Estimation of Systemic Complement C3 Activity in Age-Related Macular Degeneration

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Objectives: To determine the role of systemic complement activation in the pathogenesis of age-related macular degeneration and to examine whether serum C3a des Arg reflects systemic complement activation, independent of individual complement component levels.

Methods: Plasma complement C3a des Arg levels and a single nucleotide polymorphism at position 402 of the complement factor H gene (CFH) were determined in 3 groups of subjects: 42 subjects with early age-related maculopathy, 42 subjects with neovascular (wet) age-related macular degeneration, and a control group of 38 subjects with no clinical evidence of age-related changes at the macula.

Results: The median (range) of plasma complement C3a des Arg levels in the age-related maculopathy and neovascular age-related macular degeneration groups were 52.6 (2.8-198.1) ng/mL and 60.9 (3.1-173.1) ng/mL, respectively. The levels were significantly raised compared with the control group (n=38), which had a median (range) plasma complement C3a des Arg level of 40.3 (6.1-81.7) ng/mL (analysis of variance, \( P = .02 \)). The concentration of plasma C3a des Arg did not differ significantly between those with different CFH genotypes (\( P = .07 \)).

Conclusion: Systemic activation of the complement system may contribute to the pathogenesis of age-related macular degeneration independent of CFH polymorphism.

Clinical Relevance: The results of this study may be relevant to aiming new treatment strategies toward reducing systemic low-grade inflammation.

Arch Ophthalmol. 2007;125:515-519

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may also be associated with reduced risk of early or late AMD, although the link between the total serum cholesterol concentration and AMD remains unproven.

By estimation of plasma complement C3a des Arg levels in subjects with AMD compared with age-matched controls, it is possible to indirectly determine the role of systemic complement activation with particular emphasis on the pathway that links the host defense to the adipocyte biology in the pathogenesis of AMD.

**METHODS**

This research adhered to the tenets of the Declaration of Helsinki. Institutional ethics committee approval was obtained and all of the patients gave their full informed consent.

Eighty-four persons with a clinical diagnosis of AMD were included in the study. The control group comprised 38 healthy subjects without AMD (defined as the absence of drusen, pigmentary abnormalities, and neovascular AMD). All of the enrolled subjects underwent a complete ophthalmic examination by the recruiting retinal specialist (S.S. or N.V.C.); visual acuity, slitlamp examination results, and retinal examination results after pupil dilation were documented. Each subject had 30° color stereo fundus photographs of both eyes taken. Fluorescein angiography was also performed if coexistent fundus pathological abnormalities or subjects with ungradable photographs were included in the study.

**GRADING OF AMD**

Color fundus photographs of the subjects were graded by 2 graders (S.S. and T.A.) using the nomenclature and classification recommended by the International ARM Epidemiological Study Group. Table 1 shows the classification used in this cohort.

Subjects with stages 0a and 0b were categorized as the control group. Early age-related maculopathy was defined as the presence of stage 2b or 3. Neovascular AMD included subjects with newly diagnosed CNV. The graders were masked of the age and clinical history of the participants. Double grading for intraobserver and interobserver variability was performed. Discrepancies were resolved by discussion. If the grades in the 2 eyes were different, the subject was categorized according to the severity of changes in the worse eye.

**BLOOD SAMPLES**

Venous blood was collected from all of the subjects in heparin tubes (BD Diagnostics, Oxford, England). It was centrifuged for 15 minutes at 2000g at 4°C, and the plasma was aliquoted and stored at −70°C within an hour of collection and then thawed when required. The C3a des Arg at −70°C was stable up to 3 months from the date of collection. The samples were randomized so that the scientist (S.S. or T.A.B.) who analyzed the samples was masked to the clinical history of the subjects.

**COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY**

A commercially available competitive enzyme-linked immunosorbent assay (ELISA) kit (Metachem Diagnostics, Ltd, Northampton, England) was used for the assay of plasma C3a des Arg. A brief outline of the assay design is explained here. Plasma proteins were precipitated from the sample with 10 N hydrochloric acid and 9 N sodium hydroxide, as whole proteins compete with the complement in the assay. The supernatant was then diluted 1:20—fold in fresh tubes.

Microtiter plates coated with goat antibody specific to rabbit IgG were used in this competitive ELISA. First, 100 µL of the serially diluted standards and the diluted samples were placed in each well in duplicate. Controls included zero standard (B₀) and assay buffer only (for nonspecific binding). Then, 50 µL of alkaline phosphatase conjugated with C3a des Arg was added to each well except the blank wells. The capture antibody used was 50 µL of rabbit polyclonal antibody to C3a des Arg. The plate was then incubated at room temperature for 2 hours at 300 rpm. After that, 3 washes with 200 µL of wash buffer (0.5 mL v/v 0.05% Tween in 1 L of tris-buffered saline) to each well were performed. Then, 200 µL of p-nitrophenylphosphate substrate solution (p-nitrophenylphosphate in buffer) was added to each well and incubated at 37°C for 1 hour followed by the addition of 50 µL of stop solution (trisodium phosphate in water) to each well. Absorbance was read at 405 nm using an automated plate reader (Dynex Technologies, West Sussex, England).

**CALCULATION OF RESULTS**

The net optical density (OD) bound for each standard and sample was calculated by subtracting the average nonspecific binding OD from the average bound OD. The percentage bound was calculated as net OD/net B₀, OD × 100.

The standard curve was then plotted on a logarithmic graph of percentage bound vs concentration of human C3a des Arg for standards. The concentrations of plasma C3a des Arg in the samples were determined by interpolation. The correlation coefficient for the standard curve was 0.989. The mean intra-

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>Mean (SD), ng/mL</th>
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<tbody>
<tr>
<td>0a</td>
<td>No signs of ARM</td>
<td>40.3 (23.4)</td>
</tr>
<tr>
<td>0b</td>
<td>Hard drusen (&lt;63 µm) only</td>
<td>52.6 (59.2)</td>
</tr>
<tr>
<td>1a</td>
<td>Soft distinct drusen (≥63 µm) only</td>
<td>60.9 (44.0)</td>
</tr>
<tr>
<td>1b</td>
<td>Pigmentary abnormalities only</td>
<td>63 µm) only</td>
</tr>
<tr>
<td>2a</td>
<td>Soft indistinct drusen (≥125 µm) or reticular drusen only</td>
<td>63 µm) only</td>
</tr>
<tr>
<td>2b</td>
<td>Soft distinct drusen (≥63 µm) with pigmentary abnormalities</td>
<td>125 µm) only</td>
</tr>
<tr>
<td>3</td>
<td>Soft indistinct drusen (≥125 µm) or reticular drusen with pigmentary abnormalities</td>
<td>125 µm) or reticular drusen only</td>
</tr>
<tr>
<td>4</td>
<td>Atrophic or neovascular AMD</td>
<td>63 µm) only</td>
</tr>
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</table>

**Table 1. Stages of Age-Related Macular Degeneration**

<table>
<thead>
<tr>
<th>Disease State</th>
<th>Median (Range), ng/mL</th>
<th>Mean (SD), ng/mL</th>
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<tbody>
<tr>
<td>Control (n = 38)</td>
<td>40.3 (6.1-81.7)</td>
<td>35.8 (23.4)</td>
</tr>
<tr>
<td>ARM (n = 42)</td>
<td>52.6 (2.8-198.1)</td>
<td>67.0 (59.2)</td>
</tr>
<tr>
<td>Neovascular AMD (n = 42)</td>
<td>60.9 (3.1-173.1)</td>
<td>68.3 (44.0)</td>
</tr>
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</table>

**Table 2. Plasma C3a des Arg and C3 des Arg Levels in the 3 Disease States**

Abbreviations: AMD, age-related macular degeneration; ARM, age-related maculopathy.
The assay and interassay coefficients of variation were 11.2% and
28.8%, respectively.

The ELISA was repeated thrice on the same day and on 3 sepa-
rate days to note the precision and reproducibility of the ELISA.
The minimum detectable limit for C3a des Arg concentration was
0.120 ng/mL. The cross reactivity value with complement C3 was
1.28%. All of the randomized samples were assayed in triplicate
using a total of 4 assay kits and performed in 2 days.

AMPLIFLUOR GENOTYPING TECHNIQUE FOR
SINGLE NUCLEOTIDE POLYMORPHISM TYPING

Genomic DNA was extracted from peripheral blood leuko-
cytes by using a standard protocol. Primers to identify the CFH
Tyr402His single nucleotide polymorphism variant (rs1061170)
were designed using an ampifluor assay (KBioSciences, Hert-
fordshire, England).

STATISTICAL ANALYSIS

The SPSS version 11.0 statistical software (SPSS, Inc, Chicago,
Ill) was used for the analysis. The data were not normally dis-
tributed. Results of plasma C3a des Arg concentrations were re-
ported as medians in the 3 groups and tested by analysis of
variance. To assess the significance of the association between
plasma C3a des Arg concentration and disease in each geno-
type category, linear regression analysis was performed after
square root transformation of the outcome variable. Disease state
was treated as a 3-level categorical variable with the control
group as the baseline. One-way analysis of variance was performed
to test the association between genotype and serum C3 levels.
Statistical significance was set at 95% (P<.05).

RESULTS

CHARACTERISTICS OF SUBJECTS

Plasma complement C3a des Arg concentrations were ana-
yzed in 122 subjects, including 42 with age-related macu-
lopathy, 42 with neovascular AMD, and 38 controls. Age and
sex distributions were similar in both groups of AMD
and controls. The CNV group was evenly distributed in
the 3 genotype groups (P=.72).

Figure 1. Box plot (A) and scatterplot (B) showing the levels of C3a des Arg in the subjects with age-related maculopathy (ARM), subjects with choroidal
neovascularization (CNV), and controls. Error bars indicate interquartile range; horizontal lines outside of error bars, outliers.

<table>
<thead>
<tr>
<th>Table 3. Plasma C3a des Arg Levels by Genotype</th>
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<tbody>
<tr>
<td>Genotype</td>
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<tr>
<td></td>
</tr>
<tr>
<td>All</td>
</tr>
<tr>
<td>CC</td>
</tr>
<tr>
<td>CT</td>
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<tr>
<td>TT</td>
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Abbreviation: IQR, interquartile range.

ESTIMATION OF COMPLEMENT
C3a des Arg CONCENTRATION

The standard curve was consistent with the manufactur-
er’s values. The ELISA was reproducible and accurate.
The median and range of plasma complement C3a des
Arg concentrations in the 3 groups are shown in Table 2
and Figure 1. The plasma complement C3a des Arg con-
centrations in the age-related maculopathy and neovas-
cular AMD groups were significantly raised compared with
the control group (analysis of variance, P=.02).

PLASMA C3a des Arg IN THE 3 CFH GENOTYPES

The genotyping of CFH in our cohort was classified as
CC, CT, and TT. The mean plasma C3a des Arg concen-
trations in the 3 CFH genotypes did not differ signifi-
cantly (P=.64) according to disease state. One-way analy-
thesis of variance was performed and showed that there was
no significant association between genotype and plasma
C3a des Arg levels (P=.13) (Table 3 and Figure 2).

The results of this study suggest that plasma complement
C3a des Arg concentration increases in subjects with AMD
compared with age-matched controls. The estimation of
plasma C3a des Arg concentration reflects systemic

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complement activation, independent of individual complement component levels. The liver is the main source of complement synthesis, and the complement molecules constitute approximately 5% of the total serum proteins. Many extrathepatic cells such as monocytes, endothelial cells, epithelial cells, glial cells, and neurons also produce complements. Although plasma C3a des Arg concentration is only an indirect estimation of systemic complement activation, our study suggests that systemic activation of the complement system may play a role in the pathogenesis of AMD. Products of the complement cascade serve as a powerful chemotactic stimulus, reinforcing the inflammatory process. In addition, sublethal injury by complement proteins permits the release of growth factors.

Evidence from several studies shows that complements are involved in the pathogenesis of AMD. Drusen, the hallmark of AMD, constitute many components of the complement cascade including C3 complement fragments. Components of chronically sequestrated debris in AMD may be potential activators of the proteolytic cascade, including apoptotic cells, nuclear fragments, and membrane-bound vesicles. The results of our study suggest that systemic metabolic end products may also serve as powerful chemotactic stimuli for leukocytes via the complement cascade.

Complement activation has also been shown in a murine model of laser-induced CNV in C57BL/6 mice. Nozaki et al provided evidence that resident retinal pigment epithelial cells are the primary source of complement activation in a mouse model of CNV. Our study suggests that increased systemic C3 activation may augment the disease process in AMD.

Complement factor H is the main regulator of the activation of C3. Therefore, complement factor H deficiency would allow unhindered activation of C3. However, the results of our study showed that systemic C3 activation is not significantly influenced by the CFH genotype in AMD. It may be that the effects of the CFH polymorphism in AMD are manifested locally.

The major weakness of this study is that C3a des Arg concentration is an indirect measure of C3 activation. Although to our knowledge this is the first study that reveals an association of systemic complement activation with AMD, it is difficult to make wide-ranging conclusions or assumptions based on these observations in view of the extreme variability in normative data of serum complements. However, this is an important starting point. Larger-scale future studies will be required to clarify the emerging relationship between C3 activation and AMD.

Submitted for Publication: June 4, 2006; final revision received July 22, 2006; accepted August 17, 2006.

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Financial Disclosure: None reported.

REFERENCES