Complement Activation by Bisretinoid Constituents of RPE Lipofuscin

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PURPOSE. Studies implicate activation of complement among the processes involved in the pathogenesis of age-related macular degeneration (AMD). Questions pertain to the trigger(s) responsible for the complement-associated events. The authors previously reported that photooxidation products of A2E can activate complement. Here they have further explored these events.

METHODS. In vitro assays using human serum as a source of complement were used, and the C3 split product iC3b was measured by enzyme immunoassay. Serum was placed in contact with ARPE-19 cells and polarized human fetal retinal pigment epithelium that had accumulated A2E and were irradiated (430 nm). Serum was also incubated in wells precoated with bisretinoid pigments of lipofuscin and their oxidized forms. iC3b generation in normal human serum (NHS) was compared with that in factor B-depleted and C1q-depleted human serum.

RESULTS. iC3b levels were elevated in NHS placed in contact with A2E-laden retinal pigment epithelium that were irradiated to generate A2E photooxidation products. iC3b was also increased in serum incubated in wells precoated with peroxy-A2E, the lipofuscin pigment all-trans-retinal dimer, and oxidized forms of all-trans-retinal dimer. Substitution of NHS with factor B-depleted sera abrogated these increases in iC3b. Complement activation was also suppressed by the addition of C-reactive protein and by a C3 cleavage inhibitor.

CONCLUSIONS. The authors suggest that bisretinoid pigments of retinal pigment epithelium lipofuscin, subsequent to photoactivation and cleavage, serve to activate complement. Complement activation by this mechanism is dependent on the alternative pathway and can be modulated by an inhibitor of C3 cleavage.

Links between the complement system, an arm of innate immunity, and age-related macular degeneration (AMD) have been provided by analyses of the composition of the extracellular deposits that form subretinally as drusen1–4 and by molecular genetic studies.5–15 In the latter, genetic association studies based on case-control cohorts and single nucleotide polymorphisms (SNPs) have demonstrated that DNA sequence variants in the genes encoding complement factor H (CFH), factor B/C2, and complement component C3 confer increased risk for AMD. The complement system is activated through three enzyme cascades, the classical, lectin, and alternative pathways, which converge at the step involving cleavage of C3 to C3a and C3b. Complexing of C3b with the Bb fragment of factor B generates the C3 cleavage enzyme C3bBb, which, by further C3 cleavage, establishes the complement amplification loop. In general, the classical pathway of complement is activated by antigen-antibody complexes and C-reactive protein (CRP), whereas the alternative pathway is activated through direct contact with foreign particles or cells. Complement activation is tightly regulated at the cell membrane and within body fluids with an important fluid phase negative regulator, CRH.16 Although several haplotypes of the CFH gene are associated with either increased or decreased susceptibility to AMD,18 the most studied is the Y402H variant. Longitudinal prospective population-based studies with the power to determine the magnitude of the role played by CFH have confirmed the highly significant association between the CFH 402H polymorphism (genotype 1277CC) and risk for AMD.17–19 For instance, an allele dose effect can be observed in persons carrying two copies of the 402H risk allele, making them 3.92 times more at risk for AMD. Analysis of the participants in the Age-Related Eye Disease Study (AREDS) revealed that the risk for progression to advanced AMD (neovascularization or central geographic atrophy) was 2.6 times greater in persons carrying the CC risk variant of CFH.19 Population-attributable risk for late AMD was estimated to be 54% in the presence of 1 allele or 2 alleles.

Prompted by an interest in identifying mechanisms linking factors implicated in AMD pathogenesis, including inflammation, oxidative damage, drusen, and retinal pigment epithelial (RPE) lipofuscin, we previously explored the possibility that products of the photooxidation of RPE lipofuscin pigments activate the complement system.20 The lipofuscin of RPE cells accumulates with age, is generated primarily as a byproduct of retinoid cycling, and includes the bisretinoid compounds A2E, isoA2E, all-trans-retinal dimer, all-trans-retinal dimer-ethanolamine (all-trans-retinal dimer-E), and all-trans-retinal dimer-phosphatidylethanolamine (all-trans-retinal dimer-PE).21–24 These lipofuscin constituents are photoreactive molecules that absorb light in the visible range of the spectrum, generate reactive forms of oxygen, become oxidized, and undergo photo-induced fragmentation.21–24 All these lipofuscin pigments have been detected and measured in human retinal pigment epithelium and in eye cups of mice with null mutations in Abca4/Abcr,25–28 the gene responsible for recessive Stargardt disease.29 Mono- and bis-oxygenated photoproducts of A2E and all-trans-retinal dimer (furan-A2E, peryxo-A2E, furano-all-trans-retinal dimer, peryxo-all-trans-retinal dimer) have also been identified.27,28 With the use of cell-based and noncellular assays, we previously showed that photooxidized forms of A2E can activate complement,30 and we suggested this as a mechanism for inciting low-grade inflammation at the retinal pigment epithe-

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lumen-Bruch membrane interface. In the study described here, we explored complement activation by other bisretinoid pigments of lipofuscin in addition to A2E. We also tested for involvement of the classical and alternative pathways, investigated a modulating role for CRP, and probed the effectiveness of a C3 inhibitor.

**Methods**

**Synthesis of Compounds**

A2E and all-trans-retinal dimer were synthesized as published. Peroxy-A2E and peroxyl-all-trans-retinal dimer were synthesized by incubating the respective parent compound with 1,4-dimethylphthalene endoperoxide in CD3OD, and furano-A2E and furano-all-trans-retinal dimer were synthesized by incubation in MCPBA (11.6 mg; Sigma-Aldrich, St. Louis, MO) in methanol.

**Human Serum**

All procedures were conducted in accordance with the tenets of the Declaration of Helsinki. For normal human serum (NHS), blood samples were obtained, in the absence of anticoagulant, from a single human donor with approval from the institutional review board (IRB), as previously described. C1q-depleted human serum and factor B-depleted human serum were obtained commercially (Quidel Corp., San Diego, CA). Calcium and magnesium (20 mM) were added to the depleted sera, and for some experiments factor B protein (120 μg/mL) was added back to the respective depleted sera.

**Cell Culture**

Human adult retinal pigment epithelium (ARPE-19; American Type Culture Collection, Manassas, VA) lacking endogenous A2E were grown to confluence in eight-well slide chambers, as reported previously. Cells were grown in 35-mm dishes (Corning Incorporated, Corning, NY) in culture medium containing 5% fetal bovine serum at 37°C for 30 minutes, after which sheets of RPE cells were teased from the choroid.

**Complement Activation Assays**

Retinal pigment epithelium that had accumulated A2E and A2E-free cells were washed with phosphate buffered-saline (PBS), overlaid with 50 μL undiluted serum, irradiated at 430 nm (2.6 mW/cm², 30 minutes), and incubated at 37°C for 2 hours, as described. Serum incubated at 37°C in otherwise empty wells was used for background subtraction (typically 70–80 μg/mL iC3b), and suspensions ofzymosan A (0.25 mg/50 μL serum; Sigma-Aldrich) incubated in serum (37°C, 2 hours) were used as positive control. For cells grown in transwell chambers, serum was introduced below the polyester membrane, the medium in the upper chamber was replaced with PBS (200 μL), and the cells were irradiated through the upper chamber.

To precoat wells of 96-well plates, the indicated compounds solubilized in methanol (50 μg/50 μL methanol) or methanol alone (control) were added to the wells, and the plates were incubated in the dark (24°C, 90 minutes) to allow solvent evaporation. Once dried, undiluted human serum (50 μL) was placed in each well, and incubation was continued for 2 hours with gentle agitation. The concentrations of coated compound were calculated to be 156 μg/cm². Complement split product iC3b was measured in the serum samples by enzyme immunoassay (Quidel Corporation) with horseradish peroxidase conjugated anti-human iC3b as a chromogenic enzyme and with spectrophotometric monitoring at 405 nm, as previously described.

Samples were assayed in duplicate. Data were analyzed by one-way ANOVA and with the Newman-Keuls multiple comparison test (Prism; GraphPad Software, San Diego, CA).

**C-Reactive Protein**

Human recombinant CRP (pentamer of 115 kDa; USB, Cleveland, OH) in buffer (20 mM Tris, 2 mM CaCl₂, 140 mM NaCl, and 0.05% NaN₃, pH 7.5) was added to NHS (48 μL) in a 2-μL volume to achieve final exogenous protein concentrations of 1 and 10 μg/mL. For non-CRP samples, an equal volume (2 μL) of buffer was added to the serum.

Endogenous CRP in the donor NHS was quantified by ELISA (Alpha Diagnostics, San Antonio, TX). Serum in the presence and absence of exogenous CRP was then introduced to wells precoated with peroxyl-A2E or A2E, as described. As vehicle control, methanol was added to a well and allowed to evaporate.

**Preparation of C3 Inhibitor**

The C3 inhibitor compstatin and the potent analog POT-4 were developed in the laboratory of John D. Lambiris at the University of Pennsylvania (Philadelphia, PA). POT-4 was received as a gift from Potentia Pharmaceuticals (Louisville, KY). POT-4 (MWt 1627 g/mol) was prepared as a 2.5-mM stock solution in 20 mM Tris buffer containing 2 mM CaCl₂ and 140 mM NaCl (pH 7.5). Further dilutions in buffer were carried out such that POT-4 was added in a 2-μL volume to 48 μL human serum to achieve working concentrations of 0.1, 1, 10, and 100 μM. An equal volume of buffer (2 μL) was added to serum (48 μL) in control wells. The inactive mutant (negative control) G8A (MWt 1641.9) was used at a final concentration of 100 μM (with 1% dimethyl sulfoxide), also delivered as a 2-μL volume in 48 μL serum.

**Etoposide-Induced Apoptosis**

Confluent ARPE-19 cells were incubated with etoposide (100 μM in culture medium for 48 hours; BioVision, Mountain View, CA), a topoisomerase II inhibitor that induces apoptosis in several types of human cells. The cells were subsequently incubated with Cy3-labeled Annexin V (Annexin V Apoptosis Detection Kit; BioVision) for 5 minutes (1/100 dilution). After washing, the cells were fixed with 2% formaldehyde for 30 minutes, and nuclei were stained with DAPI (300 nM). Cy3-positive apoptotic cells were counted in 10 fields (approximately 1-mm² area) and were expressed as a percentage of total cells (DAPI stained). Annexin V is a marker for phosphatidylserine redistribution in the plasma membrane, an early event in apoptotic cell death.
RESULTS

Complement Activation Suppression in Factor B-Depleted Serum

For these experiments we used a previously established in vitro assay with human serum as a source of complement in cell-based and noncellular assays. The serum donor for these studies was homozygous for CFH Tyr402 variant (genotype 1277TT; SNP rs 1061170). To monitor C3 activation in the serum, we measured iC3b by enzyme immunoassay. The cleavage fragment, iC3b, is a product of C3 activation that forms in the alternative pathway when C3b is cleaved by factor I. As previously reported, we found that iC3b was elevated in NHS overlying irradiated (430 nm) ARPE-19 cells that had previously accumulated A2E (Fig. 1); under these conditions of irradiation, we previously showed that intracellular A2E undergoes photooxidation and photodegradation. This elevation in iC3b was realized by comparison with control cells that had accumulated A2E but were not irradiated and cells that had not accumulated A2E and were not irradiated (P > 0.01). Moreover, replacement of NHS with factor B-depleted serum abrogated the increase in iC3b observed in association with irradiated (430 nm) A2E-laden ARPE-19 cells (Fig. 1A). Elevations in iC3b generated in the presence of zymosan, a glycan derived from yeast cell walls, were also inhibited in factor B-depleted serum. Conversely, the use of C1q-depleted serum was associated with elevated levels of iC3b that were not different from those observed with normal serum (Fig. 1B). Zymosan added to C1q-depleted serum also conferred increased iC3b production. Zymosan is commonly recognized as an efficient activator of the alternative pathway. That complement activation by zymosan is exclusive to the alternative pathway is suggested by the finding that in mice in which the alternative pathway is inoperative because of a null mutation in factor B, zymosan does not activate complement.

To probe for complement activation under conditions involving direct contact with an oxidized form of A2E, we synthesized the previously characterized peroxy-A2E and precoated wells with the compound. Compared with the solvent-only (methanol) control, iC3b levels were increased in normal serum incubated in wells coated with peroxy-A2E (Fig. 2A) and furano-A2E (data not shown). In contrast, when BF-depleted serum containing 20 mM Ca2+ and Mg2+ was substituted for normal serum, the increase was inhibited. Reversal of the inhibition was achieved by adding 120 μg/mL factor B protein.
serum content of iC3b. In particular, statistically significant incubation with NHS as a source of complement led to elevated prescribed and were used to precoat wells. As shown in Figure 3, furano-all-
dimer, we undertook to test these photoproducts as activators mono- and bis-oxygenated photoproducts of all-
trans retinal dimer, all-
trans retinal dimer-PE and all-
trans retinal dimer. Because we found that unconjugated all-
trans retinal dimer is a more efficient generator of singlet oxygen and is more reactive with singlet oxygen and because we identified mono- and bis-oxygenated photoproducts of all-
trans retinal dimer, we undertook to test these photoproducts as activators of complement. To this end, peroxy-all-
trans retinal dimer and furano-all-
trans retinal dimer were generated as previously described and were used to precoat wells. As shown in Figure 3, incubation with NHS as a source of complement led to elevated serum content of iC3b. In particular, statistically significant increases in iC3b were observed when NHS was incubated in wells coated with peroxy-all-
trans retinal dimer and with furano-all-
trans retinal dimer compared with the solvent control. Serum iC3b was also increased when C1q-depleted human serum was incubated with peroxy-all-
trans retinal dimer and furano-all-
trans retinal dimer. However, when NHS was substituted with factor B-depleted serum, the increase in iC3b was attenuated.

C-Reacti ve Protein and Complement Activation
CRP is a pattern recognition molecule of the innate immune system that binds a number of ligands, including phosphoethanolamine and phosphocholine. Most people in the United States have plasma levels lower than 3 μg/mL; the endogenous level of CRP in the serum used in our experiments was 2 μg/mL. In AMD studies, CRP levels have been reported to be 10 μg/mL or lower. Therefore, we added exogenous CRP to NHS at concentrations of 1 μg and 10 μg/mL. To assay CRP involvement in complement activation in our system, we pre-coated wells with peroxy-A2E or A2E and then incubated it with NHS in the absence and presence of exogenous CRP. Given that these compounds are delivered to the wells using methanol as solvent, we included vehicle-control wells to which methanol had been added and evaporated. As shown in Figure 4, measurement of serum iC3b, a fluid-phase product of C3 activation, revealed increased amounts of iC3b in serum incubated with peroxy-A2E compared with A2E. The difference was similar to that previously observed (Fig. 4). The addition of CRP at concentrations of 1 μg/mL and 10 μg/mL attenuated this elevation in iC3b production (Fig. 4). On the other hand, the generation of iC3b was similar whether serum was incubated in a vehicle-exposed well or an A2E-coated well in the presence of 10 μg/mL exogenous CRP or when serum was incubated in the presence of 10 μg/mL exogenous CRP protein only.

C3 Cleavage Inhibitor
POT-4 is a small peptide analog of compstatin that inhibits the cleavage of C3 to the active fragments C3a and C3b, probably by interfering with protein-protein interactions during C3 convertase formation. To examine the ability of this C3 inhibitor to suppress C3 cleavage in our cell-based assay, we monitored

**FIGURE 3.** iC3b generation in serum incubated with all-
trans retinal dimer (atRAL dimer), peroxy-all-
trans retinal dimer (peroxy-atRAL dimer), and furano-all-
trans retinal dimer (furano-atRAL dimer). Wells were precoated with compound (in methanol) and incubated with normal human serum or human serum depleted of C1q (C1q-depleted) or factor B (factor B-depleted). iC3b levels measured in sera incubated in an otherwise empty well were subtracted as background. Mean ± SEM; three experiments. P values pertain to comparisons to control (solvent only).
iC3b generation in the presence and absence of POT-4. Consistent with previous results, iC3b levels in serum overlying irradiated-A2E-laden ARPE-19 cells were approximately 30% higher than in serum placed in contact with nonirradiated-A2E-laden ARPE-19 cells (Fig. 5). This elevation was reduced by POT-4 in a concentration-dependent manner. In these experiments, the mean background production of iC3b measured in serum incubated in empty wells was 75.07 μg/mL. POT-4 not only inhibited C3 activation attributable to irradiated-cell associated-A2E, it inhibited the background production of iC3b that likely occurs when serum is in contact with the polystyrene surface of the well (mean background measured in serum incubated in empty wells, 75.07 μg/mL). The latter inhibition was evidenced by a decrease in iC3b to levels below those observed in association with untreated RPE cells. To probe for inhibition of complement activation when serum is placed in direct contact with photooxidized A2E, we also coated wells with peroxyA2E and incubated with serum. Adding POT-4 to the serum suppressed iC3b production; the decreases were 22% at a concentration of 0.1 μM and 40% at a concentration of 10 μM (Fig. 5).

Controlling for Onset of Apoptosis

As an additional control, we also tested for whether the onset of apoptosis could influence complement activation in our cell-based assays. Accordingly, we induced apoptosis by treating ARPE-19 cells with the topoisomerase II inhibitor etoposide (100 μM for 48 hours). Subsequent incubation of etoposide-treated ARPE-19 cells with serum for 2 hours, a period equal to the postirradiation interval over which we measured complement activation, did not lead to elevated iC3b levels (mean ± SEM μg/mL iC3b: etoposide-treated, 116.6 ± 4.5; nonirradiated, 109.7 ± 2.0; P > 0.05; three experiments). In these experiments we also monitored cell death by labeling with Annexin V, a marker of redistributed phosphatidylserine in the plasma membrane, an early event in apoptotic cell death. Etoposide treatment (100 μM for 48 hours) resulted in 7.4% (±0.4%) Annexin V-labeled cells. This level of Annexin V labeling was slightly greater than the percentage of Annexin V labeling observed for A2E-laden ARPE-19 2 hours after 430 nm irradiation (3.14% ± 0.16%). The percentage of Annexin V labeling under other conditions was as follows: nonirradiated A2E-laden ARPE-19, 0.12% ± 0.04%; A2E-laden ARPE-19 immediately after 450 nm irradiation, 0.18% ± 0.05%; A2E-laden ARPE-19 2 hours after 430 nm irradiation, 3.14% ± 0.16%; A2E-laden ARPE-19 6 hours after 430 nm irradiation, 30.6% ± 1.6%. These results suggest that apoptosis per se does not activate complement. The possibility of a role for other intermediates is the subject of continued investigation.
DISCUSSION

We previously provided evidence that products of the photooxidation of RPE lipofuscin pigments can serve to trigger activation of complement and suggested that a stimulus such as this could generate the low-grade complement activation that, over time, contributed to the chronic inflammatory processes underlying AMD. In this follow-up study, we have shown that complement can be activated on the basal side of polarized differentiated cultures of fetal RPE cells, and we find that oxidized products of bisretinoid lipofuscin pigments other than A2E can also generate complement. Moreover, depletion of factor B from human serum inhibited the complement activation associated with photooxidized products of A2E and all-trans-retinal dimer. Given that factor B participates in the formation of the C3 convertase C3bBb and is specific to the alternative pathway, the present results suggest that complement activation by photooxidation products of bisretinoid pigments of RPE lipofuscin involves the alternative pathway. These results are significant because recent genetic studies have also implicated CFH of the alternative pathway in the aberrant complement regulation linked to AMD. A common haplotype containing genes for C2 and BF of the classical and alternative pathways, respectively, has also been reported to have an association. The associated variants in C2 and BF are in linkage disequilibrium; therefore, the genetic studies cannot discriminate between them, and involvement of the classical pathway cannot be excluded. Similarly, even in the case of a stimulus that activates complement through the classical pathway, amplification is mediated by the alternative pathway. Other sources of oxidative damage might initiate inflammation in this way.

CRP is used as a clinical biomarker of inflammation, yet its physiological function is not fully understood. Reports of an association between AMD and levels of CRP have been inconsistent, with modest increases in CRP indicated by some studies but not others. Variants in the CRP gene that alter serum levels of the protein are not associated with AMD risk. One important activity of CRP is to activate the classical complement cascade through interaction with C1q. Importantly, however, CRP also binds to CFH, thereby suppressing activation of the alternative complement pathway that would otherwise amplify the complement cascade. Recent compelling evidence indicates that the AMD-associated CFH-402Y variant; therefore, CFH-CRP interaction would have been functional and CRP would have been expected to downregulate the alternative pathway amplification loop. It is likely, for this reason, that iC3b levels were reduced when exogenous CRP was added to the serum.

Compstatin is a 13-amino acid peptide that is circularized by a disulfide bond and that binds C3 at the C-terminal half of the b-chain of the protein (C3c portion) and inhibits C3 cleavage to C3a and C3b possibly by preventing access of C3 to the convertase complexes. Compstatin has been studied in several in vitro models of complement activation using human serum and has been shown to inhibit heparin/protamine-induced complement activation in a baboon model of cardiopulmonary bypass. This inhibitor, which itself is nonimmuno- genic, exhibits greater inhibitory activity within the alternative pathway compared with the classical pathway, probably because the inhibitor binds not only C3 but also C3bBb. Compstatin has undergone a number of optimizations since its initial identification; one of these analogs is POT-4. Here we showed that POT-4 is also an effective inhibitor of complement activation when the latter is initiated by photooxidation products of RPE lipofuscin. POT-4 is the subject of clinical trials aimed at examining treatment efficacy when the compound is delivered by intravitreal injection in patients with neovascular AMD.

The most consistent environmental risk factor for atrophic and neovascular forms of AMD is cigarette smoking. Cigarette smoke harbors a variety of oxidants. The association between smoking and AMD is taken to be indicative of a role for oxidative mechanisms in disease pathogenesis. Support for this notion was also provided by the report from AREDS, which revealed that high doses of the antioxidants vitamins E and C and zinc reduce progression from intermediate to advanced AMD. Other clinical studies report similar findings. Interestingly, Wu et al. also observed that exposing retinal pigment epithelium to the oxidant hydrogen peroxide attenuated IFN-γ-induced stimulation of CFH, an important modulator of complement activation. Moreover, a number of naturally occurring antioxidants, including vitamins E and C, anthocyanins, and sulforaphane, have been shown to offer protection against A2E photooxidation.

AMD has its onset in the elderly years yet likely develops over multiple decades before diagnosis. Thus, contributions to AMD pathogenesis resulting from aberrant complement activation are likely to be long term. As a stimulus for the complement-associated events, we suggest that molecular fragments released by photooxidative processes within retinal pigment epithelium lipofuscin can play a role.

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