shown to inhibit microglial activation following lipopolysaccharide (LPS) injection (Hausse-Wegrzyniak et al, 1999).

Minocycline, a tetracycline derivative, was chosen as it had been shown to suppress microglial activation following global brain ischemia (Tikka et al, 2001), 6-hydroxydopamine administration (He et al, 2001) and excitotoxicity (Yrjanheikki et al, 1998). Dexamethasone was used as it is a potent glucocorticosteroid which is known to inhibit the cyclooxygenase and lipoxygenase inflammatory pathways as well as induce a general state of immunosuppression (Schimmer and Parker, 2001).

Dexamethasone was found to be the most efficacious anti-inflammatory for inhibition of microglial activation due to anti-A β antibodies; NCX-2216 had a moderate effect while minocycline appeared to have very little effect on the activation of microglia (Paper 2; Figure 1 and Figure 2A). Interestingly, inhibition of microglial activation had no effect on removal of diffuse amyloid deposits, in all cases where anti-A β antibodies were injected diffuse deposits were reduced to the same extent (Paper 2; Figure 1 and Figure 2B). In dexamethasone treated mice, however, there was no apparent reduction in compact, thioflaine-S positive, amyloid deposits demonstrating that the inhibition of microglial activation had also inhibited the removal of compact amyloid deposits. Mice treated with NCX-2216, which had a moderate effect on microglial activation showed modest reductions in compact amyloid deposits while those mice treated with minocycline which failed to affect microglial activation showed reductions in compact amyloid deposits comparable to those observed in mice receiving no anti-inflammatory treatment (Paper 2; Figure 1 and Figure 2C). These data strongly suggest that microglial activation is necessary for the removal of compact amyloid deposits by anti-A β antibodies but is not necessary for removal of diffuse amyloid deposits.

To determine whether the microglial activation by anti-A β antibodies occurred via the Fc γ receptor and to also determine whether it was this receptor responsible for removal of compact amyloid deposits we made F(ab')₂ fragments from an anti-A β ₂₈₋₄₀ IgG. These F(ab')₂ fragments lack the Fc portion of IgG, therefore rendering them unable to interact with Fc γ receptors on effector cells like microglia yet fully capable of binding to A β in the same way as the complete IgG. We injected these anti-A β F(ab')₂ fragments into the frontal cortex and hippocampus of aged Tg2576 APP transgenic mice and killed them 72 hours following injection. We found that the F(ab')₂ fragments were unable to activate microglia as effectively as the complete IgG is and the activation levels were comparable to that observed in the mice receiving either control IgG or control F(ab')₂ fragments (Paper 2; Figure 3 and Figure 4A). We also found that anti-A β F(ab')₂ fragments were capable of reducing diffuse amyloid deposits as effectively as the complete IgG (Paper 2; Figure 3 and Figure 4B). However, anti-A β F(ab')₂ fragments were significantly worse in removing compact amyloid deposits than the complete IgG although there was small reductions observed in the hippocampus (Paper 2; Figure 3 and Figure 4C). These data suggest that although some removal of compact amyloid deposits may be possible with anti-A β F(ab')₂ fragments, removal is much more efficient when the Fc portion of IgG is present. This further demonstrates that not only is microglial activation necessary for compact amyloid reduction by anti-A β antibody treatment but also it appears that the Fc receptor is the component of microglia mediating this effect.

Knowing that once anti-A β antibodies enter the brain parenchyma they remove A β by mechanisms independent of microglial activation and also dependent upon microglial activation via the Fc receptor we systemically injected anti-A β antibodies as a

form of passive immunization since this is more clinically relevant. We injected anti-A β ₂₈₋₄₀ antibodies weekly for 1, 2 or 3 months such that all the mice were 22 months of age at killing. Mice received Y-maze testing during the week prior to killing to test for any cognitive benefit. We found significant improvement in Y-maze performance following three months of treatment by both an increase in alternations and a decrease in the number of arm entries (Paper 3; Figure 1) suggesting that the antibody treatment had improved cognitive function. We had previously demonstrated cognitive improvement following active immunization (Morgan et al, 2000) but this is the first time we had shown improvement following passive immunization. We also found evidence for peripheral action of anti-A β antibodies. Following intraperitoneal anti-A β antibody injection we found that circulating A β levels in the serum were increased 100-fold following 1 month of treatment and remained significantly elevated following 2 and 3 months of treatment despite a slight decline compared to 1 month (Paper 3; Figure 2B). Importantly, we showed that when we stained brain tissue for mouse IgG and counterstained with Congo red to detect amyloid deposits we found that plaques were decorated with mouse IgG following anti-A β antibody treatment but no staining was observed in those mice receiving control IgG (Paper 3; Figure 3). This suggests that anti-A β antibodies administered systemically are able to cross the blood-brain barrier and bind to A β in amyloid plaques in the brain parenchyma. We also found that A β was significantly reduced following two months of anti-A β antibody treatment with a further slight reduction following three months of treatment (Paper 3; Figure 4) as were compact amyloid deposits as detected by Congo red staining (Paper 3; Figure 5).

It was apparent in this passive immunization study that there is an associated complex microglial response. The first microglial marker to be increased was the Fcγ receptors II and III, which were dramatically increased following one month of anti-Aβ antibody treatment by approximately 100-fold. The expression of these receptors fell only slightly between one and two months but were reduced to control levels following three months of treatment (Paper 3; Figure 6). CD45 expression was not significantly increased until two months of anti-Aβ antibody treatment and was reduced to control levels following three months of treatment (Paper 3; Figure 7). We later examined expression of phospho-p38 MAPK and phospho-p44/42 MAPK (also known as ERK1/2), which have been shown to be increased in microglia during activation. Phospho-p38 MAPK was high in APP transgenic mice receiving control antibody for three months indicating activation of microglia due to the presence of amyloid plaques. However, following treatment with anti-Aβ antibodies for two and three months we observed a decrease in expression in microglia despite observing increases in other microglial activation markers at these time-points (Appendix C Figures 3 and 4). The phospho-p44/42 MAPK showed an opposite effect from the phospho-p38 MAPK which was that it was low in APP transgenic mice receiving control antibody for 3 months but was increased significantly following two and three months of anti-Aβ antibody treatment, in fact it was the only microglial marker which was still significantly increased following three months of anti-Aβ antibody treatment (Appendix C; Figures 1 and 2).

This complex microglial reaction observed following systemic administration of anti-Aβ antibodies suggests that activation of microglia may not be a simple on-off phenomena but rather there may exist multiple states of activation dependent upon the

stimulus and these states may result in different functional consequences. One potential hypothesis for different activation states is that microglia may exist in two states; antigen presenting and phagocytic, as exists in peripheral macrophages. In peripheral macrophages it has been shown that phosphorylation of p38 MAPK results in production of IL-1 β (Baldassare et al, 1999), IL-6 (Vanden Berghe et al, 1998) and IL-8 (Hobbie et al, 1999) and causes upregulation of other proinflammatory molecules such as COX-2, iNOS and TNF- α by upregulating NF- κ B driven gene expression (Carter et al, 1999; Kostinaho and Kostinaho, 2002). All of these processes associated with phospho-p38 MAPK are proinflammatory in nature and are associated with an antigen-presentation phenotype. Phagocytosis of apoptotic cells by peripheral macrophages results in an anti-inflammatory phenotype with down regulation of pro-inflammatory chemokines, possibly via phosphorylation of p44/42-MAPK which has been shown to inhibit phosphorylation of p38-MAPK and therefore contribute to the anti-inflammatory characteristics of this state (Xiao et al, 2002). Using human polymorphonuclear neutrophils it has been shown that phagocytosis of microbes is inhibited when phosphorylation of p44/42 MAPK is inhibited (Zhong et al, 2003). Since microglia are known to be derived from peripheral macrophages it is feasible that this same scenario is occurring in microglial cells. Little is currently known about the function of p38 and p44/42 MAPKs in microglia. In human AD postmortem tissue it has been shown that phospho-p38 MAPK is highly expressed in microglia around amyloid plaques (Hensley et al, 1999). This upregulation of phospho-p38 MAPK has also been shown in the transgenic mouse model of amyloid deposition APP751 (Koistinaho et al, 2002). In microglia it has been shown that Fc γ receptor mediated phagocytosis is inhibited by PP2, a Src inhibitor and piceatannol, a Syk

inhibitor which both ultimately inhibit phosphorylation of p44/42, further suggesting that phosphorylation of p44/42 is necessary for phagocytosis (Song et al, 2004).

The inflammation hypothesis of AD proposed that inflammation via activation of microglia and astrocytes in response to amyloid deposits in the brain actually contributes to the disease process. Activated microglia are capable of producing numerous cytokines, chemokines, complement components and other inflammatory mediators. Activated astrocytes are also capable of producing similar molecules. All of these inflammatory molecules are capable of eliciting numerous effects in the brain such as neuronal dysfunction and death as well as causing further inflammation via activation of additional microglia and astrocytes. Despite all of the detrimental effects of inflammation in AD it has been shown that microglia are capable of eliciting beneficial effects in the AD brain as well. In APP transgenic mice crossed with TGF- β 1 overexpressing mice there was significant activation of microglia and also a reduced amyloid burden when compared to the APP transgenic mice alone, however, vascular amyloid levels were increased (Wyss-Coray et al, 2001). The same group also showed that mice expressing soluble complement receptor-related protein y (sCrry), a complement inhibitor, crossed with APP transgenic mice showed reduced microglial activation, increased amyloid plaque load and neurodegeneration (Wyss-Coray et al, 2002). Lipopolysaccharide (LPS) injection into the hippocampus of aged APP transgenic mice has been shown to result in significant microglial activation and dramatic reductions in A β (DiCarlo et al, 2001; Herber et al, 2004). Finally there is the evidence from papers 1-3 here that microglia are necessary for effective removal of compact amyloid deposits by microglia and also are activated in response to systemic anti-A β injection.

Following the three month time-course study in which we observed an improvement in Y-maze performance following three months of anti-A β antibody treatment we took an older cohort of APP transgenic mice and treated them weekly for a period of five months and examined their cognitive function using the much more robust method of the radial-arm water maze. Following three months of treatment mice were tested in a rapid two-day radial-arm water maze task and number of errors was measured. A significant impairment was observed in the APP transgenic mice receiving control antibody while the APP transgenic mice receiving anti-A β antibody performed as well as the nontransgenic mice and were significantly better than the control antibody treated group (Paper 4; Figure 1A). Mice were tested two months later having received a total of five months of treatment and the benefit of the anti-A β antibody treatment was still present to the same degree (Paper 4; Figure 1B). This data suggests that complete reversal of cognitive deficits in APP transgenic mice is possible following systemic anti-A β antibody treatment.

Histopathological analysis of Congo red and A β showed the same result as had been observed in the previous time course study; dramatic reductions in both compact and diffuse deposits were observed following anti-A β antibody treatment (Paper 4; Figure 2A and Table 1). During image analysis of the Congo red and A β it was observed that some of the animals appeared to have high levels of amyloid in the vasculature. To quantify this we took the images used for quantification of total Congo red and ran the analysis again manually deselecting the parenchymal amyloid deposits and therefore quantifying only the amyloid in the vasculature. There was a dramatic increase in vascular amyloid load following five months of anti-A β antibody treatment. When these

values were subtracted from the total amyloid load the result was parenchymal amyloid load which now showed an even more dramatic decrease due to anti-A β antibody treatment (Paper 4; Figure 2A). Once we obtained this data we decided to perform the same analyses on the three month time-course tissue. The same observation was made; vascular amyloid load increased in concert with the decreases in parenchymal amyloid. Following two months of anti-A β antibody treatment vascular amyloid burden showed a significant increase with a slight further increase following three months of treatment. Parenchymal amyloid burden showed a significant decrease following two months of treatment with a further slight decrease following three months of treatment (Paper 4; Figure 2 B, C and D). Despite this increase in cerebral amyloid angiopathy (CAA) due to anti-A β antibody treatment the mice showed tremendous cognitive benefit and so at this point the CAA is not compromising their cognitive ability, however, if the increase in CAA were to continue it may be predicted that at some point this would cause a decline in cognition as it does in the human condition. It is important to note that not all amyloid is redistributed to the vessel, as there is still a significant decrease in total Congo red staining, although some of the amyloid is clearly redistributed into the vasculature.

Due to the increase in CAA in our passive immunization studies and the observation by Pfeifer and colleagues (Pfeifer et al, 2002) that anti-A β antibody treatment in hemorrhage prone APP transgenic mice resulted in a significant increase in the number of microhemorrhages present we decided that we should stain the tissue for hemosiderin using a Prussian blue stain to examine for microhemorrhages. We found that there was a significant increase in the number of Prussian blue positive profiles following two, three and five months of anti-A β antibody treatment. There was a dramatic increase between

one and two months of anti-A β antibody treatment after which the number of profiles increased linearly with increasing duration of antibody treatment (Paper 4; Figures 4 and 5). Importantly, all vessels showing microhemorrhage were also positive for CAA, although not all CAA positive vessels showed microhemorrhage.

The mechanism by which anti-A β antibody therapy results in increased CAA remains unclear. One hypothesis is that the microglia are capable of phagocytosing the amyloid plaques but are unable to degrade it so transport the amyloid to the vessels where they dispose of it into the vessel wall. Support for this hypothesis comes from CD45 immunohistochemistry which showed that despite overall microglial activation being reduced to control levels following three months of anti-A β antibody treatment there appeared to be high levels of microglial activation around those vessels containing amyloid (Paper 4; Figure 3E and F). We were unable to quantify this observation due to the diffuse nature of the microglial staining; we could not manually deselect all non-vessel associated staining. It is also plausible that anti-A β immunotherapy is removing more A β_{1-42} , since this is the more prevalent species in both human AD and APP transgenic mice, and therefore shifting the ratio towards more A β_{1-40} , thus resulting in amyloid deposition in the vasculature. It has previously been shown that APPDutch transgenic mice and human hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) have a significantly higher ratio of A β_{40} to A β_{42} than that observed in APP transgenic mice or human AD brain. A β_{1-40} predominates in vascular amyloid in AD and APP transgenic mice and thus both the APPDutch transgenic mice and the human HCHWA-D have high levels of CAA, microhemorrhage and a perivascular microglial reaction. When APPDutch transgenic mice were crossed with PS1 transgenic mice

known to increase A β ₁₋₄₂ production it was observed that now the mice developed parenchymal amyloid deposits with much less prominent CAA (Herzig et al, 2004). These data suggest that a shift in the ratio of A β ₄₀:A β ₄₂ may influence the location of deposition.

Table 1 summarizes the evidence we found for each mechanism of β -amyloid removal by anti-A β antibody therapy. We have demonstrated here by intracranial injection that anti-A β antibodies in the brains of aged APP transgenic mice removes A β by two distinct mechanisms. Diffuse amyloid deposits are removed rapidly, within 24 hours, and this removal is independent of microglial activation. Compact amyloid deposits are removed between 24 and 72 hours, this removal is dependent upon microglial activation and Fc γ receptor activation. When anti-A β antibodies were administered systemically as a passive immunization the antibodies crossed the blood brain barrier and bound to amyloid plaques in the parenchyma. A β removal was associated with microglial activation, Fc γ receptor upregulation and increased A β in the serum. Also, systemically administered anti-A β antibodies provided complete reversal of cognitive deficits following just three months of treatment. It was also observed that there were increased levels of vascular amyloid and multiple microhemorrhages in the brains of APP transgenic mice administered anti-A β antibodies for two or more months. Overall, we have shown evidence for three distinct mechanisms of A β removal by immunotherapy which appear to occur in concert to produce robust pathological effects. These mechanisms are direct dissolution of amyloid fibrils, microglial phagocytosis via the Fc γ receptor and a peripheral sink mechanism which results from a shift in the concentration gradient of A β between brain and plasma.

There are many more questions to be answered if anti-A β antibody therapy is to be successful in the clinic given the pathological effects observed in the current studies. The main issue that needs to be overcome is the increased vascular amyloid and associated microhemorrhage. The first question is whether dose of antibody and frequency of dosing would alter the pathological consequences of anti-A β antibody therapy. It is plausible that a lower dose may not produce such a robust microglial response and as such, if indeed the microglia are responsible for the increase in vascular amyloid, the increased microhemorrhage and CAA may be avoided. The first way to approach this would be to establish a detailed dose response with doses ranging from 1 to 10 mg/kg for a period of three months with radial-arm water maze testing at the end. An optimal dose would provide cognitive benefit as well as reductions in parenchymal amyloid loads without an increase in CAA or microhemorrhage. In the current studies mice were injected weekly with 10mg/kg anti-A β antibody. It is conceivable that dosing as little as once every four weeks may be sufficient for amyloid reductions and cognitive benefit if the study were to be extended to six and nine months as opposed to three months. A study to address this would use a 10mg/kg dose and inject at intervals ranging from two to four weeks with radial-arm water maze testing at six and nine months. Ideally, there would be an interval which produces significant cognitive benefit and amyloid reductions with minimal increases in CAA and microhemorrhage.

What contribution each of the three mechanisms make to the improved cognition, amyloid reductions and increases in CAA and microhemorrhage is currently unknown. Conjugating a large, polar molecule to anti-A β antibodies would prevent blood-brain barrier passage but would still permit binding of A β in the plasma and therefore only the

peripheral sink mechanism would be functioning. Treating the mice in exactly the same way as with the standard antibody would permit comparisons of efficacy to be made. If the peripheral sink mechanism alone is sufficient to provide cognitive benefit and amyloid reductions it is likely that this would be a safe alternative if microglial phagocytosis is responsible for the increased CAA and microhemorrhage.

If the peripheral sink mechanism alone is found not to be sufficient to obtain optimal effects another approach would be to modify antibodies so that they still cross the blood-brain barrier, can still act peripherally but are unable to effectively activate microglia. One such antibody is a deglycosylated anti-A β antibody. IgG molecules have carbohydrates attached and these are critical to the recognition of IgGs by the Fc γ receptor (Radaev and Sun, 2001). If these carbohydrates are removed the IgG molecule would maintain its pharmacokinetics as the carbohydrates contribute very little to the overall molecular weight and would also not interact with the Fc γ receptor. Another option would be to systemically administer anti-A β F(ab')₂ fragments similar to those used in paper 2. These, again would not interact with the Fc γ receptor however, due to their reduced molecular weight, their half-life in serum would be much lower as they now would be filtered out by the kidney. This means that they would need to be administered more frequently. It has been shown that attachment of polyethylene-glycol groups to F(ab')₂ fragments, a process called PEGylation, results in comparable half-lives to the whole IgG molecule (Weir et al, 2002). It has been shown that PEGylation of an anti-interleukin-8 F(ab')₂ results in longer serum half-life and retention of comparable bioactivity (Koumenis et al, 2000). It is unclear whether pegylated F(ab')₂ fragments will

cross the blood-brain barrier however, if they do not, this would be an alternative way to test the peripheral sink mechanism.

It is possible that microglial phagocytosis via the Fc γ receptor is necessary for effective amyloid removal and cognitive improvement and that only targeting the peripheral sink and the direct dissolution mechanisms is insufficient for clinically relevant benefits. If this is the case, attenuating the microglial response using anti-inflammatory drugs may minimize redistribution of amyloid to the vasculature and therefore reduce the incidence of microhemorrhage. We showed in paper 2 that an NSAID such as NCX-2216 partially inhibits microglial activation and yet significant reductions in compact amyloid deposits were observed. Co-administration of an NSAID with the anti-A β antibody therapy may attenuate the microglial response sufficiently to prevent increases in CAA and microhemorrhage while providing sufficient removal of compact amyloid deposits to demonstrate cognitive benefit. Since the addition of a nitric oxide donor group to flurbiprofen in the NCX-2216 compound provides gastrointestinal protection this drug would be safe for long-term daily administration to elderly AD patients receiving the immunotherapy. NCX-2216 has also been shown to inhibit microglial reaction to intracranial infusion of lipopolysaccharide (Haus-Wegrznjak et al, 1999). Administration of glucocorticosteroids such as dexamethasone would likely not be effective for this approach as we showed in paper 2 that these completely inhibit microglial reaction and also completely inhibit the ability of anti-A β antibodies to remove compact amyloid deposits.

The isotype of the antibody may be critical in determining clinical efficacy and prevalence of increased CAA and microhemorrhage. In papers 3 and 4 we used an IgG1

antibody with an epitope recognizing amino acids 28-40 of the A β molecule. Microglia express three different classes of Fc γ receptors; Fc γ RI is a high affinity receptor while Fc γ RII and Fc γ RIII are low affinity receptors (Ravetch and Kinet, 1991). It is known that in mice IgG2a binds Fc γ RI and III with the highest affinity while IgG1 and IgG2b bind with a lower affinity (Radaev and Sun, 2001). In fact, in a study using both in vivo and ex vivo methods, it was shown that IgG2a anti-A β antibodies are more effective in removing amyloid than are IgG1 or IgG2b antibodies of the same epitope (Bard et al, 2003). In the ex vivo study the authors examined the effects of isotype on plaque removal from PDAPP brain sections by primary cultured microglial cells. In this study IgG2a antibodies were much more effective than IgG1 or IgG2b antibodies of the same epitope. If microglial phagocytosis via the Fc γ receptor is responsible for not only removal of parenchymal amyloid but is also responsible for the increased CAA and microhemorrhage then it is possible that an IgG2a isotype antibody may in fact cause more CAA and microhemorrhage as this has a much higher affinity for the Fc γ receptors than the IgG1 antibody used in paper 4.

The epitope may be another important issue to be addressed in determining efficacy. In a study using in vivo and ex vivo methods it was shown that antibodies directed against the N-terminal of A β are most effective in A β removal (Bard et al, 2003). However, we show in papers 3 and 4 that C-terminal antibodies are highly effective in the removal of A β . Also, a mid-domain antibody has been shown to significantly reduce brain amyloid (DeMattos et al, 2001) and reverse cognitive deficits (Dodart et al, 2002). Since mid-domain antibodies are able to bind soluble A β but are unable to bind A β in amyloid plaques these antibodies are highly effective for the peripheral sink mechanism

but would be unable to trigger effective microglial phagocytosis. It was recently shown that mid-domain antibodies do not cause microhemorrhage while N-terminal antibodies do (DeMattos et al, 2004).

Overall, we have shown evidence that three different mechanisms are acting in concert to reduce amyloid burden in transgenic mice following anti-A β antibody therapy. We have also shown that one undesirable consequence of anti-A β antibody therapy is increased CAA and microhemorrhage. This will need to be overcome if anti-A β immunotherapy is to be successful clinically.

Table 1: Summary of the evidence found for the different mechanisms of β -amyloid removal.

Mechanism	Paper 1	Paper 2	Paper 3	Paper 4
Microglial phagocytosis via Fcγ receptors	Microglial activation peaks at the same time-point as reductions in compact amyloid deposits are observed.	Inhibition of microglial activation inhibits removal of compact amyloid deposits. F(ab') ₂ fragments are unable to produce effective reductions in compact amyloid deposits.	Microglial expression of Fc γ receptors and CD45 is increased following systemic antibody administration. Phospho-p44/42 expression is also increased.	Microglia are activated around vascular amyloid deposits.
Direct dissolution	Diffuse amyloid deposits are reduced early with no associated microglial activation.	Diffuse amyloid deposits are removed regardless of microglial inhibition or F(ab') ₂ fragments.		
Peripheral sink			100-fold increase in circulating A β levels following systemic anti-A β antibody administration.	Increased vascular amyloid could potentially be a result of the peripheral sink mechanism.

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APPENDICES

**APPENDIX A:
NUMBER OF A β INOCULATIONS IN APP+PS1 TRANSGENIC
MICE INFLUENCES ANTIBODY TITERS, MICROGLIAL ACTIVATION AND
CONGOPHILIC PLAQUE LEVELS.**

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APPENDIX A (continued)

Abstract

There have been several reports on the use of beta-amyloid (A β) vaccination in different mouse models of Alzheimer's disease (AD) and its effects on pathology and cognitive function. In this report histopathology of the APP+PS1 doubly transgenic mice is compared after 3, 5 or 9 A β inoculations. The number of inoculations influenced the effects of vaccination on Congo red levels, microglia activation and anti-A β antibody titers. After 3 inoculations, the antibody titer of transgenic mice was substantially lower than that found in nontransgenic animals. However, after 9 inoculations, the levels were considerably higher in both genotypes, and no longer discriminable statistically. The number of inoculations influenced CD45 expression, an indicator of microglial activation. There was an initial up-regulation, which was significant after 5 inoculations, but by 9 inoculations, microglial activation was equivalent to mice given control vaccinations. Along with this increased CD45 expression there was a correlative reduction in Congo red staining, which stains compact plaques. When mice from all groups were combined, there was a significant correlation between activation of microglia and Congo red levels, suggesting that microglia play a role in clearance of compact plaque.

Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder characterized by accumulation of senile plaques consisting of beta-amyloid (A β) protein of which there are 2 forms, A β ₁₋₄₀ and A β ₁₋₄₂. This is thought to be the key step in the pathogenesis of Alzheimer's disease (Selkoe, 1991). The disorder is also characterized by

APPENDIX A (continued)

the formation of neurofibrillary tangles consisting of tau protein, and by the initiation and proliferation of a brain-specific inflammatory response (Akiyama et al, 2000). Transgenic mouse models of Alzheimer's disease have been an invaluable source of information regarding the pathological progression of AD and a vehicle in which to test possible therapeutic interventions. Here we use a transgenic mouse model of AD carrying two transgenes; amyloid precursor protein (APP) and presenilin-1 (PS1) previously described (Duff et al, 1996, Hsiao et al, 1996, Holcomb et al, 1998, 1999, Gordon et al, 2001a, Gordon et al, 2001b).

Schenk et al initially described the effects of $A\beta_{1-42}$ immunization in the PDAPP mouse. In a report published in 1999 they demonstrated the ability of their vaccination regimen to reduce $A\beta$ deposits in the brain. More recently, $A\beta$ vaccination has also been shown to prevent the cognitive decline in some transgenic mice (including the APP+PS1) in addition to reducing $A\beta$ load (Morgan et al, 2000, Janus et al, 2000). The data presented in this report examines the effect of increasing numbers of $A\beta_{1-42}$ immunizations on the pathology of the APP+PS1 mouse, specifically, anti- $A\beta$ antibody titers, the reduction in congophilic plaque load and the activation of microglia.

Of particular note in this study was the observed activation of microglial cells, which are central to the inflammatory process in AD, along with a concomitant reduction in congophilic plaque.

APPENDIX A (continued)

Materials and Methods

Vaccination Protocol.

Tg2576 APP mice (Hsiao et al, 1996) were bred with PS1 line 5.1 mice (Duff et al 1996) to obtain the double transgenic mice. These mice were then randomly assigned to groups receiving vaccination with either human A β ₁₋₄₂ peptide (Bachem) or with keyhole limpet hemocyanin (KLH) as described previously (Morgan et al, 2000). Briefly, 100 μ g of A β ₁₋₄₂ or KLH were dissolved in water at 2.2 mg/ml, mixed with PBS and incubated overnight. One day later this suspension was mixed with Freund's adjuvant (complete for the primary inoculation, incomplete for the next 4 inoculations, and mineral oil for the remaining inoculations). Three vaccination groups were used. The first group was administered 3 inoculations starting at an average age of 13 months. These mice were killed at an average of 16 months of age, 13 days after the final inoculation. A second group of mice were given 5 inoculations starting at an average age 14.5 months. These mice were killed at an average of 19.75 months of age, 10 days after the last inoculation. A third group given 9 inoculations started at an average age of 7.5 months. They were killed at an average of 16.25 months of age, 6 weeks after the last inoculation. In addition, nontransgenic mice were vaccinated with A β peptide in the 3 and 9 inoculation groups. Antibody titers were measured by ELISA as described previously (Morgan et al, 2000, Dickey et al, 2001). The KLH immunized mice are herein referred to as control mice for these experiments.

APPENDIX A (continued)

Histopathology.

Mice were overdosed with pentobarbital, perfused with 0.9% saline and the brain removed. The right side of the brain was rapidly dissected over ice and the left side fixed for 24 hours in freshly prepared, buffered 4% paraformaldehyde. Following cryoprotection through increasing concentrations of sucrose solutions at 24-hour intervals, frozen sections were taken on a sliding microtome at a 25 μ m thickness and stored in DPBS (Dulbecco's phosphate buffered saline) with sodium azide to prevent microbial growth. Sections were stained using floating immunohistochemistry for total A β (rabbit antiserum primarily reacting with the N terminal of the A β peptide, 1:10000) and CD45 (Serotec, 1:10,000) as described previously (Holcomb et al, 1999, Gordon et al, 1997). Sections were also mounted on slides and stained for Congo red (Sigma-Aldrich) (Gordon et al, 2001a). The area of hippocampus and frontal cortex occupied by positive stain was measured with a Videometric V150 image analysis system (Oncor) on a Nikon Microphot FX microscope as described in detail previously (Gordon et al, 2001a). Percentage area was measured and analyzed. Data were collected from 8-16 equally spaced horizontal sections. The values for all sections from one mouse were averaged to represent a single sample for statistical analysis.

Statistical Analysis.

Data were first analyzed by comparing A β vaccinated and control mice within each group by one-way ANOVA using the Statview software program (SAS). Because of differences in the age of kill, the results from each A β vaccinated mouse were normalized to the mean value of their respective control vaccinated group (percent of control) to

APPENDIX A (continued)

correct for differences in age at kill and overall staining intensity for each inoculation group. These were then analyzed by performing a simple regression analysis on the Statview program.

Results

Antibody titers increase with increasing numbers of inoculations in both transgenic and nontransgenic mice (Fig 1). Initially, doubly transgenic mice have lower anti-A β titers than similarly treated nontransgenic mice. However, after the ninth vaccination the transgenic mice produced antibody titers that were similar to non-transgenic mice.

In general, the Congo red staining in hippocampus was reduced as a result of A β vaccination (Fig 2A,B). In the group receiving five inoculations this reduction was almost 50% and was significantly reduced in relation to control mice ($p < 0.002$; Figure 3). In the groups receiving three or nine inoculations, the reduction in Congo red was not statistically significant. The results from frontal cortex also follow these trends although no significance is found at any time-points (Table 1).

CD45 expression in hippocampus was increased almost 2 fold in the hippocampus of mice given 5 inoculations (Figure 2C,D; Figure 4; $p < 0.001$). There was a similar trend for CD45 up-regulation in the group receiving three inoculations although not statistically significant. However, the vaccinated mice were equivalent to the control mice in the group receiving nine inoculations. The same trend was seen in frontal cortex although not to the same degree (Table 1).

APPENDIX A (continued)

When data from all three groups are combined, there is a correlation between CD45 expression and Congo red staining in the hippocampus as illustrated in Figure 5. This relationship shows that mice with elevated CD45 expression had less Congo red staining ($r=0.691$, $p=0.002$). This result did not occur because of a biased positioning of one of the inoculation groups. By plotting the data from each group with a different symbol one can see that in each inoculation group there are mice with high CD45 staining and other mice with little, which was less than the control average. This bimodal distribution of CD45 staining has been observed in most groups of mice we have examined including the control mice in the present study.

Discussion

CD45 expression is indicative of microglial activation. Here, we have shown that CD45 expression in transgenic mice administered A β vaccination is up-regulated, after 5 inoculations. However, on average, the expression was no longer elevated after a 9th inoculation. This suggests that the microglial activation resulting from A β vaccination is transient. This likely represents a desensitization to the circulating antibodies because; even though the interval between the last inoculation and kill after 9 inoculations was 6 weeks the antibody titers were still high at necropsy. Perhaps the most interesting data is the high correlation between Congo red staining levels in hippocampi of transgenic mice and activation of microglia. These data add to a growing body of literature suggesting that in transgenic mice activation of microglia leads to clearance of A β deposits. Ongoing work from our group shows reduction in amyloid deposits in association with microglial

APPENDIX A (continued)

activation in several circumstances using the transgenic mouse model of amyloid deposition. Administration of a flurbiprofen derivative which slowly releases nitric oxide (NCX-2216) causes dramatic activation of microglia and substantial reduction in Congo red staining (Jantzen et al, 2001). In another study, intrahippocampal injections of lipopolysaccharide, a pro-inflammatory agent, simultaneously reduced A β load and activated microglia (Di Carlo et al, 2001). Even in the normal time course of amyloid accumulation there is a stabilization of the congophilic deposits in doubly transgenic mice between 12 and 15 months, the age at which microglial activation becomes most pronounced (Gordon et al, 2001b).

It has been well demonstrated that microglia in culture are readily capable of internalizing A β ₁₋₄₂ aggregates (Paresce et al, 1996; Webster et al, 2001). The data reported here are consistent with several other reports regarding A β vaccination and microglial activation. In the original A β vaccination report, the vaccine was found to result in activated microglia around the few deposits that did remain (Schenk et al, 1999). Bard et al, 2000, also demonstrated that microglia can be activated to clear tissue amyloid deposits by Fc receptor mediated phagocytosis in vitro. In a very direct experiment, Bacskai et al, (2001), demonstrated that injection of anti-A β antibodies into transgenic mouse brain induced a rapid disappearance of A β associated with a florid microglial reaction. These results together with those from our research group indicate that activation of microglia can have benefit in clearing A β deposits from the brains of transgenic mice. It is unclear whether excessive activation of microglia can ultimately cause autotoxic inflammatory reactions in this model or whether the mouse brain is

APPENDIX A (continued)

somehow resistant to the development of such a reaction. It is intriguing that the microglial activation in mice with 9 inoculations had largely subsided. This suggests that under these circumstances the microglia can develop tolerance to the activating stimuli. If so, vaccination may be one mechanism to, perhaps paradoxically, reduce an autotoxic reaction in the AD brain.

The observation that the doubly transgenic mice are slower to mount an immune response after A β vaccination than their non-transgenic counterparts is a significant one. There are several plausible explanations for this impaired antibody response. One explanation is that A β is a “self-antigen” in the transgenic mice (expressing human APP), and thus, they do not mount as significant a humoral response to the injected human A β ₁₋₄₂. The murine A β sequence is slightly different than the human sequence, thus nontransgenic mice would not identify the human A β peptide as an autoantigen. Another possibility that the transgenic mice are by some means “immune compromised” as is seen in older humans; thus, they are slower to mount a significant immune response. A third explanation is absorption of the serum antibodies by circulating A β , which interferes with antibody binding to the ELISA plate. This might be most evident when the antibody titers are low and antibody concentrations are stoichiometrically similar to that of A β . In any event, repeated vaccinations ultimately overcome this restriction of antibody generation in the transgenic mice. This may have significance for treatment of human populations, with multiple vaccinations required to activate a vigorous antibody response.

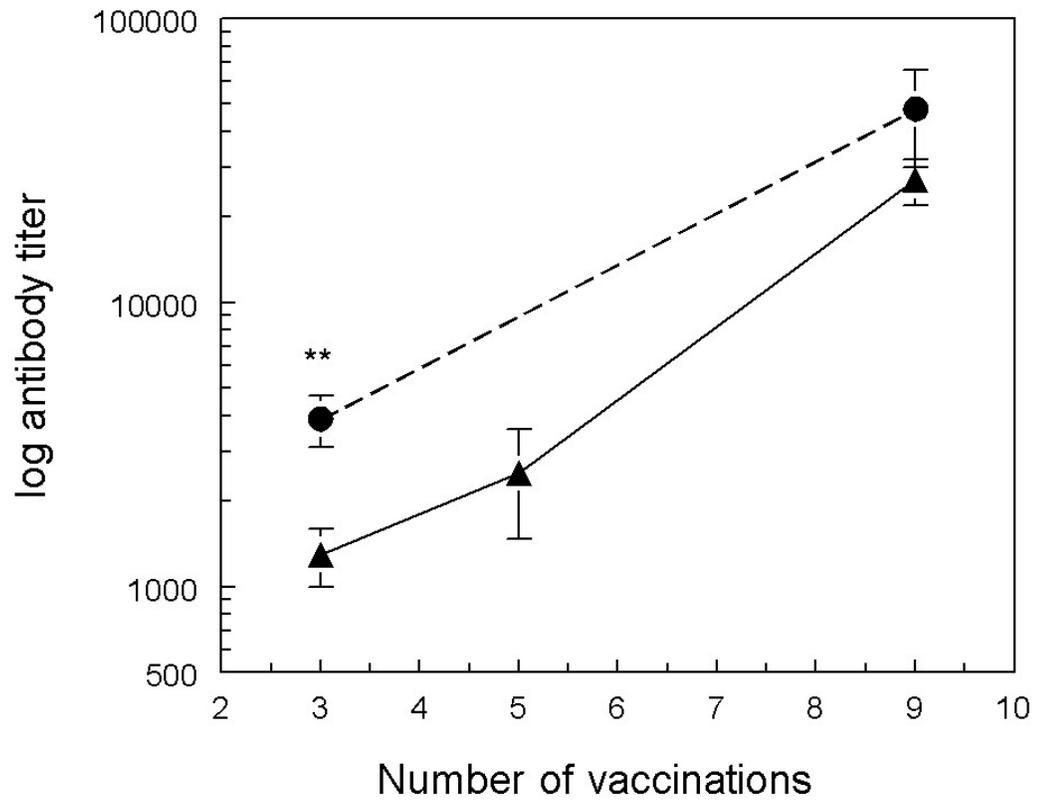
APPENDIX A (continued)

In conclusion, the data here are consistent with the argument that the A β vaccination results in plaque clearance primarily through activation of microglial cells. Still, we continue to entertain the possibility of antibodies dissolving plaques directly (Solomon, this volume), or antibodies binding circulating A β , reducing the effective plasma A β concentration, and increasing the concentration gradient between brain and blood leading to more rapid A β removal from the CNS. Finally, we believe that multiple inoculations are likely to be required if A β vaccination demonstrates efficacy in the treatment or prevention of AD.

APPENDIX A (continued)

Figure 1: Antibody titer averages as a function of number of inoculations in transgenic and non-transgenic mice. Figure legends: ●: Non-transgenic, ▲: Transgenic. There is a highly significant difference between transgenic and non-transgenic ($p=0.0002$) after 3 inoculations, indicated by **, but not by 9 inoculations.

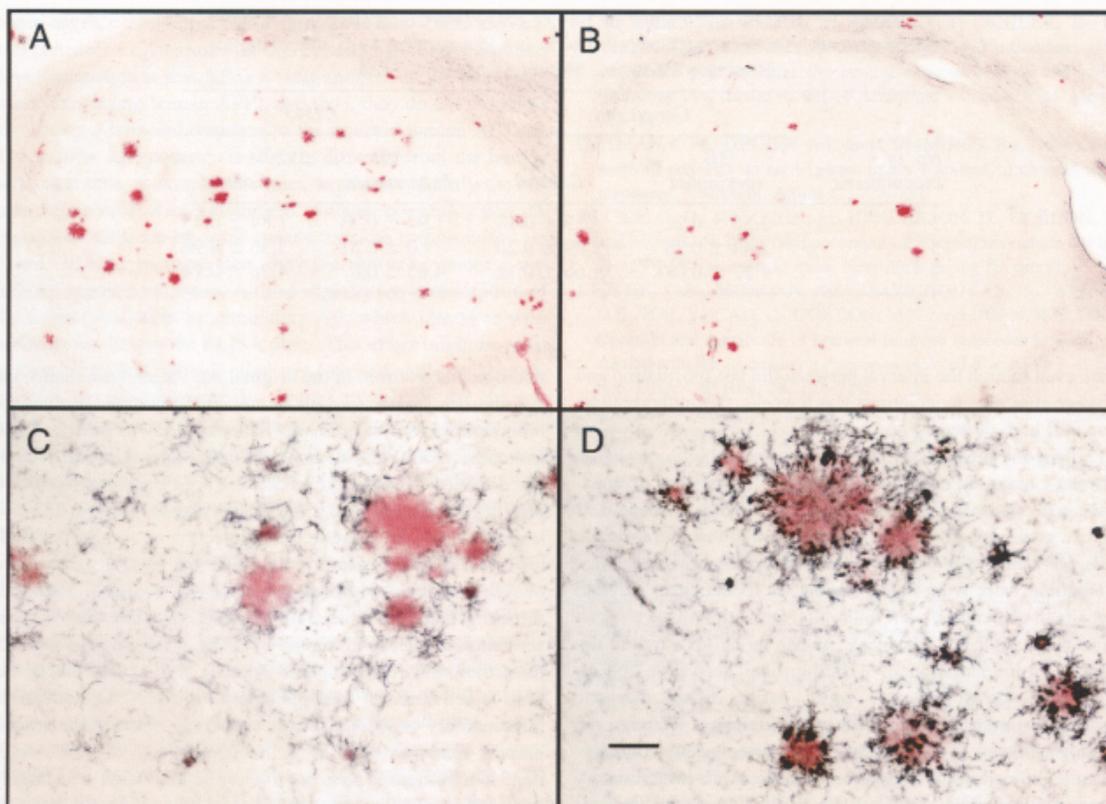
APPENDIX A (continued)



APPENDIX A (continued)

Figure 2: A, B: Congo red staining in the hippocampus of the mice receiving 5 A β inoculations (40X magnification). C, D: CD45 staining, counterstained with Congo red in the hippocampus of the mice receiving 5 A β inoculations (200X magnification). A and C: Control immunized mice. B and D: A β immunized mice. A and B; scale bar: 250 μ m. C and D; scale bar: 50 μ m.

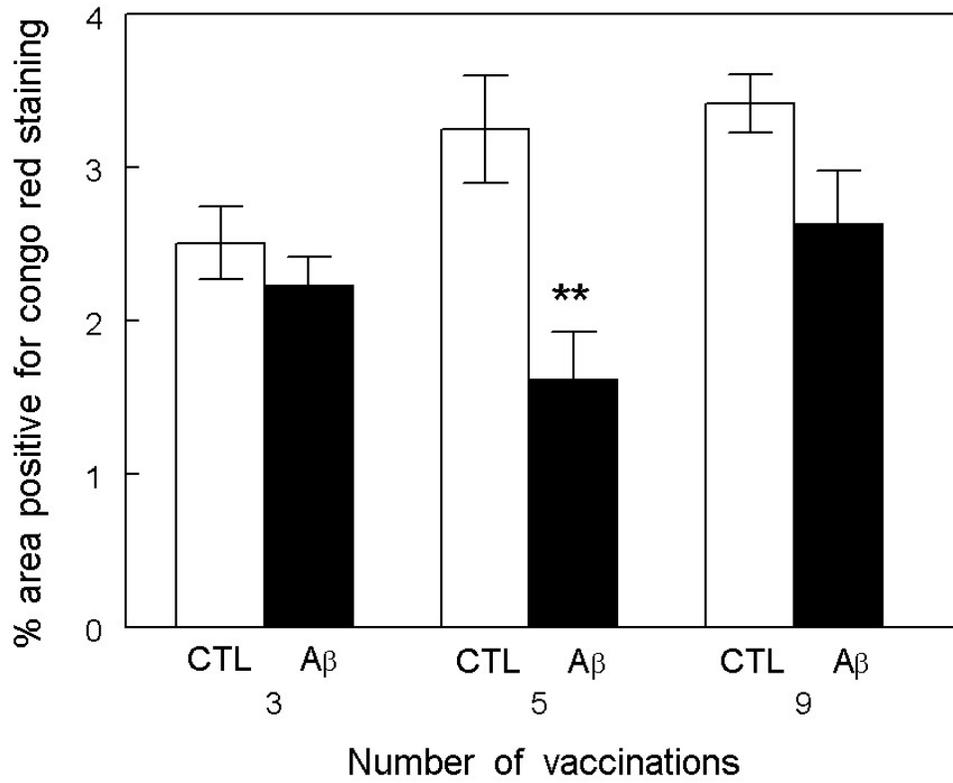
APPENDIX A (continued)



APPENDIX A (continued)

Figure 3: Congo red levels in hippocampus relative to number of inoculations for vaccinated and control mice. All mice for each group were averaged and are shown here and are all APP+PS1 transgenic mice. ** Indicates high significance ($p < 0.002$).

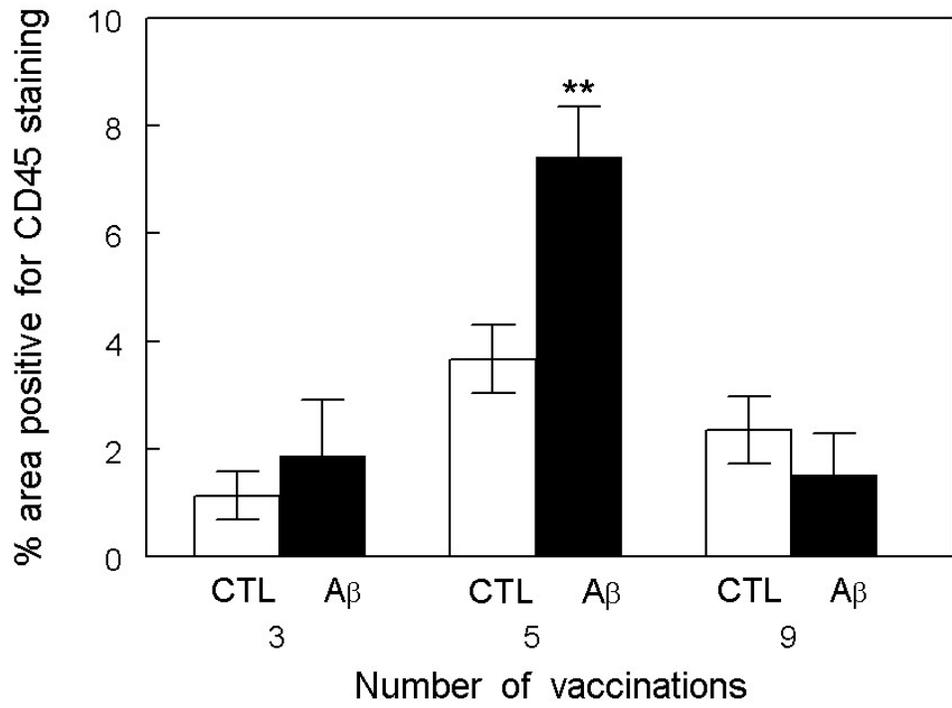
APPENDIX A (continued)



APPENDIX A (continued)

Figure 4: CD45 expression in hippocampus relative to number of inoculations for vaccinated and control mice. All mice for each group were averaged and are shown here and are all double transgenic. ** Indicates high significance ($p < 0.001$).

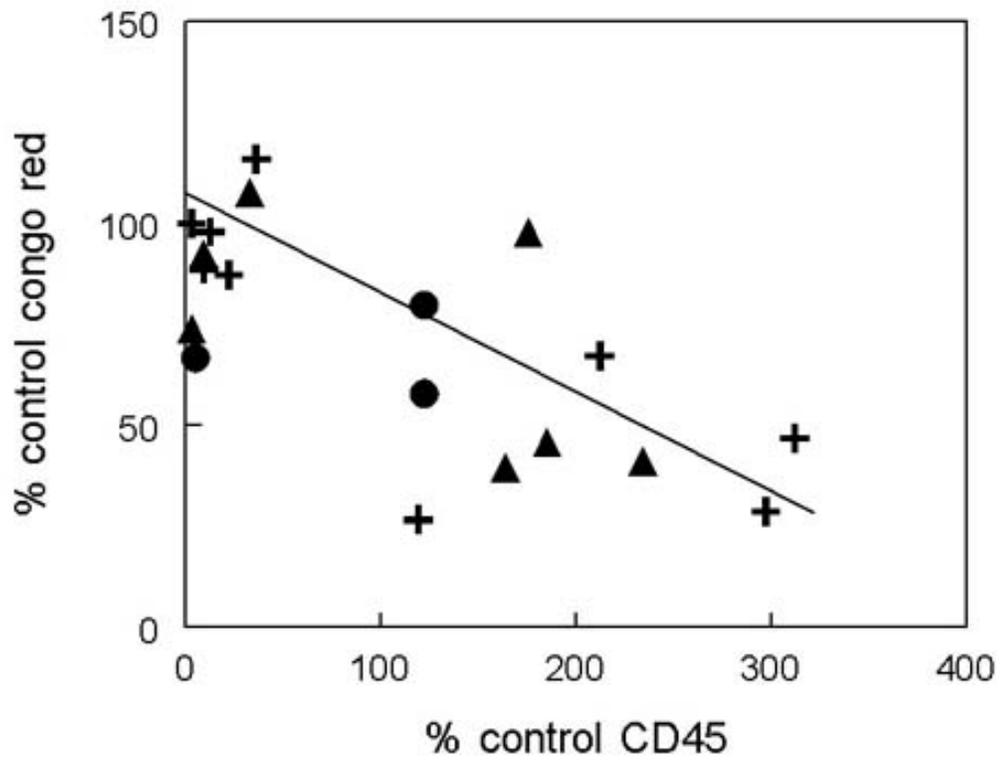
APPENDIX A (continued)



APPENDIX A (continued)

Figure 5: Correlation of Congo red levels and CD45 expression both shown as percent control in hippocampus. $R=0.69$ and $p=0.002$. Figure legends: **+**: 3 inoculations, **▲**: 5 inoculations, **●**: 9 inoculations.

APPENDIX A (continued)



APPENDIX A (continued)

Table 1: Effect of number of inoculations on Congo red and CD45 staining in the frontal cortex. Data are percent of area occupied by reaction product, shown as mean \pm SEM.

Number of inoculations	Congo red		CD45	
	Aβ vaccinated	Control	Aβ vaccinated	Control
3	2.56\pm 0.22	2.3\pm 0.18	1.94\pm 0.33	1.84\pm 0.45
5	2.44\pm 0.28	2.99\pm 0.20	7.0\pm 0.81	5.56\pm 0.96
9	1.32\pm 0.08	1.66\pm 0.12	4.63\pm 0.67	4.96\pm 0.74

APPENDIX A (continued)

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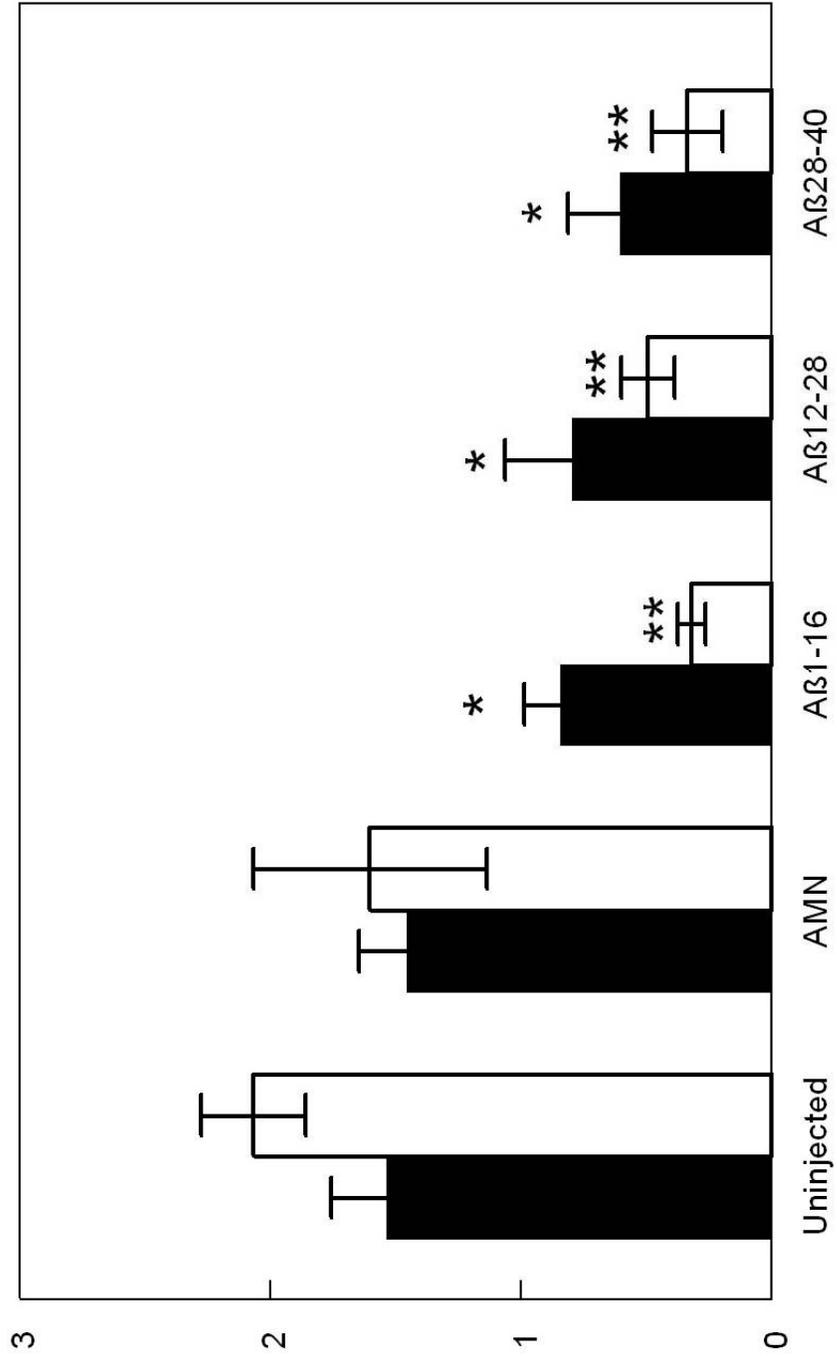
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APPENDIX B

Figure 1: Total A β is significantly reduced by anti-A β antibodies regardless of their epitope. The graph shows the ratio of right injected side: left uninjected side. Mice were injected with one of anti-drosophila amnesiac antibodies (AMN), anti-A β_{1-16} antibodies (A β_{1-16}), anti-A β_{12-28} antibodies (A β_{12-28}) or anti-A β_{28-40} antibodies (A β_{28-40}). Solid bars indicate values for the frontal cortex while open bars indicate values for the hippocampus. *indicates P<0.05, **indicates P<0.01 when compared to both uninjected and AMN groups.

APPENDIX B (continued)

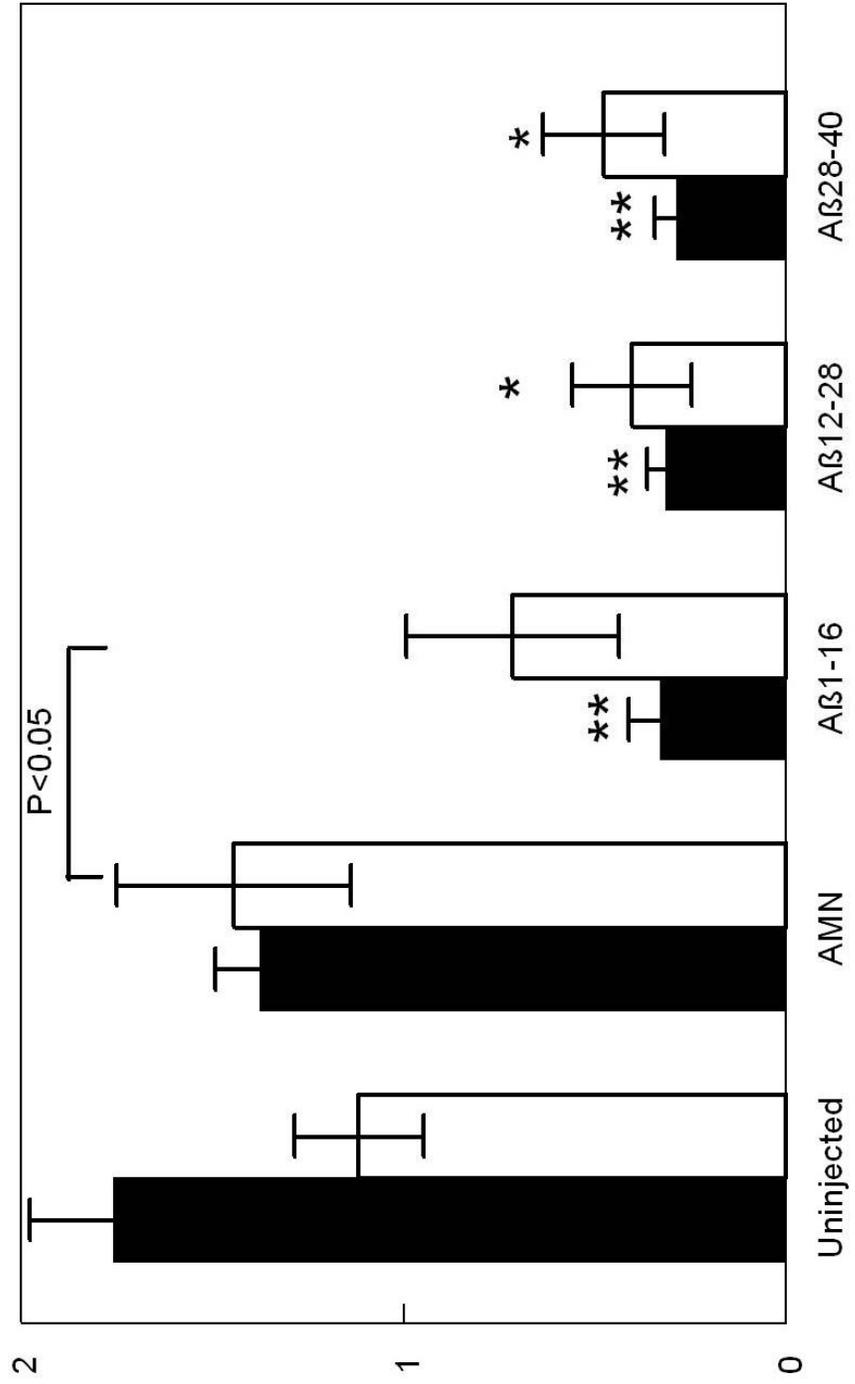


APPENDIX B (continued)

Figure 2: Thioflavine-S staining is significantly reduced by anti-A β antibodies regardless of their epitope. The graph shows the ratio of right injected side: left uninjected side.

Mice were injected with one of anti-drosophila amnesiac antibodies (AMN), anti-A β_{1-16} antibodies (A β 1-16), anti-A β_{12-28} antibodies (A β 12-28) or anti-A β_{28-40} antibodies (A β 28-40). Solid bars indicate values for the frontal cortex while open bars indicate values for the hippocampus. *indicates $P < 0.05$, **indicates $P < 0.01$ when compared to both uninjected and AMN groups.

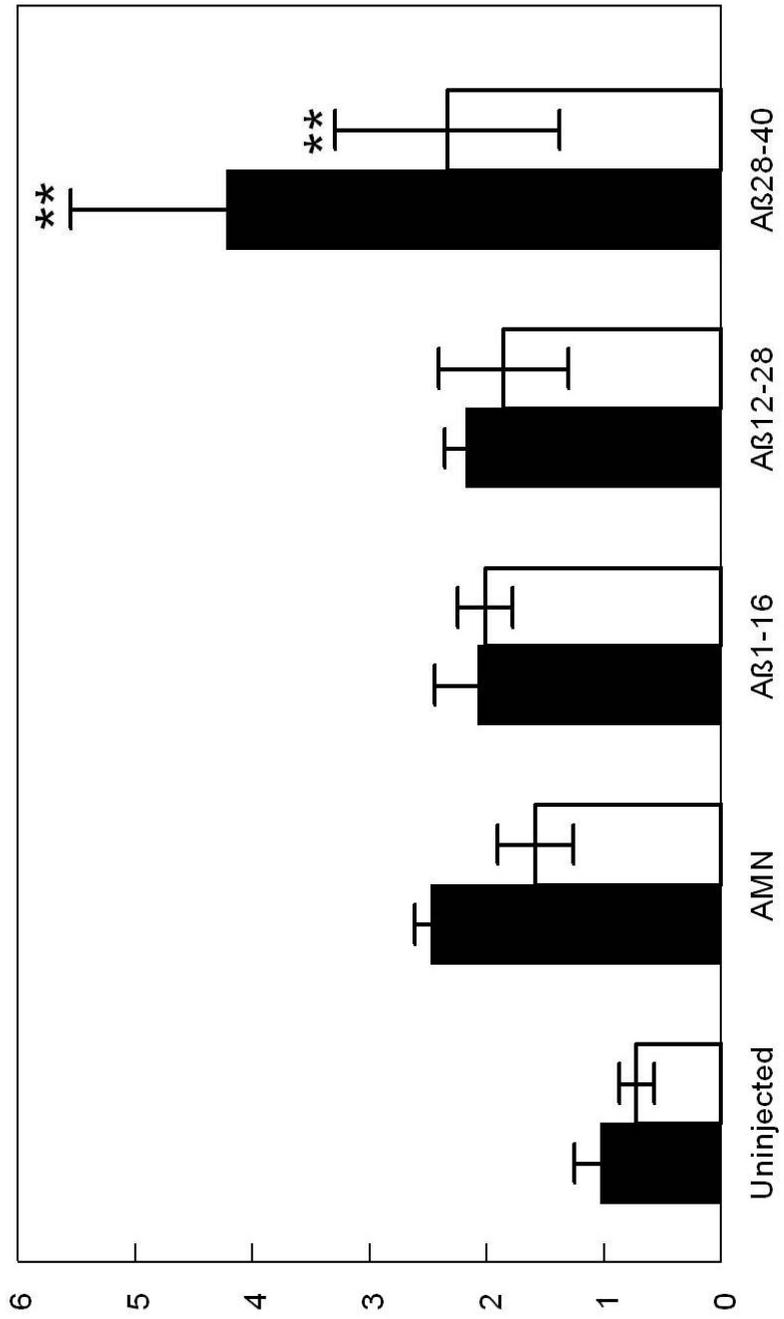
APPENDIX B (continued)



APPENDIX B (continued)

Figure 3: CD45 immunohistochemistry is increased by anti- $A\beta_{28-40}$ antibodies. The graph shows the ratio of right injected side: left uninjected side. Mice were injected with one of anti-drosophila amnesiac antibodies (AMN), anti- $A\beta_{1-16}$ antibodies ($A\beta_{1-16}$), anti- $A\beta_{12-28}$ antibodies ($A\beta_{12-28}$) or anti- $A\beta_{28-40}$ antibodies ($A\beta_{28-40}$). Solid bars indicate values for the frontal cortex while open bars indicate values for the hippocampus. **indicates $P < 0.01$ when compared to both uninjected and AMN groups.

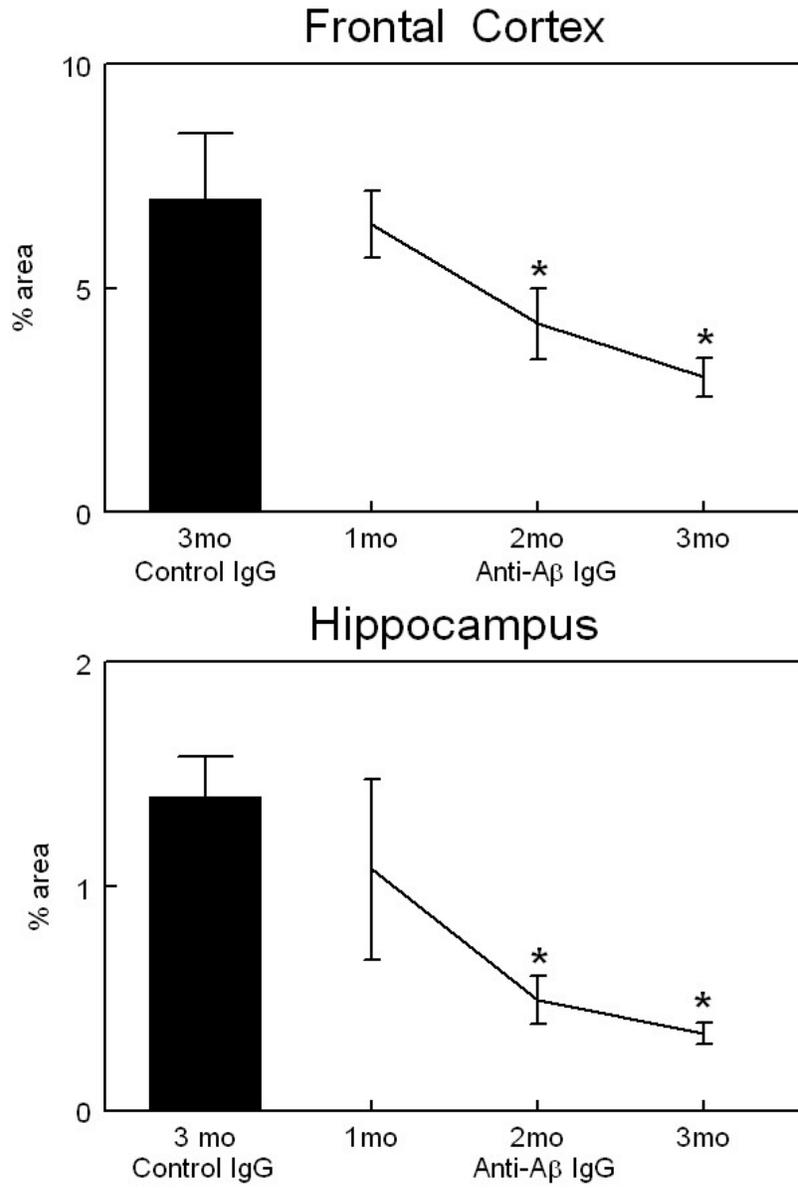
APPENDIX B (continued)



APPENDIX C

Figure 1: Phospho-p38 MAPK expression is decreased in microglia with increasing duration of anti-A β antibody treatment. The upper graph shows data for frontal cortex while the lower graph shows data for the hippocampus. All data are shown as percent area occupied by positive stain. The bar indicates values for APP transgenic mice treated with control antibody anti-AMN for three months. The line indicates values for APP transgenic mice receiving anti-A β_{28-40} antibodies for 1, 2 and 3 months. *indicates $P < 0.05$.

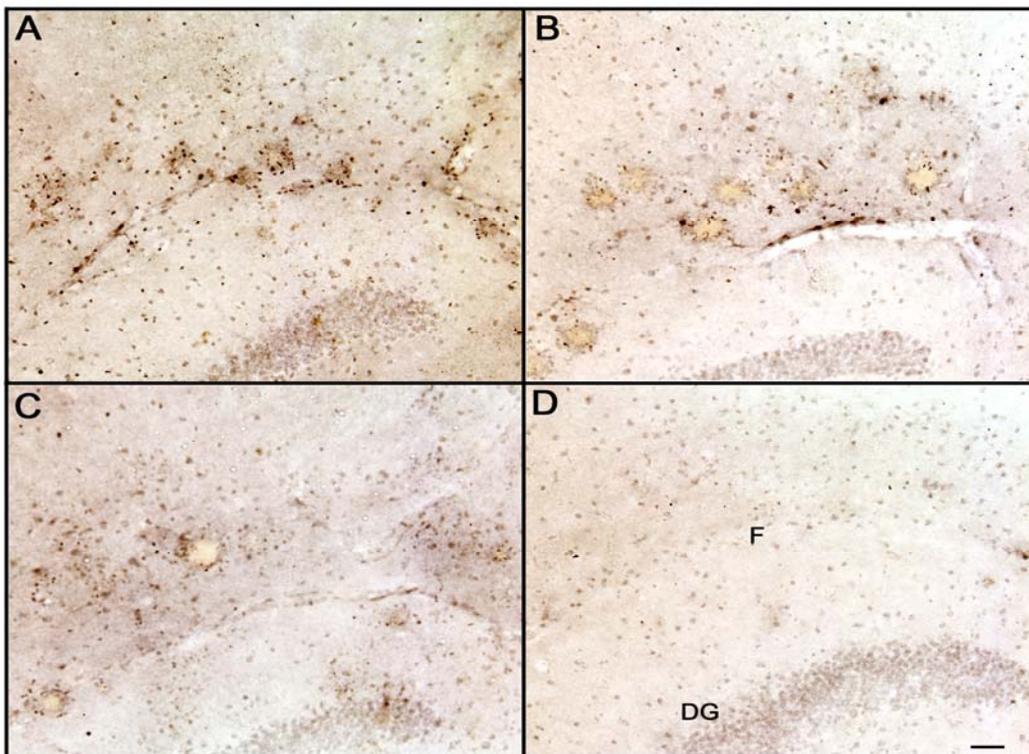
APPENDIX C (continued)



APPENDIX C (continued)

Figure 2: Phospho-p38 MAPK immunohistochemistry shows microglial expression around amyloid plaques which is decreased with increasing duration of anti-A β antibody treatment. Panels A-D show phospho-p38 MAPK immunohistochemical staining around the hippocampal fissure (F in panel D) and in the dentate gyrus (DG in panel D). APP transgenic mice were treated for 3 months with control IgG (A) or with anti-A β_{28-40} IgG for one (B), two (C) or three (D) months. Magnification = 100X. Scale bar panel D = 50 μ m.

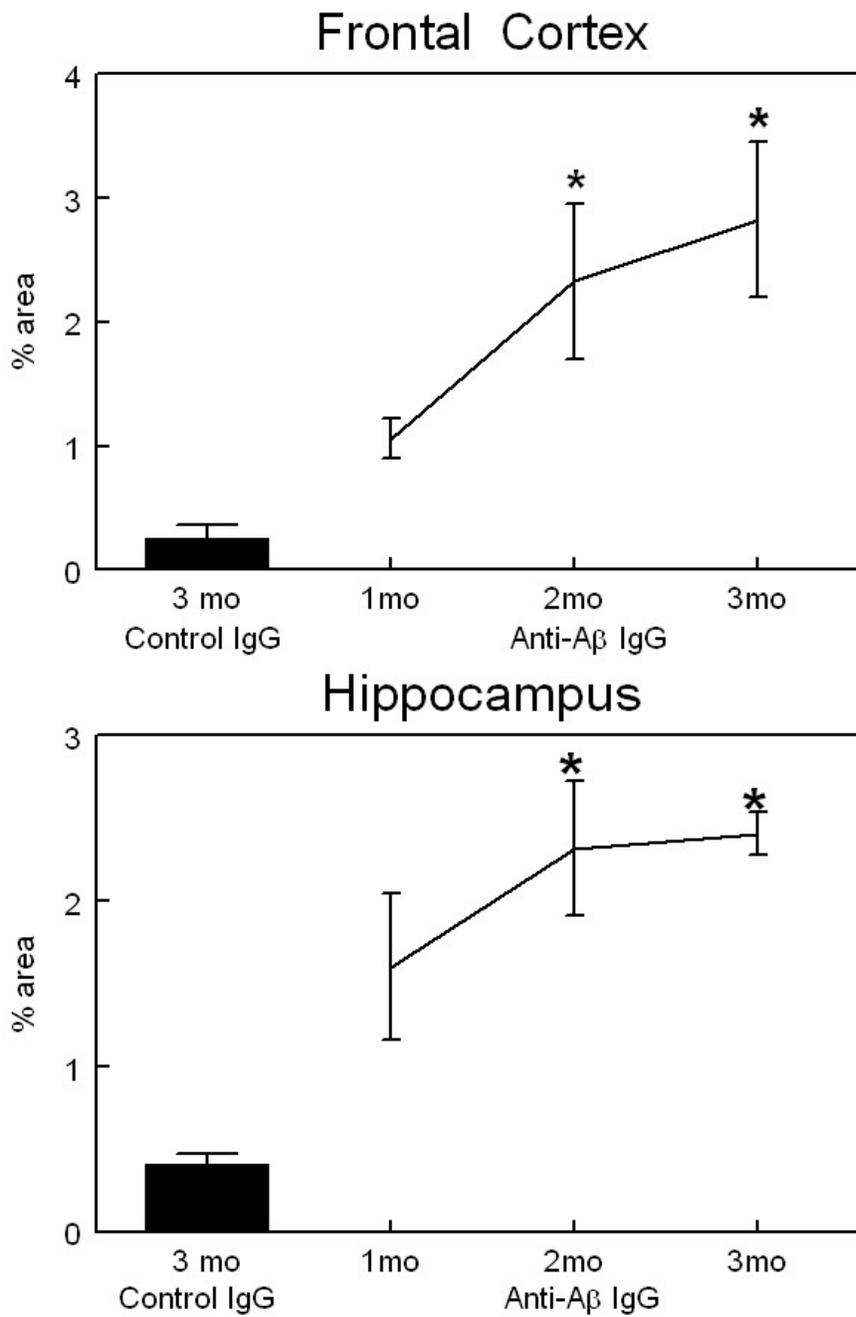
APPENDIX C (continued)



APPENDIX C (continued)

Figure 3: Phospho-p44/42 MAPK expression is increased in microglia with increasing duration of anti-A β antibody treatment. The upper graph shows data for frontal cortex while the lower graph shows data for the hippocampus. All data are shown as percent area occupied by positive stain. The bar indicates values for APP transgenic mice treated with control antibody anti-AMN for three months. The line indicates values for APP transgenic mice receiving anti-A β_{28-40} antibodies for 1, 2 and 3 months. *indicates P<0.05 when compared to control IgG treated animals.

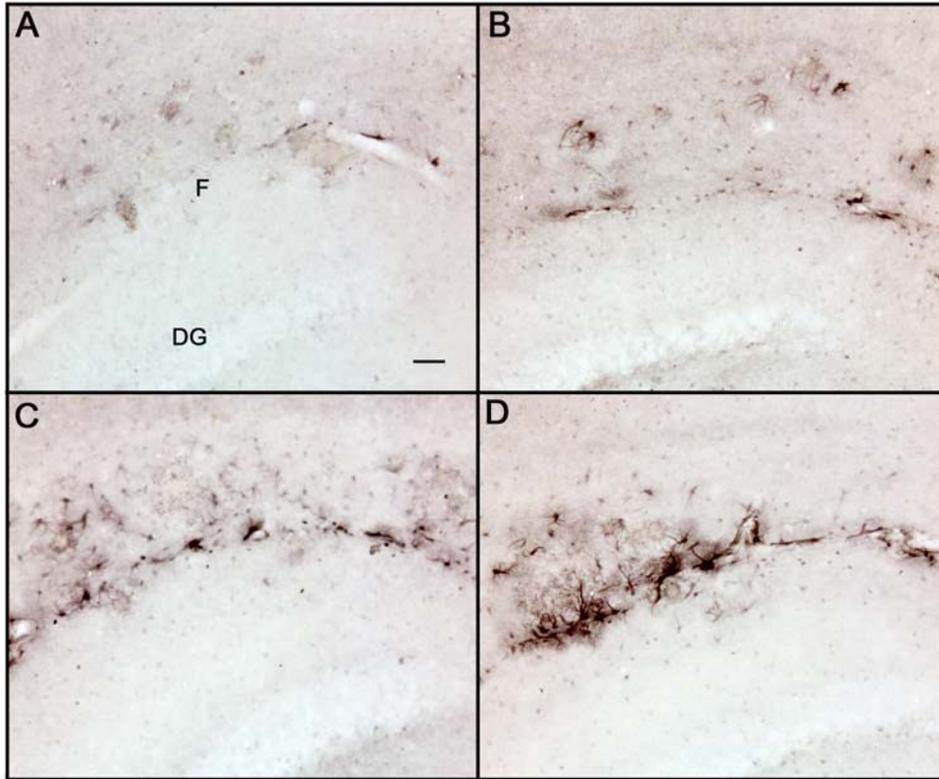
APPENDIX C (continued)



APPENDIX C (continued)

Figure 4: Phospho-p44/42 MAPK immunohistochemistry shows microglial expression around amyloid plaques which is increased with increasing duration of anti-A β antibody treatment. Panels A-D show phospho-p44/42 MAPK immunohistochemical staining around the hippocampal fissure (F in panel A) and in the dentate gyrus (DG in panel A). APP transgenic mice were treated for 3 months with control IgG (A) or with anti-A β_{28-40} IgG for one (B), two (C) or three (D) months. Magnification = 100X. Scale bar panel A = 50 μ m.

APPENDIX C (continued)



ABOUT THE AUTHOR

Donna M Wilcock received her Bachelor's degree in Pharmacology from Cardiff University in the United Kingdom in 1999 and her Master's degree in Medical Sciences from the University of South Florida in 2003. During her undergraduate years she worked in the laboratory of Dr. John Davies studying epilepsy and also completed her undergraduate thesis under Dr. John Wilson studying left ventricular hypertrophy. Donna worked as a research technician for Drs. Dave Morgan and Marcia N. Gordon in the Alzheimer's research laboratory at the University of South Florida following the completion of her Bachelor's degree where she was involved in animal husbandry and processing mouse brain tissue. Donna started Graduate school in the fall of 2001 at the University of South Florida and pursued her Ph.D. work under the tutelage of Dr. Dave Morgan studying immunotherapy for the treatment of Alzheimer's disease. She successfully defended her doctoral dissertation in January 2005 at the University of South Florida.