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Complement C3 and C4 expression in C1q sufficient and deficient mouse models of Alzheimer's Disease

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Abstract

Alzheimer's Disease (AD) is a neurodegenerative disease resulting in progressive cognitive decline. Amyloid plaque deposits consisting specifically of A β peptides that have formed fibrils displaying β -pleated sheet conformation are associated with activated microglia and astrocytes, are colocalized with C1q and other complement activation products, and appear at the time of cognitive decline in AD. APP transgenic mouse models of AD, that lack the ability to activate the classical complement pathway display less neuropathology than do the APPQ^{+/+} mice, consistent with the hypothesis that complement activation and the resultant inflammation may play a role in the pathogenesis of AD. Further investigation of the presence of complement proteins C3 and C4 in the brain of these mice demonstrate that both C3 and C4 deposition increase with age in APPQ^{+/+} transgenic mice, as expected with the age-dependent increase in fibrillar A β deposition. In addition, while C4 is predominantly localized on the plaques and/or associated with oligodendrocytes in APPQ^{+/+} mice, little C4 is detected in APPQ^{-/-} brains consistent with a lack of classical complement pathway activation due to the absence of C1q in these mice. In contrast, plaque and cell associated C3 immunoreactivity is seen in both animal models and, surprisingly, is higher in APPQ^{-/-} than in APPQ^{+/+} mice, providing evidence for alternative pathway activation. The unexpected increase in C3 levels in the APPQ^{-/-} mice coincident with decreased neuropathology provides support for the hypothesis that complement can mediate protective events as well as detrimental events in this disease. Finally, induced expression of C3 in a subset of astrocytes suggests the existence of differential activation states of these cells.

Keywords

complement; C3; C4; transgenic models; A β plaques; Alzheimer's Disease

Introduction

Complement proteins of both the classical and alternative pathways (such as C1q, C4, C3, and Factor B) have been colocalized with fibrillar amyloid plaques and cerebral vascular amyloid in the cerebral cortex and hippocampus of AD patients (Strohmeyer *et al.* 2000; Eikelenboom and Stam 1984; Stoltzner *et al.* 2000). The C5b-9 membrane attack complex has been found associated with myelin and membranes in AD brain (Webster *et al.*

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1997), demonstrating that in this disorder the entire complement cascade is activated. *In vitro*, A β fibrils (fA β) activate both the classical complement pathway by directly binding to C1q (Rogers *et al.* 1992; Jiang *et al.* 1994) and the alternative pathway via interactions with C3 (Bradt *et al.* 1998; Watson *et al.* 1997). Thus, it was hypothesized that *in vivo* fA β activates the complement cascade and contributes to local inflammation, particularly by recruiting glia into the area of the plaque, resulting in neurotoxicity and dementia (Tenner 2001; Cooper *et al.* 2000; Eikelenboom and Veerhuis 1996). This hypothesis was examined and supported in subsequent *in vivo* studies using a mouse model of AD with a complete deficiency of the complement protein C1q (APPQ $^{-/-}$) and thus unable to activate the classical complement pathway. While this APP C1q $^{-/-}$ transgenic mouse demonstrated age-dependent amyloid plaque deposition, there was a 50–60% reduction glial activation (GFAP, MAC-1) surrounding the plaques and a similar significant increase in neuronal markers in the CA3 region of the hippocampus (Fonseca *et al.* 2004). These data suggest that, at ages when the fibrillar plaque pathology is present, C1q contributes a detrimental effect on neuronal integrity, most likely through the activation of the classical complement cascade and the enhancement of inflammation. However, the source of the residual pathology remains unknown. Potential mechanisms include a complement-independent pathway and/or the activation of the alternative pathway of complement by A β , a process which would be unaltered by the deficiency of C1q and which would also lead to the generation of the chemotactic factors, C3a and C5a and recruitment of glia to the plaques.

Interestingly, in another murine model the over-expression of an inhibitor of complement C3, Crry, resulted in increased pathology, suggesting that some complement activation fragments (such as C3b, C3a or C5a) may decrease the neuropathology in mouse models of inflammation including those over expressing mutant APP (Wyss-Coray *et al.* 2002; Mukherjee and Pasinetti 2000) or limit the detrimental responses to neurodegenerative stimuli in other injury models (Van Beek *et al.* 2003). In addition, recent *in vitro* studies from this lab demonstrated a neuroprotective effect of C1q on primary neurons in culture in the absence of any other complement components (Pisalyaput and Tenner 2008). Thus, complement components may also be neuroprotective.

To further investigate complement protein expression in our murine models of AD, immunohistochemistry followed by quantitative image analysis and western blot analysis were used to assess the presence and localization of C3 and C4 in the brains of these mice using antibodies that recognize the murine C4 and that differentiate native C3 and C3 cleaved as a result of activation of the complement cascade.

Material and methods

Animals

Tg (HuAPP605.K670N-M671L)2576 mice from K. Hsiao-Ashe (Hsiao *et al.* 1996) were crossed with C1q knockout mice (C1qa $^{-/-}$) (Botto *et al.* 1998), and mice with APPQ $^{-/-}$ genotype were generated (Fonseca *et al.* 2004). Non-transgenic littermates or B6/SJL wild type mice were used as age-matched controls. APPPSQ $^{-/-}$ animals were obtained by crossing Tg2576 APP or PS1 (line6.2 on a SW/B6D2F1/J background from University of South Florida) (Holcomb *et al.* 1998) with C1q $^{-/-}$. APPQ $^{+/-}$ or APPQ $^{-/-}$ and/or PSQ $^{-/-}$ mice were intercrossed until APPPSQ $^{+/+}$ and APPPSQ $^{-/-}$ were generated. (All genotypes were confirmed by PCR.)

Tissue collection and immunohistochemistry

Mice at different ages (3, 6, 9, 12, 16 months) were deeply anesthetized with an overdose of pentobarbital (150mg/kg, IP) and then transcardially perfused with cold phosphate-buffered

saline (PBS). After dissection, one half of the brain was immediately frozen on dry ice (for Western blots) and the other half fixed overnight with 4% paraformaldehyde in PBS, pH 7.4. Thereafter, fixed tissue was stored in PBS/0.02% Sodium azide (NaN_3) at 4°C until use. Fixed brain tissue was sectioned (40 μm) with a vibratome, and coronal sections were collected in PBS (containing 0.02% sodium azide), and stored at 4°C before being stained.

Immunohistochemistry (IHC) was performed on free-floating brain sections. Sections were pretreated with 50% formic acid for 5 min (to enhance subsequent staining of β amyloid) or microwaved (700w) in antigen unmasking solution (Vector Laboratories, Burlingame, CA) (for C3, C4 and CNPase antigens) for 2~5 min. Endogenous peroxidase in tissue was blocked by treating with 3% H_2O_2 in PBS, 20 min. at room temperature. Nonspecific background staining was blocked by a 2 hour incubation in 2% BSA with 0.3% Triton X-100 (TX). Sections were then incubated with primary antibodies (Table 1) overnight at 4°C, rinsed in PBS with 0.1%TX and incubated with biotinylated secondary antibody (Vector) and streptavidin-horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) for 1 hour each at room temperature. Finally, the sections were incubated for approximately 2~5 min with diamino-benzidine (DAB) (Vector). Sections were mounted on slides, dehydrated in a series of graded ethanol, cleared with xylene, and then coverslipped with DPX (BHD, Biomedical Specialties, CA). In double-labeling experiments, bound antibodies were detected using Fab'2 FITC-, CY3-, or TRITC-conjugated anti-IgG (Jackson ImmunoResearch, PA). As controls, sections were incubated in parallel without primary antibody or with control IgG of the corresponding species and these sections failed to develop specific staining.

Image analysis

Immunostaining was observed under a Zeiss Axiovert-200 inverted microscope (Carl Zeiss, Thornwood NY) and images acquired with a Zeiss AxioCam high-resolution digital color camera (1300 \times 1030 pixel) using Axiovision 3.1 software. Digital images were analyzed using KS300 analysis program (Zeiss). Percentage of immunostained area (field area of immunostaining/total image area \times 100) was determined for all the markers studied by averaging several images per section that cover all or most of the region of study (cortex and hippocampus). Optical sections ($z=1\mu\text{m}$) of fluorescently labeled specimens were captured using a FLUOVIEW confocal Microscope (Olympus). All experiments were repeated at least twice, with $n=3-6$ animals per group per age per marker. All quantitative comparisons were performed on sections processed at the same time.

Western Blot

Tissue samples were prepared as previously indicated (Fonseca *et al.* 2004). Briefly, fresh frozen half brain (including only cortex and hippocampus) was homogenized in 10 volumes of Tris-buffered saline (TBS, pH 7.4) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 $\mu\text{g}/\text{ml}$ aprotinin, leupeptin and pepstatin and 1mM EDTA; all inhibitors obtained from Sigma). Homogenates were briefly sonicated and centrifuged at 15,000 g for 30 min. Pellets were extracted with 2% SDS in TBS with protein inhibitors and centrifuged at 15,000g for 30 min. Protein concentration in the supernatants was determined with BCA protein assay (Pierce, Rockford, IL). Samples (30 μg protein per lane) were run on 10% SDS polyacrylamide gel under reducing conditions (100 mM DTT). Proteins were transferred to PDVF (Amersham Biosciences, Piscataway, NJ) (300 mA for 2 h). Membranes were blocked with 3% dry milk in 0.1% Tween/TBS overnight, then incubated with primary antibodies for 2 h at RT at the dilutions indicated (Table 1). After washing, blots were incubated with the corresponding HRP-labeled secondary antibodies (1:2000 – 1:5000 dilution) for 1 h. Labeling was detected using ECL system (Amersham Biosciences). Blots were stripped following manufacturer's instructions (Amersham) and

subsequently labeled with actin antibody (1:10,000 Sigma) following same procedures as above to verify equal loading.

Statistical analysis

Single ANOVA statistical analysis was used to assess the significance of the differences in plaque area, and anti C3 and anti C4 reactivity among the animal groups (B6/SJL, APPQ^{+/+} and APPQ^{-/-}). Standard deviation (S.D.) of the mean is indicated by the error bars on the graphs.

Results

C1q deposition is age-dependent in APP mice brain

As observed in AD brain, C1q immunoreactivity was seen associated with thioflavine-positive A β plaques only in the Tg2576 animals (Fonseca *et al.* 2004) and APP/PS mice models (Matsuoka *et al.* 2001). To verify and expand these results, brain tissues from APP transgenic mice with (APPQ^{+/+}) and without a functional C1q gene (APPQ^{-/-}) and the nontransgenic control, B6/SJL, were assessed for detectable C1q at 3, 6, 9, 12, and 16 months of age by immunohistochemical analysis. C1q immunoreactivity quantitatively increases with age and amyloid plaque deposition, as shown in Figure 1A for the Tg2576 (APPQ^{+/+}) model. No C1q staining was seen in APPQ^{-/-} animals at any age ((Fonseca *et al.* 2004) and Figure 1A) nor in the B6/SJL nontransgenic animal (data not shown). Western blot analysis on SDS-extracted brain tissue using a polyclonal anti-mouse C1q confirmed the absence of C1q in the APPQ^{-/-} mice homozygous for the ablated C1q gene (Figure 1B).

C4 deposition correlates with age in APPQ^{+/+} transgenic mice but not APPQ^{-/-}

Brain sections from mice at 6, 9, 12 and 16 months of age, were then stained with a monoclonal anti-mouse C4 (HBT) that recognizes various forms of C4 α -chain via an epitope in the C4d region of the molecule. (The C4d region contains the thioester which, upon activation of complement and cleavage of C4 by activated C1, can mediate covalent linkage to the activator.) Distinct C4 immunoreactivity was first detected in the APPQ^{+/+} mice at 12 months, and this reactivity was increased in 16 month old mice, temporally correlating with the appearance of C1q associated with thioflavine-positive plaques (Figure 1A, 2A, 2C). In contrast, at 12 and 16 months little to no C4 immunoreactivity was seen in APPQ^{-/-} mice. Image analysis of the 16 mo animals results in average % field area of 0, 1.2 and 0.15 for B6/SJL (wild type), APPQ^{+/+} and APPQ^{-/-} respectively (Figure 2C). Results in 16 mo APPPS1Q^{+/+} vs APPPS1Q^{-/-} mice also show the lack of immunoreactivity for C4 in APPPS1Q^{-/-} confirming the lack of classical pathway activation in the C1q^{-/-} mice (Figure 2B).

Western blot analysis was used as an alternative approach to assess C4 levels in extracts from brain tissue of 16 mo old mice. Using both a polyclonal anti-murine C4 antibody and the same monoclonal antibody against C4d used in the immunohistochemical investigations above, uncleaved C4 was detected in the nontransgenic B6/SJL mice, the APPQ^{+/+} and the APPQ^{-/-} animals (data not shown). However, the 42,000 Mr activation-induced cleavage fragment of C4, C4d, was clearly present at increased levels in the APPQ^{+/+} brain extracts relative to the nontransgenic and APPC1q^{-/-} mice (Figure 2D and data not shown). These observations are consistent with the requirement for C1q in C1 to induce cleavage of C4 (and thus allow deposition of C4b/d on plaques).

Some C4d immunoreactivity is seen in extracts of brains of 16 mo nontransgenic mice (Figure 2D), data suggesting an amyloid-independent baseline level of this complement

fragment since no amyloid deposits are present in the nontransgenic animals (data not shown). In addition, levels of C4d in the APPQ^{-/-} mice were similar to the nontransgenic mice, levels that may be due to amyloid-independent baseline fragment deposition, similar to the non-transgenic mice. C4d staining is not seen in the brain vasculature in any of the animals studied. However, it is also possible that some C4d in the APPQ^{-/-} (that is clustered as seen in Figure 2A) may be the result of activation of the contact system by β -amyloid as previously seen in *in vitro* systems (Bergamaschini *et al.* 1999).

C4 Immunoreactivity is associated with plaques and oligodendrocytes

C4 reactivity was localized with amyloid plaques and cellular structures surrounding the plaque. To identify the C4 labeled structures, sections were double labeled with antibodies specific for astrocytes, microglia and oligodendrocytes as well as thioflavine to assess plaque associated C4. C4 was not associated specifically with astrocytes (Figure 3A) or microglia (Figure 3B) alone, but was found in thioflavine positive plaque areas (data not shown) containing microglia and surrounded by astrocytes (Figure 3A and B). Confocal microscopy following double labeling for C4 and CPNase, an oligodendrocyte specific marker, demonstrated cell-associated C4 reactivity colocalized with oligodendrocytes. The C4 labeled oligodendrocytes were predominantly found in the APPQ^{+/+} animals (Figure 4, top panel), but some immunopositive cells are present in sections from APPQ^{-/-} animals (Figure 4, bottom panel). There was no C4 detected in association with oligodendrocytes in age matched wild type (B6/SJL) controls.

C3 reactivity is greater in APPQ^{-/-} than in APPQ^{+/+} mice

We previously reported that while both astrocytic and microglia accumulation and loss of neuronal markers was significantly reduced in APPQ^{-/-} animals, some gliosis and loss of synaptophysin and MAP2 in the CA3 region remained, relative to nontransgenic animals. This observed pathology could be due to either a complement-independent pathway or activation of the alternative pathway of complement by β -amyloid, as reported by Cooper and colleagues *in vitro* (Bradt *et al.* 1998). Such alternative pathway activation would be unaltered by the deficiency of C1q. If β -amyloid activation of the alternative pathway was occurring in the APPQ^{-/-} brain, C3 could be covalently linked to the plaques via activation exposed thioester (Bradt *et al.* 1998). Quantitative image analysis of immunopositive staining with anti-murine C3 showed C3 reactivity in all APP mice starting at 12 mo, but was surprisingly significantly elevated in APPQ^{-/-} relative to APPQ^{+/+} at 16 mo. Interestingly, this was detected with three distinct anti-C3 monoclonal antibodies (Figure 5A–D), one of which detects full length, native C3 (HBT anti-C3), while anti-C3 monoclonal antibodies 2/16 and 2/11 detects only activated (cleaved) C3 (Mastellos *et al.* 2004).

In APP mice native C3 is upregulated in astrocytes whereas C3 activation cleavage fragment is plaque associated

Morphologically, the staining of these antibodies differed (Figure 5A, 5C) suggesting native and activated C3 associated with different structures. Confocal microscopy imaging of immunofluorescent double labeling demonstrated that native C3 (HBT, Netherlands) was colocalized with the astrocyte marker GFAP (Figure 6A & 6B). In contrast, anti-C3 monoclonal antibodies to activated C3 (2/11 and 2/16) were associated with thioflavine positive plaques (Figure 7 and data not shown). Finally, using a polyclonal anti-C3 (Cappel), Western blot analysis of brain homogenates confirmed the increase in native C3 (C3 α -chain) in APPQ^{-/-} animals relative to the APPQ^{+/+} (Figure 5E), and further verified the identification of C3 in the brains of these transgenic animals. Thus, the data accordingly suggest both alternative pathway activation by amyloid plaques and an upregulation of C3 synthesis in astrocytes (consistent with reports of others assessing C3 synthesis in cultured

astrocytes in response to injury (Levi-Strauss and Mallat 1987; Rus *et al.* 1992)). This C3 expression appears to be more robust in the classical complement pathway deficient mouse (APPQ^{-/-}) than in the complement sufficient APP mice.

Discussion

In the C1q-deficient Tg2576 APP transgenic model of amyloid pathology, the decreased level of inflammatory glial cells and the reduced loss of neuronal integrity in CA3 region of the hippocampus (while levels of both total and fibrillar amyloid deposits remain unchanged) suggested a detrimental role for the complement cascade in this neurodegenerative disease (Fonseca *et al.* 2004). Here we show that while complement activation by the classical pathway was clearly absent in brain of these Tg2576 C1q^{-/-} animals (Figure 1 and 2), fibrillar plaque associated C3b/iC3b, the cleavage product of all three complement activating cascades, was found to be elevated in the classical pathway deficient animals (Figure 5). Since C4 deposition, which could result from either classical or lectin pathway activation, was reduced to baseline levels, this C3 deposition could not be the result of lectin pathway activation. These data are consistent with the activation of the alternative pathway of complement by β -sheet fibrillar amyloid deposits, providing the *in vivo* correlate of the *in vitro* A β activation of the alternative complement pathway (Bradt *et al.* 1998; Watson *et al.* 1997). However, the diminished glial activation and protection of neuronal markers in the C1q-deficient animals indicates that alternative pathway activation does not fully compensate for the lack of classical pathway activation in terms of detrimental consequences. This leads to the question of whether the remaining plaque associated glial activation and/or loss of synaptophysin and MAP2 seen in the C1q^{-/-} Tg2576 mice is due to the activation of the alternative complement pathway and/or to complement-independent events with the activated alternative pathway lacking a detrimental function in this system.

It is important to appreciate that in the absence of C4 deposition (via its thioester), additional “plaque-associated” sites for C3 thioester binding are available and thus the amount of C3 bound to plaques may not quantitatively correlate with the absolute amount of complement activation between these two genotypic models. As a result of this, and given the additional fact that any C3 associated with plaques is long lived due to its covalent nature, deposition of C3 activation fragments may be only loosely correlated with the concentration of C5a, C3a, and/or C5b-9 generated at any given time. Thus, the partial but substantial protection observed in the absence of classical pathway activation (Fonseca *et al.* 2004) that occurs even when C3 is present on plaques may reflect a difference in the kinetics and/or the extent of complement activation and consequent generation of effector molecules in these models.

While a detrimental role of complement in neurodegeneration has been demonstrated in this and other studies, there is evidence for beneficial effects arising from complement activity which may limit the detrimental responses to neurodegenerative stimuli in certain injury models (Pasinetti *et al.* 1996; Rus *et al.* 2005) as reviewed in (Tenner and Pisalyaput 2008). The complement activation products C3a (Van Beek *et al.* 2001; Boos *et al.* 2004; Boos *et al.* 2005) and C5a (Osaka *et al.* 1999; Mukherjee and Pasinetti 2000; Farkas *et al.* 2003; van *et al.* 2003; Sewell *et al.* 2004; Morgan *et al.* 2004; Reiman *et al.* 2005; Woodruff *et al.* 2006) have been reported to have both protective and detrimental effects in the CNS. Cleavage of C3 in APPQ^{-/-} mice, presumably via alternative pathway activation, and the generation of C3a and C3b may result in neuroprotective activities, thereby contributing to the decreased pathology observed in the APPQ^{-/-} mice in comparison to APP mice. This hypothesis is consistent with the increased pathology observed following inhibition of C3 (Wyss-Coray *et al.* 2002) by over-expressing Crry, an inhibitor of C3 cleavage and thus also of further downstream complement activation events. These authors suggested a protective role for

complement activation, perhaps via the generation of C3b and its opsonizing activity which may contribute to amyloid clearance (Wyss-Coray *et al.* 2002). If C5a, which is chemotactic for microglia and astrocytes, is generated via the alternative pathway C5 convertase, glia would presumably be recruited into the area of the complement activating fibrillar plaques. However, the influence of C5a in neurodegeneration in AD models is still under debate. Evidence of terminal complement pathway cleavage of C5 and thus generation of C5a and the C5b-9 has not yet been demonstrated in these models. Indeed, the presence of C5a alone in the brain does not necessarily induce or exacerbate inflammation (Reiman *et al.* 2005). While a direct measure of C5a generation in the brain is problematic (particularly post mortem) as the molecule is short-lived, future investigation of the role of C5a in the progression of disease in these animal models could be approached using C5a receptor antagonists or investigating the effect of a deficiency in either of the C5a receptors (CD88 or the more recently described C5L2).

In another model of neurodegeneration, Fan, et al. demonstrated C1q, C3 and C4 were elevated and localized to areas with fibrillar amyloid deposits in a mouse model of cerebral amyloid angiopathy (Tg-SwDI) (Fan *et al.* 2007). These results are consistent with the data presented here and with the possibility of a common mechanism of neurotoxicity based on A β dependent complement-mediated inflammation and/or neurotoxicity which can result in cognitive loss modeled in each of these systems. This group demonstrated a 2- and 5-fold upregulation of C3 and C1q mRNA, respectively, in the amyloid rich thalamic region of the brains from these transgenics (not in wild type animals). Interestingly, however, in the Fan report, cellular associated C3 immunoreactivity was exclusively localized to microglia (Fan *et al.* 2007), in contrast to the astrocyte localization in the present study. While it is known that C1q and C3 can be synthesized by neurons, microglia, and astrocytes in the injured brain, the factors involved in the cell and tissue specific regulation of that induced expression are currently an area of active investigation, and ultimately should shed light on the basis for the different apparent cellular sites of synthesis suggested in these studies.

Although C1q plays a central role in the activation of the classical complement cascade, this protein alone has been shown to have multiple effects in disease and homeostasis. For example, it is known that C1q enhances phagocytosis (Botto *et al.* 1998; Mitchell *et al.* 1999; Bobak *et al.* 1987) (and reviewed in (Bohlsón *et al.* 2007)), down regulates the production of proinflammatory molecules in myeloid cells (Fraser *et al.* 2006; Fraser *et al.* 2007; Nauta *et al.* 2004; Yamada *et al.* 2004), and *in vitro* has a direct neuroprotective effect on injured neurons (Pisalyaput and Tenner 2008). Neuronal synthesis of C1q has been seen in several injury models (Spielman *et al.* 2002; Thomas *et al.* 2000; Fan and Tenner 2005; Rozovsky *et al.* 1994; Shen *et al.* 1997) often in the absence of detected induction of other C1 subcomponents C1r and C1s (Veerhuis *et al.* 1999). These data are consistent with a protective role for C1q in early stages of neuronal injury by mediating the rapid clearance of apoptotic neurons and synaptic debris and/or suppressing the progression of an inflammatory state, hypotheses which are currently under investigation. While clearly more data is needed, if the absence of C1q leads to less modulation (ie higher expression) of proinflammatory gene expression in the brain, the lack of C1q may be the reason that C3 expression in astrocytes is elevated in the APPQ $^{-/-}$ animals.

Upon induction of synthesis of the remaining components of the cascade and the emergence of complement activators (such as fibrillar amyloid plaques in AD (Afagh *et al.* 1996; Cummings *et al.* 1996), or amyloid containing drusen in age related macular degeneration (Johnson *et al.* 2002) or other misfolded or aggregated proteins such as in other dementias (Rostagno *et al.* 2002)), complement activation could occur and contribute to progression of disease. Indeed, synthesis of most complement factors occurs within the AD brain (Johnson *et al.* 1992; Shen *et al.* 1997), and also in “normal” aged brain to a lesser

extent (Walker and McGeer 1992). Receptors for complement activation products C5a and C3a are on neurons as well as on microglia and astrocytes [reviewed in (Nataf *et al.* 1999)], and signature markers (C1q, C4b, C3b/iC3b, C5b-9) indicate that complement activation does occur in the AD brain (Eikelenboom and Stam 1982; Webster *et al.* 1997; Loeffler *et al.* 2008) (and data presented here). The consequences of this activation are the generation of C3b, which may facilitate phagocytosis or result in bystander damage due to release of toxic enzymes and oxidative radicals from activated phagocytes [“frustrated phagocytosis”] (Gardiner *et al.* 1994). C5a is also generated, resulting in recruitment of phagocytic cells to the plaque area and these cells may then be activated by plaque components. Finally, generation of the C5b-9 complex can occur and cause cell lysis if present in high enough concentration or if the expression of the host membrane inhibitor, CD59, is decreased (Yang *et al.* 2000). However, sublytic C5b-9 has also been shown to be neuroprotective, perhaps via its anti apoptotic effects on oligodendrocytes (Rus *et al.* 2006). The importance of the balance between activation of complement and the effective regulation of the cascade in neurodegenerative disorders has been most recently evident in the association of specific single nucleotide polymorphisms in complement components C3, C2, Factor B, and Factor H, the latter a complement regulator protein, which together have been shown to contribute >80% of the risk of developing aged related macular degeneration (Hageman *et al.* 2005; Gold *et al.* 2006; Maller *et al.* 2007). Clearly, both the nature of the activators and the local environment also influence development of disease, as these degenerative processes are not seen in all tissues and/or ages.

While the kinetics of all these processes may differ in genetically diverse individuals as well as in the different genetic backgrounds of model mouse strains, the variety of contributing factors may also provide multiple and/or specific targets for regulation of these disease processes both in AD and other neurodegenerative diseases. Given the number of activities as well as regulators of the complement effector system, much more is to be learned of the balance/imbalance that influences health and disease. An recent example of novel functions for these proteins is the interesting data reported by Stevens and colleagues that demonstrates a critical role for C1q and C3 in proper synapse pruning during development but a possible detrimental role in glaucoma (Stevens *et al.* 2007).

Therapeutic interventions in murine models of Alzheimer’s Disease that target inflammation and oxidative stress have proven successful in reducing amyloid plaque burden/pathology as well as the proinflammatory cytokine IL-1 β and indicators of oxidative stress (Yao *et al.* 2004). If complement activation contributes significantly to the inflammation and subsequent loss of cognitive function, then a specific inhibitor of the C1q interaction site **on the activator** would be valuable as it would block the pathogenic activation but should not affect the systemic protective functions of the complement system. However, if the early complement activation products (C3b, C3a) have beneficial consequences (such as enhanced clearance and promotion of neurogenesis (Rahpeymai *et al.* 2006)), an inhibitor of the complement system downstream of these events (such as C5a generation or interaction with cellular receptors) or the upregulation of the beneficial activities, would be optimal (Figure 8). Such therapeutic interventions should be particularly beneficial for individuals entering what has been called the “catastrophic” phase of AD, after cognitive dysfunction becomes clinically apparent, the rate of decline appears to accelerate, and neuritic plaques are observable upon autopsy (Cotman *et al.* 1996). Although multiple potential targets exist for therapeutic intervention in AD, including immunization to prevent accumulation and/or promote clearance of A β (Moore and O’Banion 2002; Zhou *et al.* 2005; Lemere *et al.* 2003; Selkoe and Schenk 2003) and/or inhibition of oligomeric amyloid formation, multi point interventions will likely be beneficial at different stages of the disease, and thus a cocktail of therapeutic reagents may be more successful in delaying CNS degeneration and the cognitive impairment characteristic of AD.

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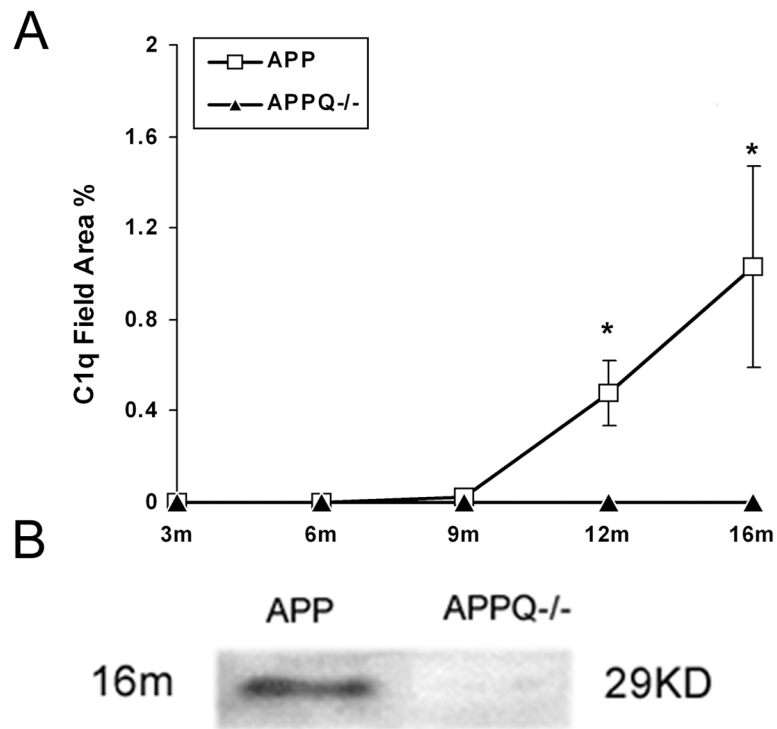


Figure 1. C1q deposition is age-dependent in APP Q^{+/+} mice brain, and absence in APPQ^{-/-} mice

A. Quantification of C1q immunostaining in animals at different ages (3mo–16mo). Data points represent group means \pm SD (n=4 animals per age group except n=6 for 16 months, 5 fields/animal including cortex and hippocampus). 12m and 16m APP mice C1q staining was significantly different from 9 month old animals (*, $p < 0.01$). B. Western blot analysis of 16mo APPQ^{+/+} and APPQ^{-/-} brain extracts using a polyclonal anti-mouse C1q antibody as described in Materials and Methods. The C1q immunoreactive band is only seen in APPQ^{+/+} mice.

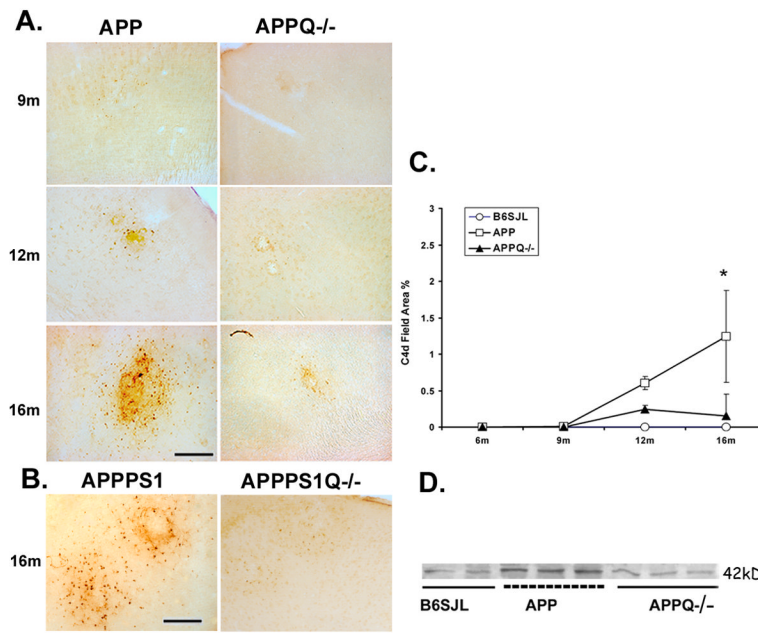


Figure 2. APPQ^{-/-} have less C4 reactivity than APPQ^{+/+} mice

A. Representative pictures of cortex of APPQ^{+/+} (left) and APPQ^{-/-} (right) mice at different ages showing immunostaining with anti-mouse C4 (C4d) (HBT). Scale bar: 50 μ m. B. Sections from cortex of 16 mo old double transgenic APPPS1 Q^{+/+} and APPPS1Q^{-/-} were stained with anti-mouse C4 as in A. C. Quantification of C4 reactivity by image analysis shows that C4d staining in APPQ^{+/+} (-□-) mice is significantly higher (* p<0.02) than in APPQ^{-/-} (-▲-) at 16 mo. (n=3 or 4 mice for each genotype at each age). There was no reactivity in the wild type B6/SJL (-○-). Data points represent group means \pm SD. D. Western blot analysis of brain extracts from 16m B6SJL, APPQ^{+/+} and APPQ^{-/-} mice probed with polyclonal anti-mouse C4 (Ogata, Table 1) detect C4 activation fragment, C4d at 42,000 Mr.

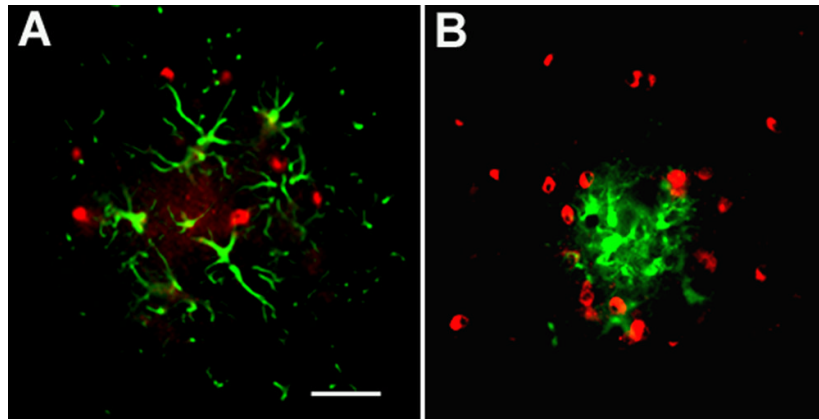


Figure 3. C4 is not associated with astrocytes or microglia

Immunofluorescent double labeling of C4 (red, Ogata anti mouse C4) and either astrocytes (GFAP, green) (A), or microglia (CD45, green) (B) in cortex of 16m APPQ^{+/+} mice. Scale bar: 25 μ m.

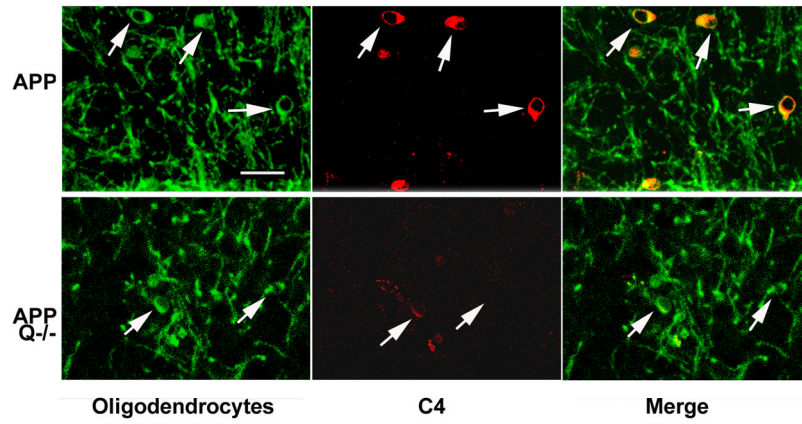


Figure 4. C4 colocalizes with oligodendrocytes in APP transgenic mouse brain

Confocal images in brain cortex show colocalization of C4 (Ogata polyclonal anti-mouse C4, red,) with oligodendrocyte cell bodies (anti-CNPase monoclonal, green) in 16mo APPQ^{+/+} (upper panels). In APPQ^{-/-} (lower panels), C4 immunostaining is lower but also present in oligodendrocyte cell bodies. Right panels are merged confocal images. Scale bar: 25 μ m.

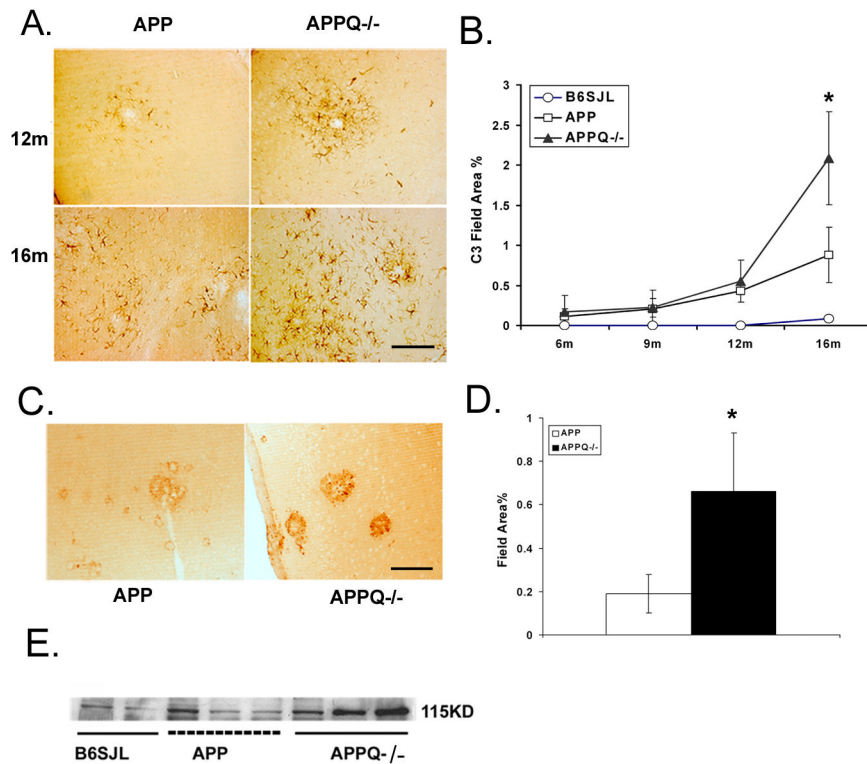


Figure 5. C3 immunoreactivity is greater in APPQ^{-/-} than in APPQ^{+/+}
 Anti-C3 staining (HBT) in cortex of APPQ^{+/+} (left) and APPQ^{-/-} (right) at 12m and 16m shows increased cellular immunoreactivity in APPQ^{-/-} mice. Scale bar: 50 μ m. B. Quantification of C3 immunoreactivity in cortex and hippocampus in APPQ^{+/+} (-□-), APPQ^{-/-} (-▲-) and B6/SJL (-o-) at 6, 9, 12, 16 mo (6m n=3; 9m n=3; 12m n=4; 16m n=4 each genotype) shows significantly higher levels of C3 (anti-C3, HBT) in APPQ^{-/-} than APP at 16 months (* p<0.02). Data points are group means \pm SD. C. Anti-C3c (Lambris 2/16) staining in 16m APPQ^{+/+} (left) and APPQ^{-/-} (right) in plaque-like structures. Scale bar: 50 μ m. D. Image analysis of anti-C3c (2/16) staining. (n=3 animals each genotype; * p < 0.05). Data shown are group means \pm SD. E. Western blot of brain extracts from B6/SJL, APPQ^{+/+} and APPQ^{-/-} at 16 months probed with anti-mouse C3 antibody (Cappel) shows greater reactivity to anti native C3 α chain (115,000 Mr) in the APPQ^{-/-} animals.

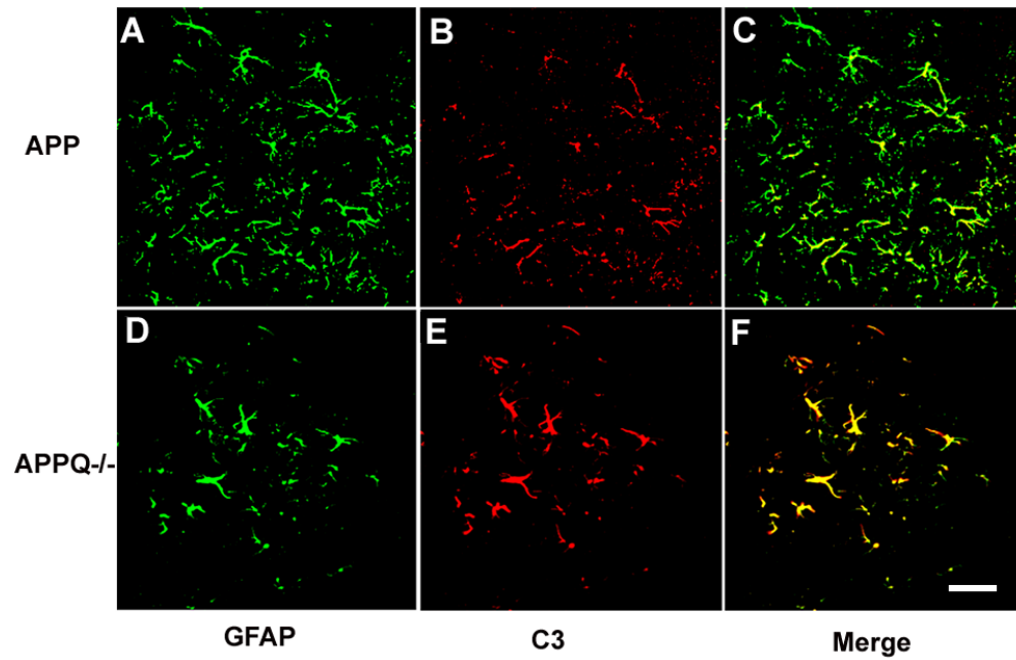


Figure 6. C3 colocalizes with astrocytes in APPQ^{+/+} and APPQ^{-/-}
 Confocal images of GFAP (green) (A,D) and C3 (red, HBT) (B,E) double labeling in cortex of 16 mo APPQ^{+/+} (A,B,C) and APPQ^{-/-} (D,E,F) mice. Note fewer astrocytes surrounding plaques in APPQ^{-/-} mice (D) than in APPQ^{+/+} (A), but the C3 staining appears stronger (E) than in APPQ^{+/+} (B) Merge of GFAP and C3 (C,F) demonstrates that astrocytes are positive for C3. Scale bar: 25 μ m

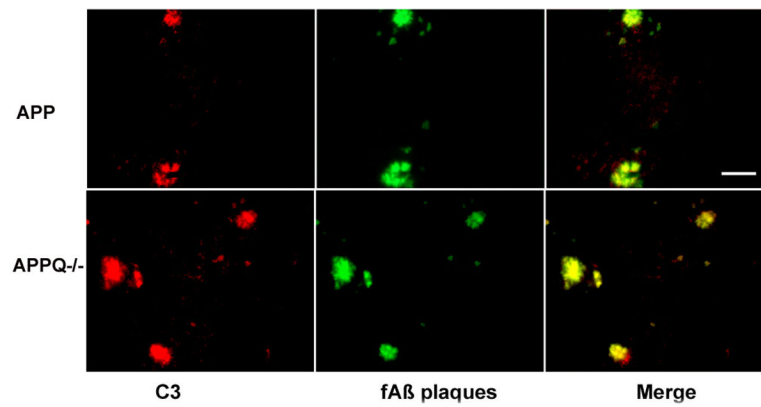


Figure 7. Activated C3 colocalizes with fibrillar amyloid plaques in both APPQ^{+/+} and APPQ^{-/-} mice

Representative pictures of 16 mo APPQ^{+/+} and APPQ^{-/-} sections from cortex that were immunostained with rat anti-mouse C3c (Lambris, 2/11) (red) and with thioflavine (β -sheet structure, green). Scale bar: 25 μ m.

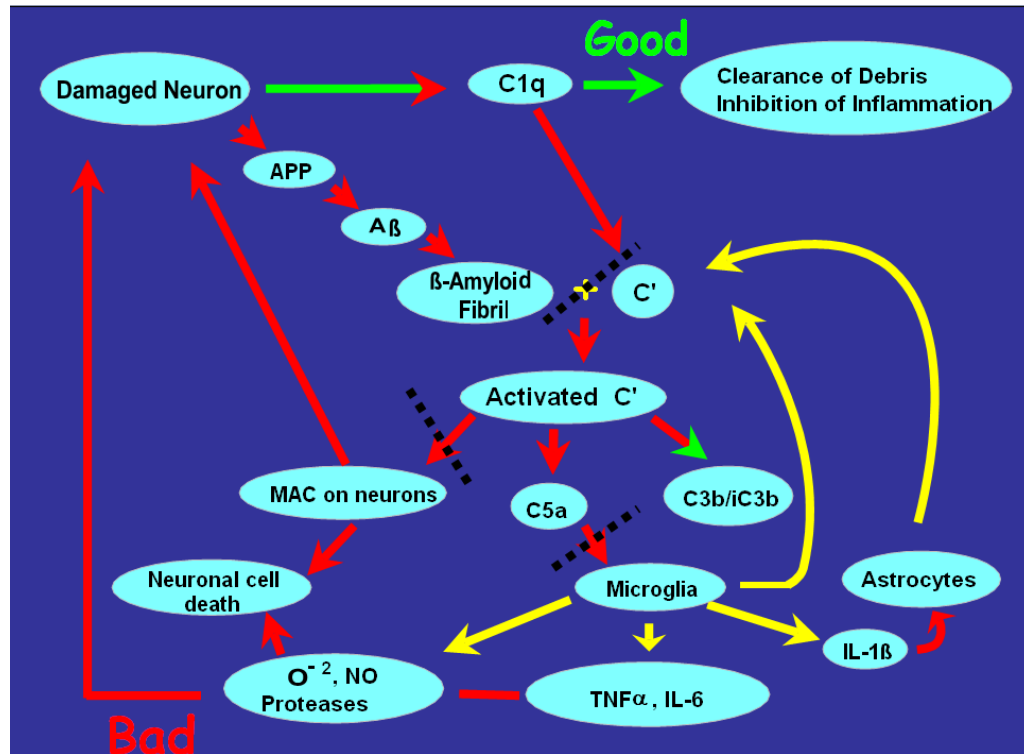


Figure 8. Schematic diagram of potential therapeutic drug targeting

Fibrillar A β activates the classical (and alternative) pathway of complement, resulting in C3b/iC3b deposition on plaques, glial recruitment and generation of neurotoxic molecules, and potential lysis by the Membrane attack complex (MAC/C5b-9) of complement. The red and green arrows signify pathways that are probably detrimental and protective respectively. The yellow arrows represent events that are suggested to occur in response to complement activation in vivo but have yet to be definitively proven. The black dotted lines indicate potential targets for intervention/inhibition of detrimental pathways. Modified from (Tenner 2001).

Table 1

Summary of antibody used in this study

Antibody	Antigen	Type	Source	Dilution ¹	Reference
C1q	C1q (mouse)	Goat polyclonal	F. Petry	IHC: 16ug/ml	(Fonseca <i>et al.</i> 2004)
C1q(1151)	C1q (mouse)	Rabbit polyclonal	A.J.Tenner	IHC: 4ug/ml WB: 5ug/ml	(Huang <i>et al.</i> 1999)
C3(11H9)	C3/C3b/iC3b (mouse)	Rat monoclonal	Cell Sciences (HBT)	IHC: 3.3ug/ml	(Kremmer <i>et al.</i> 1990)
C3(2/16)	C3/iC3b/C3c (mouse)	Rat monoclonal	J. D. Lambris	IHC: 1:1000	(Mastellos <i>et al.</i> 2004)
C3(2/11)	C3b/iC3b/C3c (mouse)	Rat monoclonal	J. D. Lambris	IHC: 1:1000	(Mastellos <i>et al.</i> 2004)
C3	C3 (mouse)	Goat polyclonal	Cappel	WB: 1:2000	
C4	C4/C4b/C4d (mouse)	Rat monoclonal	Cell Sciences (HBT)	IHC: 3.3ug/ml WB: 2ug/ml	(Kremmer <i>et al.</i> 1990)
C4	C4/C4d (mouse)	Rabbit polyclonal	R. Ogata	IHC: 2.4ug/ml WB: 24 ug/ml	(Ogata <i>et al.</i> 1994)
MAC-1	CD11b (mouse)	Rat monoclonal	Serotec	IHC: 10ug/ml	(Anderson <i>et al.</i> 1986)
CD45	CD45 (mouse)	Rat Monoclonal	Serotec	IHC: 1ug/ml	(Brewer <i>et al.</i> 1989)
CNPase	CNPase (human)	Mouse monoclonal	Chemicon	IHC: 1ug/ml	(Ehtesham <i>et al.</i> 2002)
GFAP	Glial Fibrillary acidic protein (bovine)	Rabbit polyclonal	Dako	IHC: 4ug/ml	(Viale <i>et al.</i> 1991)
6E10	A β 1-17 (human)	Mouse monoclonal	Senetek	IHC: 1ug/ml	(Jung <i>et al.</i> 1999)

¹ IHC, immunohistochemistry; WB, Western Blot