Defining the Complement Biomarker Profile of C3 Glomerulopathy

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Abstract

Background and objectives C3 glomerulopathy (C3G) applies to a group of renal diseases defined by a specific renal biopsy finding: a dominant pattern of C3 fragment deposition on immunofluorescence. The primary pathogenic mechanism involves abnormal control of the alternative complement pathway, although a full description of the disease spectrum remains to be determined. This study sought to validate and define the association of complement dysregulation with C3G and to determine whether specific complement pathway abnormalities could inform disease definition.

Design, setting, participants, & measurements This study included 34 patients with C3G (17 with C3 glomerulonephritis [C3GN] and 17 with dense deposit disease [DDD]) diagnosed between 2008 and 2013 selected from the C3G Registry. Control samples (n=100) were recruited from regional blood drives. Nineteen complement biomarkers were assayed on all samples. Results were compared between C3G disease categories and with normal controls.

Results Assessment of the alternative complement pathway showed that compared with controls, patients with C3G had lower levels of serum C3 (P<0.001 for both DDD and C3GN) and factor B (P<0.001 for both DDD and C3GN) as well as higher levels of complement breakdown products including C3d (P<0.001 for both DDD and C3GN) and Bb (P<0.001 for both DDD and C3GN). A comparison of terminal complement pathway proteins showed that although C5 levels were significantly suppressed (P<0.001 for both DDD and C3GN) its breakdown product C5a was significantly higher only in patients with C3GN (P<0.05). Of the other terminal pathway components (C6–C9), the only significant difference was in C7 levels between patients with C3GN and controls (P<0.01). Soluble C5b-9 was elevated in both diseases but only the difference between patients with C3GN and controls reached statistical significance (P<0.001). Levels of C3 nephritic factor activity were qualitatively higher in patients with DDD compared with patients with C3GN.

Conclusions Complement biomarkers are significantly abnormal in patients with C3G compared with controls. These data substantiate the link between complement dysregulation and C3G and identify C3G interdisease differences.


Introduction

The term “C3 glomerulopathy” (C3G) has evolved to describe a set of diseases with distinct renal biopsy findings: dominant glomerular C3 fragment deposition on immunofluorescence and characteristic densities on electron microscopy (EM) (1–3). The emergence of this term has been driven by a number of key developments, including (1) the identification of well characterized pedigrees that have led to the first glimpses of disease definition and spectrum, (2) a movement to accurately group patients into a pathologic category that may have both mechanistic and treatment significance, (3) advances in the sophistication and availability of assays of complement function and, (4) improved accessibility of comprehensive screens of the complement genes.

Two major subgroups of C3G are recognized: dense deposit disease (DDD) and C3 glomerulonephritis (C3GN). DDD and C3GN by definition have in common C3 dominance on renal biopsy immunofluorescence. Although the term “dominance” has yet to be fully agreed upon, it generally implies a C3 fragment immunofluorescence intensity of at least two orders of magnitude greater than any other immune reactants (i.e., IgG) on a scale of 0–3 (including 0, trace, 1+, 2+, and 3+) (4). There is no light microscopy requirement in the current definition of C3G.

EM distinguishes DDD from C3GN (Figure 1). The complexity and location of the electron-dense deposits in C3G are varied; however, it is generally accepted that the character of the intramembranous immune deposits on EM differs between DDD and C3GN. In DDD, the glomerular intramembranous deposits are very dense, band-like, or sausage-like shapes interspersed with more normal-appearing glomerular basement
of a reactive thiol ester in the C3 molecule located in the thiol ester domain of the protein.

Spontaneous hydrolysis of the thiol ester converts C3 to C3(H₂O) (also known as iC3) in a process known as "tick-over." C3(H₂O) reacts with factors B (FB) and D to form C3(H₂O)Bb, an intermediate enzyme and the initial C3 convertase of the alternative pathway. C3(H₂O)Bb cleaves C3 to C3b, forming the nascent C3 convertase C3bBb. Tight control of C3 convertase activity by the regulators of complement activity prevents excessive activity and unintentional complement-mediated damage. However, on pathogenic surfaces or in the absence of appropriate control on self-tissue, large amounts of C3 convertase form and complement activity continues unchecked.

C3 is one of the most abundant plasma proteins (approximately 2% of total plasma protein), and tremendous amounts of C3b are generated with C3 convertase dysregulation. Excess C3b is handled by one of two concurrent pathways: (1) C3b is cleaved in multiple sequential steps by factor I-mediated activity to generate inactive C3b and ultimately the C3 breakdown products C3d and C3g; and (2) C3b combines with extant C3bBb to form the C5 convertase C3bBbC3b, which is the second important enzyme of the complement pathway. C5 convertase cleaves C5 to C5a and C5b. C5b triggers the terminal complement cascade, which culminates in formation of the membrane attack complex (Figure 2).

C3G is driven by dysregulation of the C3 and C5 convertases. Because there have been no comprehensive assessments of the degree of dysregulation of the convertases in C3G compared with controls, we sought to determine whether we could define and/or distinguish the complement activity in DDD and C3GN by biomarker profiling. We hypothesized that specific biomarkers may provide a more detailed and mechanistic understanding of the underlying complement pathology and therefore add to disease definition. By defining a complement pathway signature, we predict that biomarkers may be useful metrics of disease activity and/or severity in the clinical setting.

Materials and Methods

Study Population

Thirty-four patients with biopsy-proven C3G (17 with DDD and 17 with C3GN) diagnosed between 2008 and 2013 were selected from our C3G Registry for inclusion in this study based on the availability of sufficient sera and plasma samples to complete all assays multiple times, as well as histopathologic data (light microscopy, immunofluorescence, and EM) to review the pathologic diagnosis (Figure 3). All patients had biopsy-proven disease consistent with the description provided in the C3G consensus article, and all qualifying biopsies were confirmed by the research team and an independent pathologist (4). Posteculizumab samples were not included in this study. The University of Iowa Institutional Review Board approved all procedures and all patients gave informed consent before donating samples.

Serum and Plasma Samples

Serum and plasma samples were prepared by centrifugation (1000×g/10 minutes) of whole blood allowed to clot, respectively, in either plain (untreated) tubes for 45 minutes or EDTA tubes for <20 minutes. Collected samples were
immediately frozen and stored at \(-80^\circ\text{C}\). Upon receipt by the Molecular Otolaryngology and Renal Research Laboratories, samples were thawed, aliquoted on wet ice into screw-top Eppendorf tubes for future use (serum samples, 250 \(\mu\text{l}\); plasma samples, 50 \(\mu\text{l}\)), and stored at \(-80^\circ\text{C}\). Samples used for complement testing were never refrozen; the unused portion was discarded. Normative data were generated from serum and plasma samples from 100 healthy blood donors (1:1 ratio of men to women; 97% of participants were European American and 3% were of other ethnicity).

**Complement Protein Assays**

Plasma samples were analyzed using a variety of commercially available assays (Supplemental Table 1). Direct sandwich ELISA (detection antibody directly coupled with horseradish peroxidase) was used to measure \(\text{Ba}, \text{Bb}, \text{C}3\alpha, \text{C}3\beta, \text{C}3\gamma, \text{and soluble C}5\text{b-9 (sC5b-9)}\) (Quidel Corporation, San Diego, CA; ProGen Biologics, Saint Louis, MO). Indirect sandwich ELISA (biotinylated detection antibody followed by streptavidin-conjugated horseradish peroxidase) was used for properdin, \(\text{FB}, \text{C}5, \text{C}6, \text{C}7, \text{C}8, \text{and C9 (Abcam, Cambridge, MA; Hycult Biotech, Plymouth Meeting, PA).}

Total \(\text{C3}\) was measured by radial immunodiffusion (The Binding Site, Birmingham, UK) and \(\text{C3c}\) was measured as described (7). Results were interpreted using a microplate plate reader at \(A450\) (Bio-Rad Life Science, Hercules, CA) and calculated by Microsoft Excel software or by four-parameter logistic regression (www.myassay.com). \(R^2\) exceeded 0.98 for each ELISA assay.

**Autoantibody Assays**

Serum samples were analyzed for \(\text{C3 nephritic factors (C3Nefs)}\) and factor \(\text{H autoantibodies as described (8).}\)

**DNA Extraction and Genetic Testing**

Genomic DNA was extracted from blood samples using standard protocols (PAXgene Blood DNA Kit; Qiagen, Valencia, CA). Coding regions and intron-exon boundary junctions of multiple complement genes were amplified and screened using bidirectional sequencing as described (9).

**Statistical Analyses**

Data were collected in triplicate on different testing days using aliquots of a single serum or plasma sample. All data were presented as box plots with values greater than 1.5\times the interquartile range (IQR) considered as outliers. The \(t\) test or the Mann–Whitney \(U\) test was performed using SigmaPlot (Systat Software Inc, San Jose, CA). A \(P\) value <0.05 was considered significant.

**Results**

**Study Population**

Demographic details and basic clinical information are listed in Table 1. Complement assays were performed on...
34 patients and 100 controls. The age range for patients was 7–62 years (mean 23 ± 13). The mean duration of disease at the time of specimen procurement was approximately 5 years. All but two study patients were European American. Patients with C3GN were predominantly women and patients with DDD were predominantly men.

**Complement Assays**

**Alternative Pathway.** In both DDD and C3GN, serum C3 levels were significantly lower than in controls (DDD: median 0.52 g/l [IQR, 0.2–0.82]; C3GN: 0.71 g/l [0.44–1.06]; controls: 1.34 g/l [1.21–1.49]; P < 0.001 for both). Serum FB, a component of the C3 convertase, was also reduced (DDD: 167 mg/l [IQR, 135–207]; C3GN: 143 mg/l [126–179]; controls: 203 mg/l [188–219]; P < 0.001 for both), whereas its breakdown products Ba (DDD: 1.0 mg/l [IQR, 0.49–1.71]; controls: 0.63 mg/l [0.49–0.72]) and Bb (DDD: 1.28 mg/l [IQR, 1.14–1.52]; C3GN: 1.33 mg/l [1.16–3.10]; controls: 0.85 mg/l [0.75–1.03]) were significantly elevated in C3GN and DDD (P < 0.01 and P < 0.001, respectively, for Ba; P < 0.001 for both for Bb). Properdin binds to and stabilizes C3bBb, increasing its t1/2 approximately 10-fold. Whereas properdin serum levels were reduced in both DDD and C3GN, properdin levels were generally lower in C3GN compared with DDD (DDD: 17.4 mg/l [IQR, 12.4–20.2]; C3GN: 10.2 mg/l [7.4–14]; controls: 21.5 mg/l [17.5–25.2]; P < 0.05 for DDD versus controls; P < 0.001 for C3GN versus controls). Interdisease comparison of DDD and C3GN for these complement proteins identified only the decrease in properdin as significant between diseases (DDD versus C3GN, P < 0.01). Measured breakdown products of C3 included C3a, C3c, and C3d (see Figure 2), with only the increase in C3d significantly different in DDD and C3GN versus controls (DDD: 0.88 mg/l [IQR, 0.71–1.52]; C3GN: 1.05 mg/l [0.86–1.14]; controls: 0.47 mg/l [0.44–0.54]; P < 0.001 for both). There were no significant interdisease differences for any measured cleavage products of C3 (Figure 4A).

**Terminal Pathway.** Activation of the terminal complement pathway is initiated by activity of the C5 convertase, C3bBbC3b, which cleaves C5 to C5a and C5b. In both DDD and C3GN, C5 levels were reduced compared with controls (DDD: 70 mg/l [IQR, 59–77]; C3GN: 65 mg/l [54–79]; controls: 91 mg/l [76–102]; P < 0.001 for both) and although C5a levels tended to be elevated, only differences between C3GN and controls were significant (DDD: 9.6 µg/l [6.0–14.2]; C3GN: 12.9 µg/l [7.9–16.9]; controls: 8.8 µg/l [4.3–9.9]; C3GN versus controls, P < 0.05). With the sequential addition of C6–C9 to C5b, the membrane attack complex and soluble C5b-9 are formed. C6, C8, and C9 levels were not reduced in either DDD or C3GN, but serum levels of C7 were decreased in C3GN compared with both controls and DDD (DDD: 79 mg/l [65–103]; C3GN: 69 mg/l [55–78]; controls: 83 mg/l [73–93]; C3GN versus controls, P < 0.01; C3GN versus DDD, P < 0.05). Soluble C5b-9 was increased in both diseases, although the increase was greater in C3GN (DDD: 0.18 mg/l [0.19–0.92]; controls: 0.14 mg/l [0.05–0.19]; C3GN versus controls, P < 0.001; C3GN versus DDD, P < 0.05) (Figure 4B).

**Autoantibodies.** C3Nefs are autoantibodies to the C3 convertase that can be detected using direct and indirect assays. Direct assays test patient-purified Igs after their isolation using a Melon Gel IgG purification kit (Pierce Protein Biology Products, Thermo Fisher Scientific Inc, Rockland, IL) in assays of C3 convertase activity with or without the addition of properdin (C3 convertase stabilizing assay with or without properdin [C3CSAP and C3CSA, respectively]). Indirect assays, by contrast, measure the activity of C3 convertases (presumed to be stabilized by C3Nefs) by detecting C3b breakdown products when patient and control sera are mixed (immune-fixation electrophoresis [IFE]). C3Nefs were significantly present in patients with C3G compared with controls. In the three assays we used (C3CSAP, C3CSA, and IFE), C3Nef activity was always greater in DDD compared with C3GN (DDD versus C3GN: P < 0.001 for C3CSAP, P < 0.001 for C3CSA, and P < 0.01 for IFE). No FH autoantibodies were detected in either group (Figure 4C, Supplemental Tables 2 and 3).

**Genetic Testing**

Three patients with DDD (17.6%) carried genetic variants. In two patients, three FH variants were identified (heterozygous c.2821G>A, p.V941F; compound heterozygous c.2509G>A, p.V837I; and c.3435G>C, p.E1145D), whereas the third patient carried a F1 variant (heterozygous c.1217G>A, p.R406H). One patient with C3GN (5.9%) carried an FH variant (heterozygous c.1699A>G, p.R567G). There was no difference in the frequency of FH and C3 risk alleles between groups (DDD versus C3GN: P > 0.05) (Supplemental Tables 2 and 3).

**Discussion**

The recently adopted disease designation C3G emerged in part because it has become clear that pedigrees with glomerular disease associated with identifiable complement

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**Table 1. Demographic parameters for the study cohort**

<table>
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<th>Parameter</th>
<th>C3GN</th>
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<th>Controls</th>
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<td>100</td>
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<td>Age, yr</td>
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<td>4.9±3.6</td>
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</table>

Data are presented as the mean±SD unless otherwise indicated. DDD, dense deposit disease; N/A, not applicable.
mutations have a specific immunofluorescence pattern on renal biopsy. In addition, it is now understood that historical renal pathology descriptions were not sufficiently informative about renal disease (i.e., patients with membranoproliferative GN II did not always have membranoproliferative GN). The final advance, the expanded development and availability of both complement functional assays and complement gene analyses, was a necessary advancement in making it reasonable to rethink disease descriptions.

Immunofluorescence findings that are strongly positive for C3 and negative or only weakly positive for Igs suggest an abnormality in the complement system and thus, as recommended by consensus, identify a group of patients in whom genetic and functional testing of the alternative pathway and terminal complement system are warranted (4). Our study confirms the validity of this recommendation in the first comprehensive attempt to characterize in a stepwise fashion the unique complement abnormalities in patients with C3G. It is our expectation that this type of granular approach to identifying the specifics of a given patient’s complement abnormality will not only be informative for pathobiology but will also prove integral to the development of effective on-target treatment options. The ultimate goal will be to aid the clinician in choosing a tailored therapy as additional anticomplement therapeutics become available.

Given the history of C3G and our a priori assumption that C3G is a complement-mediated disease, it was not surprising that serum C3 was significantly reduced in C3G compared with normal controls. Although the reduction in C3 tended to be greater in DDD, the difference between DDD and C3GN was not statistically significant. Also in keeping with the belief that a continuously active alternative pathway is central to disease, it was not unanticipated that there was an apparent consumptive decrease in FB with an increase of FB split products Ba and Bb. The reduction in properdin found in both diseases is noteworthy because properdin stabilizes the C3 convertase (C3bBb), increasing its t1/2 about 10-fold and favoring formation of the C5 convertase (C3bBbC3b). The intergroup difference in properdin reduction suggests that the generation of C5 convertase is greater in C3GN than in DDD (Figure 4A) (10).

Figure 4. | Complement biomarker assessments in the C3G cohort. (A) Eight alternative pathway biomarkers (C3, FB, properdin, and their associated degradation products C3a, C3c, C3d, Ba and Bb) were assessed in 17 patients with C3GN, 17 patients with DDD, and 100 controls (normal). Serum C3 and FB levels were significantly lower in patients with C3G than in controls, whereas the breakdown products C3d, Ba, and Bb were significantly elevated. Properdin serum levels were reduced in both DDD and C3GN although properdin levels were generally lower in C3GN compared with DDD. (B) Seven terminal pathway biomarkers (C5–C9 and the activation products C5a and sC5b-9) were assessed in the same cohorts. C5 levels were reduced compared with controls in both DDD and C3GN. Although C3a levels tended to be elevated in both diseases, only the elevation in C3GN versus controls was significant. Whereas C6, C8, and C9 levels were not reduced in DDD or C3GN, serum levels of C7 were decreased in C3GN compared with both controls and DDD. Soluble C5b-9 was elevated in both diseases, with the elevation being greater in C3GN. (C) C3Nefs were measured by two direct assays (C3CSA and C3CSAP) and one indirect assay (IFE) (8). No control was positive for any C3Nef assay. In all assays, C3Nef activity was greater in DDD compared with C3GN. For all box-and-whisker plots, the first and third quartiles are represented by the bottom and top ends of the box, the median is represented by the horizontal line in the interior of the box, and the whiskers represent 1.5× the interquartile range. Open circles are outliers. *P<0.05; **P<0.01; ***P<0.001. C3CSA, C3 convertase stabilizing assay; C3CSAP, C3 convertase stabilizing assay with properdin; FB, factor B; IFE, immunofixation electrophoresis; NL, normal; sC5b-9, soluble C5b-9.
although its split product C5a is elevated only in C3GN. In addition, C7 is consumed, although more so in C3GN than in DDD. Finally, and arguably the most important finding from a clinical perspective given the mechanism of the currently available anticomplement therapeutic option, soluble C5b-9 is more likely to be elevated in C3GN than in DDD (C3GN versus normal, \( P<0.001 \); C3GN versus DDD, \( P<0.05 \)). The combination of an elevated C5a, a potent proinflammatory anaphylotoxin, and an elevated C5b-9 level may define a group of patients more likely to respond to anti-C5 therapeutics as suggested by Bomback et al. (11).

C3Nef activity was significantly elevated in patients with C3G compared with normal controls. Because C3Nefs stabilize the C3 convertase, this finding suggests a relative upregulation of this enzyme, which appeared to be more pronounced in DDD compared with C3GN.

In aggregate, our data can be interpreted to mean that C3G is characterized by dysregulation of the C3 and C5 convertases. Because these convertases are extremely similar (C3 convertase=C3bBb; C5 convertase=C3bBbC3b), we do not believe that a “second hit” needs to be invoked for dysregulation of both convertases to occur. Rather, we propose a model in which the initiating factor or factors (genetic and/or acquired) have a differential effect on the two convertases given their similarity, with the observed differences between DDD and C3GN being defined, at least in part, by differences in the degree of convertase dysregulation.

Disregulation of the C3 convertase tends to be greater in DDD, whereas dysregulation of the C5 convertase tends to be greater in C3GN. The consequence of C3 convertase dysregulation (only or predominantly) appears on EM as the darker/denser, sausage-like, or band-like midlayer deposits and may represent a constant, slow build-up of C3. Dysregulation of both convertases to occur. Rather, we propose a model in which the initiating factor or factors (genetic and/or acquired) have a differential effect on the two convertases given their similarity, with the observed differences between DDD and C3GN being defined, at least in part, by differences in the degree of convertase dysregulation.

In summary, we have shown that complement biomarkers are significantly abnormal in patients with C3G compared with controls. These abnormalities expand our understanding of C3G by substantiating the link between complement dysregulation and C3G and identifying C3G interdisease differences.

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Disclosures

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