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A Quantitative Lateral Flow Assay to Detect Complement Activation in Blood

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Abstract

Complement is a major effector arm of the innate immune system that responds rapidly to pathogens or altered self. The central protein of the system, C3, participates in an amplification loop that can lead to rapid complement deposition on a target and, if excessive, can result in host tissue damage. Currently, complement activation is routinely monitored by assessing total C3 levels, which is an indirect and relatively insensitive method. An alternative approach would be to measure downstream C3 activation products such as C3a or iC3b. However, *in vitro* activation can produce falsely elevated levels of these biomarkers. To circumvent this issue, a lateral flow immunoassay system was developed that measures iC3b in whole blood, plasma and serum and avoids *in vitro* activation by minimizing sample handling. This assay system returns results in 15 minutes and specifically measures iC3b while having minimal cross-reactivity to other C3 split products. While evaluating the potential of this assay, it was observed that circulating iC3b levels can distinguish healthy individuals from those with complement activation-associated diseases. This tool is engineered to provide an improved method to assess complement activation at point-of-care and could facilitate studies to monitor disease progression in a variety of inflammatory conditions.

Keywords

Complement activation; iC3b biomarker; lateral flow assay; lupus; intracerebral hemorrhage (ICH)

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Introduction

The complement system is a phylogenetically ancient branch of the innate immune system that primarily serves to eliminate foreign pathogens from the host [1, 2]. A second function of the complement system is to recognize and mark altered self, such as apoptotic or necrotic cells, for clearance [3]. The complement system is activated via three distinct pathways, the classical, lectin, and alternative. While the classical and lectin pathways are initiated by antibodies recognizing antigens and lectins binding sugars, respectively, the alternative pathway (AP) is spontaneously triggered at a continuous, low rate in the blood through a process known as tick-over (reviewed in [4]). The three activation cascades converge at the central step of activation of component 3 (C3) (Figure 1). Additionally, recent studies have identified an extrinsic pathway, which allows activation of C3 (and the downstream C5) via enzymes of the coagulation pathway and other proteases [5, 6].

C3 is the most abundant protein of the complement system and its key opsonic protein [7]. Proteolytic activation of C3 leads to two split products, C3a and C3b. Deposition of C3b marks pathogens and waste material for clearance by phagocytic cells through immune adherence and ingestion [3]. Release of the anaphylatoxins C3a and the downstream C5a lead to recruitment of inflammatory cells such as neutrophils to a site of infection. Furthermore, initiation of the terminal pathway leads to membrane perturbation and subsequent target cell lysis by the membrane attack complex (MAC).

C3 is activated by the C3 convertases, enzymatic complexes formed via all three pathways which convert C3 to C3a and C3b (Figure 1A). During this conversion, the protein undergoes a dramatic conformational change that results in exposure of the thioester bond [8, 9]. This is a highly reactive species that enables the transfer of the protein from the fluid phase to nearby targets through a covalent interaction. C3b is itself a component of the AP C3 convertase and thus participates in its own activation. This results in a powerful positive feedback loop (the AP amplification loop) that can result in the rapid deposition of many copies of C3b on a target (reviewed in [10]).

To prevent damage to the host, complement activation is stringently regulated (Figure 1A). Once produced, C3b can be rapidly converted to iC3b by the serine protease Factor I (FI) and a cofactor protein, which releases a small fragment of 18 amino acids, C3f, into the fluid phase (reviewed in [11, 12]). iC3b cannot participate in the amplification loop of the AP and is normally cleared from circulation.

While iC3b does not participate in the complement activation cascade, it does have important immunological roles. iC3b binds to complement receptors 2, 3 and 4 (CR2, CR3 and CR4). Upon binding to CR2 on B-cells, iC3b contributes to the humoral response by serving as a costimulatory signal. CR3 and CR4 are expressed on myeloid cells, particularly neutrophils and macrophages, respectively. When iC3b engages either of these receptors it leads to adhesion as well as phagocytosis. Deficiency of CR3/CR4 leads to leukocyte adhesion deficiency syndrome (reviewed in [13]).

The initiating C3 convertase [C3(H₂O)Bb] of the AP is thought to be generated through the tick-over process. This system continuously surveys the host environment for danger and, if found, initiates a response within seconds. When generated, C3b deposits on any nearby surface with nucleophilic groups, whether it is a foreign surface or self [14]. In healthy individuals, there is a balance between activation and regulation which allows for constant surveillance for targets while preventing excessive activation on healthy host cells. However, if imperfectly regulated, the system also allows for chronic, non-specific activation and can contribute to the pathology of chronic inflammatory disease [4]. If C3 comes in contact with non-biologic surfaces such as dialysis tubing or plasticware used in the laboratory, it can deposit on those surfaces as well, causing further *in vitro* activation through the feedback loop and shifting the balance towards further C3b generation (Figure 1B). Similarly, if samples are not handled carefully or stored properly, *in vitro* activation can occur which may mask the *in vivo* signal, cause falsely elevated levels of split products, and confound the interpretation of the patient's complement activation status.

Complement activation is associated with many diseases including systemic lupus erythematosus (SLE) [15], atypical hemolytic uremic syndrome (aHUS) [16], membranoproliferative glomerulonephritis I (MPGN I) [17], dense deposit disease (DDD) [18, 19], age-related macular degeneration (AMD) [20], myocardial infarction [21], and preeclampsia (PE) [22]. Current clinical tests for complement measure either functional activity by total hemolytic activity (CH50) or total C3 antigen levels (intact C3 plus activation breakdown products) with an immunoassay. Although these tests are established and used routinely, they may not accurately reflect C3 activation status and lack the sensitivity to identify lower levels of activation. The normal range for both functional assessments and immunoassays is fairly wide, and therefore a substantial change is required before C3 activation is reliably detected by these measures. As C3 is an acute phase reactant, during times of inflammation C3 may be consumed while production by the liver is increased. Consequently, a continuous moderate level of activation, although pathogenic, may not be detected. A biomarker that serves as an accurate measure of disease activity would have significant clinical utility. iC3b is a good candidate biomarker, because its presence is a result of complement activation. Unlike the C3a and C3b fragments, which have shorter half-lives (Table 1), iC3b is not cleared as quickly, and thus may provide a more reliable measure for current complement activation status [23]. C3d is another cleavage product of C3 that is generated downstream of iC3b and has an even longer half-life. However, its longer half-life (days) may make it a less than ideal marker for determining the degree of complement activation that is occurring at that moment. Furthermore, there is a paucity of antibodies that specifically recognize C3d, without recognizing iC3b (unpublished data). Despite the potential utility of iC3b as a biomarker for complement activation, it has not been adopted to date, in part due to *in vitro* activation associated with sample handling and the assay method which may obscure the physiologic levels of iC3b.

In this study, a new lateral flow assay (LFA) system was developed for both C3 and iC3b and was utilized to measure the levels of these proteins in blood, serum and plasma of healthy donors and patients with complement-associated disease. The results demonstrate

the potential for utility of this assay system to accurately measure total C3 and iC3b and avoid the *in vitro* activation seen in traditional methods such as ELISA. Finally, these studies show that circulating iC3b levels are very low in healthy donors, but often elevated in disease.

Materials and Methods

Materials

Purified C3, C3b, and iC3b were obtained from Complement Technologies (Tyler, TX). Monoclonal antibodies to C3 fragments were obtained from Quidel (San Diego, CA). Polyclonal C3 antibodies were obtained from Dako (Carpinteria, CA) and MP Biomedicals (Solon, OH). The MicroVue iC3b EIA Kit was obtained from Quidel.

Normal donor samples

Whole blood was obtained under a protocol approved by the Washington University School of Medicine institutional review board from healthy donors by venipuncture or finger stick, and immediately analyzed in some applications. Plasma was prepared immediately after blood collection employing K3-EDTA Vacutainer tubes (Becton Dickinson, USA) by centrifugation at $2,000 \times g$ for 10 min. For serum preparation, blood was incubated at RT for 30 min in serum Vacutainer tubes (Becton Dickinson), followed by centrifugation at $2,000 \times g$ for five min at RT.

Patient samples

Serum samples were obtained from patients with active SLE enrolled in studies at Johns Hopkins University and Beth Israel Deaconess Medical Center. For the purpose of this study, active SLE was defined as a SLEDAI ≥ 4 and low C3 and C4. The Johns Hopkins University School of Medicine approved the Hopkins Lupus Cohort on a yearly basis and all patients gave written informed consent. Whole blood samples were obtained from intracerebral hemorrhage (ICH) patients according to the protocol approved by the Columbia University institutional review board and informed consent was obtained. Whole blood from normal control volunteers was collected under the same protocol. The samples from ICH patients were collected within 48 hours of admission concurrent with glucose monitoring. Data on ICH volume, location and edema volume were also recorded. Serum samples were stored at -80°C prior to analysis and whole blood was analyzed immediately after collection.

Lateral flow assays

Assay principle—The tests are based on LFA design which utilizes two compatible antigen-specific antibodies to form an immunochromatography assay [24, 25]. The assay methodology includes a visible detection antibody conjugated to colloidal gold and a capture antibody bound to a nitrocellulose membrane strip to form a solid-phase enzyme immunoassay. The basic assembly for each test consists of a blood cell pad to filter out blood cells, followed by a conjugate pad containing the gold-conjugated detection antibody. Next, a nitrocellulose membrane contains immobilized capture antibodies that make up the test and control lines. Finally, there is an absorbent pad for wicking (Figure 2A). The final

assembled strip is contained in a cassette with two openings: the first (smaller) opening is for loading the sample of interest, while the second opening is where the test or assay develops visually in a concentration-dependent manner. After applying the sample to the test, the fluid mixes with the antibody-gold conjugate to create an antigen-antibody-gold complex. The immobilized capture antibody forms a test line on the exposed nitrocellulose by capturing the antigen-antibody-gold complexes in a concentration and time-dependent manner. High concentrations of antigen (iC3b or total C3) may allow the test line to become visible within 2 min of sample application. Over the course of 10-30 min, more antigen-antibody-gold complex accumulates to form visible lines. A second line, the control line, forms an easily visible line regardless of the amount of biomarker present and usually becomes visible within a minute of sample application. This control line is comprised of anti-IgG antibodies and captures any gold-conjugated antibodies, thus demonstrating that the reaction conditions and technology worked. After passing these reaction zones, the fluid enters the final absorbent pad that simply acts as a waste container.

Assay procedure—The test procedure was carried out as follows. The serum, plasma or whole blood sample was serially diluted 1:200 and 1:10,000 for the iC3b and C3 tests, respectively, in sample diluent buffer (BioAssay Works, ISOT-003, Ijamsville, MD). Diluted sample (100 μ l) was added to the cassette. After a 15 min (for the C3 test) or 30 min (iC3b test) incubation at RT, the cassette was inserted into the LFA reader. The concentration of total C3 and iC3b was determined in each sample using the test-specific polynomial equations and correction for the dilution factor.

LFA reader—For quantitative analysis of the LFA, the reflectance reader from LRE Medical (München, Germany) was utilized. The reader employs light reflectance technology to scan the test strips for visible gold complexes and generate values in a concentration-dependent manner. The results are reported as reflectance units.

Nephelometry—The MININEPH™ Human C3 Kit (The Binding Site; ZK023.R) was used according to the manufacturer's instructions and analyzed via the MININEPH instrument. Briefly, serum samples were diluted 1:11 into sample diluent before being transferred into a cuvette, which was then placed into the MININEPH instrument. The MININEPH C3 antiserum and C3 buffer were immediately added to the diluted serum, mixed, and then analyzed via nephelometry 30 s later. Each serum sample was assayed in duplicate, and the average C3 concentration was calculated using the calibration curve provided with the instrument. The measurement range of the Minineph is 0.275-4.44 g/L (using the recommended 1:11 sample dilution).

iC3b ELISA—For the in-house iC3b ELISA, Immulon 1B microtiter plates (Thermo Scientific) were coated with a mouse anti-human iC3b monoclonal antibody (Quidel) for capture while goat anti-C3 polyclonal HRP antibody was employed as the detection antibody. Wells were blocked with StartingBlock Buffer (Thermo Scientific). PBS (1 $\times$) with 5.0 mM EDTA was utilized for sample dilution and 1 $\times$ PBS, 0.05% Tween-20 was used for washing the wells. Plasma and serum samples were diluted into a non-protein binding 96-well microtiter plate (Thermo Scientific) before transferring to the iC3b ELISA plate. The

samples were initially diluted 1:10, followed by serial 1:2 dilutions. They were incubated for the specified times in the anti-iC3b antibody coated wells before washing with buffer. Then, HRP-conjugated anti-C3 detection antibody was applied for 30 min. Following washing, a 1:1 solution of detection solution (Peroxide Solution and Peroxidase Substrate TMB; Thermo Scientific) was used to develop the assay for up to 4 min before addition of 1M H₂SO₄ to stop the color-forming reaction. Absorbance at 450 nm was determined with a BMG Labtech POLARstart Omega plate reader (Ortenberg, Germany).

In other experiments, the commercially available MicroVue iC3b ELISA (Quidel) was performed according to the manufacturer's instructions.

Statistical Analysis—To measure the agreement between the various concentration measurements (LFA, ELISA, nephelometry, and those provided by the supplier), intraclass correlation coefficients (ICC) were used. All statistical analyses were performed using the R environment for statistical computing (R Development Core Team, Vienna, Austria, 2013), and the R package irr [26].

Results

C3 and iC3b tests on the lateral flow assay platform are specific and have a wide dynamic range

The lateral flow assay (LFA) utilizes matched pair Abs to specifically identify its target analyte (Figure 2A). The C3 cassette is composed of an anti-C3c polyclonal antibody (Dako) as the gold-labeled detection antibody and a polyclonal goat anti-C3 (MP Biomedicals) antibody as the test line. The iC3b cassette consists of an anti-iC3b neoepitope specific gold-labeled monoclonal antibody and the test line uses a C3d neoepitope specific monoclonal antibody (both from Quidel). For both tests, the purified protein was diluted into LFA buffer and assayed in triplicate. For the total C3 test standard curve, C3 protein was diluted to concentrations between 6.25 and 800 ng/ml and tests were allowed to develop for 15 min before analysis via the reader. For the iC3b test standard curve, purified iC3b protein was diluted from 1.0 to 128 ng/ml and allowed to develop for 30 min before reader analysis. The coefficients of variation (CVs) were generally between 1-10% for both tests across the range except for the very low end of the iC3b detection range where it was 10-20%. Thus, for whole blood, plasma and serum, the total C3 LFA has a quantitation range of 6.25 to 400 ng/ml and the iC3b test can quantitate protein from 4 to 128 ng/ml (Figure 2B and C).

The lateral flow assay platform can detect total C3 levels in 15 minutes with the same accuracy as nephelometry

Blood and serum samples from 10 normal donors were diluted 1:10,000 into LFA buffer for the total C3 test. The samples were assayed immediately by applying 100 µl of diluted sample to each test, which were run in duplicate or triplicate, and incubated for 15 min before analysis using the reader. C3 levels detected in serum by LFA were substantially equivalent to those obtained by nephelometry in a side-by-side comparison (Table 2).

ELISA leads to *in vitro* activation

Measurement of iC3b by the in-house ELISA was performed on normal donor plasma and serum samples using a range of capture times. A comparison between 15 and 60 min capture times demonstrated that there was an increase in the concentration of iC3b at 60 min, when compared to the purified iC3b control protein used as the reference at each time point (Figure 3A). To further investigate the dynamics of iC3b generation, the ELISA was repeated with capture times from 5 to 15 min. The level of iC3b detected in the serum (Figure 3B) and plasma (not shown) samples showed a progressive increase in iC3b levels from 5 to 15 min. This result suggests that C3 activation occurs during the ELISA, leading to the production of iC3b *in vitro*, despite the presence of 5 mM EDTA in the assay buffer. Although surprising, the inability of EDTA to prevent complement activation over time has been previously demonstrated [27, 28].

iC3b LFA detects iC3b in control samples

To confirm that the iC3b LFA was able to accurately detect iC3b levels in serum, contrived samples were prepared with purified iC3b (concentration determined by absorbance at 280 nm and an extinction coefficient of $E^{0.1\%/280}=1.03$) spiked into C3 depleted serum (Complement Technologies). This type of sample eliminates autoactivation as there is no native C3 in the mixture. The samples were analyzed by LFA and by the commercially available MicroVue iC3b ELISA (Quidel), and the results are shown in Figure 4. Regression analysis demonstrated that there was good correlation between the LFA and ELISA results ($R^2=0.996$).

To determine whether there was a differential matrix effect between blood, plasma and serum, titration curves were performed using all three sample types (Supplementary Figure 1). The dilution curves of each sample demonstrated similar shape and dilution effect, indicating the different matrices were not having a significant effect on the results.

To confirm the specificity of the iC3b LFA, samples were prepared by spiking purified C3, C3b, iC3b, C3c or C3d into the sample buffer at known concentrations and analyzed by iC3b LFA. Results of this analysis indicate that there is minimal cross reactivity for other C3 fragments (Table 3). The C3b sample demonstrated some cross-reactivity, but as C3b is very short-lived in serum ($t_{1/2}=2$ min) and not present at an appreciable concentration (Sim et al., 1981), it should not contribute to iC3b measurements in serum. These data demonstrate that the iC3b LFA is capable of selectively assessing iC3b levels while avoiding cross-reactivity with other C3 fragments.

LFA detects iC3b in normal donor serum

To determine complement activation in normal donors, iC3b was measured by LFA in blood, plasma and serum samples from the 10 normal donors (Table 2). The iC3b concentrations were consistently low, 1-3 $\mu\text{g/ml}$ in blood. The levels of iC3b in plasma and serum were somewhat higher (2-4 $\mu\text{g/ml}$), likely corresponding to the change in volume when plasma or serum is processed from whole blood. A comparison of the average iC3b level in the serum compared to plasma demonstrated increased levels in the serum (4.0 vs 3.1 $\mu\text{g/ml}$, serum vs plasma) that is possibly due to a small amount of complement activation

during coagulation [28]. The MicroVue iC3b ELISA was performed in parallel and iC3b concentrations were calculated using a standard curve provided with the kit (Table 2, Supplementary Figure 2). The iC3b concentrations determined by LFA were similar to those determined by ELISA.

In vitro activation of serum complement can be evaluated using the iC3b LFA

To assess the level of *in vitro* activation of C3 that can occur with improperly handled biological samples, stability studies were performed. Serum and plasma samples were collected from normal donors and assayed immediately via the iC3b LFA before being stored under different conditions (-80°C, -20°C, 4°C) for one week. The samples were then reanalyzed with the iC3b LFA (Table 4). Storage at -80°C had minimal effect on the iC3b levels, whereas storage at -20 or 4°C led to increased iC3b levels in both plasma and particularly serum (up to a 25-fold increase).

iC3b levels are elevated in samples from patients with SLE and ICH

To evaluate if differences in iC3b levels could be appreciated in patient populations, two disease states were evaluated. It is well established that complement activation is associated with SLE [15, 29]. C3, C4, and total complement hemolytic activity are commonly measured at the time of diagnosis and serial C3 and C4 measurements are utilized as a guide to disease activity in a subset of patients (reviewed in [30, 31]). The second condition assessed was intracerebral hemorrhage (ICH). Complement activation has been associated with increased cerebral edema in ICH [32], but the utility of complement monitoring in this indication has received much less scrutiny. Levels of iC3b were measured by LFA in the serum of 10 normal controls and 110 SLE patients (Figure 5A) or the blood of 8 normal controls and 10 ICH patients (Figure 5B). While normal donors demonstrate low iC3b levels in a narrow range (1-3 µg/ml), the disease samples had higher levels of iC3b and a wider range of concentrations. In the SLE patients, iC3b levels were also compared to C3 and C4 nephelometry (when available). This analysis demonstrated that the higher levels of iC3b were found predominantly in patients who also had low C3 and C4 levels (Supplementary Figure 3).

Discussion

In this study, two new quantitative lateral flow assays (LFAs) for complement proteins were evaluated. Both LFAs were developed to facilitate minimal handling and manipulation of samples. This allows samples to be applied directly to the assay system with minimal manipulation, enabling time to results within 15-30 minutes of blood draw. The total C3 LFA gives equivalent C3 values compared to the current standard nephelometry methodology but with the benefit of a much faster turn-around time to results because it can be performed on whole blood immediately upon collection. While the nephelometry technology itself is also fast, samples need to be processed to generate serum, therefore the time to results is longer than the 30 s required to perform the test.

The second LFA measures the C3 activation product iC3b in the same benefits of speed and minimal sample manipulation. The iC3b test is specific and has a wide dynamic range

measuring from 0.8 µg/ml to 25.6 µg/ml in whole blood, plasma or serum. In "spiking" experiments, the iC3b levels generated by the LFA and MicroVue ELISA were in agreement. Experiments analyzing different incubation times of serum in the in-house ELISA demonstrated that iC3b is likely being generated in the ELISA wells during the capture.

Using the LFA, this study determined that the concentration of iC3b in normal blood is between 1-3 µg/ml. Previous reports have suggested that it is higher [33, 34], but these levels were determined by ELISA, so there is the caveat that experimental *in vitro* activation is likely to have occurred during measurement. The possibility of *in vitro* activation leading to artificially high iC3b levels in past studies is supported by data presented here demonstrating increased iC3b in samples that were improperly stored. A recent report described a newly developed C3c ELISA that defined the level of C3c in normal donors as around 4 µg/ml in plasma which is very similar to what we report here for iC3b despite the fact the two biomarkers are reported to have markedly different half-lives [35]. It is possible that there will be more than one clinically useful biomarker of complement activation and further clinical utility studies will help to shed light on this question.

Complement activation has historically been problematic to assess in the laboratory [28, 36]. Sample handling, storage conditions, cryoproteins, assay conditions and plasticware can all lead to *in vitro* activation. Further, the amplification loop of the AP has the capacity to amplify any spurious activation. Because of these issues, specific activation signatures associated with various pathogenic states have not been well defined. The iC3b LFA test described in this report offers the potential to study this biomarker in near real-time while avoiding the *in vitro* activation that occurs in other assay systems. Additionally, the iC3b LFA provides the opportunity to test the hypothesis that the level of the iC3b biomarker in blood is proportional to the extent of injury or severity of disease. Thus, having a rapid method to define these signatures and monitor iC3b levels could facilitate assessment of alterations in complement activation and eventually lead to earlier decision making.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Walport MJ. Complement. First of two parts. *N Engl J Med*. 2001; 344:1058–1066.10.1056/NEJM200104053441406 [PubMed: 11287977]
2. Trouw LA, Daha MR. Role of complement in innate immunity and host defense. *Immunol Lett*. 2011; 138:35–37.10.1016/j.imlet.2011.02.014 [PubMed: 21333684]
3. Gasque P. Complement: a unique innate immune sensor for danger signals. *Mol Immunol*. 2004; 41:1089–1098.10.1016/j.molimm.2004.06.011 [PubMed: 15476920]
4. Nilsson B, Nilsson Ekdahl K. The tick-over theory revisited: is C3 a contact-activated protein? *Immunobiology*. 2012; 217:1106–1110.10.1016/j.imbio.2012.07.008 [PubMed: 22964236]

5. Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, et al. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med.* 2006; 12:682–687.10.1038/nm1419 [PubMed: 16715088]
6. Amara U, Flierl MA, Rittirsch D, Klos A, Chen H, Acker B, et al. Molecular Intercommunication between the Complement and Coagulation Systems. *J Immunol.* 2010; 185:5628–5636.10.4049/jimmunol.0903678 [PubMed: 20870944]
7. Lambris JD. The multifunctional role of C3, the third component of complement. *Immunol Today.* 1988; 9:387–393. [PubMed: 3076413]
8. Janssen BJC, Christodoulidou A, McCarthy A, Lambris J, Gros P. Structure of C3b reveals conformational changes that underlie complement activity. *Nature.* 2006; 444:213–216. [PubMed: 17051160]
9. Gros P, Milder FJ, Janssen BJC. Complement driven by conformational changes. *Nat Rev Immunol.* 2008; 8:48–58.10.1038/nri2231 [PubMed: 18064050]
10. Harboe M, Mollnes TE. The alternative complement pathway revisited. *J Cell Mol Med.* 2008; 12:1074–1084.10.1111/j.1582-4934.2008.00350.x [PubMed: 18419792]
11. Liszewski MK, Fang CJ, Atkinson JP. Inhibiting complement activation on cells at the step of C3 cleavage. *Vaccine.* 2008; 26(8):I22–27. [PubMed: 19388160]
12. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol.* 200910.1038/nri2620
13. Holers VM. Complement and Its Receptors: New Insights into Human Disease. *Annu Rev Immunol.* 2014; 32:433–459.10.1146/annurev-immunol-032713-120154 [PubMed: 24499275]
14. Nilsson B, Ekdahl KN, Mollnes TE, Lambris JD. The role of complement in biomaterial-induced inflammation. *Mol Immunol.* 2007; 44:82–94.10.1016/j.molimm.2006.06.020 [PubMed: 16905192]
15. Alper CA, Rosen FS. Alper CA, Rosen FS: Studies of the in vivo behavior of human C'3 in normal subjects and patients. *J Clin Invest.* 1967; 46:2021–2034.10.1172/JCI105691 [PubMed: 6074005]
16. Warwicker P, Goodship TH, Donne RL, Pirson Y, Nicholls A, Ward RM, et al. Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int.* 1998; 53:836–844.10.1111/j.1523-1755.1998.00824.x [PubMed: 9551389]
17. Perrin LH, Lambert PH, Miescher PA. Complement breakdown products in plasma from patients with systemic lupus erythematosus and patients with membranoproliferative or other glomerulonephritis. *J Clin Invest.* 1975; 56:165–176.10.1172/JCI108065 [PubMed: 1141431]
18. Spitzer RE, Vallota EH, Forristal J, Sudora E, Stitzel A, Davis NC, et al. Serum C'3 lytic system in patients with glomerulonephritis. *Science.* 1969; 164:436–437. [PubMed: 4180576]
19. Hiramatsu M, Balow JE, Tsokos GC. Production of nephritic factor of the alternative complement pathway by Epstein Barr virus-transformed B cell lines derived from a patient with membranoproliferative glomerulonephritis. *J Immunol Baltim Md* 1950. 1986; 136:4451–4455.
20. Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res.* 2001; 20:705–732. [PubMed: 11587915]
21. Griselli M, Herbert J, Hutchinson WL, Taylor KM, Sohail M, Krausz T, et al. C-reactive protein and complement are important mediators of tissue damage in acute myocardial infarction. *J Exp Med.* 1999; 190:1733–1740. [PubMed: 10601349]
22. Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L, et al. Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. *PLoS Med.* 2011; 8:e1001013.10.1371/journal.pmed.1001013 [PubMed: 21445332]
23. Sim E, Wood AB, Hsiung LM, Sim RB. Pattern of degradation of human complement fragment, C3b. *FEBS Lett.* 1981; 132:55–60. [PubMed: 6457754]
24. Posthuma-Trumpie GA, Korf J, van Amerongen A. Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. *Anal Bioanal Chem.* 2009; 393:569–582.10.1007/s00216-008-2287-2 [PubMed: 18696055]

25. Wong, RC.; Tse, HY. Lateral flow immunoassay. Springer; New York, NY: 2009. <http://public.eblib.com/EBLPublic/PublicView.do?ptiID=417533> [accessed December 12, 2013]
26. Garner, M.; Lemon, J.; Sing, P. irr: Various coefficients of interrater reliability and agreement (Version 0.83). 2010. software Available from <http://CRAN.R-project.org/package=irr>, n.d
27. Newell S, Gorman JC, Bell E, Atkinson JP. Hemolytic and antigenic measurements of complement. A comparison of serum and plasma samples in normal individuals and patients. *J Lab Clin Med*. 1982; 100:437–444. [PubMed: 7108352]
28. Mollnes TE, Garred P, Bergseth G. Effect of time, temperature and anticoagulants on in vitro complement activation: consequences for collection and preservation of samples to be examined for complement activation. *Clin Exp Immunol*. 1988; 73:484–488. [PubMed: 2463123]
29. Sturfelt G, Truedsson L. Complement and its breakdown products in SLE. *Rheumatol Oxf Engl*. 2005; 44:1227–1232.10.1093/rheumatology/keh719
30. Manderson AP, Botto M, Walport MJ. The role of complement in the development of systemic lupus erythematosus. *Annu Rev Immunol*. 2004; 22:431–456.10.1146/annurev.immunol.22.012703.104549 [PubMed: 15032584]
31. Leffler J, Bengtsson AA, Blom AM. The complement system in systemic lupus erythematosus: an update. *Ann Rheum Dis*. 2014.10.1136/annrheumdis-2014-205287
32. Ducruet AF, Zacharia BE, Hickman ZL, Grobelny BT, Yeh ML, Sosunov SA, et al. The complement cascade as a therapeutic target in intracerebral hemorrhage. *Exp Neurol*. 2009; 219:398–403.10.1016/j.expneurol.2009.07.018 [PubMed: 19632224]
33. Negoro N, Okamura M, Takeda T, Koda S, Amatsu K, Inoue T, et al. The clinical significance of iC3b neoantigen expression in plasma from patients with systemic lupus erythematosus. *Arthritis Rheum*. 1989; 32:1233–1242. [PubMed: 2803326]
34. Porcel JM, Ordi J, Castro-Salomo A, Vilardell M, Rodrigo MJ, Gene T, et al. The Value of Complement Activation Products in the Assessment of Systemic Lupus Erythematosus Flares. *Clin Immunol Immunopathol*. 1995; 74:283–288.10.1006/clin.1995.1040 [PubMed: 7859418]
35. Palarasah Y, Skjodt K, Brandt J, Teisner B, Koch C, Vitved L, et al. Generation of a C3c specific monoclonal antibody and assessment of C3c as a putative inflammatory marker derived from complement factor C3. *J Immunol Methods*. 2010; 362:142–150.10.1016/j.jim.2010.09.024 [PubMed: 20869965]
36. Bronfenbrenner J. A Biochemical study of the Phenomena known as Complement-Splitting: First Paper: Splitting of the Complement Associated with Globulin Precipitation. *J Exp Med*. 15(1912): 598–624.10.1084/jem.15.6.598 [PubMed: 19867549]
37. Alper CA, Rosen FS. Alper CA, Rosen FS: Studies of the in vivo behavior of human C'3 in normal subjects and patients. *J Clin Invest*. 1967; 46:2021–2034.10.1172/JCI105691 [PubMed: 6074005]
38. Carpenter CB, Ruddy S, Shehadeh IH, Müller-Eberhard HJ, Merrill JP, Austen KF. Complement metabolism in man: hypercatabolism of the fourth (C4) and third (C3) components in patients with renal allograft rejection and hereditary, angioedema (HAE). *J Clin Invest*. 1969; 48:1495–1505.10.1172/JCI106116 [PubMed: 4894302]
39. Petz LD, Fink DJ, Letsky EA, Fudenberg HH, Müller-Eberhard HJ. In vivo metabolism of complement. I. Metabolism of the third component (C'3) in acquired hemolytic anemia. *J Clin Invest*. 1969; 47:2469–2484.10.1172/JCI105929 [PubMed: 4975569]
40. Bokisch VA, Müller-Eberhard HJ. Anaphylatoxin inactivator of human plasma: its isolation and characterization as a carboxypeptidase. *J Clin Invest*. 1970; 49:2427–2436.10.1172/JCI106462 [PubMed: 4098172]
41. Nilsson B, Ekdahl KN. Complement Diagnostics: Concepts, Indications, and Practical Guidelines. *Clin Dev Immunol*. 2012; 2012:1–11.10.1155/2012/962702
42. Charlesworth JA, Williams DG, Sherington E, Lachmann PJ, Peters DK. Metabolic Studies of the Third Component of Complement and the Glycine-Rich Beta Glycoprotein in Patients with Hypocomplementemia. *J Clin Invest*. 1974; 53:1578–1587.10.1172/JCI107708 [PubMed: 4830223]
43. Bedwell AE, Elson CJ, Carter SD, Dieppe PA, Hutton CW, Czudek R. Isolation and analysis of complement activating aggregates from synovial fluid of patients with rheumatoid arthritis using monoclonal anti-C3d antibodies. *Ann Rheum Dis*. 1987; 46:55–64. [PubMed: 3492971]

A. *In vivo* activation

B. *In vitro* activation

Figure 1. Diagram of C3 activation

A. *In vivo* C3 is commonly activated to C3b via the C3 convertases following which it can covalently attach to a target. The resulting C3b may either participate in an amplification loop or be inactivated via regulatory proteins. **B.** *In vitro* C3 activation may occur during exposure to plasticware, improper storage, freeze-thaw cycles or activity of clotting or other proteolytic enzymes. *In vitro* activation leads to more C3b generation and then this may be amplified. See Introduction for further explanation. AP, alternative pathway; CP, classical pathway; LP, lectin pathway; red line, covalent bond.

Figure 2. Design of total C3 and iC3b lateral flow assays

A. Schematic diagram of LFA design; see Materials and Methods for a more detailed explanation. **B** and **C.** Standard curves of total C3 and iC3b LFAs. Purified proteins were serially diluted in LFA buffer and analyzed in triplicate. Results are expressed as reflectance units (RU) (mean $\pm$ SD) and are representative of three separate experiments.

Figure 3. *In vitro* generation of iC3b in ELISA wells

A. Generation of iC3b was assessed with capture time of 15 or 60 min. Shown is the mean $\pm$ SEM of four experiments. **B.** Serum samples were diluted and incubated in ELISA wells coated with anti-iC3b antibody for the capture times indicated. Following washing, iC3b concentrations were determined and compared to a standard curve of purified iC3b. Representative of four similar experiments.

Figure 4. iC3b levels in contrived samples determined by LFA and ELISA

iC3b levels were determined in samples with known concentrations of iC3b by LFA and ELISA and a regression analysis was performed.

Figure 5. iC3b levels in patient populations

iC3b levels were measured by LFA in serum from patients with SLE (A) and blood from ICH patients (B) and compared with normal controls. The median and range for each population are shown.

Table 1
Half-lives of C3 and C3 split-products

	$t_{1/2}$	Reference
C3	40-70 hrs	[37–39]
C3a (C3a des arg)	~2 m (30 m-4 hours)	[40, 41]
C3b	2 m	[23]
iC3b	90 m	[23]
C3c	24 hrs	[42]
C3d	50 hrs	[43]

Table 2
C3 and iC3b levels in 10 normal donors

	Total C3 ¹ (mg/ml)		iC3b (µg/ml)	
	LFA	Nephelometry	LFA	ELISA ²
Blood	0.93±0.3	--	2.0 ± 0.5	--
Plasma	--	--	3.1 ± 0.7	3.4 ± 1.3
Serum	1.3 ± 0.2	1.3 ± 0.2	4.0 ± 1.1	4.2 ± 1.5

Values correspond to the mean ± SD from 10 normal donors.

¹Total C3 was only measured in blood and serum with the LFA and serum only by nephelometry.

²The iC3b ELISA was not designed for whole blood.

Table 3
iC3b LFA measurement of samples containing C3 or C3 fragments

Protein ^a (1000 µg/ml)	iC3b LFA measurement ^b (µg/ml)	iC3b to target protein ratio
C3	12	0.01
C3b	102	0.10
iC3b	988	1
C3c	16	0.02
C3d	0	--

Commercially available purified C3 and C3 fragments (1 mg/ml) were utilized to assess cross-reactivity in the iC3b LFA.

^a Samples were diluted 1:1,000, and 1:10,000 (C3, C3b, C3c and C3d) or 1:10,000 and 1:100,000 (iC3b) in LFA assay buffer and assayed in triplicate.

^b Calculated concentration after correction for dilution factor. Representative of three similar experiments.

Table 4
iC3b levels in plasma and serum stored at different conditions

Sample type	Storage condition	Average iC3b level
Plasma	Initial measurement	4 ± 0.23
Plasma	-80° C	4 ± 0.39
Plasma	-20° C	7 ± 1.24
Plasma	4° C	$50 \pm 12.54^*$
Serum	Initial measurement	4 ± 0.35
Serum	-80° C	5 ± 0.45
Serum	-20° C	51 ± 22.36
Serum	4° C	$46 \pm 9.13^\dagger$

*
p<0.0001 by one-way ANOVA

[†]
p=0.0023 by one-way ANOVA