

Complement-dependent Proinflammatory Properties of the Alzheimer's Disease β -Peptide

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Summary

Large numbers of neuritic plaques (NP), largely composed of a fibrillar insoluble form of the β -amyloid peptide (A β), are found in the hippocampus and neocortex of Alzheimer's disease (AD) patients in association with damaged neuronal processes, increased numbers of activated astrocytes and microglia, and several proteins including the components of the proinflammatory complement system. These studies address the hypothesis that the activated complement system mediates the cellular changes that surround fibrillar A β deposits in NP. We report that A β peptides directly and independently activate the alternative complement pathway as well as the classical complement pathway; trigger the formation of covalent, ester-linked complexes of A β with activation products of the third complement component (C3); generate the cytokine-like C5a complement-activation fragment; and mediate formation of the proinflammatory C5b-9 membrane attack complex, in functionally active form able to insert into and permeabilize the membrane of neuronal precursor cells. These findings provide inflammation-based mechanisms to account for the presence of complement components in NP in association with damaged neurons and increased numbers of activated glial cells, and they have potential implications for the therapy of AD.

Key words: Alzheimer's disease • amyloid • C3 • complement • inflammation

We and others (1–3) have noted that the pathological changes which characterize Alzheimer's disease (AD)¹ could all result from complement activation in neuritic plaques (NP), since this effector system has the ability to activate various cell types with release of cytokines and secondary mediators; to induce directed migration of these cells toward the complement activator; to alter cellular functions; and to damage cells (4, 5). Potential complement involvement in the brain is not dependent on disruption of the blood–brain barrier, since neurons, astrocytes, microglia, and oligodendrocytes synthesize most, and likely all, of the proteins of the complement system (6).

Since activation is a prerequisite for manifestation of all of the biological activities of the complement system, the β -amyloid peptide (A β) or another component of NP must possess the ability to activate complement in order for complement to be involved in mediating the pathologic cellular characteristics of AD. In this regard, we and others

previously showed that fibrillar forms of A β bound the first reacting factor of the classical complement pathway (CCP), C1q (3, 7), and depleted the activity of the fourth complement component (C4) as well as whole complement activity (CH50), when incubated with human serum as a complement source in vitro (3, 7). Residues 14–26 of the collagen-like portion of the A polypeptide chain of the C1q molecule were implicated in binding fibrillar A β (7). Recent studies have confirmed this suggestive evidence of CCP activation by aggregated A β (8–12), and have also emphasized the critical role of the β -pleated structure of A β in mediating these effects (9, 11). Inhibition studies have implicated A β residues 1–11 in C1q binding (11, 12). The complement depletion, inhibition, and cleavage assays used in these various studies have provided suggestive evidence for CCP activation by fibrillar A β ; however, as indirect assays, they are subject to other interpretations. In this context, we recently presented preliminary evidence suggesting that A β forms complexes with C3 after incubation of fibrillar A β with a complement source (9).

In the course of these studies, we found that the addition of fibrillar A β to a complement source led to the generation of covalent ester-linked complexes of A β with C3 activation fragments, providing unequivocal evidence for complement activation by A β , since covalent attachment

¹Abbreviations used in this paper: ACP, alternative complement pathway; AD, Alzheimer's disease; A β , β -amyloid peptide; CCP, classical complement pathway; dd, double distilled; MAC, membrane attack complex; NP, neuritic plaques; NHS, normal human serum; SOD, superoxide dismutase.

of C3 activation fragments to complement activators represents a fundamental tenet of complement action. We also found that fibrillar A β possesses the ability to activate the alternative complement pathway (ACP) in serum, as well as in mixtures of the six purified proteins of the alternative pathway in physiologic concentrations, providing the first indication that A β independently activates both complement pathways. Additionally, we observed that such activation is highly specific for A β and completely independent of oxidative processes. These studies are described here. Finally, we also report for the first time that A β -mediated complement activation is biologically significant, as it leads to generation of the cytokine-like C5a complement-activation fragment, and mediates formation of the proinflammatory C5b-9 membrane attack complex (MAC), in functionally active form able to insert into and permeabilize the membranes of neuronal precursor cells.

Materials and Methods

A β -Mediated Complement Activation and Complement Activation ELISA Assays. A β 1–40 and 1–42 (Bachem California, Torrance, CA; Bachem Bioscience, Inc., King of Prussia, PA; Quality Controlled Biochemicals, Inc., Hopkinton, MA; Anaspec, Inc., San Jose, CA; and California Peptide Research, Inc., Napa, CA) were dissolved in either 100% DMSO at 10 mg/ml, or double distilled (dd)H₂O, gradually diluted in ddH₂O to 2 mg/ml and then brought to 1 mg/ml in 0.1 M Tris buffer, pH 7.4. A β was used immediately (nonaggregated) or permitted to aggregate by incubation at room temperature for 48 h (A β 1–42) to 72 h (A β 1–40); further incubation for 2 wk did not decrease complement-activating potential. Preaggregated A β preparations were incubated with an equal volume of 1:5 normal human serum (NHS), complement-depleted sera (Advanced Research Technologies, Inc.), or the six purified proteins of the ACP (Advanced Research Technologies, Inc.) in physiologic ratios (13). Dilutions were in veronal buffered saline, pH 7.4, containing calcium and magnesium. Inhibition studies were carried out with preaggregated A β 1–42 in the presence of deferoxamine, glutathione, dimethylthiourea, catalase, or superoxide dismutase (SOD), all purchased from Sigma Chemical Co. (St. Louis, MO). After 1 h at 37°C, EDTA was added to stop further complement activation, and the samples were diluted (1:20–1:300) and added, in replicate, to microtitration wells precoated overnight (4°C) with 1 μ g (100 μ l) of mAb to A β (10D5), mAb to a C3b neoantigen (clone 129), or mAb to an iC3b neoantigen (Quidel, San Diego, CA) at pH 7.4, and then blocked (BLOTTO; Pierce Chemical Co., Rockford, IL). After 1 h at room temperature, bound A β –C3b/iC3b complexes were detected with rabbit Ab to C3 or to A β , horseradish peroxidase-conjugated anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and ABTS (Kirkegaard & Perry Laboratories). C3 standard curves were generated with dilutions of purified iC3b (Advanced Research Technologies, Inc.) captured on wells precoated with anti-iC3b (Quidel). C5b-9 was captured on wells precoated with mAb to a C5b-9 neoantigen located in the poly C9 portion of the complex (Quidel) and detected with polyclonal goat Ab to C6 followed by horseradish peroxidase-conjugated mouse anti-goat IgG (Accurate Chemical and Science Corp., Westbury, NY) and ABTS. Values for the C5b-9 assay were back-calculated to the values in undiluted NHS. In some experiments, neuropeptide Y-porcine, urotensin I, or exendin 3

(all from California Peptide Research, Inc.); insulin B chain (Sigma Chemical Co.); amyloid precursor protein peptide (657–676 (Bachem California); and adenovirus penton base 50-residue fragment (residues 317–366; reference 14), as well as preaggregated A β 1–42 preparations and monomeric A β 1–42, were dissolved in DMSO or ddH₂O and aged at room temperature, exactly as described above for A β . In some studies, peptides, after dissolution as just described but diluted in Hepes-buffered NaCl at pH 7.4, were covalently cross-linked at a concentration of 200 μ M with the primary amine reactive agent, bis(sulfosuccinimidyl)suberate (BS³; Pierce Chemical Co.), at a concentration of 5 mM. After 30 min, the reaction was terminated by quenching. All peptides were incubated with NHS for 1 h at 37°C. After dilution, complement activation was assessed by the conventional CH50 assay (15) by quantitating residual functional C3 using C3-depleted serum according to product instructions (Advanced Research Technologies, Inc.), or by evaluating C5b-9 formation.

Studies with Hydroxylamine. After capture of complexes onto 10D5-coated wells, replicate wells were treated with 0.1 M Tris, pH 9.5, or 1 M hydroxylamine in 0.1 M Tris, pH 9.5, for 2 h at 37°C. After washing, remaining bound C3 was detected as described above. Residual A β was detected with rabbit Ab to A β , as described above. The formation of covalent complexes of A β with C3 activation products was also evaluated using the Western blotting procedure on SDS-PAGE gels. Replicate samples of aggregated A β 1–42 or A β 1–40 were incubated with human serum for 1 h at 37°C in the presence or absence of EDTA, and the reaction mixtures were then microfuged, washed in Tris buffer, and incubated for 3 h at 37°C with 0.1 M Tris at pH 7.4, 0.1 M Tris at pH 9.5, or 1 M hydroxylamine in 0.1 M Tris at pH 9.5. The samples were again microfuged, and washed with Tris at pH 7.4 followed by the same buffer containing 0.1% SDS. The samples were then subjected to SDS-PAGE under nonreducing conditions and electroblotted, and bands were detected with rabbit Ab to C3 followed by goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) and the SuperSignal system (Pierce Chemical Co.). After stripping, A β was detected with 6E10 mouse Ab to A β (Senetek PLC, St. Louis, MO) followed by goat anti-mouse IgG (Kirkegaard & Perry Laboratories) and the SuperSignal system. In some experiments, electroblotted gels were reacted first with rabbit Ab to A β (generated in this laboratory) or with 6E10 mAb to A β (Senetek PLC) followed by goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) or goat anti-mouse IgG (Kirkegaard & Perry Laboratories), stripped, and reacted with mAb anti-iC3b (Quidel) or rabbit Ab to C3 (generated in this laboratory), followed by goat anti-mouse IgG or goat anti-rabbit IgG. Quantitation was with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Mass Spectroscopy. After solubilization in 70% formic acid, samples were analyzed by MALDI spectroscopy (Perseptive Voyager ELITE; Perseptive Biosystems, Inc., Framingham, MA).

C5a. C5a (and C5a des-Arg) was detected in diluted samples with the Biotrak radioimmunoassay kit (Amersham Corp., Arlington Heights, IL); the samples were subjected to acid precipitation before analysis (16). Values were back-calculated to the concentrations in undiluted NHS.

C5b-9 Membrane Insertion. Ntera2/D1 (NT2) cells (Stratagene Inc., La Jolla, CA) were grown to subconfluence, released with nonenzymatic cell dissociation solution (GIBCO BRL, Gaithersburg, MD), washed, and resuspended (2×10^7 cells/ml). NT2 cells (100 μ l) were incubated with 50 μ l NHS in the presence or absence of EDTA and 50 μ l preaggregated A β . After 15 min at 37°C, the MAC was detected with rabbit Ab (Advanced Re-

search Technologies, Inc.) or mAb (Quidel) to C5b-9 neoantigens, followed by FITC anti-rabbit or -mouse Ig and propidium iodide. Readings were performed on a FACScan® and analyzed with CellQuest software (Becton Dickinson, San Jose, CA).

Results and Discussion

Sandwich-type ELISAs showed that complexes containing A β and C3b/iC3b were generated in NHS, as a complement source, after incubation with aggregated A β 1–42. Complexes were demonstrable after capture with mAbs to activation-dependent neoantigens in the first (C3b) or second (iC3b) C3 cleavage products and detection with rabbit Ab to A β (Fig. 1, *a* and *b*), as well as after capture with mAb to A β and detection with rabbit Ab to C3 (Fig. 1 *c*) or C3d (not shown). EDTA, which blocks complement activation by chelating calcium and magnesium, prevented complex formation (Fig. 1 *c*). ELISAs in which complexes were captured with mAb to A β and detected with Ab to C3 were used for most of the studies, since such ELISAs permitted quantitation by reference to included standard curves generated with purified C3 captured on wells coated with mAb to C3 and detected with rabbit Ab to C3 (Fig. 1 *d*). Complement activation was detectable to $\sim 1 \mu\text{M}$ A β 1–42 (Fig. 1 *d*). 10 different preaggregated A β 1–40 and 20 different preaggregated A β 1–42 preparations from 5 manufacturers generated such complexes. A β 1–42 was generally 5–10-fold more active than A β 1–40 in this regard. The cation-dependent formation of A β –C3b/iC3b complexes after incubation of aggregated A β with NHS provides unequivocal evidence for complement activation by aggregated A β .

A β –C3b/iC3b complex formation was evident after incubation of aggregated A β 1–42 with NHS lacking factor B, an essential component of the ACP (Fig. 1 *e*); such sera contain an intact CCP, but do not permit ACP activation. A significant reduction in complex formation was also evident in C1q-depleted serum compared with NHS (Fig. 1 *e*). These data show that the CCP mediates complex formation by aggregated A β , findings that were anticipated from the results of the complement depletion assays described earlier. Unexpectedly, however, the ACP also mediated the formation of complexes, since they were also generated after the addition of aggregated A β 1–42 to NHS lacking factor C1q, and complex formation was reduced in factor B–depleted serum compared with NHS (Fig. 1 *e*), a result replicated in four additional experiments with different A β 1–42 preparations. The ability of A β 1–42 to activate the ACP was confirmed in three studies in which preaggregated A β 1–42 was incubated with a mixture of the six purified proteins of the ACP (factors B, D, H, and I, properdin, and C3) in physiological ratios (reference 13; Fig. 1 *f*). These data document the ability of aggregated A β not only to activate the CCP, but also to independently activate the ACP. This is the first indication that aggregated A β activates the ACP; it had been presumed that complement activation by A β was exclusively via the CCP because of the absence of ACP components (factor B and properdin) in NP (1, 17, 18). The failure to detect ACP components in NP may be due to the extreme lability of the ACP C3 convertase.

Multiple different aggregated A β 1–42 preparations activated complement, as determined by the classical CH50 complement consumption technique (Fig. 1 *g*). The aging

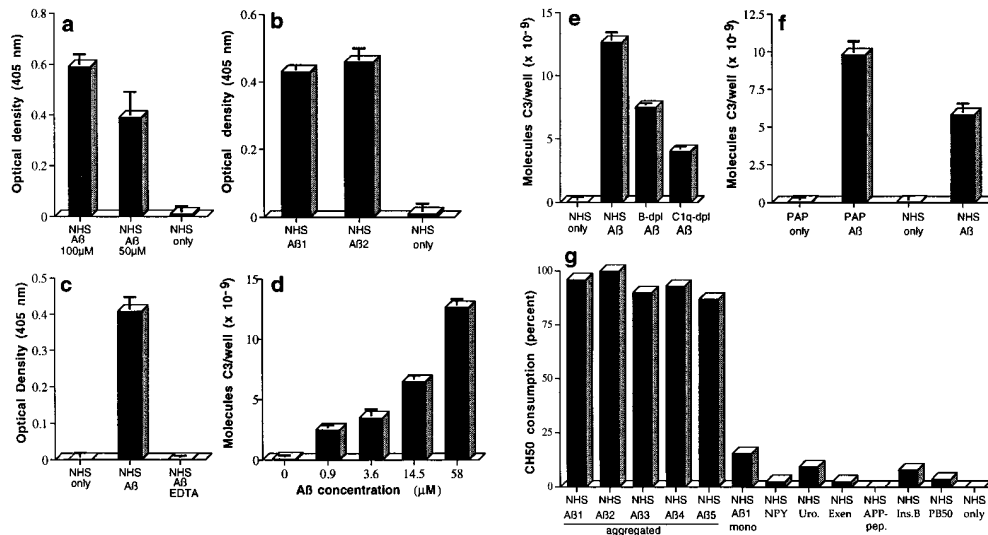


Figure 1. ELISA demonstration of complement-mediated formation of complexes of A β with C3 activation fragments (*a–d*). Complexes were captured, detected, and quantitated as described in Materials and Methods. NHS or purified ACP proteins only do not contain A β . (*a*) Preaggregated A β 1–42 (100 μM and 50 μM) was incubated in NHS, captured with mAb to C3b, and detected with rabbit Ab to A β . (*b*) Two preaggregated A β 1–42 preparations (A β 1 and A β 2, at 20 μM) were incubated in NHS, captured with mAb to iC3b, and detected with rabbit Ab to A β . (*c*) Preaggregated A β 1–42 (58 μM) was incubated in NHS, or in NHS containing 10 mM EDTA, captured with mAb to A β , and detected with rabbit Ab to C3. (*d*) Preaggregated A β 1–42 (58 μM) was incubated in NHS, or in NHS containing 10 mM EDTA, captured with mAb to A β , and detected with rabbit Ab to C3. (*e*) Preaggregated A β 1–42 (58 μM) was incubated in NHS, factor B–depleted NHS (*B-dpl*), or C1q–depleted NHS (*C1q-dpl*), captured with mAb to A β , and detected with rabbit Ab to C3. (*f*) Preaggregated A β 1–42 (58 μM) was incubated with the six purified ACP proteins (*PAP*) or NHS, captured with mAb to A β , and detected with rabbit Ab to C3. Background levels obtained in EDTA controls containing A β and purified ACP proteins or NHS, in the various experiments described above, were subtracted. (*g*) Specificity of complement activation. Preaggregated A β preparations (20 μM) and the same concentrations of monomeric A β (*mono*), insulin B chain (*Ins.B*), neuropeptide Y–porcine (*NPY*), urotensin I (*Uro.*), exendin 3 (*Exen.*), amyloid precursor peptide 657–676 (*APP-pep.*), and adenovirus penton base 50-residue peptide (*PB50*) were incubated with NHS. Complement activation was assessed by the CH50 method. Correlation coefficients for the CH50 determinations ranged from 0.995 to 1.000.

detected with rabbit Ab to C3. (*d*) Preaggregated A β 1–42 was incubated in NHS at the indicated final concentrations, captured with mAb to A β , and detected with rabbit Ab to C3. (*e*) Preaggregated A β 1–42 (58 μM) was incubated in NHS, factor B–depleted NHS (*B-dpl*), or C1q–depleted NHS (*C1q-dpl*), captured with mAb to A β , and detected with rabbit Ab to C3. (*f*) Preaggregated A β 1–42 (58 μM) was incubated with the six purified ACP proteins (*PAP*) or NHS, captured with mAb to A β , and detected with rabbit Ab to C3. Background levels obtained in EDTA controls containing A β and purified ACP proteins or NHS, in the various experiments described above, were subtracted. (*g*) Specificity of complement activation. Preaggregated A β preparations (20 μM) and the same concentrations of monomeric A β (*mono*), insulin B chain (*Ins.B*), neuropeptide Y–porcine (*NPY*), urotensin I (*Uro.*), exendin 3 (*Exen.*), amyloid precursor peptide 657–676 (*APP-pep.*), and adenovirus penton base 50-residue peptide (*PB50*) were incubated with NHS. Complement activation was assessed by the CH50 method. Correlation coefficients for the CH50 determinations ranged from 0.995 to 1.000.

procedure used to aggregate A β generates β -pleated fibrils (9). Nonfibrillar "amorphous" aggregates of A β are devoid of complement-activating ability (9). In contrast to the aggregated preparations, A β used immediately after dissolution had limited ability to activate complement (Fig. 1 *g*). These data document the important role of fibril formation for complement activation by A β in vitro. Amylin, another peptide which spontaneously forms β -pleated fibrils, was also tested for complement-activating ability in these studies. The 37-residue amylin polypeptide represents the principal constituent of the amyloid deposits in type 2 diabetes. On SDS-PAGE gels, aged amylin migrated primarily as large SDS-insoluble stained bands. However, this fibrillar peptide did not significantly activate complement at a concentration of 100 μ M (7% CH50 consumption).

The specificity of complement activation by A β was also evaluated by determining whether complement was activated by other small peptides (20–50 amino acids) containing multiple residues able to mediate covalent linkage to the glutamate residue of the hydrolyzed thioester of C3 (serine, tyrosine, threonine, lysine) and expressing similar overall charge to A β . All of the peptides were processed and aged in the same manner as A β . None of the peptides, including the insulin B chain (30 residues), neuropeptide Y-porcine (36 residues), urotensin I (41 residues), exendin 3 (39 residues), amyloid precursor peptide 657–676 (20 residues), and the adenovirus penton base fragment (50 residues) significantly activated complement at a concentration of 20 μ M, as assessed by the classical CH50 technique (Fig. 1 *g*). The peptides also showed little or no ability to activate complement in other assays, including the ability to deplete residual functional C3 and form the SC5b-9 complex (not shown). To determine whether peptide aggregation would increase complement-activating ability, urotensin I, neuropeptide Y-porcine, and exendin 3 were covalently cross-linked with the primary amine reactive reagent BS³ before evaluating their ability to activate complement by the CH50 technique. Cross-linked urotensin I and neuropeptide Y-porcine gave a ladder of Coomassie-stained bands on SDS-PAGE analyses, but exendin 3 gave no stained bands, possibly due to the formation of very large aggregates. These three cross-linked peptides did not significantly activate complement (<10% CH50 depletion) at a concentration of 20 μ M. In another study, cross-linked urotensin I exhibited 7% CH50 consumption at a concentration of 100 μ M, whereas aggregated A β 1–42 showed 45% consumption. These data cumulatively demonstrate the marked specificity of complement activation by fibrillar A β .

C3 preferentially binds to activators via ester bonds, although amide linkage has been described (19, 20); such bonds form between the reactive γ -carbonyl group of the glutamate residue of the activation-cleaved internal thioester bond in C3, and hydroxyl (ester) or amino (amide) groups on the activator (19). To evaluate possible ester linkage, complexes were captured with mAb to A β and incubated with Tris buffer containing 1 M hydroxylamine at pH 9.5 for 2 h at 37°C, a treatment which disrupts ester but not amide bonds (19, 20). Approximately 50% of the bound

C3, but none of the A β , was removed from the captured complexes by this treatment (Fig. 2, *a* and *b*), a result replicated in two additional studies with A β 1–40 and 1–42.

The formation of covalent complexes of A β with C3 activation products was also independently demonstrated using a Western blotting approach. In these studies, aggregated A β 1–40 was incubated with serum, and the complexes of insoluble fibrillar A β with C3 activation fragments were then sedimented, washed, and incubated with either 1 M hydroxylamine at pH 9.5 or control buffers. After washing, a prominent band with a molecular mass of \sim 180 kD, the molecular mass of C3, as well as several higher molecular mass bands, were detected with Ab to C3 (Fig. 2 *c*). After stripping, the same bands were also found to react with Ab to A β , although the gels were darker due to the presence of large amounts of aggregated A β (Fig. 2 *c*). Bands of the same molecular masses reactive with Abs to both A β and C3 were also observed when the blotting studies were performed in the reverse direction, i.e., blotting first with either mAb or polyclonal Ab to A β followed, after stripping, by blotting with rabbit Ab or mAb to C3 (not shown). The 180-kD band and the larger bands, which contain both C3 and A β , undoubtedly represent complexes of A β monomers with C3b monomers and oligomers, since they were not evident in the reactions carried out in the presence of EDTA or in the absence of fibrillar A β (Fig. 2 *d*).

C3 was also detected in the large A β aggregates on the top of the gels (except for the EDTA lane) on longer exposure (not shown). The lesser reactivity of C3 in the larger A β aggregates at the top of the gels, compared with the C3 monomers and oligomers within the gels, indicates that not all A β monomers bear a molecule of C3b; this is not surprising, since A β is in large aggregates and, in addition, in molar excess over C3. It may also be that A β molecules bearing covalently bound C3b dissociate from the aggregates, in analogy to the dissociation of immune complexes by the covalent binding of C3b (21, 22).

Hydroxylamine treatment disrupted approximately half of the complexes (Fig. 2 *c*). Quantitative scanning of the C3 Western blot showed that the treatment with 1 M hydroxylamine at pH 9.5 removed 42% of the bound C3 compared with the pH 9.5 control; the pH 9.5 buffer treatment removed only trivial amounts (5.1%) of the bound C3 compared with the pH 7.4 treatment. The A β Western blot could not be satisfactorily scanned due to the large background, but visual inspection reveals the same pattern (Fig. 2 *c*). Identical results were obtained with A β 1–42 (not shown). These two independent assay systems both show that ester bonds, in part, mediate covalent attachment of C3 activation fragments to A β . A β 1–42 contains two serines, at positions 8 and 26, and a tyrosine, at position 10, which could mediate ester linkages with C3 activation fragments.

With regard to the bond(s) responsible for the nonester-linked A β -C3b/iC3b complexes, A β alone has been reported to generate free radicals (23) upon incubation in aqueous solution, and oxidative processes have been associated with A β denaturation, fragmentation, and oxidation (24, 25). Because of the potential relevance of these pro-

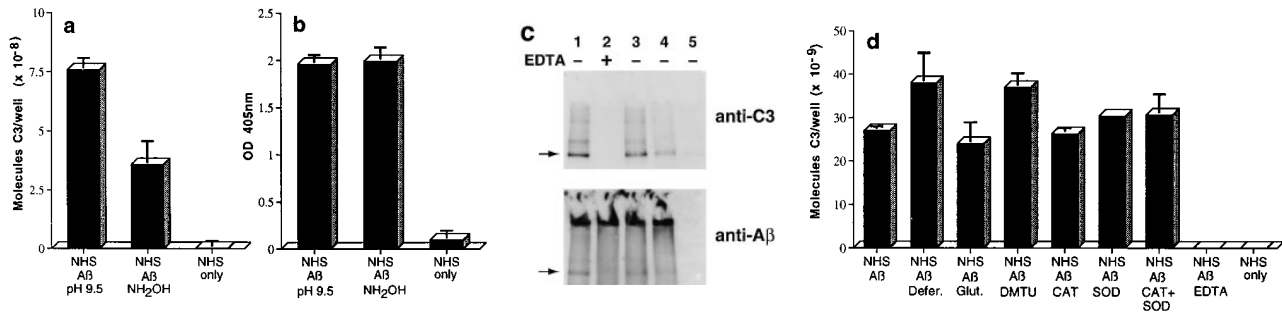


Figure 2. Assessment of bonds mediating binding of A β to C3 activation fragments. (a) Preaggregated A β 1–42 (25 μ M) was incubated in NHS, and complexes were captured on 10D5-coated wells; NHS only does not contain A β . Replicate samples were treated with pH 9.5 buffer, or with the same buffer containing 1 M hydroxylamine (NH₂OH), and remaining bound C3 was then detected and quantitated as described in Materials and Methods. The control containing A β , NHS, and EDTA has been subtracted. (b) Replicate wells subjected to treatment with the pH 9.5 buffer or hydroxylamine were evaluated for residual bound A β as described in Materials and Methods. (c) Preaggregated A β 1–40 was incubated in NHS in the presence or absence of EDTA; lane 5 contains NHS but no A β . After centrifugation and washing, fibrillar A β pellets were incubated with pH 7.4 buffer (lanes 1 and 2), pH 9.5 buffer (lane 3), or 1 M hydroxylamine in pH 9.5 buffer (lane 4). After further washing, samples were subjected to SDS-PAGE under nonreducing conditions followed by blotting for the presence of C3 and, after stripping, for A β . Arrow, The C3 band at \sim 180 kD. (d) Preaggregated A β 1–42 (50 μ M) was incubated in NHS alone, and in the presence of deferoxamine (*Defer.*; 1 mM), glutathione (*Glut.*; 1 mM), dimethylthiourea (*DMTU*; 30 mM), catalase (*CAT*; 2×10^4 U/ml), SOD (10 μ M), or catalase plus SOD, and the A β complexes with C3 activation fragments were then detected as described in Materials and Methods.

cesses to the formation of complexes of A β with C3 activation fragments, A β 1–42 was assessed by MALDI mass spectroscopy after aging from 0 to 10 d. Aggregates are not detected in these assays, since the samples are dissolved in 70% formic acid for mass spectroscopic analysis. The molecular mass of the major peak in the various samples ranged from 4510 to 4514, and no other peaks were present, ruling out significant oxidation, fragmentation, and covalent cross-linking of A β . To determine whether oxidative processes mediated the formation of complexes of A β with C3 activation fragments, complement activation was carried out in the presence of deferoxamine, glutathione, dimethylthiourea, catalase, SOD, and catalase plus SOD. Since none of these antioxidants or free radical scavengers inhibited the formation of or interfered with the detection of complexes (Fig. 2 d), it is unlikely that free radical-mediated or oxidative processes are involved in the formation of complexes of A β with C3 activation fragments. In all likelihood, amide bonds are responsible for the remaining A β –C3b/iC3b complexes. A β 1–42 contains two lysine residues, at positions 16 and 28, which could mediate such linkages.

Additional studies showed that A β triggered activation of the terminal, proinflammatory portion of the complement-reaction sequence in NHS. C5a, a cytokine-like activation cleavage product of C5 with numerous biological properties, was efficiently generated by aggregated A β 1–42 in NHS, as determined by a specific radioimmunoassay which detects C5a and C5a des-Arg (lacking the COOH-terminal arginine residue) (reference 26; Fig. 3 a). A sandwich ELISA in which an mAb to a C5b-9 neoantigen located in poly C9 served as the capture Ab, and polyclonal Ab to C6 served as the detection Ab, showed that A β -mediated complement activation led to formation of the C5b-9 complex (Fig. 3 b). This ELISA detects C5b-9 as well as SC5b-9 complexes; the latter are formed in NHS in the absence of

cells, as a consequence of the binding of S protein, a complement control protein, to the complex. A β 1–42 was generally more efficient in generating C5b-9 than A β 1–40 (Fig. 3 b). In contrast, another group recently reported that A β -mediated complement activation does not lead to generation of the C5b-9 complex (10). The reason(s) for their failure to demonstrate C5b-9 formation is not known. One possibility is the well-known variability in the properties of different A β preparations. In this regard, we have observed more variability in the ability of various A β 1–40 and A β 1–42 preparations to trigger C5b-9 complex formation, than in their ability to generate A β –C3b and A β –iC3b complexes. Other explanations could lie in slight differences in experimental conditions. For example, their C5b-9 formation experiments were carried out in NHS diluted 1:10 in phosphate-buffered NaCl; this combination provides suboptimal concentrations of calcium and magnesium, which are required for CCP and ACP activation. In this regard, we obtained 10-fold higher levels of SC5b-9 formation than they obtained with 1 μ M aggregated IgG in their control studies (not shown).

The C5b-9 complex generated by A β 1–42-mediated complement activation was able to insert into the membranes of NT2 cells, a committed neuronal precursor cell line, when such cells were included in reaction mixtures with aggregated A β and NHS (Fig. 4 a). Depicted are flow cytometric analyses with rabbit Ab to activation-specific neoantigens in the C5b-9 MAC. C5b-9 membrane insertion was likely proportional to the extent of complement activation, since it was dependent on the concentration of A β 1–42. mAb to C5b-9 neoantigens gave the same result (not shown). Identical A β 1–40 concentrations mediated lower levels of C5b-9 membrane insertion (not shown), probably because of the significantly lower levels of C5b-9 formation with A β 1–40. NT2 cells and other neuronal cell lines are resistant to complement-dependent cytolysis,

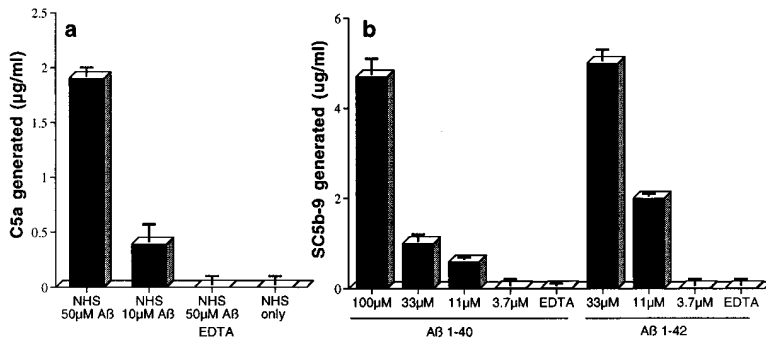


Figure 3. Aβ-mediated complement activation generates C5a and the MAC. (a) Preaggregated Aβ 1–42 was incubated in NHS, and C5a generation was then quantitated as described in Materials and Methods. (b) C5b-9 was quantitated after NHS was incubated with varying concentrations of preaggregated Aβ 1–42 or 1–40. SC5b-9 formation was quantitated as described in Materials and Methods.

likely because of the presence of CD59 (27), a complement regulatory protein, a finding confirmed here. Nevertheless, C5b-9 insertion into NT2 cell membranes mediated an increase in the permeability of the cells to propidium iodide that was dependent on the concentration of Aβ 1–42 (Fig. 4 b). These data indicate that C5b-9 generated by Aβ-mediated complement activation is functionally competent, since it inserts into the membranes of neuronal precursor cells and renders them permeable to small molecules.

Thus, Aβ directly and independently activates the ACP as well as the CCP, leading to the formation of covalent Aβ–C3b and Aβ–iC3b complexes; generates C5a; and mediates assembly of functionally active C5b-9 complexes *in vitro*. These findings have potential implications for understanding the mechanisms which lead to continuing neuronal damage and altered glial functions in the vicinity of NP, and thus to the progression of AD. First, they provide an explanation for the association of bound C3 with Aβ in NP (1–3), since covalently bound C3b molecules in NP would remain bound and provide a nidus for chronic complement activation. Second, C5a generated by Aβ-mediated complement activation could be responsible for the increased numbers of activated astrocytes and microglia

around NP compared with diffuse Aβ plaques (28), since these cells possess C5a receptors and are activated and migrate in response to C5a (6, 29–31). C5a could also trigger the release of proinflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α) from glial cells, as it does from other cell types (26, 32); proinflammatory cytokines are increased in the AD brain (2, 28, 33). These cytokines could further activate glial cells and alter neuronal and glial functions (28, 32). Third, incoming activated glial cells could bind and remain adherent, via their complement receptors, to C3 activation fragments attached to Aβ (6). Fourth, C5b-9 insertion into cell membranes provides an explanation for the association of this complex with dystrophic neurites in NP (2, 3). Although not likely to be directly cytotoxic for neurons, since they bear CD59 (6, 34), C5b-9 as well as C5b-7 and C5b-8 complexes could alter neuronal functional properties over time by chronic low-level triggering of various cellular signaling pathways (35). If this inflammation-based scenario is verified, complement inhibitors should be evaluated for use in AD. Such inhibitors would need to pass the blood–brain barrier, target both complement activation pathways, and prevent C5b-9 activation.

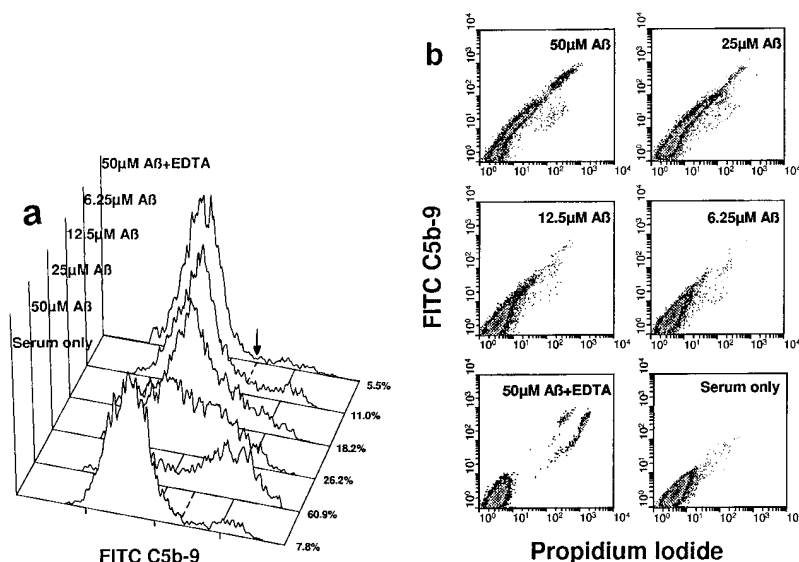


Figure 4. C5b-9 generated by Aβ 1–42-mediated complement activation is functionally active. (a) NT2 cells were incubated with preaggregated Aβ 1–42 at the designated concentrations in NHS, or in NHS containing 10 mM EDTA; serum only lacks Aβ. Flow cytometric analyses with a rabbit Ab to C5b-9 neoantigens are shown. Numbers (right), Percentage of C5b-9+ cells, as determined by their relationship to the marker (arrow, dashed line). (b) Density plot analyses of propidium iodide and C5b-9 (FITC C5b-9) reactivities are shown. For clarity, only live cells are depicted.

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